Characterisation of the mechanisms of tumour-induced dysfunction of clonal T cell expansions in multiple myeloma

A Thesis Submitted for the Degree

of

Doctor of Philosophy

by

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Hayley Suen

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PREFACE

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Publications

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Suen H, Brown R, Yang S, Ho PJ, Joshua D, Gibson J (2015) The failure of immune checkpoint blockade in multiple myeloma with PD-1 inhibitors in a phase 1 study. *Leukemia* **29,** 1621-1622.

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Bryant C, **Suen H**, Brown R, Yang S, Favaloro J, Aklilu E, Gibson J, Ho PJ, Iland H, Fromm P, Woodland N, Nassif N, Hart D, Joshua D. Long-term survival in multiple myeloma is associated with a distinct immunological profile, which includes proliferative cytotoxic T-cell clones and a favourable Treg/Th17 balance. Blood Cancer Journal 2013; 3: e148.

Conference papers and presentations

Suen H, Brown R, Joshua D, Yang S, Weatherburn C, Nassif N, Woodland N, Hsu J, Bryant C, Hart D, Ho P, Gibson J. Multiple myeloma causes T cell ageing (immunosenescence) in tumour induced T cell clones (2015). Proceedings of the Annual Scientfic Meeting of the HAA.

Suen H, Joshua, DE, Brown RD, Yang S, Barbaro PM, Ho, PJ, Gibson, J (2014) Protective Cytotoxic Clonal T-Cells in Myeloma Have the Characteristics of Telomere-Independent Senescence Rather Than an Exhausted or Anergic Phenotype: Implications for Immunotherapy. *Blood,* **124,** 3367-3367. Proceedings of the American Society of Hematology.

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Awards and Prizes

- **Young Investigator Awarrd**

Awarded to the best oral presentation at the 33rd Combined Health Sciences New Horizons Conference

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LIST OF ABBREVIATIONS

AIF	Apoptosis-inducing factor
AIHW	Australian Institute of Welfare and Health
ALL	Acute lymphoblastic leukaemia
Allo-SCT	Allogeneic stem cell transplant
Anti-	Antibody
APAF1	Apoptotic protease-activating factor
APC	Antigen presenting cell
Auto SCT	Autologous stem cell transplant
Bak	Bcl-2-antagonist/killer
Bax	Bcl-2 associated X
Bcl	B cell lymphoma
BCMA	B cell maturation antigen
BID	BH3-interacting death domain agonist
BM	Bone marrow
Bort	Bortezomib
B_2M	Beta 2 microglobulin
C	Constant
Ca	Calcium
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CDKI	cyclin-dependent kinase inhibitor
CDR	Complementarity determining region
CFSE	Carboxyfluorescein succinimidyl ester
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CRAB	Hypercalcaemia, renal failure, anaemia and bone lesions
CRP	C reactive protein
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
CyBORD	Cyclophosphamide, bortezomib and

	dexamethasone
DAPI	4’,6-diamidino-2-phenylindole
DC/s	Dendritic cell/s
Dex	Dexamethasone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EMRA	Effector memory T cell expressing CD45RA
ERK	Extracellular signal-related kinase
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death-domain protein
FoxP3	Forkhead box P3
FISH	Fluorescence in situ hybridisation
FLIP	FADD-like IL-1β-converting enzyme-inhibitory protein
FMO	Fluorescence minus one
FR	Framework regions
G-CSF	Granulocyte colony stimulating factor
g/L	Grams/litre
GM-CSF	Granulocyte macrophage colony stimulating factor
h	hour
HePTP	Haematopoietic protein tyrosine phosphatase
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMiDs	Immunomodulatory drugs
IMWG	International Myeloma Working Group
ISS	International Staging System
ITAMS	Immunoreceptor tyrosine-based activation motifs
JNK	c-Jun N terminal kinase
K	Kinase
L	Leader sequence

LAG-3	Lymphocyte activation gene-3
Lat	Linker for activation of T cells
Lck	Lymphocyte specific protein tyrosine kinase
LDH	Lactate dehydrogenase
Len	Lenalidomide
LGL	Large granular lymphocytic leukaemia
M protein	Monoclonal protein; paraprotein
MAPK	Mitogen activated protein kinases
Mcl-1	Myeloid-cell leukaemia sequence 1
MDS	Myelodysplasia
MDSC	Myeloid derived suppressor cells
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
Min	Minutes
MM	Multiple myeloma
mRNA	Messenger ribonucleic acid
MSAG	Medical Scientific Advisory Group
mSMART	Mayo stratification of Myeloma and Risk Adapted Therapy
n	(sample) number
N/A	Not applicable
NF-κB	Nuclear factor kappa B
ng	Nanograms
NK cells	Natural killer cells
NT	Not available for testing
nTreg	Natural T regulatory cells
p16	p16INK4a
p21	p21CIP1/WAF1
PB	Peripheral blood
PBMC/s	Peripheral blood mononuclear cell/s
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem cell
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein-1 or CD274

PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
Pom	Pomalidomide
pRb	Retinoblastoma tumor suppressor
PTP	Protein tyrosine phosphatase
PTPN7	Protein tyrosine phosphatase non-receptor type 7
qPCR	Quantitative polymerase chain reaction
RPAH	Royal Prince Alfred Hospital
RT	Room temperature
SARA	SMAD anchor for receptor activation
SASP	Senescence associated secretory phenotype
SCT	Stem cell transplant
SHP-2	Src homology 2 (SH2) domain containing protein tyrosine phosphatase (PTP)
SM	Smouldering myeloma
SMAD	Homologs of the *Caenorhabditis elegans* protein SMA and the *Drosophilia* protein, mothers against decapentaplegic (MAD)
SPE	Serum protein electrophoresis
STAT3	Signal transducer and activator of transcription
T cells	T lymphocytes
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TGF-β	Transforming growth factor β
Th	T helper cell
T-LGL	T-large granulocytic l
TNF	Tumour necrosis factor
Treg	T regulatory cell
T/S	Telomeric DNA quantity/single copy gene DNA quantity
U	International Units
U&E	Urea and electrolytes
UV	Ultraviolet
V	Variable

Vβ	Beta chain of the variable region (of the TCR)
WHO	World Health Organisation
WM	Waldenström macroglobulinaemia
ZAP-70	ζ-chain associated protein kinase of 70kDa
α	Alpha
β	Beta
γ	Gamma
δ	Delta
K	Kappa
λ	Lambda
°C	Centrigrade (degrees Celsius)
µL	Microlitres

ABSTRACT

Multiple myeloma is a cancer involving malignant plasma cells in the bone marrow. Despite advances in therapy, relapse is inevitable due to residual disease and myeloma remains incurable. New therapies are required to remove residual disease and maintain long term survival. Expanded clones of cytotoxic T cells have been detected in myeloma and their presence is associated with improved survival, suggesting a role in anti-tumour immunity. However, these cells are dysfunctional as they do not proliferate. Thus, tumour-induced dysfunction of T cell clones may be a tumour evasion mechanism that contributes to immune escape. The primary aim of this thesis was to elucidate the mechanism/s responsible for the observed dysfunction of these T cell clones, which may allow future development and implementation of novel strategies to restore clonal T cell function.

T cell clones were detected in 75% of a new cohort of myeloma patients (n=103) and their presence was associated with an improved survival, despite being non-proliferative. T cell clones were present in 100% of long term survivors of myeloma, providing further evidence that these cells prolong survival. In contrast, T cell clones from 10 year survivors were proliferative. Phospho-flow technology was used to investigate the differences in cell signalling pathways between T cell clones of 10 year and non-10 year survivors. The dysfunction in these cells was related to the upregulation of the SMAD pathway, promoting T cell inactivation and downregulation of the ERK pathway, which blocks proliferation of T cells.

Classification of T cell clones into an anergic, exhausted or senescence phenotype was carried out to determine if dysfunction is reversible, since reversal of dysfunction is phenotype dependent. The cells exhibited a senescent secretory effector phenotype: KLRG-1+/CD57+/CD160+/CD28- with normal telomere lengths for age, suggesting telomere-independent senescence. Importantly, the results demonstrate that dysfunction is potentially reversible. The p38-MAPK, p16 and p21 signaling pathways, which are known to induce

senescence were not upregulated. However, elevated telomerase levels may explain how senescent T cells maintain normal telomere lengths.

This thesis expands our understanding of the biology and clinical significance of T cell clones. It is the first to describe the dysfunction of T cell clones as telomere independent senescence, which is potentially reversible. Additionally, it has identified two novel mechanisms by which tumour cells induce dysfunction in T cell clones. These findings have implications for reversing tumour-induced dysfunction of T cell clones in patients with myeloma.

CHAPTER 1 INTRODUCTION

1.1 Multiple Myeloma

1.1.1 Introduction to Multiple Myeloma

Multiple myeloma (MM) is a cancer involving the proliferation of malignant clonal plasma cells in the bone marrow (BM) (Figure 1.1A). It is also known as plasma cell myeloma in the World Health Organisation (WHO) classification of tumours of haematopoietic and lymphoid tissues (Swerdlow *et al* 2008). According to data collected by the Australian Institute of Welfare and Health (AIHW), MM accounts for 1.3% of all cancers and 10% of the haematological cancers (AIHW 2016). In Australia, approximately 1200 new cases of MM are detected each year with a median age of diagnosis in the early 60s (Joshua 2005). The likelihood of MM increases with age and the incidence is higher amongst males than females, occurring at a ratio of 1.5:1 (AIHW 2016). Morbidity is primarily related to bone disease, with other major clinical manifestations including anaemia, hypercalcaemia, renal failure and an increased risk of infection due to a compromised immune system (Rajkumar 2011a).

1.1.2 Clinical Presentation of MM

The excessive malignant BM plasma cells produce a single type of immunoglobulin known as a monoclonal protein, M protein or paraprotein. The paraprotein can be detected in the serum or urine through serum protein electrophoresis (Figure 1.1B), immunofixation (for detection of isotype; Figure 1.1C), or serum free light chain assays (Kyle 1999, Katzmann *et al* 2005). An paraprotein is present in the serum of 97% of patients and the remaining 3% of patients that do not secrete a paraprotein, are classified as non-secretory MM (Kyle *et al* 2003). The paraprotein can be one of the different classes of immunologbulin (Ig) including IgA, IgD, IgE, IgD and IgM and are either kappa (K) or lambda (λ) restricted. The most common type is the IgG type, followed by the IgA type. The IgD and IgE types are less common while the IgM type is very rare. About 1 in 5 people with MM do not actually produce intact Ig and only produce the light chain portion of the Ig. This is known as light chain myeloma (Abraham *et al* 2002).

As the malignant plasma cells produce excessive amounts of a single type of Ig (monoclonal protein), there is a reduction in the production of the normal repertoire of polyclonal immunoglobulins required for the humoral immune system (Nucci and Anaissie 2009). This contributes to the compromised immune system seen in patients with MM and they are more susceptible to bacterial and viral infections (Frassanito *et al* 1998, Nucci and Anaissie 2009). Interactions between BM plasma cells and surrounding stromal cells also increases the activity of osteoclasts normally involved in bone resorption, leading to osteolytic bone lesions in MM patients (Figure 1.1D). The infiltration of BM with malignant plasma cells affects normal haemopoiesis. Decreased numbers or dysfunctional white blood cells may contribute to compromised immunity and a reduction in the number of red blood cells may cause the patient to become anaemic. Other causes of anaemia include abnormal iron utilisation, low serum erythropoietin levels, decreased response to erythropoietin and haemolysis (Caers *et al* 2008). MM can also cause severe end-organ effects such as renal failure due to the increased load on the kidneys to remove the abnormal levels of paraprotein (Swerdlow *et al* 2008).

The initial diagnostic workup process for MM is shown in Table 1.1. A range of tests are suggested in order to ascertain the diagnosis, stage of disease and prognostic markers which may influence treatment choices for patients (Quach and Prince 2012).

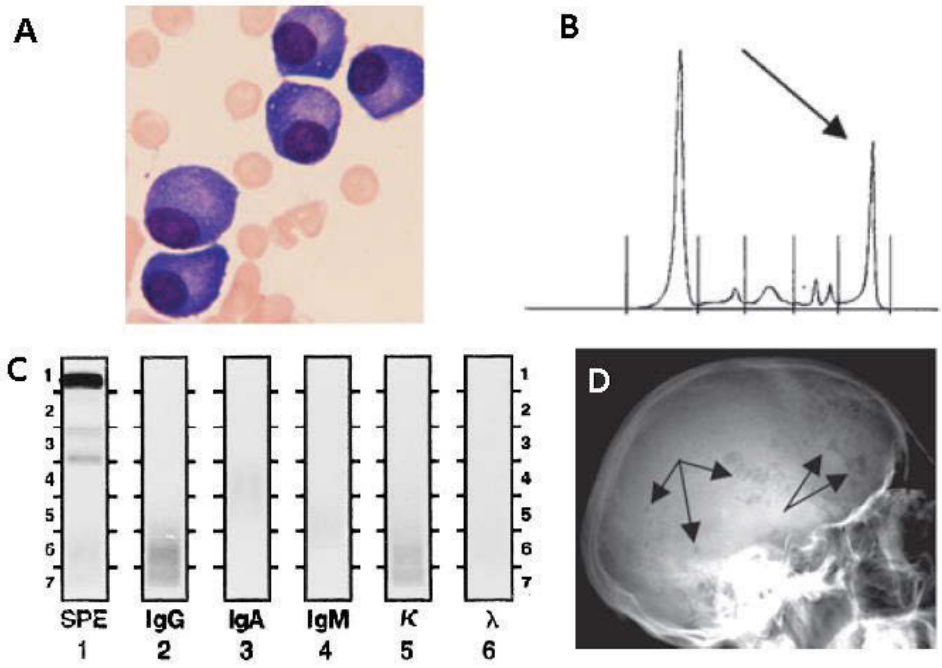

Figure 1.1 Diagnostic features of MM
(A) MM involves the proliferation of malignant plasma cells in the BM. **(B)** The monoclonal protein (indicated by arrow) produced by the plasma cells in MM patients can be detected using serum protein electrophoresis (SPE). **(C)** Example of an immunofixation assay demonstrating a MM patient with a κ restricted IgG monoclonal protein. **(D)** Bone lesions (indicated by arrows) observed in an X-ray of an MM patient. (Adapted from Caers *et al* 2008).

Abbreviations: Ig: Immunoglobulin; κ: Kappa; λ: Lambda.

Table 1.1 Initial diagnostic workup on suspicion of MM or other monoclonal gammopathies.

Department	Investigations
Haematology	— Full blood count — Differential count — Blood film
Biochemistry	— U&E, Ca++, PO_4/Mg++, urate — β_2M, LDH, CRP — Serum protein electrophoresis — Immunofixation electrophoresis — 24 hour urine collection — Serum free light chain
Cellular Pathology	— Morphology — Cytogenetics — FISH
Flow cytometry	– CD138, CD19, CD56, κ and λ light chain expression
Imaging	– Skeletal survey

Abbreviations: U&E: urea and electrolytes; Ca: calcium, PO_4: phosphate; Mg: magnesium; β_2M: Beta 2 microglobulin; LDH: lactate dehydrogenase; CRP: C reactive protein; FISH: fluorescence *in situ* hybridisation (Adapted from Quach and Prince 2012).

1.1.3 Diagnosis of MM

Figure 1.2 outlines the current diagnostic criteria for MM, which have been established and revised by the International Myeloma Working Group (IMWG) and Mayo Clinic (Rajkumar *et al* 2014). The first diagnostic criteria for MM is the identification of 10% or more clonal plasma cells in the BM. Figure 1.1A illustrates the morphological examination of BM from a MM patient, demonstrating the clonal proliferation of malignant plasma cells. Classification as MM also requires one or more 'myeloma defining events', that includes evidence of end organ damage that occurs as a result of the underlying plasma cell clone (Figure 1.2). This includes 4 symptoms denoted by the acronym 'CRAB': hypercalcemia (elevated serum calcium), renal insufficiency, anaemia or osteolytic bone lesions. If myeloma defining events are not present, the presence of any one or more biomarkers of malignancy are sufficient for the diagnosis of MM. These biomarkers are listed in Figure 1.2.

The presence of end-organ damage distinguishes MM from two pre-malignant, asymptomatic conditions: monoclonal gammopathy of undetermined significance (MGUS) and asymptomatic smouldering myeloma (SM). Most patients present firstly with the benign condition MGUS which can progress to MM at a rate of 1% per year (Kyle and Rajkumar 2006). MGUS is characterised by the presence of a serum monoclonal protein at a concentration of <30g/L but with less than 10% clonal plasma cells observed in the BM with no end organ damage. SM is the asymptomatic stage that bridges MGUS and MM. For a diagnosis of SM, the serum monoclonal protein concentration must be >30g/L with >10% plasma cells in the BM but no end organ damage detected, hence the disease is asymptomatic.

***Panel:* Revised International Myeloma Working Group diagnostic criteria for multiple myeloma and smouldering multiple myeloma**

Definition of multiple myeloma

Clonal bone marrow plasma cells ≥10% or biopsy-proven bony or extramedullary plasmacytoma* and any one or more of the following myeloma defining events:

- Myeloma defining events:
  - Evidence of end organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically:
    - Hypercalcaemia: serum calcium >0·25 mmol/L (>1 mg/dL) higher than the upper limit of normal or >2·75 mmol/L (>11 mg/dL)
    - Renal insufficiency: creatinine clearance <40 mL per min† or serum creatinine >177 μmol/L (>2 mg/dL)
    - Anaemia: haemoglobin value of >20 g/L below the lower limit of normal, or a haemoglobin value <100 g/L
    - Bone lesions: one or more osteolytic lesions on skeletal radiography, CT, or PET-CT‡
  - Any one or more of the following biomarkers of malignancy:
    - Clonal bone marrow plasma cell percentage* ≥60%
    - Involved:uninvolved serum free light chain ratio§ ≥100
    - >1 focal lesions on MRI studies¶

Definition of smouldering multiple myeloma

Both criteria must be met:

- Serum monoclonal protein (IgG or IgA) ≥30 g/L or urinary monoclonal protein ≥500 mg per 24 h and/or clonal bone marrow plasma cells 10–60%
- Absence of myeloma defining events or amyloidosis

PET-CT=^{18}F-fluorodeoxyglucose PET with CT. *Clonality should be established by showing κ/λ-light-chain restriction on flow cytometry, immunohistochemistry, or immunofluorescence. Bone marrow plasma cell percentage should preferably be estimated from a core biopsy specimen; in case of a disparity between the aspirate and core biopsy, the highest value should be used. †Measured or estimated by validated equations. ‡If bone marrow has less than 10% clonal plasma cells, more than one bone lesion is required to distinguish from solitary plasmacytoma with minimal marrow involvement. §These values are based on the serum Freelite assay (The Binding Site Group, Birmingham, UK). The involved free light chain must be ≥100 mg/L. ¶Each focal lesion must be 5 mm or more in size.

Figure 1.2 Revised IMWG diagnostic criteria for MM and SM

The diagnostic criteria for the diagnosis of multiple myeloma and smouldering myeloma. Adapted from Rajkumar *et al* (2014).

1.1.4 Classification and Staging of MM

1.1.4.1 The Durie-Salmon Clinical Staging System

The Durie Salmon clinical staging system was implemented in 1975 and takes into consideration the clinical features exhibited by patients, response to treatment and also correlates survival with measured tumour cell burden (Durie and Salmon 1975). The presenting clinical features involved in this system were specifically chosen for their significant correlation to the measured myeloma cell mass. The only exception is serum creatinine, which, while correlative with survival, did not significantly correlate with the measured cell mass. Its inclusion in the system is an indication of renal function and categorises the stages into A (serum creatinine <20 g/L) or B (serum creatinine >20 g/L). Patients were categorised as stage I, II or III according to the degree of anaemia, hypercalcemia, serum and urine monoclonal protein levels and bone lesions (Table 1.2)

1.1.4.2 The International Staging System

In 2005, the International staging system (ISS) replaced the Durie-Salmon clinical staging system. It is comprised of two laboratory tests, the serum β_2 microglobulin (β_2M) and serum albumin, classifying the patients into three stages (Greipp *et al* 2005). In 2015, the revised ISS (R-ISS) took into consideration two extra parameters: chromosomal abnormalities by fluorescence *in situ* hybridisation (FISH) and serum lactate dehydrogenase (LDH) (Palumbo *et al* 2015). Table 1.3 lists the criteria for the three stages and also provides the median survival in months for each stage for the original ISS. The ISS classifies Stage 1 as β_2M < 3.5mg/L and albumin ≥ 35 g/L; stage II as neither stage I nor stage III; and stage III as β_2M ≥ 5.5 mg/L. It also lists the factors utilised in the R-ISS.

Table 1.2 The Durie-Salmon Clinical Staging System

Stage	Criteria	Measured MM cell mass (cells x $10^{12}/m^{2)}$
I	All of the following: 1. Haemoglobin value >100 g/L 2. Serum Calcium value normal 3. Normal bone structure or solitary bone plasmacytoma only 4. Low M-Component production rates a) IgG value <50 g/L b) IgA value <30 g/L c) Urine light chain M component on electrophoresis < 4 g/24 hours	<0.6 (low)
II	Fitting neither stage I or stage III	0.6-1.20 (intermediate)
III	One or more of the following: 1. Haemoglobin value <85 g/L 2. Serum calcium value >120 mg/100mL 3. Advanced lytic bone lesions 4. High M-component production rates a) IgG value >70 g/L b) IgA value >50 g/L c) Urine light chain M component on electrophoresis > 12 g/24 hours	>1.20 (high)
Sub-classification		**Serum Creatinine value**
A	Relatively normal renal function	<20 g/L
B	Abnormal renal function	>20 g/L

Adapted from Durie and Salmon 1975.

Table 1.3 The Revised International Staging System

Prognostic Factor	Criteria
ISS stage	
I	Serum β_2-microglobulin $<$ 3.5 mg/L, serum albumin $\geq$ 3.5 g/dL
II	Not ISS stage I or III
III	Serum β_2-microglobulin $\geq$ 5.5 mg/L
CA by iFISH	
High risk	Presence of del(17p) and/or translocation t(4;14) and/or translocation t(14;16)
Standard risk	No high-risk CA
LDH	
Normal	Serum LDH $<$ the upper limit of normal
High	Serum LDH $>$ the upper limit of normal
A new model for risk stratification for MM	
R-ISS stage	
I	ISS stage I and standard-risk CA by iFISH and normal LDH
II	Not R-ISS stage I or III
III	ISS stage III and either high-risk CA by iFISH or high LDH

Abbreviations: CA, chromosomal abnormalities; iFISH, interphase fluorescent in situ hybridization; ISS, International Staging System; LDH, lactate dehydrogenase; MM, multiple myeloma; R-ISS, revised International Staging System.

Adapted from Palumbo *et al* 2015.

1.1.1 T cells

Lymphocytes can be classified into three groups: T cells, B cells and natural killer (NK) cells (Figure 1.3). T cells and B cells play a major role in the adaptive immune system, being responsible for the recognition of specific antigens and the initiation of the adaptive immune response. NK cells on the other hand, are involved in innate immunity. They lack antigen specific receptors and, instead, recognise and execute cytotoxic activity against tumour or virus infected cells through antibody dependent cell-mediated cytotoxicity.

The T cells are further subdivided into two major subtypes based on the expression of CD4 or CD8 surface markers (Figure 1.3). T helper (Th) cells (CD4+), upon activation, differentiate into different Th subsets and promote different immune responses. Cells within the Th1 subset secrete interleukin (IL)-2 and interferon (IFN)-γ and are involved in aspects of cellular immunity, including cytotoxicity and local inflammatory responses whilst the Th2 cell subset produces IL-4, IL-5, IL-6 and IL-10, which stimulate B cells to proliferate and produce antibodies, and are therefore involved in promoting humoral immunity. CD4 cells may also differentiate into pro-inflammatory Th17 and regulatory T cell (Treg) subtypes.

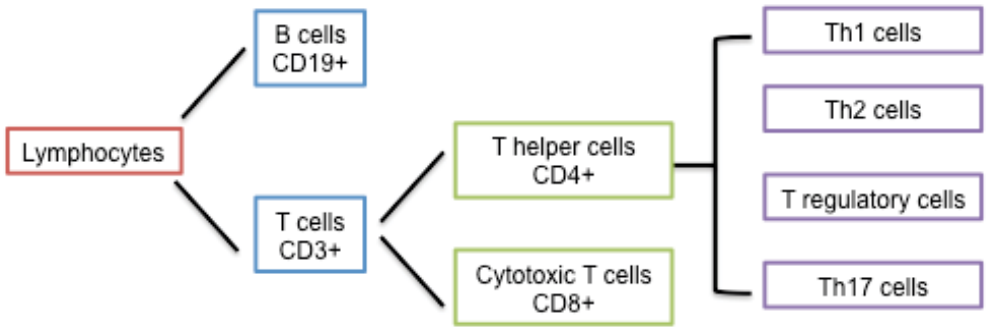

Figure 1.3 Hierarchy of human lymphocytes and their subsets.
Lymphocytes can be divided into two major groups: B cells (CD19+) and T cells (CD3+). T cells are further subdivided into T helper cells (CD4+) and cytotoxic T cells (CD8+). Depending on the cytokine environment, CD4+ Th cells may differentiate into Th1 cells or Th2 cells which are involved in cellular immunity and humoral immunity, respectively. CD4+ T cells can also differentiate into regulatory T cells or pro-inflammatory T helper 17 (Th17) cells.

The use of lymph node homing receptors CCR7 and CD62L in conjunction with CD45RA/CD45RO and/or CD27/CD28 can be used to distinguish different naïve and memory T cell subsets. Effector memory T cells (TEM) are cells that lack constitutive expression of CCR7, are heterogenous for CD62L expression and display rapid effector function with the expression of high levels of perforin. TEM are also predominantly CD8+ whilst CD4+ T cells are usually central memory T cells (TCM) which express both CCR7 and CD62L. Both these subsets also express CD45RO. Another effector memory T cell subset, known as TEMRA, also lacks CCR7 and has heterogenous CD62L expression but differs from TEM due to its positivity for CD45RA rather than CD45RO.

1.1.1.1 Structure of the T cell receptor

All T cells have a T cell receptor (TCR) which is a heterodimer of two transmembrane glycoprotein chains: either α and β, or γ and δ, which account for 90-95% and 5-10% of blood T cells, respectively. The extracellular portion of each glycoprotein chain consists of the variable (V) region and the constant (C) region (Roitt *et al* 2001) (Figure 1.4). The variable domain gives rise to the antigen binding site and the constant domain is responsible for coordinating effector functions (Schatz *et al* 1992).

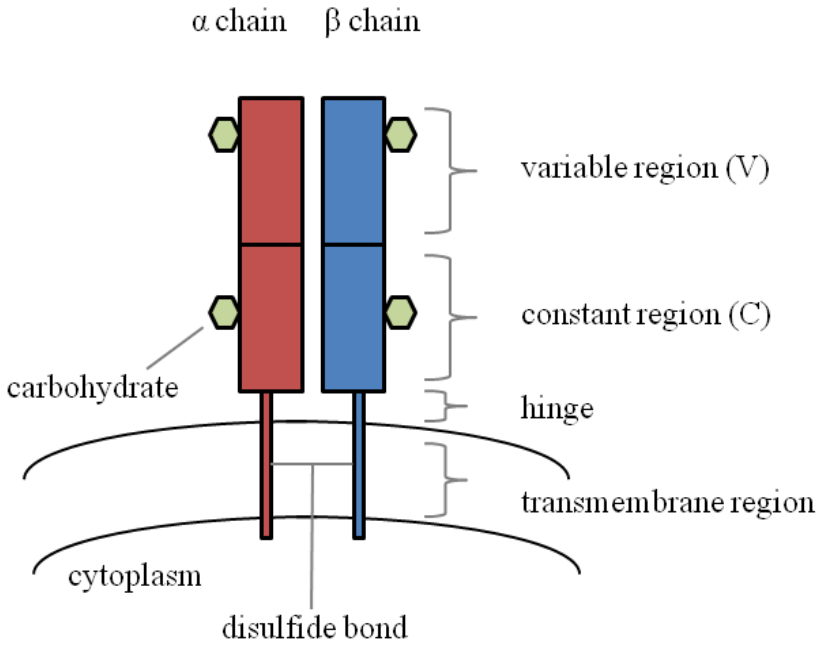

Figure 1.4 Structure of the T cell receptor
The T cell receptor consists of a heterodimer of two transmembrane glycoprotein chains, α and β, which are linked by a disulphide bond. The extracellular component of the chain is composed of two domains which resemble the variable (V) and constant (C) domains on immunoglobulins. Both chains have carbohydrate attachments. The hinge region connects the two chains as it contains the disulfide bond involved in the formation of the interchain disulfide bond. (Adapted from Roitt *et al* 2001 and Janeway *et al* 2005).

1.1.1.2 Generation of the T cell receptor

The *TCRα* and *TCRβ* genes are generated during T cell development when distinct genetic segments are joined together through a process known as somatic recombination. The organisation of the TCRα and TCRβ loci are shown in Figure 1.5. There are 4 genes involved in the synthesis of the TCR: variable (V), the diversity (D) and joining (J) genes, which encode the antigen and MHC binding sites and constant (C) genes which are responsible for coding of the non-variable TCR polypeptide chain (Maeurer 2005). This process is responsible for the generation of the diversity of TCRs required in humans (Schatz *et al* 1992). The process of TCR synthesis is demonstrated in Figure 1.6. For the generation of the α chain, a *Vα* gene segment rearranges to a *Jα* gene segment, generating a functional Vα region exon. Transcription and splicing of the Vα exon to Cα generates the messenger ribonucleic acid (mRNA), which is translated to produce the TCRα chain protein. In the case of

the β chain, the variable domain is encoded by *Vβ, Dβ* and *Jβ* gene segments. A functional VDJβ V region exon is generated through recombination and is transcribed and spliced to join to Cβ. The mRNA is translated to give rise to the TCRβ protein. The α and β chains come together to yield the functional αβ TCR heterodimer (Janeway *et al* 2005).

1.1.1.3 Junctional diversity and TCR specificity

TCR diversity is achieved from both the variation in the exact positions at which the junctions between gene segments occur and through junctional diversity where different numbers of P- and N-nucleotides are added between the V, D and J gene segments of the rearranged TCRβ gene (Janeway *et al* 2005).

The N region diversity occurs as a result of the deletion of nucleotides at the fringe of the coding V, D and J genes through the activation of an exonuclease and the random addition of nucleotides by the action of terminal deoxynucleotidyl transferase (TdT). The TdT enzyme preferentially adds G and C nucleotides at these junctions, known as the third hypervariable loop complementarity determining region (CDR)3 (Maeurer 2005). The CDR3 region contributed to by the D and J gene segments of the TCR α and β chains is situated in the centre of the antigen-binding site of a TCR. It is flanked by the other two hypervariable loops, CDR1 and CDR2. Junctional diversity mainly leads to a variation in the CDR3 length at the centre of the TCR as the other two hypervariable loops are encoded within the germline segments (Janeway *et al* 2005). The Vβ CDR3 length can vary by up to nine amino acids as a result of junctional diversity. In human peripheral blood, the minimum number of TCRs is approximately 2.5×10^7 (Arstila *et al* 1999). The CDR3 region in humans is approximately 11 amino acid residues in length. The sequence of the CDR3 region and its length define the specificity of the TCR and therefore are responsible for the diversity of the TCR repertoire.

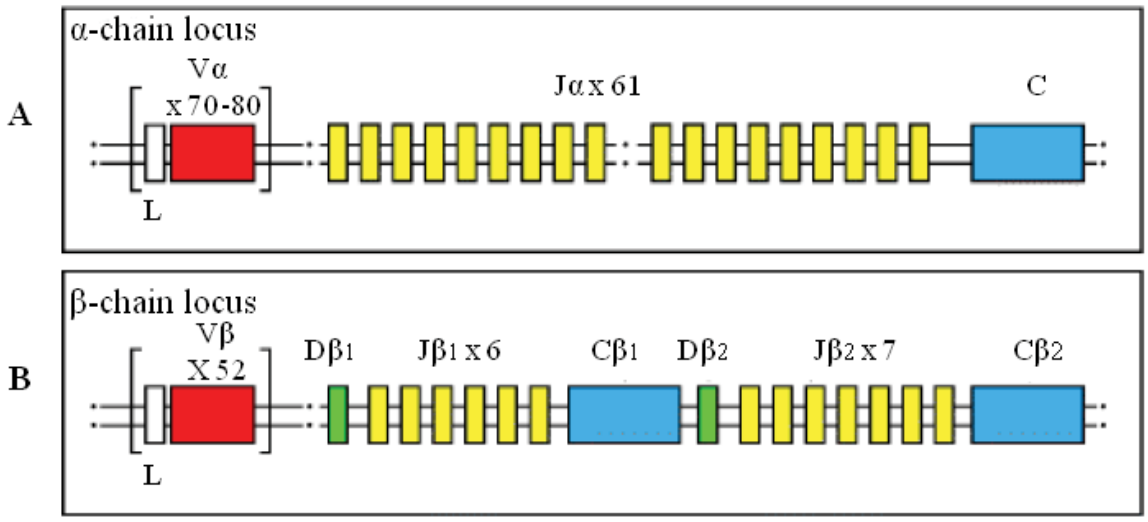

Figure 1.5 The genomic organisation of the TCR α and β loci
There are separate variable (V), diversity (D), joining (J) gene segments and constant (C) genes. **(A)** The TCRα locus is located on chromosome 14 and there are 70-80 Vα gene segments, which follows the exon encoding the leader sequence (L). 61 Jα gene segments are clustered further downstream, and are followed by a single C gene. **(B)** The TCRβ locus is located on chromosome 7 and it has a different structure compared to the TCRα locus. It begins with the L sequence followed closely by a group of 52 Vβ gene segments, located a considerable distance from two separate clusters, each containing a single D gene segment, six or seven J gene segments and finally, a single C gene. (Adapted from Janeway *et al* 2005).

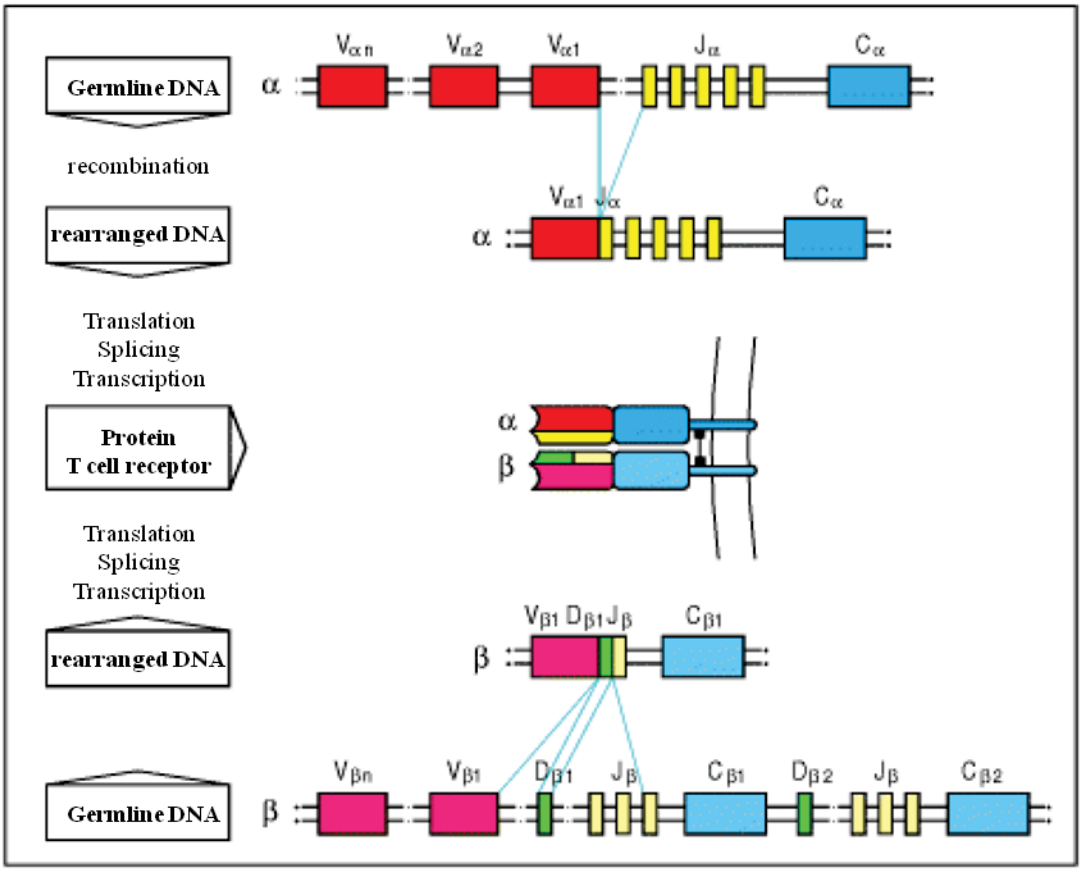

Figure 1.6 Germline rearrangement of the TCR-α and TCR-β genes
The TCR-α and TCR-β genes are generated from the joining of segments during T cell development in the process known as somatic recombination. The upper part of the figure demonstrates the generation of the α chain. A Vα gene segment rearranges to a Jα segment, generating a functional V region exon. The VJα exon joins with the Cα region through transcription and splicing to create mRNA which becomes translated to form the TCR α chain protein. In the lower part of the figure, the β chain is generated from the joining of three gene segments. Rearrangement of the gene segments creates the functional VDJβ V region exon that becomes transcribed and spliced to β. The resultant mRNA is translated to produce the TCR-β chain. The α and β chains pair up to produce the α:β TCR heterodimer. (Adapted from Janeway *et al* 2005).

1.2 The Defective Immune System in MM

1.2.1 The Immune System

The immune system is the body's defence against infection and disease. It consists of two components, the innate and adaptive immune system. The innate immune system is the body's first line of defence against infection (Parkin and Cohen 2001, Janeway *et al* 2005, Kindt *et al* 2006). Innate defence mechanisms are pre-existing and non-specific and can be activated within

minutes of encountering an infectious agent (Janeway *et al* 2005). The adaptive immune system is involved in recognising specific antigens, leading to the generation of antigen-specific effector cells that drive different immune responses (Parkin and Cohen 2001, Janeway *et al* 2005). Another aspect of this system is the generation of immunologic memory, whereby, upon second encounter with the same antigen, a heightened immune response is elicited (Parkin and Cohen 2001, Kindt *et al* 2006). Cells such as lymphocytes, granulocytes, monocytes and dendritic cells all play a role in immunity.

It is now well documented that numerical and phenotypical abnormalities are present in the T cell compartment in MM (Brown *et al* 2012b). Tumour cells employ many mechanisms to evade the host immune response, one of which is the suppression of tumour-specific cytotoxic T cells (Kim *et al* 2007). Cytotoxic T cells are the predominant type of effector cell involved in cancer immune destruction. Therefore, reversing tumour-induced cytotoxic T cell dysfunction is an important and growing consideration for cancer immunotherapies.

1.2.2 TCR-Vβ subfamilies

The V gene encodes the TCR variable region, which is the area that comes into contact with antigens. Each V gene consists of five regions: two CDRs and three framework regions (FRs). The CDRs generate α loops that interact with antigens and the FRs form β sheets responsible for providing structural support to CDRs. There are a large number of V genes in comparison to D and J genes and the sequence diversity among the V genes is mostly responsible for generating TCR diversity (Baran *et al* 2001). The TCR-Vβ region is located on chromosome 7 and contains Vβ genes that can be divided into 26 Vβ subfamilies with 75% nucleotide sequence homology (Arden *et al* 1995).

1.2.2.1 Identification of clonal T cell expansions by screening the TCR-Vβ repertoire

As the TCR is responsible for T cell specificity, the different types of TCR that are present in the TCR repertoire can be a mirror of what antigens have been experienced. The presence of a monoclonal expansion of T cells may indicate an antigen-driven process such as a T cell encountering its cognate antigen,

resulting in the rapid expansion and proliferation of T cells with the same TCR as the parent cell (Maeurer 2005). As the CDR3 region and its length define antigen specificity of the TCR, probing of the TCR-Vβ repertoire in T cells allows the determination of T cell clonality. Analysis of the TCR-Vβ repertoire diversity has been used to assess clonality in patients with lymphoproliferative disorders. T cell clonality can be determined using 4 different techniques: Southern blotting to detect rearrangements in the TCR-β chain, qualitative analysis of Vβ families in T cells using flow cytometry, CDR3 length analysis or spectratyping and through sequencing of the CDR3 region.

1.2.2.2 Southern blotting

One of the earlier detection methods for clonal T cells was the detection of rearrangements in the TCR-β chain by Southern blotting (Figure 1.7). Deoxyribonucleic acid (DNA) was firstly extracted from cells of interest and then digested with different restriction enzymes to produce DNA fragments. The digests were electrophoresed onto agarose gels for separation of fragments according to molecular weight and then transferred to a positively charged nylon membrane for hybridisation with a TCR-β-specific probe. After hybridisation, unhybridised probes were washed away and only probes with complementary sequence to the region of interest stay bound on the blot and are detected (Brown *et al* 2001). Patients showing rearrangements after the use of at least two different restriction enzymes were considered to have rearranged TCR-β chains (Brown *et al* 1997).

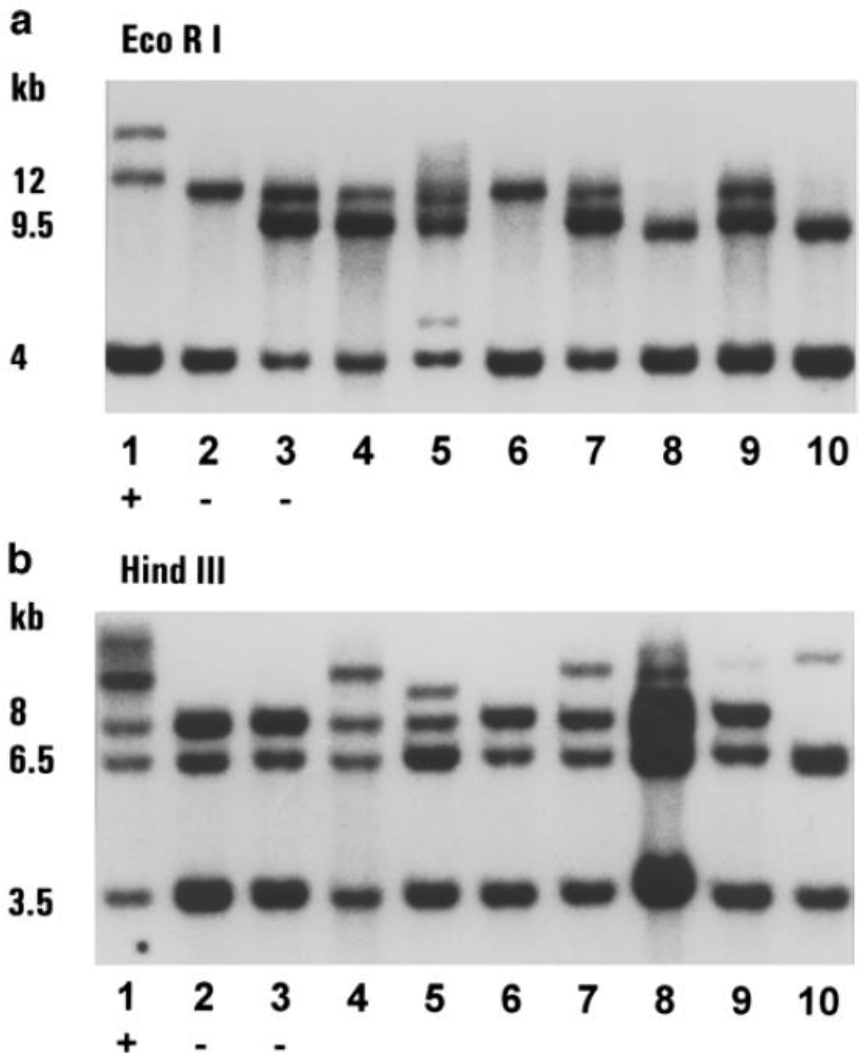

Figure 1.7 Detection of TCR β rearrangements by Southern blot analysis. Southern blot analysis of human genomic DNA digested with the restriction enzymes **(A)** *Eco*R1 and **(B)** *Hin*dIII**.** Immobilised DNA was hybridised with cDNA probes specific for the TCR b region**.** Lane 1 is the positive control and lanes 2 and 3 are the negative controls. Patients with rearrangements appearing after digestion with at least two different restriction enzymes were considered to be rearranged. **(A)** Sample in Lane 5 has an additional band. **(B)** For another group of patients, rearrangements are detected in Lanes 4, 5, 7, 8, 9 and 10 (Brown *et al* 1997).

1.2.2.3 Analysis of Vβ families in T cells using flow cytometry

Antigen driven expansion of a specific T cell clone leads to an increase in the number of T cells with that specificity so clonal T cell expansions can be detected by measuring the percentage of T cells expressing a particular TCR-Vβ family. Through multi-parametric flow cytometry, approximately 70% of the human TCR-Vβ repertoire can be quantified with different monoclonal antibodies targeting 24 specific Vβ families. A representative analysis of the

TCR-Vβ repertoire in a MM patient is shown in Figure 1.8A. A clonal T cell expansion is defined as an overexpression of a specific TCR-Vβ family which is mathematically defined as greater than the mean plus three times the standard deviation of the corresponding Vβ subfamily found in aged-matched normal controls (Figure 1.8B). An example of the gating strategy used in the detection of a specific clonal T cell population by flow cytometry is shown in (Figure 1.8C). (Raitakari *et al* 2000).

1.2.2.4 Analysis of CDR3 length and sequence for detection of monoclonal expansions

The TCR-Vβ flow cytometry assay, described in section 1.2.2.3 allows a quick qualitative screen of the repertoire. However, this assay only acts as a surrogate marker for the detection of abnormal T cell clonality as an increased number of TCR-Vβ expressing T cells does not necessarily indicate an expansion of T cells that is monoclonal. The most sensitive way to reveal monoclonality of a T cell population is to analyse the length of the CDR3 region followed by sequencing.

Specific oligonucleotide primers for Vβ subfamilies are available and are used in conjunction with a primer specific for Cβ region to amplify segments of the mRNA for the TCRβ chain that span the CDR3 region using polymerase chain reaction (PCR) analysis. Different populations of TCR Vβ genes will reveal a distribution of a variety of CDR3 lengths appearing as PCR products of varying lengths when resolved using polyacrylamide gel electrophoresis. From a diverse population of T cells, the resulting bands of CDR3 lengths approximately represent a Gaussian distribution. Deviations from a Gaussian distribution, caused by an increase in a particular CDR3 length, may be indicative of an expansion of a specific T cell clone (Figure 1.9) (Maeurer 2005). Sequencing of the PCR products will also reveal monoclonal or polyclonal sequences (Figure 1.10) (Sze *et al* 2001).

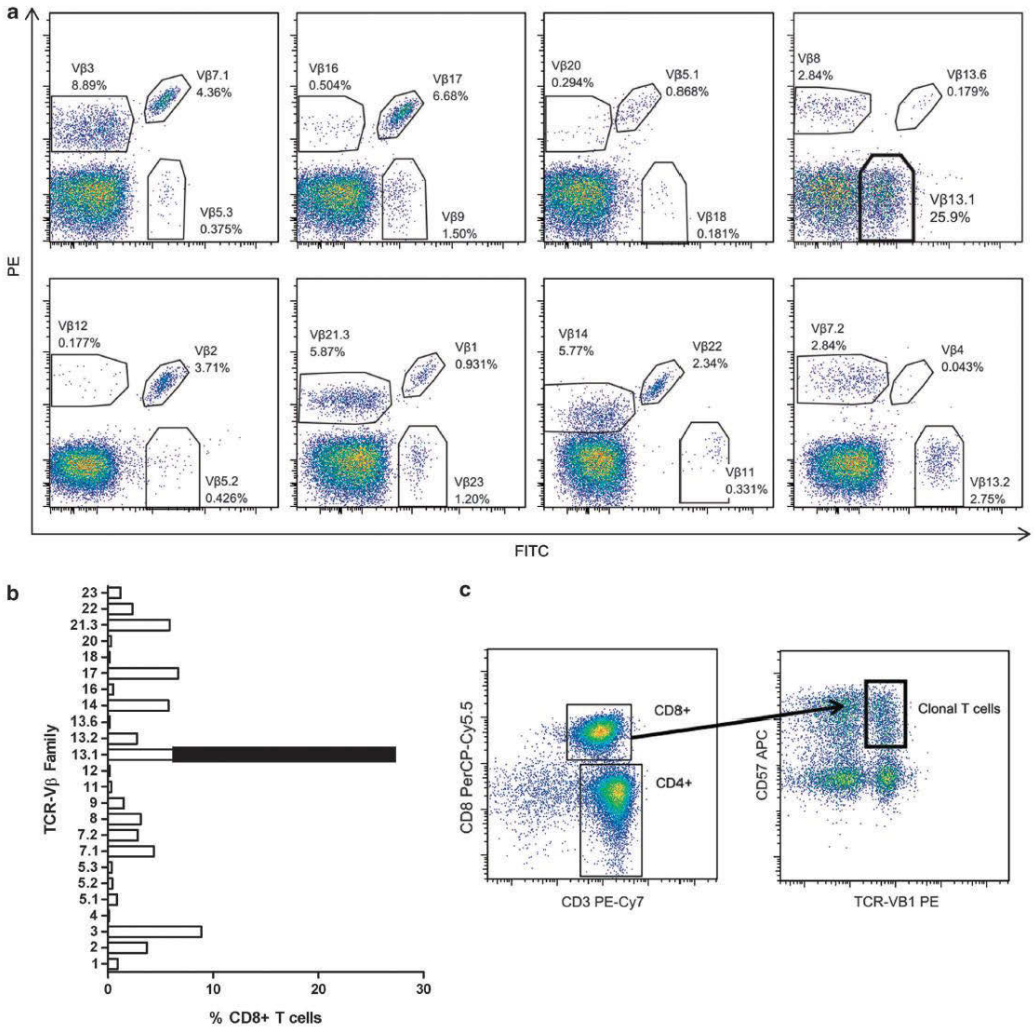

Figure 1.8 Analysis of the TCR-Vβ repertoire by flow cytometry using monoclonal antibodies against 24 Vβ families.

(A) Using flow cytometry, 24 TCR-Vβ families were analysed in an individual. Each flow cytometry plot illustrates the detection of 3 different families (circled) using 3 different monoclonal antibodies conjugated to either FITC, PE or a combination of the two. The 8 plots shown represent a study of 24 families. This individual has an expanded Vβ13.1 family (bold box). **(B)** The bar graph demonstrates the normal ranges of expression of each Vβ family (unshaded) and the black box illustrates the abnormal expansion in Vβ13.1. **(C)** Gating strategy for the identification of a clonal T cell population by flow cytometry. In this example, a TCR-Vβ1 expansion was detected from CD3+CD8+ T cells. Adapted from (Bryant *et al* 2013).

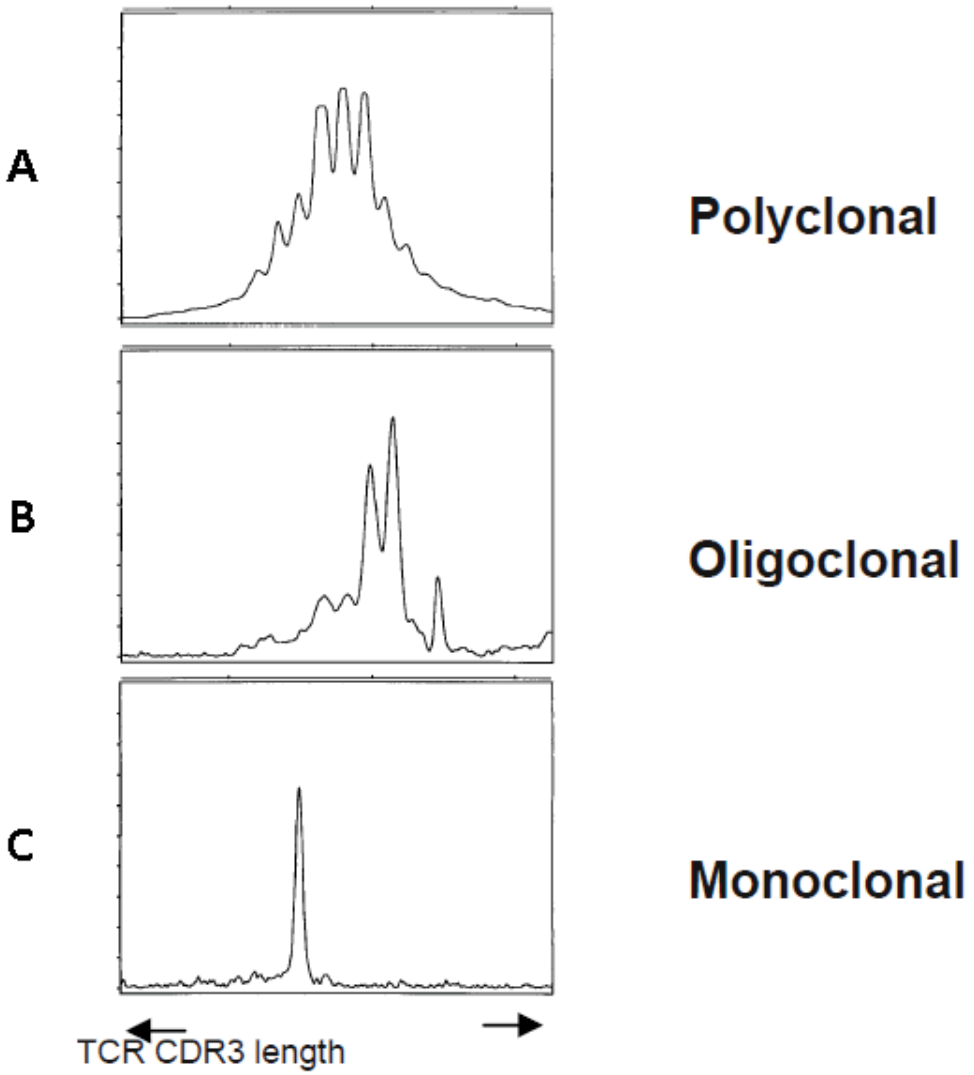

Figure 1.9 Determination of T cell clonality by TCR CDR3 length analysis. The CDR3 length of different T cells was measured using PCR with specific primers. **(A)** A variety of different CDR3 lengths are detected (representative of a Gaussian distribution), as seen by the presence of multiple peaks indicating the T cell population analysed in this sample is polyclonal. **(B)** There are 2 major peaks indicating a lower variability in the T cell population analysed, indicating an oligoclonal sample. **(C)** The T cell population of this sample is monoclonal as indicated by the presence of a single peak (Maeurer 2005).

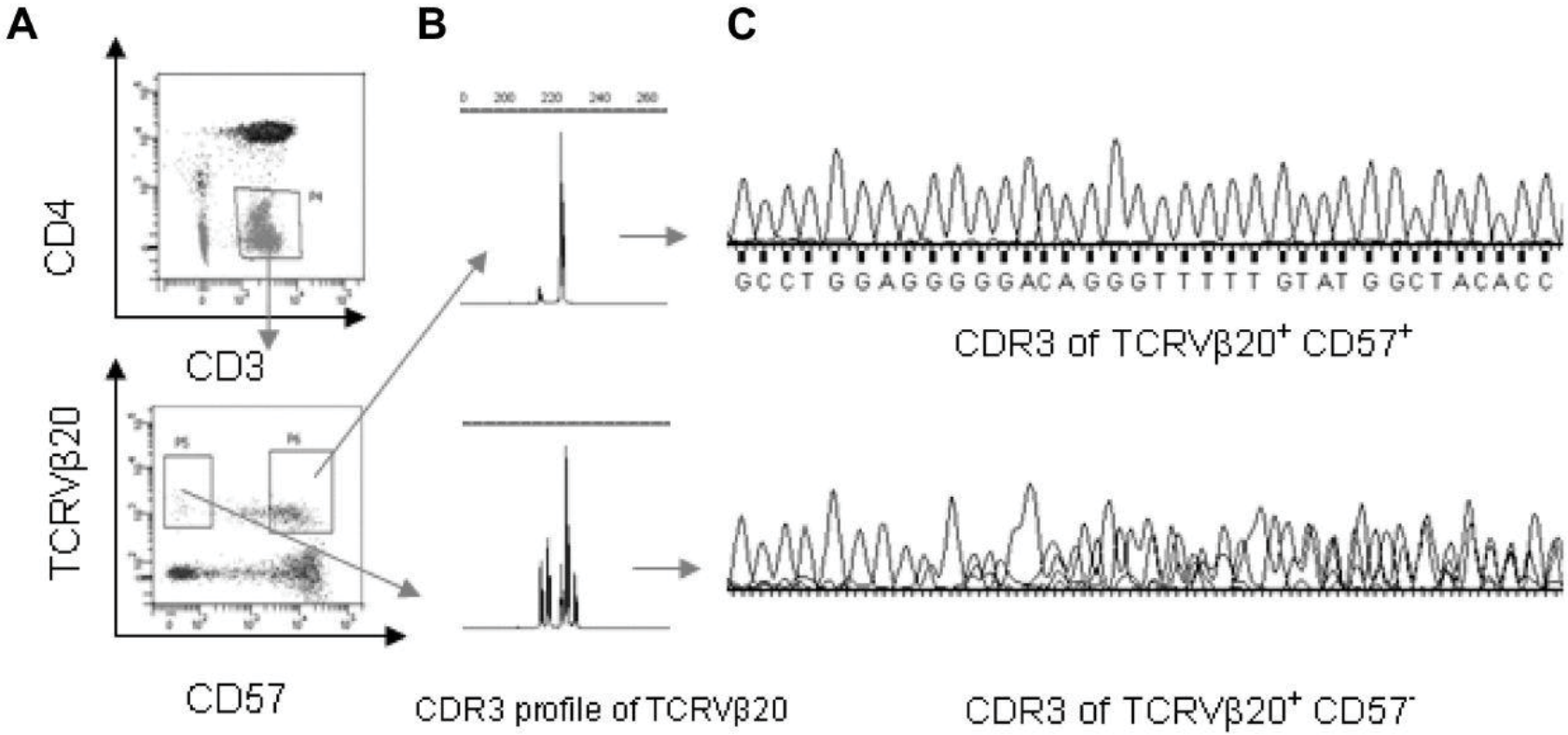

Figure 1.10 CDR3 length analysis and sequencing of PCR products of sorted T cells to determine clonality
(A) $CD3^+$, $CD4^-$, TCR-$V\beta^+$, $CD57^{+/-}$ T cells were sorted from peripheral blood mononuclear cells using flow cytometry. **(B)** The CDR3 length was analysed using specific primers. The presence of a single peak indicates that the $CD57^+$ T cells are monoclonal, whereas multiple peaks indicate the $CD57^-$ T cells are polyclonal. **(C)** DNA sequence analysis was used to confirm clonality of the T cells. The sequence generated from $CD57^+$ T cells matched the sequence of the respective TCR-Vβ subfamily sequence and length, thus demonstrating the $CD57^+$ T cells were clonal, whereas the $CD57^-$ T cells yielded a highly mixed sequence pattern and confirmed the cells were polyclonal. Adapted from Li *et al.* (2010)

1.2.3 Tumour induced suppression of the immune system in MM

Complex interactions between MM plasma cells and the surrounding tumour BM microenvironment cause deregulation of the host immune system. The myriad of immune defects is caused by both inhibitory factors and cytokines secreted by the malignant plasma cells and cells of the BM microenvironment, leading to numerical and functional abnormalities of cells involved in normal immune function (Rutella and Locatelli 2012).

The tumour microenvironment plays an active role in immunosuppression through three main mechanisms: recruitment of suppressive cell populations such as regulatory T cells (Tregs) or myeloid derived suppressor cells (MDSC), production of molecules that exert a suppressive effect such as TGF-β, IL-10 and expression of ligands, that when bound to their receptors such as cytotoxic T lymphocyte associated protein 4 (CTLA-4) or programmed cell death protein-1 (PD-1), promote inhibition of different cell types such (Abe and Macian 2013):

Our laboratory has focussed our research efforts into understanding tumour suppression of T cells in MM. Figure 1.11 illustrates the proposed mechanisms associated with tumour-mediated suppression of T cells in MM (Brown *et al* 2012a) and are discussed below in detail. Other immune subsets including B cells (Urashima *et al* 1996) and natural killer (NK) cells (Fauriat *et al* 2006, Jarahian *et al* 2007, Maki *et al* 2008) are also suppressed or defective, however this is beyond the scope of this thesis.

1.2.3.1 Dendritic cell dysfunction

Dendritic cells (DCs) are professional antigen presenting cells. These cells are responsible for processing antigens in the periphery for presentation to T cells to induce antigen-specific T cell responses (Steinman 1991). Immature DCs present in the blood and peripheral tissues efficiently process antigens and undergo a maturation process to become mature DCs. This involves the up-regulation of adhesion, co-stimulatory and major histocompatibility complex (MHC) class II molecules that are recognised by antigen-specific T cells (Bippes C *et al* 2011). The mature DCs migrate to the T cell rich areas of lymph nodes

and present processed antigenic peptides on their cell surface, in the context of MHC molecules to naïve T cells. The T cell expresses a unique antigen-binding receptor (TCR) on its membrane, which recognises specific antigenic peptides bound to MHC molecules (Parkin and Cohen 2001).

In MM, the number of DCs in the peripheral blood of patients are normal but the cells are functionally defective (Brown *et al* 2001). After stimulation with CD40 ligand, the DCs are unable to significantly upregulate the expression of the B7 co-stimulatory molecules, CD80 and CD86. As a result, DCs remain immature and are unable to provide co-stimulation to T cells during antigen presentation. This impaired antigen presentation to cytotoxic T cells leads to suppressed recognition and killing of malignant plasma cells. The functional defect is induced by transforming growth factor β (TGF-β) and interleukin (IL)-10 that are secreted by malignant plasma cells (Brown *et al* 2001). This DC defect can be reversed by the addition of exogenous IL-12 or interferon (IFN)-γ (Brown *et al* 2004).

Tumour secreted IL-6 also significantly alters the number, phenotype, function and development of DCs. IL-6 inhibits the growth of CD34+ DC progenitors, causing the cells to differentiate into CD1a- CD14+ monocytes with phagocytic function rather than antigen-presenting abilities (Ratta *et al* 2002). In addition, DCs in MM also have a decreased ability to stimulate allogeneic T cell responses and these features are hallmarks of tolerogenised DC (Rutella *et al* 2006).

The lack of dendritic cell maturation results in inefficient antigen presentation and the failure to produce an effective immune response. This results in T cell anergy or hypo-responsiveness of the T cell, tolerance or T cell death. Sub-optimal presentation of antigens due to the presence of inhibitory signals and/or poor co-stimulation also contribute to this.

1.2.3.2 Trogocytosis and acquired regulatory T cells

Trogocytosis is the term given to the transfer of membrane proteins between cells during contact and such acquisition of cell membrane proteins can alter

cell function (Joly and Hudrisier 2003). Trogocytosis occurs in MM whereby T cells acquire CD86 and human leucocyte antigen (HLA)-G antigens after contact with malignant plasma cells. The resultant T cells are associated with a poor prognosis. HLA-G+ T cells also display an acquired regulatory function, similar to that of natural Tregs and inhibited the proliferation and function of other T cells (Brown *et al* 2012a, Yang *et al* 2012).

1.2.3.3 Alteration of the balance between inhibitory Treg and pro-inflammatory T helper 17 cells

The balance between Tregs and T helper 17 (Th17) cells is significantly different in MM patients. The Treg/Th17 ratio is significantly increased in MM patients compared to patients with MGUS, Waldenström macroglobulinaemia (WM) and age matched normal controls and therefore favours a suppressive state. An increased Treg/Th17 ratio correlated with a reduced overall survival of MM patients (Favaloro *et al* 2014a).

1.2.3.4 MDSC

MDSC are a heterogeneous population of immature myeloid progenitor cells that have tumour promoting and immune suppressive properties (Youn and Gabrilovich 2010). These cells are increased in MM patients and there is a bidirectional beneficial relationship between MDSC and MM tumour cells. In co-cultures, the MDSCs inhibit autologous T cell proliferation in both the peripheral blood and BM. They also exhibit suppressive effects on immune effector cells and reduce CD4+ T cells, CD8+ T cells and NKT cell mediated anti-tumour immune responses (Görgün *et al* 2013). The granulocytic subset of MDSC with a phenotype of HLA-DR- CD33+, CD11b+, CD15+ are the most inhibitory and are significantly increased in MM patients with progressive disease. G-MDSC generate additional Treg cells and are stimulated by G-CSF during stem cell mobilisation (Favaloro *et al* 2014b).

1.2.3.5 Dysfunctional cytotoxic T cells

Changes in the way T cells encounter antigen, are activated or differentiate may potentially lead to T cell dysfunction (Schietinger and Greenberg 2014). The different mechanisms utilised by the tumour cells to evade the host immune

system also impair cytotoxic T cell function (Crespo *et al* 2013), leading to defective killing of tumour cells. It is likely that tumour-induced immunosuppression may partly be responsible for the lack of success of immunotherapy approaches in MM. It is therefore necessary to fully understand all the mechanisms of T cell suppression in order to restore T cell function and ultimately the ability of these T cells to mount an effective immune response against MM tumour cells.

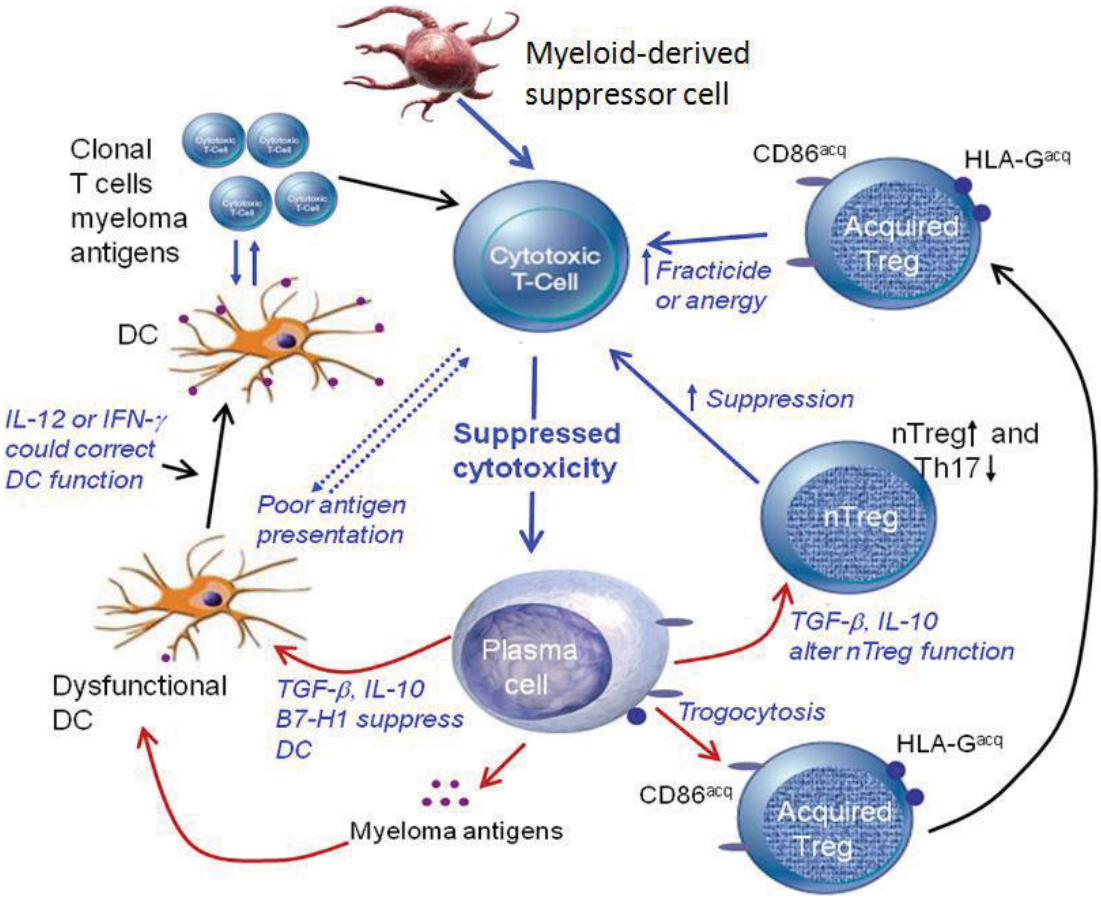

Figure 1.11 Proposed mechanisms associated with tumour-induced immunosuppression of cytotoxic T cells in MM
Chronic tumour stimulation of clonal T cells may lead to suppressed cytotoxicity against MM plasma cells. Plasma cell secreted TGF-β and IL-10 suppress co-stimulatory molecules on the DC surface, leading to ineffective antigen presentation to T cells. These cytokines can also induce the production of Tregs which suppress T cells along with the increased number of natural Tregs (nTregs) found in MM. Through trogocytosis, T cells acquire CD86 and HLA-G from plasma cells forming altered cells that can also inhibit T cells (acquired Tregs). Myeloid derived-suppressor cells inhibit autologous T cell proliferation and also suppress effector function. (Adapted from Brown *et al* 2012).

1.3 Treatment for MM

Although MM remains incurable at present, there are a wide range of treatment options that reduce tumour burden and improve the quality of life for patients (Rajkumar 2011b). The overall survival for MM has improved dramatically since the introduction of novel therapies such as the immunomodulatory drugs (IMiDs) thalidomide and lenalidomide and the proteasome inhibitors bortezomib and carfilzomib (Kumar *et al* 2008, Dimopoulos *et al* 2011). The median survival for MM patients has increased to 7 years (Dimopoulos *et al* 2011), however MM remains incurable (Rajkumar 2011a).

Either entry into a clinical trial or high dose chemotherapy followed by autologous stem cell transplantation remain the main treatments of choice (Quach and Prince 2012). Treatment for MM depends on the patient's eligibility for stem cell transplantation and also on risk stratification. In 2007, the Mayo Stratification of Myeloma and Risk Adapted Therapy (mSMART) consensus guidelines were first introduced to provide recommendations to clinicians for initial therapy, transplant and maintenance therapy in newly diagnosed MM patients (Dispenzieri *et al* 2007) with the most recent update in 2013 (Mikhael *et al* 2013). The IMWG, a collaborative group of 160 leading myeloma specialists around the world, also publishes guidelines on the diagnosis and treatment of MM. In the Australian setting, the Medical Scientific Advisory Group (MSAG) to the Myeloma Foundation of Australia has produced a Clinical Practice Guideline for MM (Quach and Prince 2012). As a result of these treatment options, an increasing number of patients achieve remission; however, relapse is common due to the development of chemotherapy resistant disease and evolving sub-clones of malignant plasma cells (Keats *et al* 2012).

1.3.1 Immunomodulatory drugs

Immunomodulatory drugs (IMiDs) include thalidomide and its analogues. Thalidomide, a teratogenic sedative drug, has immunomodulatory effects on cellular adhesion molecules (Kumar and Rajkumar 2006). The primary teratogenic target of thalidomide is cereblon (Ito *et al* 2010) and is required for the anti-myeloma activity of thalidomide and its analogs (Zhu *et al* 2011). It

enhances cytotoxic T lymphocyte proliferation, enhances IL-2 and IFN-γ production and inhibits inflammatory cytokines such as tumour necrosis factor (TNF)α and IL-6, involved in myeloma cell signalling pathways (Hus *et al* 2001, Thomas *et al* 2007). Furthermore, it stimulates NK cells which leads directly to myeloma cell lysis (Davis *et al* 2001). However, due to the concern surrounding the teratogenic effects of thalidomide, second generation analogue lenalidomide, which retains the anti-myeloma effects without the teratogenicity was developed (Kumar and Rajkumar 2006, Richardson *et al* 2009). Lenalidomide is an oral IMiD which differs from its parent drug by the addition of an amino group (Thomas *et al* 2007). It has an anti-TNFα activity 50,000 times more potent than that of thalidomide and its toxicity profile is more ideal than that of its parent drug (Richardson *et al* 2009). It also prevents the adhesion, and thus, invasion of myeloma cells to the bone marrow stroma (Palumbo *et al* 2009). Another thalidomide analog is Pomalidomide, which has immunomodulatory, anti-proliferative and anti-angiogenic effects (Richardson *et al* 2013).

1.3.2 Proteasome Inhibitors

Bortezomib, a potent dipeptide boronic acid analogue is a reversible inhibitor of the proteasome, a multi-catalytic enzyme complex responsible for the degradation of intracellular proteins and activation of nuclear factor kappa B (NF-κB), a regulator of cell cycle control (Adams *et al* 1999, Morgan *et al* 2008, Terpos *et al* 2008). By inhibiting the proteasome enzymatic activity, the degradation of key proteins is prohibited and signalling cascades within the cells are affected, eventually leading to cell death. Bortezomib also inhibits the binding of myeloma cells to bone marrow stromal cells, an interaction that is required for myeloma cell growth and survival (Terpos *et al* 2008, Curran and Mckeage 2009).

1.3.3 Autologous Stem Cell Transplantation

If the patient is younger than 70 years, an autologous stem cell transplant (auto SCT) should be considered. However other factors such as comorbidities, organ dysfunction, resources and preference need also to be considered. Tumour cells are firstly eradicated through chemotherapy before infusion with

stem cells previously harvested from the peripheral blood of the patient. After engraftment of the stem cells, haematopoiesis is established in the bone marrow and new blood cells will be produced. A peripheral blood SCT is preferred over a bone marrow transplant as engraftment occurs more rapidly and contamination of infused cells with tumour cells is less likely. Disadvantages of auto SCT include failure to eradicate myeloma and the possible contamination of autologous peripheral stem cells with tumour cells (Kyle 2001).

The goal of initial treatment for patients eligible for auto SCT is to reduce the tumour burden through induction therapy (Richardson *et al* 2009). The type of treatment potentially impacts subsequent stem cell collection so initial treatment options should depend on patient transplant eligibility. Patients are treated with 2-4 cycles of induction therapy before stem cell harvest to rapidly control the disease, allow for reversal of disease complications and to minimise the chance of early death (Kumar 2011, Rajkumar 2011a). Following that, patients can undertake frontline SCT or continue induction therapy until the SCT is required, such as at first relapse (Rajkumar 2011a).

1.3.4 Allogeneic Stem Cell Transplantation

Allogeneic stem cell transplantation (allo SCT) involves transplanting stem cells from a healthy donor, rather than an autologous donor. Allo SCT is potentially a curative treatment option for patients with MM due to the graft vs. myeloma effect and the absence of contaminating myeloma cells in the graft (Lokhorst *et al* 1997). However, there are high rates of mortality and morbidity associated with this therapeutic option, mainly due to graft vs. host disease and the use of allo-SCT also depends on patient age and donor availability (San Miguel J *et al* 1998, Kyle 2001).

1.3.5 Immunotherapy

Despite advances in therapy in the last 10 years, relapse is inevitable due to residual disease and MM remains incurable. The central problem remains the removal of residual disease after conventional therapy. Only allogeneic hematopoietic stem cell transplantation, the prototypic cellular immune therapy,

leads to a cure, however it is associated with high morbidity and mortality in MM. Immunotherapy may be a promising therapeutic strategy and involves stimulating the host immune response to fight MM. The immune system has the potential to target and eradicate MM tumour cells. Immunotherapy in conjunction with standard chemotherapy may be necessary to provide patients with long-term remission and cure (Reichardt VL *et al* 1999, Danylesko *et al* 2012). There is both direct and indirect evidence that immunological control of the malignant myeloma cells is possible. The therapeutic efficacy of allo SCT in MM patients is highly suggestive of MM tumour cells being receptive to immune recognition. This efficacy is due to the graft versus myeloma effect, which is mediated by donor lymphocytes in the graft (Giaccone *et al* 2011, Rutella and Locatelli 2012). This also led to the use of donor lymphocyte infusions, the isolation of donor T lymphocytes for infusion into patients in the hope of intensifying the graft versus myeloma effect (Lokhorst *et al* 1997). These therapies have mostly been efficacious with the induction of long-term survival and long-term disease control (Lokhorst *et al* 1997, Giaccone *et al* 2011, Roddie and Peggs 2011), however allo-SCT are still associated with a high mortality rate. Indirect evidence for host anti-tumour activity includes the detection of pre-malignancy specific effector T cells in the BM of patients with MGUS (Dhodapkar *et al* 2003), patients with disease in plateau phase, where a significant tumour load is detected but the disease remains stable, suggesting a degree of host immune control (Joshua *et al* 1994) and the correlation between expanded CD8+ T cell clones and improved survival (Brown *et al* 1997, Raitakari *et al* 2003, Brown *et al* 2009). Immunotherapy for MM primarily includes three different approaches: 1) reversal of tumour mediated immune suppression to stimulate pre-existing anti-tumour responses, including immune checkpoint inhibitors; 2) stimulation of myeloma specific immune responses with peptide or DC vaccinations or adoptive T cell therapy; and 3) elimination of the malignant MM clone with monoclonal antibodies against plasma cell antigens (Kocoglu and Badros 2016).

Another promising concept is the existence of naturally occurring clonal expansions of cytotoxic T cells that may have anti-tumour properties in MM (Brown *et al* 1997, Raitakari *et al* 2000, Sze *et al* 2001, Raitakari *et al* 2003, Sze

et al 2003, Brown *et al* 2009). The current paradigm is that these T cell clones confer a survival advantage most likely through anti-tumour properties but have simultaneously been rendered dysfunctional by tumour cells. The T cells need to be freed from MM induced dysfunction before possible use in immunotherapy.

1.4 Clonal CD8+ T cell expansions

1.4.1 Expansion of CD8+CD57+ T cells

Increased populations of CD8+ T cells are often described in the CD8+ CD57+ subset (Morley *et al* 1995). In normal individuals, the percentage of CD8+ CD57+ T cells is 5-20% of lymphocytes with a median of 15-16% (Abo and Balch 1981, Morley *et al* 1995). The CD8 expansions most likely accumulate over the course of normal immune responses to stimuli. This is supported by the predominant CD45RO+ memory phenotype of the T cell clones indicating previous antigen exposure and the fact that CD8+ clones can develop after immunisation (Hingorani *et al* 1993, Morley *et al* 1995). Another piece of supporting evidence is that at birth, basically all T cells are CD57- (Weekes *et al* 1999). Clonal CD8+ T cell expansions are well documented in healthy individuals and are known to form part of the ageing process (Ligthart *et al* 1986, Morley *et al* 1995, McNerlan *et al* 1998, Merino *et al* 1998).

The increased CD8+ CD57+ population led many groups to investigate whether they were clonal expansions and clonal expansions were in fact enriched in this particular subset (Hingorani *et al* 1993, Morley *et al* 1995, Mugnaini *et al* 1999, Degauque *et al* 2011). In a small study, CDR3 length analysis and screening of the TCR-Vβ repertoire in CD8+ CD57+ cells in 8 healthy individuals identified the presence of TCR-Vβ expansions (Degauque *et al* 2011). In a slightly larger study of 41 healthy patients, these cells were not different from other normal CD8+ cells in terms of cytokine production (IFN-γ, TNF-α and IL-2) and did not produce cytotoxic molecules (granzyme-B and perforin) (Morley *et al* 1995). The incidence of T cell expansions is lower in another study involving a slightly larger cohort where only 37% of patients had detectable CD8+ T cell expansions. The authors do acknowledge it is possible that T cell expansions

were underestimated due to a restricted monoclonal antibody panel, the use of an arbitrary value of >15% T cells being representative of an expansion and the finding that T cell expansions are not exclusive to the CD57+ subset and that CD8+ CD57- T cell expansions are possible (Morley *et al* 1995).

According to the literature, the exact incidence appears to vary between each study. This may be due to several factors such as the differences in ages of the studied cohorts, the criteria of a 'normal or healthy individual', serology status of patients studied, the arbitrary numerical value that indicates an expansion is present if studied by flow cytometry and the detection of TCR-Vβ expansions is restricted by the availability of monoclonal antibodies. Another important contributing factor may be the use of CD57 positivity to identify clonal expansions amongst the TCR-Vβ cells as CD57- expanded populations do exist and so the two populations may not be regarded as being distinct.

In general, CD8+ CD57+ cells are classified as antigen specific, clonally expanded, terminally differentiated, exhausted effector memory T cells that have progressed through multiple rounds of cell divisions as indicated by their shortened telomeres and lower levels of cell cycle regulatory gene expression (Arosa 2002, Vallejo 2005, Weng *et al* 2009a, Focosi *et al* 2010).

1.4.2 Function of CD8+ CD57+ Cells

It is unclear what causes the expansion of these cells with speculation that the stimulus could be viral, allo-immune or tumour antigen related (Morley *et al* 1995). It is however, established that common persistent viral infections with viruses such as cytomegalovirus (CMV) are largely responsible for the expansion of the CD8+ CD28- CD57+ T cell population in the elderly (Focosi *et al* 2010). Furthermore, the exact function of these cells remains largely unclear (Wood *et al* 2009), (Morley *et al* 1995) and there is conflict among the literature where this area is concerned. It is evident that CD8+ CD57+ T cell expansions are heterogeneous in their function across a variety of conditions. To generalise, CD8+ CD57+ cells can be described as having one of two possible, yet opposing functions and these are either immunoregulatory or cytotoxic, with the latter forming the majority of cases. Their role in immunoregulation may

occur through more than one mechanism including: resistance to apoptosis, release of soluble inhibitors of cytotoxic activity, expression of Fas ligand), upregulation of PD-1 and expression of inhibitory NK receptors or forkhead box P3 (FOX-P3) (Wood *et al* 2009). The cytotoxic subset expresses perforin, granzymes and granulysin, and these cytotoxic molecules tend to be present at higher levels than in their CD57- counterparts (Chattopadhyay *et al* 2009). The phenotype, incidence and proposed functions of CD8+ clonal T cell expansions with restricted TCR-Vβ expression in healthy individuals and disease are summarised in Table 1.4.

1.4.3 Clonal CD8+ T cell expansions in diseases

Clonal cytotoxic CD8+ T cell expansions have been detected in a number of haematological malignancies including MM (Brown *et al* 1997, Raitakari *et al* 2000, Sze *et al* 2001, Raitakari *et al* 2003, Sze *et al* 2003, Brown *et al* 2009, Li *et al* 2011) as well as chronic myeloid leukaemia (CML) (Li *et al* 2004, Mustjoki *et al* 2009), chronic lymphocytic leukaemia (CLL) (Terstappen *et al* 1990, Farace *et al* 1994), myelodysplasia (MDS) (Epling-Burnette *et al* 2007, Vries *et al* 2008) and WM (Li *et al* 2010). There is also a large body of evidence demonstrating that clonal CD8+ CD57+ T cell expansions occur in response to chronic viral infections such as human immunodeficiency virus (HIV) (Gupta 1986, Stites *et al* 1986, Vingerhoets *et al* 1995) and CMV infection (Würsch *et al* 1985, Wang *et al* 1993, Rossi *et al* 2007). CD8+ T cell expansions have also been documented in common variable immunodeficiency (Jaffe *et al* 1993) and post BM transplantation (Favrot *et al* 1983).

Table 1.4 Summary of the type and properties of CD8+ T cell expansions in normal aging individuals and some clinical conditions.

Condition	Phenotype of expansions*	Properties	Reference
Normal	CD57+ CD28-	– Long-term persistence – Stimulated by chronic antigen exposure (?CMV)	Ligthart *et al* 1986, Morley *et al* 1995, McNerlan *et al* 1998, Merino *et al* 1998
MM	CD57+ CD28- TCR-Vβ+ perforin+ granzyme-B+	– Anergic/exhausted/senescent? – Survival advantage – Long-term persistence	Brown *et al* 1997, Raitakari *et al* 2000, Sze *et al* 2001 Raitakari *et al* 2003, Sze *et al* 2003, Brown *et al* 2009
CLL	TCR-Vβ+ CD57+	– Not studied	Farace *et al* 1994
	CD4+ CD57+CD28-CD45RO+	– Cytotoxic? – Antigen experienced	Porakishvili *et al* 2001
CML	TCR-Vβ+ CD45RO+ CD27-CD62L-	– Not studied	Li *et al* 2001, Mustjoki *et al* 2009, Kreutzman *et al* 2010
MDS	CD57+ CD28- CD62L- NKG2D+ CD244+ perforin+ granzyme-B+	– Not studied	Eping-Burnette *et al* 2007, de Vries *et al* 2008
WM	CD57+ CD28- TCR-Vβ+ perforin+ granzyme-B+	– Anergic	Li *et al* 2010
T-LGL	CD57+ CD56-CD16+ TCR-Vαβ/γδ	– Not studied	Kaplanski *et al* 1992, Bourgault-Rouxel *et al* 2008, Feng *et al* 2010
HIV	CD57+ CD28- CD27- CD62L- CD45RA+	– Lack of proliferation – Stimulated by chronic antigen exposure? – Long-term/apoptotic persistence	Gupta 1986, Stites *et al* 1986, Vingerhoets *et al* 1995, Brenchley *et al* 2003, Petrovas *et al* 2009
CMV	CD57+	– Long-term persistence – Stimulated by chronic antigen	Würsch *et al* 1985, Wang *et al* 1993, Rossi *et al* 2007

*All expansions were CD3+CD8+ unless otherwise specified. Abbreviations: CLL: Chronic Lymphocytic Leukaemia; CML: Chronic Myeloid Leukaemia; CMV: Cytomegalovirus; HIV: MM: Multiple myeloma; TCR: T cell receptor

1.4.4 Clonal T cell expansions in MM

Clonal T cell expansions have been identified in 48% of MM patients in a nationwide Australian clinical trial (Brown *et al* 2009) and in 54% of MM patients at the Royal Prince Alfred Hospital (Raitakari *et al* 2003). The expanded T cell clones were predominantly CD8+. The incidence of these clonal expansions was much lower in normal age matched controls and was predominantly CD4+. The T cell clones are also CD57+CD28-, with restricted TCR-Vβ expression and CD45RA+ and therefore an immunophenotype of effector memory T cells (Raitakari *et al* 2000). They were also perforin+ and granzyme-B+, which suggests these cells are cytotoxic CD8+ T cells (Halapi *et al* 1997, Mugnaini *et al* 1999, Raitakari *et al* 2000, Sze *et al* 2001). There was no preferential expansion of a particular Vβ family (Figure 1.12), nor was it related to the age of the patient, class of M protein or time since diagnosis (Raitakari *et al* 2000). Representative T cell expansions from MM patients have all been proven to be truly clonal through sequencing of TCR CDR3 hypervariable regions (Figure 1.13) so it can be deduced that detection of an expanded T cell population in MM by flow cytometry is associated with a T cell clone (Sze *et al* 2001, Sze *et al* 2003, Li *et al* 2010).

True clonality has been associated with expression of CD57 as clonally expanded TCR-Vβ restricted populations preferentially expressed CD57 when compared to normal TCR-Vβ populations (Morley *et al* 1995). Therefore, CD57 expression within an expanded TCR-Vβ subfamily has been employed to potentially distinguish clonal T cell expansions from non-clonal T cell expansions (Sze *et al* 2003). CDR3 length analysis and direct sequencing of the PCR product indicated that CD57+ Vβ+ cell subsets were either monoclonal or oligoclonal whilst the $CD57^-$ counterparts were usually polyclonal (Li *et al* 2010).

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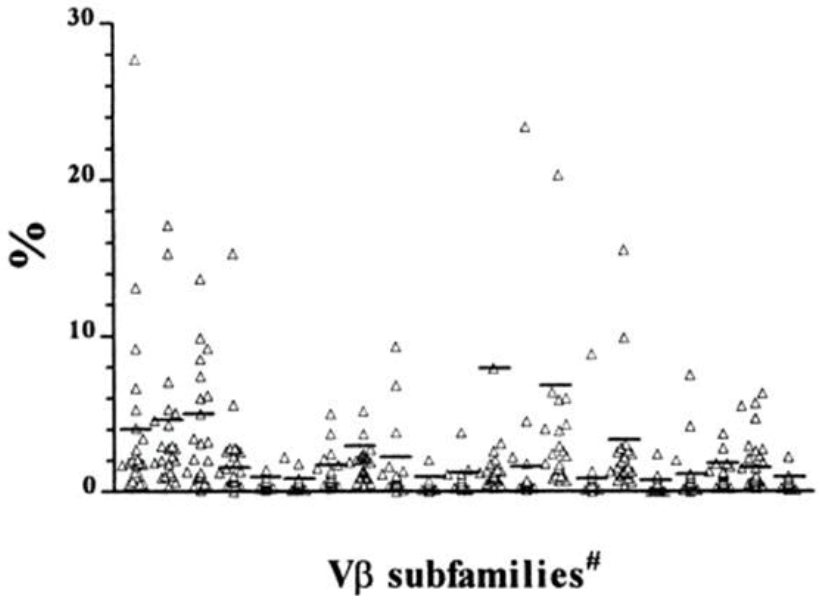


Figure 1.12 Spread of CD8+ T cell expansions in MM
The CD8+ TCR-Vβ+ T cell expansions are shown as a percentage (%) of CD3+ T cells. There was no preferential expansion of any particular Vβ subfamily.

The 21 Vβ families studied (left-right) were 1, 2, 3, 5.1, 5.2, 5.3, 7, 8, 9, 11, 12, 13.1, 13.6, 14, 16, 17, 18, 20, 21.3, 22, and 23. (Adapted from Sze *et al* 2001).

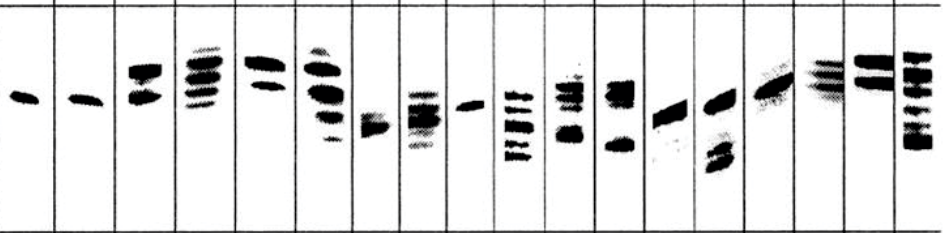
MM Patients:	A				B				C				D		E		F	
TCRVβ	Vβ1		Vβ8		Vβ1		Vβ17		Vβ14		Vβ17		Vβ5.1		Vβ20		Vβ16	
% TCRVβ in total $CD3^+$ cells	12.8		3.6		8.8		6.3		21.4		12.8		17.6		5.4		7.9	
(mean+3S.D. of age-matched controls*)	(6.2)		(8.8)		(6.2)		(9.3)		(8.2)		(9.3)		(7.7)		(4.8)		(2.5)	
% CD8 in TCRVβ	87		68		59		27		95		78		87		78		93	
% $CD57^+$ in $CD8^+$ $TCRVβ^+$ cells	62		66		82		26		81		83		92		53		90	
% $CD57^+$ in $CD8^-$ $TCRVβ^+$ cells	65		27		5		8		21		18		9		3		10	
Expression of CD57 on $TCRVβ^+$ $CD8^+$	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
TCRVβ PCR products run on 6% polyacrylamide gel																		
Direct sequencing on PCR products**	S	S	ND	ND	Fail	Fail	ND	ND	S	Fail	ND	ND	ND	ND	ND	ND	ND	ND
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

Figure 1.13 CDR3 length analysis of T cell clones in MM patients
Expanded T cells were sorted (CD8+ TCRVβ+ CD57+ or CD57-) and PCR products from these cells were used for CDR3 length analysis in order to determine clonality of the population. The resulting bands from the gel electrophoresis have been superimposed into the table for ease of comparison and are not representative of the same position on the gel. 1 or 2 bands indicate oligoclonality and more than 2 bands indicate the expanded population is polyclonal. Expanded Vβ populations are underlined (third row). (Adapted from Sze *et al* 2001).

1.4.5 Specificity of T cell clones

While there is no definitive evidence to prove clonal T cells in MM are induced by chronic tumour antigen stimulation, paraprotein detected in patients with MM indicates there are large amounts of circulating tumour antigens. Perforin and granzyme B expression have been detected in these T cell clones (Halapi *et al* 1997, Mugnaini *et al* 1999, Raitakari *et al* 2000, Sze *et al* 2001), suggesting these cells are cytotoxic. Perforin expression was highly expressed in the MM T

cell clones and correlated with CD57 expression. It is tempting to speculate that these naturally occurring T cell clones may be cytotoxic against the malignant plasma cells, however, chromium release assays have demonstrated minimal or no *in vitro* killing activity (Brown, personal communication). However, this may be attributable to their hypo-responsive nature *in vitro*, which also makes it difficult to determine the exact specificity of these T cell clones. The cells were not specific for the MM neoantigens MAGE A2 or MUC-1 tetramers (Brown, personal communication) or for idiotype, a highly tumour specific antigen in MM (Sze *et al* 2003). However, these T cell clones are not simply T cells induced with aging as they are not present in all patients with MM, where patients are predominantly elderly. They are also not viral-specific T cells as they did not correlate with common viral serology and tetramers specific for an immuno-dominant peptide from CMV lower matrix protein pp65 were negative (Sze *et al* 2003).

1.4.6 Prognostic significance of T cell clones in MM

The presence of T cell clones is associated with an improved survival in patients with MM (Figure 1.14) (Brown *et al* 1997, Brown *et al* 2009). As these cells are associated with a survival advantage, this suggests that they may play a role in anti-tumour immunity, possibly as a cytotoxic T cell specific for MM tumour cells, however this has yet to be proven. CD8+ CD57+ T cells in the BM of MM patients has also been correlated with survival, with lower CD57+ cell numbers in BM predicting lower progression free survival (Mileshkin L *et al* 2007). Collectively, this suggests that T cell clones may have an immunomodulatory role in MM, possibly with an anti-tumour effect, however this remains to be fully understood.

The median survival of MM patients has improved since the introduction of novel agents in the last decade (Kumar *et al* 2008, Dimopoulos *et al* 2011). Thalidomide can also stimulate additional T cell clones, as shown in an earlier clinical trial (Brown *et al* 2009). It is therefore of interest to know if the presence of T cell clones in MM has increased as a result of increased use of thalidomide. It is also currently not known whether IMiDs other than thalidomide and proteasome inhibitors have an effect on the incidence of T cell clones. The

patients from the first study to demonstrate prognostic significance of T cell clones, were treated with multi-agent therapy including prednisone, doxorubicin and bis-chloronitrosourea) plus or minus interferon-alpha (ALSG trial) and if they were over the age of 70, they were treated with melphalan and prednisone (Brown *et al* 1997). Patients in the second study were part of the ALLG-MM6 randomised Phase III trial who received maintenance prednisone plus or minus thalidomide post ASCT (Brown *et al* 2009). A more recent cohort of MM patients should be screened for the presence of T cell clones and in order to determine if the cells are still prognostically significant in the current era of therapy. his will be explored in Chapter 3 of this thesis.

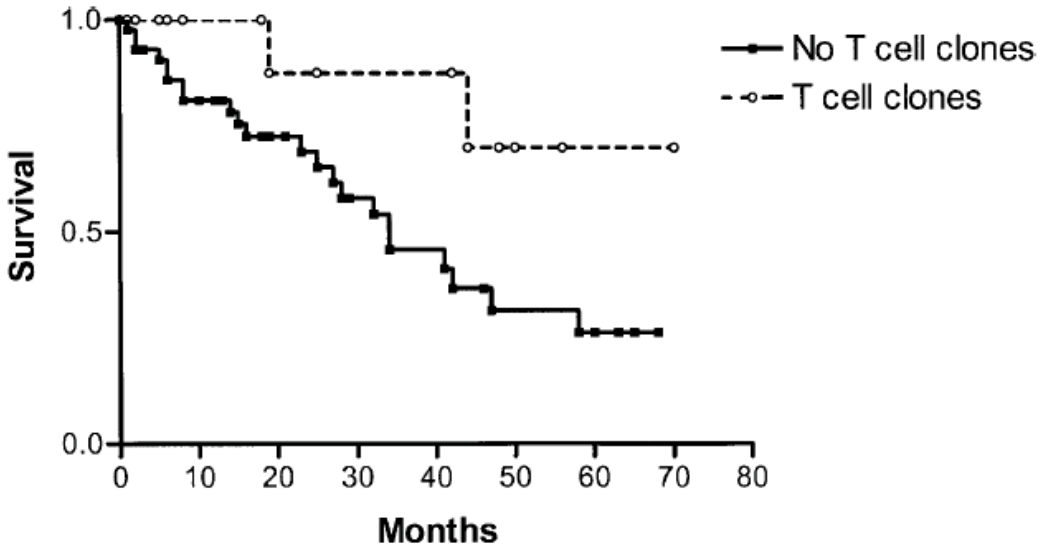


Figure 1.14 Survival curve for MM patients in the presence and absence of T cell clones.
An increased survival is observed for MM patients with detectable T cell clones at diagnosis compared to MM patients without detectable T cell clones. (Adapted from Raitakari *et al* 2003)

1.4.7 T cell clones in MM are hypo-responsive

Further work is needed to clarify the role of T cell clones in MM, however this has been hindered by the unresponsive nature of these cells *in vitro*. T cell clones from WM patients were stimulated with anti-CD3 and anti-CD28 beads for TCR ligation *in vitro* but were non-proliferative, demonstrating that T cell

clones were hypo-responsive to proliferative stimulation (Figure 1.15) (Li *et al* 2010). Investigations in MM patients have revealed a similar phenomenon (Brown and Yang; personal communication), suggesting that the T cell clones in MM are dysfunctional. As the presence of these clones correlates with improved prognosis, it is hypothesised that these cells are specific for antigen/s expressed on myeloma cells and are therefore anti-tumour T cells (Sze *et al* 2003). However, their anti-tumour function has been disabled during interactions with tumour cells and are therefore dysfunctional. The mechanisms that induce clonal T cell dysfunction in MM have not been elucidated and warrant investigation.

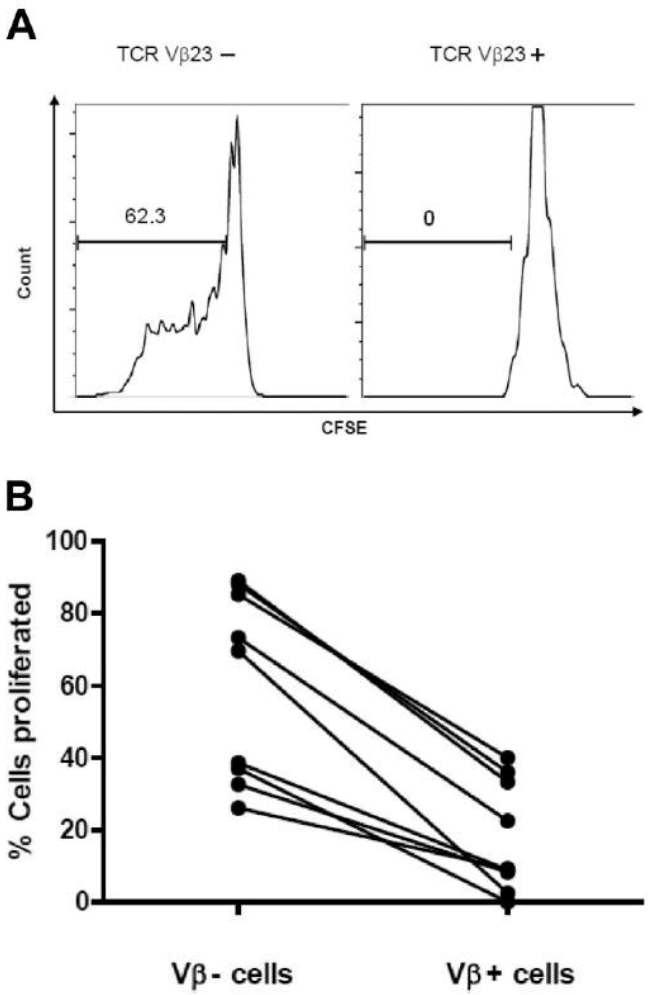


Figure 1.15 Proliferative capacity of T cells in WM patients
Representative CFSE plots from a single WM patient. TCR-Vβ+ and TCR-Vβ- cells were labelled with CFSE proliferation tracking dye. With each division, the daughter cells will inherit half of the CFSE fluorescence the parent cell possessed so

proliferation was measured as a decrease in CFSE fluorescence. (B) The TCR-Vβ+ cells were non-proliferative whereas their TCR-Vβ- counterparts were more proliferative. (Adapted from Li *et al* 2010).

1.4.8 T cell clones are present in long term survivors of MM and are responsive

The preliminary observation that a small group of long term survivors of MM (survived for more than 10 years since original diagnosis of MM) all had T cell clones that were not hypo-responsive (Bryant *et al* 2011) renewed interest in the area of the role of T cell clones in MM. This new finding provided more compelling evidence that T cell clones are beneficial in MM as these cells are present in all the long term survivors that were tested in this small study (Bryant *et al* 2011). Interestingly, the T cell clones from these long term survivors were shown to be proliferative *in vitro* (Bryant *et al* 2011). These findings need to be confirmed with a larger cohort of long term survivors in order to elucidate the relationship between the presence of T cell clones and long term survivorship. Furthermore, the proliferative nature T cell clones in long term survivors needs to be studied to confirm that they are not hypo-responsive and are functionally different. These two aspects will be explored in greater detail in Chapter 3 of this thesis.

As T cell clones from long term survivors are not hypo-responsive, this provides a platform for elucidating mechanisms of induction of hypo-responsiveness. Comparison of the signalling pathways in T cell clones from 10 year survivors to that of hypo-responsive T cell clones from non-10 year survivors may identify the mechanisms responsible for inducing dysfunction in T cell clones. This will provide insight into the search for mechanisms of overcoming the hypo-responsiveness of these cells. Signalling pathways involved in inducing T cell dysfunction will be reviewed in Chapter 4 of this thesis.

T cell dysfunction in cancer is thought to exist in the generation of different dysfunctional T cell states, namely anergy, exhaustion, senescence and stemness (Crespo *et al* 2013). It is crucial to determine the exact type of T cell dysfunction as reversal of dysfunction is phenotype dependent. The lack of

definitive biomarkers to discriminate between these states, and the observation that some of these biomarkers are shared between the different dysfunctional T cell types has led to confusion in the literature when describing dysfunctional T cells found in many malignancies (Schietinger and Greenberg 2014), including MM.

T cell clones in MM lack the co-stimulatory receptor CD28 (Raitakari *et al* 2000, Sze *et al* 2001, Sze *et al* 2003), the necessary second co-stimulation signal during antigen presentation that supports T cell proliferation (Appleman and Boussiotis 2003, Yokosuka and Saito 2009). When T cells are activated in the presence of poor or absent co-stimulation, this leads to T cell anergy. However, T cell clones predominantly express CD57 (Raitakari *et al* 2000, Sze *et al* 2001, Sze *et al* 2003), which has been described as the marker of T cell exhaustion and senescence (Brenchley *et al* 2003). These cells persist for long periods of time with remarkable stability, often observed months and even years after first detection (Mugnaini *et al* 1999, Raitakari *et al* 2000, Sze *et al* 2001), which would suggest that these cells are chronically exposed to tumour antigen stimulation (Raitakari *et al* 2000). Persistent antigenic stimulation leads to an irreversible down regulation of CD28 on T cells (Effros 1997, Vallejo *et al* 1999, Vallejo 2005) and leads to T cell exhaustion. Based on what is purported in the literature, in addition to the features listed above, MM T cell clones display features of three types of dysfunctional T cells. Whether these hypo-responsive T cells are anergic, exhausted or senescent is an important consideration that will need to be investigated as the mechanisms to reverse dysfunction are different for all 3 types. Further investigation of the phenotype of T cell clones is therefore necessary to provide insights into ways to reverse dysfunction and this is explored in Chapter 5. The literature surrounding T cell dysfunction in cancer and studies on the phenotype of dysfunctional clonal T cells in MM will also be discussed in further detail in Chapter 5 of this thesis.

1.5 Background and Aims of the Project

Understanding the mechanism/s that induce dysfunction in T cell clones are required before strategies to restore the function of MM T cell clones can be

devised and implemented. The presence of clonal T cell expansions has been well documented in MM but until recently, functional studies of these cells have been difficult due to their unresponsive nature. The responsive clones identified in long term survivors of MM now offers a unique opportunity to conduct functional assays to investigate T cell clones in MM. New techniques such as phospho-flow cytometry also provides new avenues for functional studies of signalling pathways in these cells.

The overall aim of this work, as presented in this thesis, was to characterise the dysfunction of T cell clones in patients with MM. This characterisation aimed to determine the mechanism(s) by which T cell dysfunction is/are induced in order to provide insights to how this dysfunction can be reversed.

This will be achieved through fulfilment of the following specific experimental aims:

1. To identify and determine the prognostic significance of T cell clones in a new cohort of MM patients, receiving current therapy.

T cell clones have been detected in MM patients in the past, however, it is of interest to determine if these cells maintain a role in enhancing survival in the current era of treatment. A heterogeneous cohort of MM patients (n=103) was screened for clonal T cell expansions by analysis of the TCR-Vβ repertoire by flow cytometry. Chapter 3 demonstrates the incidence of T cell clones in MM patients and validates the prognostic significance of these cells by correlating the presence or absence of clonal T cell expansions to patient survival.

2. To validate preliminary observations that T cell clones from 10 year survivors were proliferative in vitro and not hypo-responsive.

To this end, the proliferation of T cell clones in an expanded cohort of 10 year survivors was investigated in Chapter 3. As a comparator, the proliferation of T cell clones from non-10 year survivors were also analysed and confirmed previous findings that they were hypo-responsive. The results from this chapter demonstrate a functional difference between T cell clones from the two groups

of patients, with the detection of responsive clones from 10 year survivors and hypo-responsive clones in non-10 year survivors.

3. To detect any differences in the cell signalling pathways between hypo-responsive and responsive T cell clones from MM patients, in order to determine the mechanisms responsible for the functional difference observed in proliferation.

This would also provide targets that could be used to reverse dysfunction in T cell clones. Chapter 4, details an extensive study of signalling pathways in these cells using intracellular flow cytometry and phospho-flow cytometry and identified two novel mechanisms of dysfunction in these cells.

4. To understand the nature of clonal T cell dysfunction by investigating the phenotype of the cells.

Classification of T cell clones as anergic, exhausted or senescent, would help determine if the dysfunction in these cells is reversible. This would also help guide experimental means to reverse dysfunction as different methods are required for the three different phenotypes. Chapter 5 presents the results of this investigation and include surface phenotyping of T cell clones by flow cytometry and measurement of telomere length by quantitative polymerase chain reaction and flow-fluorescence *in situ* hybridisation. This analysis demonstrated that T cell clones exhibit the phenotype of telomere-independent senescent cells and have normal telomere lengths. As dysfunction was not related to shortened telomeres, it is potentially reversible. Telomere independent senescence is induced by changes to the molecular programming of the cell so the p16, p21, p38-MAPK signalling pathways that have been shown to induce senescence, were analysed by phospho-flow cytometry. While these pathways were shown not to be responsible, flow cytometric measurement of human telomerase reverse transcriptase that correlates to telomerase activity, was found to be upregulated and may explain the maintenance of normal-for-age telomere lengths in senescent T cell clones.

1.6 Clinical Significance of the Project

A further understanding of the function and role of T cell clones in MM would allow us to determine their suitability for use in immunotherapy. The ultimate aim would be to reverse clonal T cell dysfunction and free the cells from tumour induced suppression. The cells would be expanded *ex vivo* and delivered back to patients, where once functional again, could carry out their potential anti-tumour effects. Adoptive T cell immunotherapy with clonal T cells is attractive as it involves the use of the patient's own cytotoxic T cells to specifically target their own tumour cells (Brown *et al* 1997). Similar expanded T cell clones are found in other cancers and these studies therefore have significance for the understanding and/or potential treatment of many forms of cancer.

Recent advances in the field of immunotherapy has allowed for targeting of the host immune system with CAR T cells that are genetically engineered to recognise specific targets. However, as these are autologous T cells that are introduced as adoptive cell immunotherapy, they too can be suppressed by tumour cells *in vivo* (Kofler *et al* 2011, Motz and Coukos 2013). Immunosuppression is a hallmark of MM and these immunosuppressive mechanisms need to be overcome in order to increase efficacy of adoptive therapies like CAR T cells (Atanackovic *et al* 2016). Therefore, increasing our understanding of tumour-T cell interactions in MM, by exploring and elucidating mechanisms of clonal T cell dysfunction could also improve existing T cell therapies that are being trialled in MM and other types of cancer.

CHAPTER 2
MATERIALS AND METHODS

2.1 General Chemicals and Reagents

The general chemicals and reagents and their manufacturer details are listed in Table 2.1.

Table 2.1 Manufacturer details for chemicals and reagents

Chemicals and Reagents	Manufacturer
4',6-diamidino-2-phenylindole (DAPI)	Invitrogen, USA
BD Cytofix Buffer	BD Biosciences, USA
BD Cytofix/cytoperm permeabilisation solution	BD Biosciences, USA
BD Lyse/fix buffer 5X	BD Biosciences, USA
BD Perm/Wash I Buffer	BD Biosciences, USA
BD Phosflow Perm III buffer	BD Biosciences, USA
BD FACS tube	BD Biosciences, USA
BD FACS tube with cell strainer cap	BD Biosciences, USA
Brefeldin A (BFA)	BD Biosciences, USA
Carboxyfluorescein succinimidyl ester (CFSE)	Invitrogen, USA
Dako Telomere PNA Fish kit	Elitech, Australia
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, USA
Ethanol	Fronine, Australia
Ficoll-Paque™ Plus	GE Healthcare, Sweden
Foetal calf serum (FCS)	Sigma-Aldrich, USA
Hanks Balanced Salt Solution (HBSS)	
Heparin	
IO test Beta Mark TCR Vβ Repertoire Kit	Beckman-Coulter, USA
IL-2, IL-12, IL-15	R&D Systems, USA
IL-21	Invitrogen, USA
Ionomycin	Sigma-Aldrich, USA
Lenalidomide	Celgene Corporation, USA
Leucoperm	AbD Serotec, UK
Maxwell® 16 LEV DNA Purification Kit	Promega, USA
Nalgene Mr Frosty freezing container	Sigma-Aldrich, USA
Penicillin, streptomycin and glutamine	

Perfix-P cell preparation kit	Beckman Coulter, USA
Phosphate buffered saline (PBS)	Clinipure
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, USA
Propidium Iodide (PI)	R&D Systems, USA
Recombinant human interleukin-2 (rh-IL-2)	R&D Systems, USA
RiboPure blood kit	Ambion, Australia
Rotorgene SYBR Green PCR Kit	Qiagen, Germany
Roswell Park Memorial Institute (RPMI)-1640	MP Biomedicals, USA
Sodium pyruvate	
T cell activation and expansion kit	Miltenyi Biotec, Germany
Tissue culture flasks (T25)	BD Biosciences, USA
Tissue culture flasks (T75)	BD Biosciences, USA
Tissue culture plates (24 well)	BD Biosciences, USA
Tissue culture plates (48 well)	BD Biosciences, USA
Tissue culture plates (96 well)	BD Biosciences, USA
Transforming growth factor-beta	R&D Systems, USA
Trypan blue (0.4%)	
Vacutainers	BD Biosciences, USA

2.2 Monoclonal Antibodies

Table 2.2 lists the monoclonal antibodies that were used for flow cytometry experiments to investigate cell surface phenotype and intracellular phospho-flow signalling proteins or for cell sorting. The isotype controls and secondary antibodies are listed in Table 2.3.

Table 2.2 Monoclonal antibodies for flow cytometry

Antibody	Fluorochrome	Clone	Isotype	Manufacturer
Bcl-xL	PE	ab26035	IgG3	Abcam
BTLA	APC	J168-540	Mouse IgG_1 K	BD Biosciences
CCR7	Alexa Fluor® 647	150503	Mouse IgG2a K	BD Biosciences
CD3	APC, APC-H7, PE-Cy7, PerCP-Cy5.5	SK7	Mouse IgG_1 K	BD Biosciences
	V500	UCHT1	Mouse IgG_1 K	BD Biosciences
CD8	APC, V500	RPA-T8	Mouse IgG_1 K	BD Biosciences
	APC-H7, PE-Cy7, PerCP-Cy5.5	SK1	Mouse IgG_1 K	BD Biosciences
CD25	PE-Cy7	2A3	Mouse IgG_1 K	BD Biosciences
CD27	PE	L128	Mouse IgG_1 K	BD Biosciences
	FITC	M-T271	Mouse IgG_1 K	BD Biosciences
CD28	PerCP-Cy5.5	L293	Mouse IgG_1 K	BD Biosciences
CD38	APC	HIT2	Mouse IgG_1 K	BD Biosciences
CD45	V500	HI30	Mouse IgG_1 K	BD Biosciences
	APC-H7	2D1	Mouse IgG_1 K	BD Biosciences
CD45RA	PE-Cy7	H100	Mouse IgG_1 K	BD Biosciences
CD45RO	PE-Cy7	UCHL1	Mouse IgG2a K	BD Biosciences

CD57	APC, FITC, PE	NK-1	Mouse IgM K	BD Biosciences
	eFluor450	TB01	Mouse IgM K	eBioscience
	Pacific Blue	HCD57	Mouse IgM K	Biolegend
CD62L	FITC	MEL-14	Rat IgG2a K	BD Biosciences
CD69	APC	FN50	Mouse IgG_1 K	BD Biosciences
CD95 (Fas)	APC	DX2	Mouse IgG_1 K	BD Biosciences
CD134	Purified	ACT35	Mouse IgG_1 K	BD Biosciences
CD137	Purified	4B4-1	Mouse IgG_1 K	BD Biosciences
CD152 (CTLA-4)	APC	BNI3	Mouse IgG2a K	BD Biosciences
CD178 (Fas-ligand)	PE	NOK-1	Mouse IgG_1 K	Biolegend
CD247 (CD3-ζ)	Alexa Fluor® 647	K25-407.69	Mouse IgG_1 K	BD Biosciences
CD274 (PD-L1)	FITC, PE	MIH1	Mouse IgG_1 K	BD Biosciences
CD279 (PD-1)	PE-Cy7	EH12.1	Mouse IgG_1 K	BD Biosciences
HLA-A2	FITC	BB7.2	Mouse IgG2b K	BD Biosciences
hTERT	Purified	2C4	IgM	Thermo Scientific
IFN-γ	APC	4S.B3	Mouse IgG_1 K	BD Biosciences
KLRG-1	APC	2F1/KLRG1	Syrian Hamster IgG	Biolegend
LAG-3	APC	M89-61	Goat IgG	R&D systems
Perforin	APC	dG9	Mouse IgG2b K	Biolegend
p-Akt	PE	M89-61	Mouse IgG_1 K	BD Biosciences

(pS473)				
p-ERK	Alexa Fluor® 647	20A	Mouse IgG_1 K	BD Biosciences
p-Smad2/ Smad3	PE	O72-670	Mouse IgG_1 K	BD Biosciences
p-Stat3 (pY705)	Alexa Fluor® 647	4/P-STAT3	Mouse IgG2a K	BD Biosciences
p-SHP-2 (pY542)	Alexa Fluor® 647	L99-921	Mouse IgG_1 K	BD Biosciences
p-ZAP-70 (pY292)	Alexa Fluor® 647	J34-602	Mouse IgG_1 K	BD Biosciences
p16INK4	FITC	G175-1239	Mouse IgG_1 K	BD Biosciences
p21 Waf/Cip1	Alexa Fluor® 647	12D1	Rabbit IgG	Cell signalling
p38 MAPK (T180/Y182)	Alexa Fluor®488	28B10	Mouse IgG_1 K	Beckman Coulter
T-bet	PE	4B10	Mouse IgG_1 K	BD Biosciences
Tim-3	PE-Cy7	F38-2E2	Mouse IgG_1 K	Biolegend
Vβ1	FITC	BL37.2	Rat IgG_1	Beckman Coulter
Vβ2	PE	MPB2D5	Mouse IgG_1	Beckman Coulter
Vβ3	FITC	CH92	Mouse IgM	Beckman Coulter
Vβ5.1	FITC, PE	IMMU 57	Mouse IgG2a	Beckman Coulter
Vβ5.2	PE	36213	Mouse IgG1	Beckman Coulter
Vβ5.3	PE	3D11	Mouse IgG1	Beckman Coulter
Vβ7.1	FITC	ZOE	Mouse IgG2a	Beckman

				Coulter
Vβ7.2	FITC	ZIZOU4	Mouse IgG2a	Beckman Coulter
Vβ8	PE	56C5.2	Mouse IgG2a	Beckman Coulter
Vβ9	PE	FIN9	Mouse IgG2a	Beckman Coulter
Vβ11	PE	C21	Mouse IgG2a	Beckman Coulter
Vβ12	PE	VER2.32.1	Mouse IgG2a	Beckman Coulter
Vβ13.1	FITC, PE	IMMU 222	Mouse IgG2b	Beckman Coulter
Vβ13.2	PE	H132	Mouse IgG_1	Beckman Coulter
Vβ13.6	FITC	JU74.3	Mouse IgG_1	Beckman Coulter
Vβ14	FITC	CAS1.1.3	Mouse IgG_1	Beckman Coulter
Vβ16	FITC	TAMAYA 1.2	Mouse IgG_1	Beckman Coulter
Vβ17	PE	E17.5F3.15.13	Mouse IgG_1	Beckman Coulter
Vβ18	PE	BA62.6	Mouse IgG_1	Beckman Coulter
Vβ20	PE	ELL1.4	Mouse IgG2a	Beckman Coulter
Vβ21.3	FITC	IG125	Mouse IgG2a	Beckman Coulter
Vβ22	PE	IMMU 546	Mouse IgG_1	Beckman Coulter
Vβ23	PE	AF23	Mouse IgG_1	Beckman Coulter

Table 2.3 Isotype controls and secondary antibodies for flow cytometry

Isotype antibody	Fluorochrome	Manufacturer
IgG_1 κ	APC, FITC, PE, PerCP-Cy5.5, PE-Cy7	BD Biosciences
IgG2b κ	FITC	BD Biosciences
IgM κ	Purified	BD Biosciences
Goat-anti-mouse-Ig	APC	BD Biosciences

2.3 Instruments, Equipment and Software

The names of equipment and software used in this thesis, and their respective manufacturer details are listed in Table 2.4.

Table 2.4 Details for equipment and software

Instrument/Equipment/Software	Manufacturer
10% carbon dioxide (CO_2) incubator (NuAIRE Purecell 5100)	NuAIRE, USA
37°C water bath (Ratek WB14)	Ratek Instruments Pty. Ltd., Australia
BD FACs Aria II Flow Cytometer	BD Biosciences, USA
BD FACs Canto II Flow Cytometer	BD Biosciences, USA
BD Diva Software	BD Biosciences, USA
Biofuge Primo R centrifuge	Thermo Scientific, USA
Carl Zeiss light microscope	Carl Zeiss, USA
Cell-Dyn Sapphire	Abbott Diagnostics, USA
Cerner Pathnet	Cerner, Australia
Class II Biological Safety Cabinet	Email Westinghouse Pty Ltd., Australia
DAKO Telomere PNA kit/FITC for flow cytometry	Elitech, Australia
FlowJo	TreeStar, Oregon, USA

GraphPad Prism version 5.5	GraphPad, USA
Handi Vac SUC 8100	BMDi TUTA Healthcare Pty Ltd., Australia
Hettich EBA 21 centrifuge	Hettich Lab Technology, Germany
Liquid Nitrogen Tank CRYO 6000	MVE Inc., USA
Maxwell® 16 Instrument	Promega, USA
Neubauer Improved Hemocytometer	American Optical Corporation, USA
Nanodrop 2000 System	Thermo Fisher Scientific, USA
Rotor-Gene Q	Qiagen, Germany
Vortex	Ratek Instruments Pty. Ltd., Australia

2.4 Patient Selection

Ethics clearance was obtained from the institutional Ethics Review Committee for the Royal Prince Alfred Hospital (RPAH) Zone for 1) the use of residual human peripheral blood (PB) and bone marrow (BM) samples collected from MM patients undergoing routine clinical monitoring at RPAH (ethics protocol number: X12-0205) and 2) the collection of 30 mL of PB from MM patients at diagnosis and after treatment (ethics protocol number: X15-0357). Informed consent was obtained in accordance with the Declaration of Helsinki. All patients were de-identified and assigned a unique identification number and patient details could only be accessed with a secure login. Clinical details for these patients were accessible through the institutional software (Pathnet, Cerner) under a secure login. As most of the samples obtained from these patients were residual blood samples after routine investigations, it was unfeasible to utilise the same group of MM patients and healthy controls for all the different assays described in this thesis. The clinical characteristics for the different patient cohorts are listed in Table 2.5.

2.5 Selection of the 10 year MM patient cohort

Clinical data on all MM patients attending the haematology clinic at RPAH was reviewed. Patients who survived for more than 10 years with the diagnosis of MM (diagnostic criteria established by the IMWG- see section 1.1.3), were symptomatic and treated, were selected. A total of twenty patients fulfilled these criteria and formed the 10 year survivor study cohort for this project. Of the twenty patients identified, only 19 patients were available for testing and either cryopreserved or fresh samples were available. The patients were staged according to the current International Staging system (see section 1.1.4.2) and the clinical details are presented in Table 2.6.

Table 2.5 Clinical characteristics of different patient cohorts analysed in this study

	PATIENT COHORTS				
	Incidence of Clones (n=103) (Chapter 3)	**Proliferation and expansion** (n=54) (Chapter 3)	**Signalling pathways** (n=98) (Chapter 4)	**Senescent phenotype and immune checkpoints** (n=68) (Chapter 5)	**Senescent signalling pathways** (n=21) (Chapter 5)
Age, median, range	62, 27-86	65, 42-86	62, 43-85	65, 43-86	68, 50-85
Sex, n (%)					
Male	47 (46)	30 (56)	37 (38)	36 (53)	7 (33)
Female	56 (54)	24 (44)	61 (62)	32 (47)	14 (67)
Months since diagnosis median, range	38, 0-261	82, 0-261	88, 6-247	113, 0-250	103, 25-253
ISS at diagnosis, n (%)					
I	52 (50)	30 (56)	74 (76)	52 (76)	14 (67)

II	15 (15)	8 (15)	11 (11)	8 (12)	3 (21)
III	18 (17)	3 (6)	1 (1)	2 (3)	1 (5)
unknown	18 (17)	12 (22)	12 (12)	5 (7)	3 (14)
ISS at time of testing, n (%)					
I	42 (41)	20 (37)	24 (24)	16 (24)	3 (14)
II	14 (14)	16 (30)	12 (12)	15 (22)	4 (19)
III	9 (9)	3 (6)	8 (8)	2 (3)	0 (0)
unknown	38 (37)	14 (26)	53 (54)	34 (50)	14 (67)
Previous exposure to treatment, n (%)					
Conventional therapy	31 (31)	17 (31)	43 (44)	26 (38)	4 (19)
IMiDs	68 (66)	29 (54)	79 (81)	49 (72)	16 (76)
Proteasome inhibitors	45 (44)	14 (26)	53 (54)	27 (40)	7 (33)
Autologous	55 (53)	23 (43)	63 (64)	34 (50)	9 (43)
Allogeneic	2 (2)	4 (7)	9 (9)	2 (3)	0 (0)
Treatment at time of testing, n (%)					
Conventional therapy	4 (4)	1 (2)	5 (5)	1 (1)	1 (5)
IMiDs	35 (34)	14 (26)	48 (49)	38 (56)	12 (57)

Proteasome inhibitors	16 (16)	4 (7)	18 (18)	10 (15)	4 (19)
Not currently on treatment	34 (33)	17 (31)	25 (26)	13 (19)	2 (10)
Never treated	9 (9)	5 (9)	4 (4)	5 (7)	2 (10)
Disease status at time of testing, n (%)					
CR/VGPR	38 (37)	18 (33)	33 (34)	26 (38)	6 (29)
PR	12 (12)	3 (6)	18 (18)	11 (16)	5 (24)
Stable Disease	26 (25)	17 (31)	26 (27)	17 (25)	9 (43)
Progressive disease/Diagnosis	21 (20)	5 (9)	20 (20)	12 (18)	1 (5)
Smouldering	4 (4)	3 (6)	1 (1)	1 (1)	0 (0)
Unknown	0 (0)	8 (15)	0 (0)	0 (0)	0 (0)

Abbreviations: CR: complete remission; IMiDs: immunomodulatory drugs; ISS: International Staging System; n: number; PR: partial response; VGPR: very good partial response. Published in Suen *et al* 2016.

Table 2.6 Clinical characteristics of 10 year survivor cohort

R:renal disease; A: anaemia; B: bone lesions; Auto: autologous stem cell transplant; CTD= Cyclophosphamide, Thalidomide and Dexamethasone; MP= Melphalan, Prednisone; Cyclo= Cyclophosphamide; Rev: Revlimid; Dex=Dexamethasone; PCAB: Prednisone, Cyclophosphamide; Adriamycin; BCNU; Thal: Thalidomide; Allo: allogeneic stem cell transplant.

Patient ID	Sex/ age	CRAB features	B2M (mg/L)	Albumin (g/L)	M Protein Isotype	Max M protein (g/L)	Survival (years)	Therapy
10MM-1	F48	RAB	2.7	32	IgGK	50.0	16	Auto, Velcade
10MM-2	M59	B	1.6	48	IgGK	23.0	17	CTD
10MM-3	M71	B	2.7	40	IgG L	26.7	10	MP
10MM-4	F73	A	1.2	47	IgA L	NA	21	Cyclo, Pred
10MM-5	M52	B	2.0	43	IgG L	15.6	12	Auto, IFN
10MM-6	M32	BR	1.6	44	IgG K	33.0	17	Auto
10MM-7	M59	B	2.7	33	IgGK	52.7	10	Rev, Dex
10MM-8	F47	BA	1.8	37	IgGK	21.2	11	Auto, Allo
10MM-9	F52	B	1.7	41	IgG L	14.4	11	PCAB
10MM-10	M62	B	1.5	33	IgG L	34.4	15	Cyclo, Pred
10MM-11	M61	RA	1.9	29	IgA K	NA	11	Auto
10MM-12	M49	0	1.9	48	IgG K	38.7	16	Auto , CTD
10MM-13	F49	B	1.6	44	IgG K	17.5	11	Auto + IFN
10MM-14	F49	A	1.6	40	IgG K	61.0	12	Auto
10MM-15	F64	0	1.2	44	IgG K	32.9	12	Auto
10MM-16	F66	B	2.4	38	IgA K	32.0	10	Cyclo, Pred
10MM17	F67	B	3.7	42	IgGK	21.4	15	MP
10MM-18	F57	B	1.4	44	LLC	131	16	Auto , Thal, IFN, Rev,Dex
10MM-19	M28	RAB	NA	NA	IgGK	15.6	23	Allo
10MM-20	F35	RAB	NA	NA	IgGK	31.6	20	Auto + IFN

2.6 Collection of patient samples

PB samples were collected by the registered nurses working at the Haematology Clinic at RPAH. Blood was collected into BD Vacutainer 4 mL blood tubes coated with EDTA anticoagulant or 9 mL blood tubes coated with lithium heparin. For each blood sample, a full blood count was performed using the Cell-Dyn Sapphire automated haematology analyser and a manual differential count was conducted by the hospital scientists in the haematology laboratory. BM samples were collected by haematology registrars into tubes containing Hanks Balanced Salt Solution (HBSS) containing heparin and foetal calf serum (FCS).

2.7 General cell techniques

2.7.1 Isolation of PBMCs using Ficoll-Paque™

Density gradient Ficoll-Paque™ was used to separate whole blood into its various components for the isolation of peripheral blood mononuclear cells (PBMC). During centrifugation, the cells migrate through the density gradient allowing for the formation of different layers containing different blood cell types. After centrifugation, the PBMCs are located within the buffy coat and can be collected for further downstream experiments or analyses.

Whole blood was firstly diluted with 1X phosphate buffered saline (PBS) in a 10 mL round bottom tube. After thorough mixing with a sterile plastic transfer pipette, the blood was gently overlaid on the Ficoll medium at a ratio of 4 mL blood to 3 mL Ficoll medium in a fresh tube. This was then centrifuged for 15 minutes (min) at 300 g using a Biofuge Primo R centrifuge. The buffy coat containing PBMCs was harvested into a fresh tube and washed in 10 mL PBS through centrifugation for 10 min at 300g. Cells were then resuspended in PBS or media for further processing.

2.7.2 Cell washes

For all cell washes, unless otherwise stated, 2 mL of PBS was added to the cells and centrifuged for 5 min at 300 g (relative centrifugal force) in a Hettich EBA 21 centrifuge. The supernatant was discarded through decantation or aspiration with a Handi Vac SUC 8100. For cell washes in phospho-flow assays, 3 mL PBS was added to the cells and centrifuged for 6 min at 600g. A harder centrifugation is required as the cells are buoyant as a result of fixation and permeabilisation.

2.7.3 Cell counting

After PBMCs were isolated by Ficoll density gradient centrifugation, they were resuspended in a known volume of media or buffer for cell counting using a Neubauer Improved Haemocytometer and a Carl Zeiss light microscope. Cells (10 µL) were diluted 1:2 in Trypan Blue (0.4%) and 10 µL sample was loaded into the chamber of a haemocytometer. Cells falling within the 9 square central grid were counted (N). The number of cells was determined using the following formula:

$$\text{Total number of cells/mL} = N \times 10^4 \times \text{dilution factor}$$

2.7.4 Preparation of cell culture media

RPMI-1640 was supplemented with 2 mM glutamine, 1X penicillin/streptomycin, 10% FCS and 5 mL sodium pyruvate. For T cell expansions, 10% AB serum was used in place of FCS.

2.7.5 Sterility

For cell sorting, cell culture and RNA extraction and any other samples where sterility was a requirement, all associated procedures were conducted in a class II biological safety cabinet. The cabinet was wiped with ethanol and ultraviolet (UV) irradiated for 20 minutes after each usage, to maintain the sterile nature. Sterile complete media for cell culture was also prepared using the biological safety cabinet.

2.7.6 Preparation of frozen samples

PBMC and bone marrow mononuclear cells (BMMC) from MM patients were historically collected and cryopreserved in liquid nitrogen at RPA for several

decades. For cryopreservation, up to $5x10^6$ cells were resuspended in 1 mL RPMI-10 containing 10% dimethyl sulfoxide (DMSO), a cryoprotectant that preserves cell integrity. The cryovials were placed in Nalgene Mr Frosty™ freezing containers, which allow cells to cool and freeze at a rate of -1°C/min. The Mr Frosty containers were placed in a -80°C freezer for at least 24 hours and then the cryovials were transferred to liquid nitrogen.

2.7.7 Thawing of frozen PBMCs

Cryopreserved PBMC or BMMC (frozen in liquid nitrogen) were rapidly thawed in a 37°C water bath for 2 min. Cells were then transferred to a pre-warmed tube of RPMI-10. Cells were centrifuged for 5 min at 300 g to remove residual cryoprotectants such as DMSO added at the time of freezing and the supernatant was discarded. Cells were resuspended in an appropriate volume of media or PBS for subsequent analyses.

2.7.8 Cell lines

Cryopreserved Jurkat cell line ($5x10^6$ cells) and U266 cell line ($5x10^6$ cells) were removed from liquid nitrogen (CRYO 6000) and thawed in a water bath for 1 min. Cryopreserved cells were washed in pre-warmed complete media (6 mL) for 5 min at 300 g. After centrifugation, the supernatant was decanted. Fresh pre-warmed complete media (10 mL) was added to the cell pellet. After mixing well, cells were transferred to a T25 flask for culture in a 37°C incubator with 5% carbon dioxide (CO_2). Cells were checked macroscopically and microscopically every day for cell growth. At confluence, cells were resuspended in a new T25 flask at a concentration of $1x10^5$/mL. If confluence was not achieved by the fourth day, fresh media was supplemented to allow for further growth.

CEM cell line was kindly provided by Dr. Tatjana Kilo from the Children's Hospital at Westmead. The cells were cultured in a T75 flask in a 37°C incubator with 5% carbon dioxide (CO_2). Cells were checked macroscopically and microscopically every day for cell growth. At confluence, cells were resuspended in a new flask at a concentration of $2x10^5$/mL. If confluence was not achieved by the second day, fresh media was supplemented to allow for further growth.

2.8 Cell staining protocols for flow cytometry

2.8.1 Staining of Surface Antigens

For staining of cells with antibodies recognising surface antigens, the appropriate antibody volume was added to up to $1x10^6$ cells and PBS was added for a total staining volume of 100 μL. Cells were stained at room temperature (RT) for 15 min or on ice for 30 min. Following incubation, cells were washed and the supernatant was decanted.

2.8.2 Detection of intracellular antigens and cytokines

The detection of intracellular antigens and cytokines requires the cells to be fixed and then permeabilised so antibodies can enter into the cytoplasm or nucleus of the cell. There are numerous methods to fix and permeabilise cells and the method required is dependent on the type and location of the target intracellular antigen. For antigens of cytoplasmic location, a relatively mild permeabilisation buffer will suffice, however, for antigens of nuclear location, a harsher permeabilisation buffer is required to ensure sufficient permeabilisation for the antibody to enter into the nucleus. These methods will be discussed further in Chapter 4.

2.9 Flow cytometry protocols

2.9.1 Flow cytometry acquisition

Cells were analysed on a BD FACs Canto II flow cytometer or a BD FACs ARIA II flow cytometer. In general, for cell surface staining, 20,000 lymphocyte events were collected and for intracellular staining, 50,000 lymphocyte events were collected. Doublet cell exclusion was always used to remove doublet cells that interferes with analysis. These cells may arise from non-specific binding of two cell types together, and therefore have twice the height and width of a single cell. These doublet cells are easily located on a height vs. width plot of forward scatter and side scatter and are excluded from analysis (see Figure 2.1). All data were reanalysed using the FlowJo software.

2.9.2 Determination of cell viability by flow cytometry

DAPI (4'6-diamidino-2-phenylindole, dihyrdrochloride) dye is an intercalating dye that can enter through the compromised cell membranes of dead cells, whereupon binding to AT regions of DNA, emits a blue fluorescence that can be detected by the violet laser (405nm) on the BD FACS Canto II or UV laser on the BD FACS Aria II flow cytometers. This dye was added immediately prior to analysis on the flow cytometer and viable cells were determined by exclusion of this dye.

Propidium iodide (PI) is another intercalating dye that can be used to test cell viability. This dye was used instead of DAPI when the antibody panel included an antibody conjugated to a violet dye, which would interfere with the DAPI fluorescence signal.

2.9.3 Detection of clonal T cell expansions in patients

The phenotype of clonal CD8+ T cells is CD3+CD8+CD57+TCR-Vβ+. Therefore, CD3, CD8, CD57 and the specific TCR-Vβ subfamily antibody was added for the identification of the clonal CD8+ T cell population. Different fluorochrome combinations were used for these 4 antibodies and the fluorochrome choice was dependent on the availability of fluorochromes for the target antigens in each assay. The flow cytometry antibody staining panels for the different flow cytometry assays will be listed in the relevant sections.

A representative example for the detection of clonal CD8+ T cells in a MM patient is shown in Figure 2.2. Lymphocytes were firstly gated according to low forward and side scatter (FSC-A and SSC-A respectively). Doublet discrimination was then used to exclude doublet cells (not shown) and CD8+ T cells were identified as double positive for CD3 and CD8. The clonal T cells were identified as Vβ+ CD57+. This gating strategy was used in every instance to identify T cell clones. Non-clonal T cells were used as internal controls and these are cells that do not express the TCR-Vβ family but are CD57+ (Vβ-CD57+).

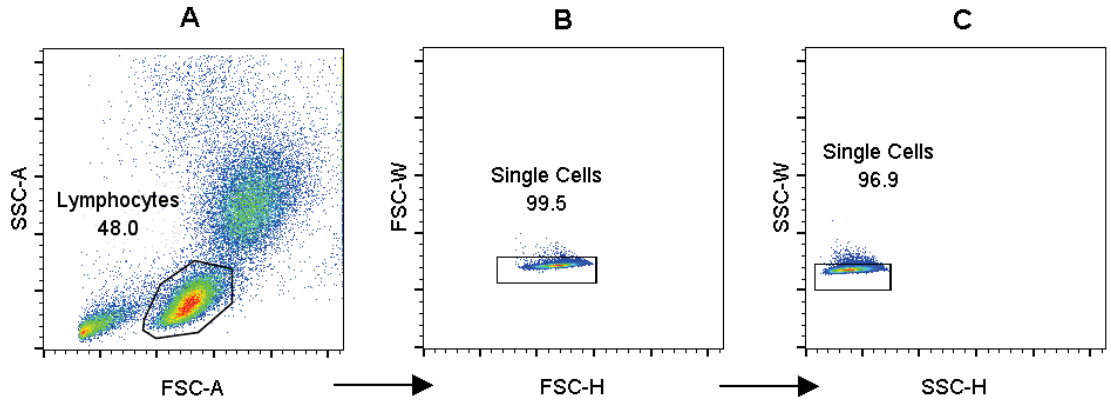


Figure 2.1 Doublet discrimination

(A) Lymphocytes were gated according to FSC and SSC properties. Doublet cells were then excluded by examining the height and width of the cells on the FSC and SSC parameters. The height and width of doublet cells or cells that adhere together will be double the size of normal cells and will be situated in a different location on the scatter plots. Singlet cells were selected prior to further gating.

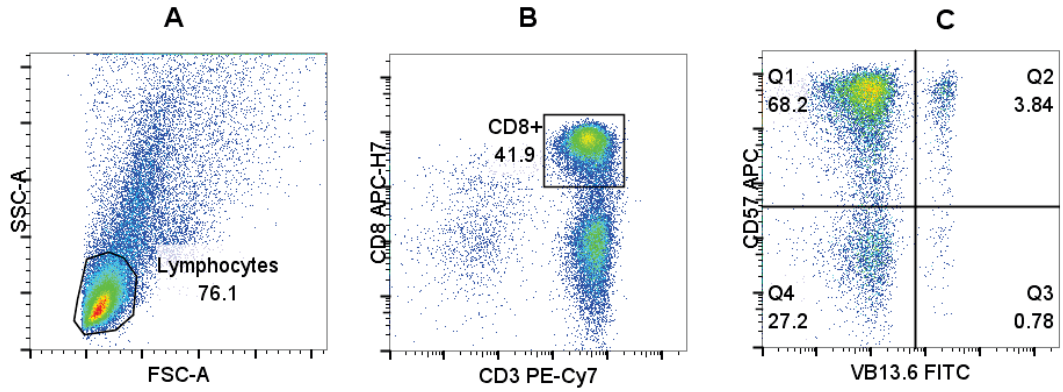


Figure 2.2 Representative gating strategy for identification of clonal T cell population in a MM patient

PBMC were harvested from a MM patient through density centrifugation and stained with antibodies to identify clonal T cells. **(A)** Lymphocytes were identified according to forward (FSC-A) and side scatter (SSC-A) properties and doublets were excluded (not shown). **(B)** CD3+ CD8+ lymphocytes were selected and the **(C)** clonal T cells were identified as TCR-Vβ+CD57+ (Q2 quadrant) and non-clonal T cells were identified as TCR-Vβ-CD57+ (Q1 quadrant).

2.9.4 Cell sorting for purification of T cell clones

For cell sorting, all samples were prepared under sterile conditions. PBMC were firstly obtained from whole blood using Ficoll density gradient centrifugation (see section 2.7.1) before staining with antibodies to identify the clonal T cell subset (CD3+CD8+CD57+Vβ+) for 30 min on ice. The non-clonal T cell subset (CD3+CD8+CD57+Vβ-) was also sorted for use as an internal control. Cells were washed and then resuspended in 2 mL PBS and filtered through a 5 mL polystyrene tube with a BD tube with cell strainer cap to remove any clumps which may interfere with cell sorting. The BD FACS Aria II flow cytometer was used to sort clonal and non-clonal T cells. Cell purity was determined after each sort and was routinely greater than 95%. A representative flow sorting gating strategy and a post sort cell purity analysis is shown in Figure 2.3.

2.10 Statistical Analysis

Statistical analyses were performed on the results using GraphPad Prism 6.0. The unpaired Student t test was used to determine significant differences between the means of two different cohorts. This test was used when the two subsets had equal variances, as measured by the F-test. If there were unequal variances, then a non-parametric Mann-Whitney U test was used to analyse the two groups. To analyse different cell subsets from paired cohorts, a paired t-test was used if the data was normally distributed and if not, a Wilcoxon matched-pairs signed rank test was used. The Pearson's chi-squared test was used to analyse categorical data. A p value less than or equal to 0.05 indicated that the differences were statistically significant.

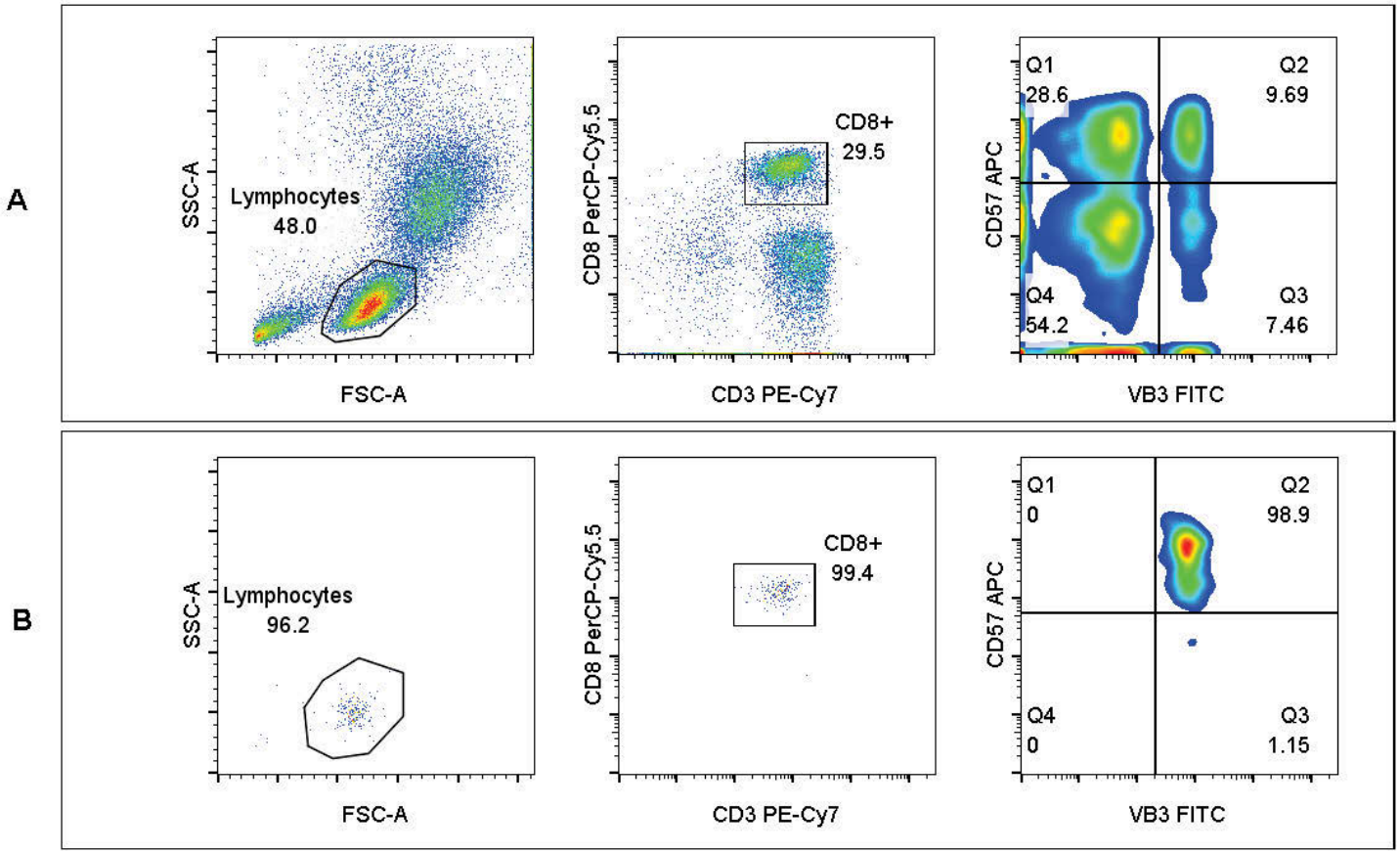


Figure 2.3 Sorting of T cell clones
PBMC were harvested from a MM patient using Ficoll density centrifugation and stained with antibodies to identify clonal T cells as described in section 2.9.3. **(A)** T cell clones (Q2 quadrant) were selected for FACS sorting. **(B)** After sorting, T cell clones were 98.9% pure.

CHAPTER 3 DETECTION AND CHARACTERISATION OF CLONAL T CELL EXPANSIONS

3.1 Introduction

3.1.1 Presence of clonal T cell expansions in MM

Clonal CD8+ T cell expansions have been identified in a number of MM patient cohorts and these are listed in Table 3.1. T cell clones were first detected in 32% of patients with MM in the early 90s using Southern blotting (Brown *et al* 1997). T cell clones were then identified by flow cytometry, and found to occur in 54% of MM patients, in an era prior to the introduction of novel therapies (Joshua *et al* 2003). Several smaller studies have also detected T cell clones in patients with MM (Raitakari *et al* 2000, Sze *et al* 2001). In the Australian MM6 trial that investigated the use of thalidomide as maintenance therapy, T cell clones were detected in 48% of patients prior to transplant. After transplant, for patients who received control maintenance (prednisolone), the T cell clone incidence remained similar, at 47%, however, in the patient group that received thalidomide maintenance, clone incidence increased to 76% (Brown *et al* 2009).

Thalidomide treatment was therefore able to stimulate additional T cell clones. As treatment with novel drugs like thalidomide can induce T cell clones, it is of interest to determine whether current MM therapies including other IMiDs or proteasome inhibitors have an impact on the presence of T cell clones. Therefore, a more recent cohort of MM patients should be investigated to determine the incidence and significance of T cell clones in the current era of therapy.

3.1.2 Clinical relevance of hypo-responsive MM T cell clones

The presence of T cell clones in MM was determined to be an independent prognostic factor by multi-variate analysis (Brown *et al* 2009). As they are related to improved survival, T cell clones have potential anti-tumour roles and are likely to be MM-specific T cells. They also exhibit an effector memory T cell phenotype (CD3+CD8+CD28-CD57+) (Sze *et al* 2001), which supports this notion. However, determining the exact specificity of these T cells has been hindered by their hypo-responsive nature (Li *et al* 2010). The cells have previously failed to respond in functional assays studying proliferation and cytotoxicity *in vitro* (Brown and Yang, personal communication). Staining of T

cell clones with tetramers specific for idiotype (patient specific paraprotein antigens) (Sze *et al* 2001) and cancer germline-specific (MAGE and MUC-1) tetramers (Brown and Yang, personal communication) has rarely resulted in tetramer-positive cells of more than 0.1% of the T cell population, indicating that these T cell clones are not specific for these antigens.

The inability of these cells to proliferate *in vitro* despite powerful stimulation with anti-CD3 and CD28 beads has led to previous suggestions that they are anergic, much like the T cell clones found in WM patients (Li *et al* 2010). As they persist for long periods of time, it was also thought that the cells arose in response to chronic antigenic stimulation. Chronic antigen stimulation of T cells results in the build-up of late differentiated, highly antigen-specific, oligoclonal T cells which are often located within the $CD8^+$ T cell compartment (Strioga *et al* 2011). A study following the Vβ repertoire of a MM patient over 18 months showed remarkable stability and persistence of the expanded Vβ population over this time (Raitakari *et al* 2000), suggesting that T cells were under chronic stimulation by an antigen, perhaps a tumour antigen. The clonal nature of the cells is also highly suggestive of an antigen-specific event that, once initiated, resulted in sustained and continual proliferation (Morley *et al* 1995). This would lead to an eventual loss of CD28 and the cells would accumulate as highly antigen experienced, terminally differentiated CD57+ T cells. CD8+ T cells recognise antigens presented by APC in the context of MHC class I (Parkin and Cohen 2001) and these are most often tumour antigens or viral antigens. Some of these antigens persist for long periods of time and this also infers that chronic antigen stimulation may be responsible for persistence of clonal T cell expansions (Mugnaini *et al* 1999). Chronic viral antigen stimulation is most often thought to be responsible for inducing clonal CD8+ T cell expansions as individuals with such expansions frequently test positive for persistent viruses such as CMV (Khan *et al* 2002, Focosi *et al* 2010). To rule out viral antigens as stimulants of CD8+ T cell expansions in MM, common viral serology and tetramers specific for an immuno-dominant peptide from CMV lower matrix protein pp65 were performed (Sze *et al* 2003). Less than 10% of the number of cells within the expanded ‘late’ differentiated CD8+ CD28- CD27- compartment, where CMV-specific CD8+ T cells are usually detected (Appay *et al* 2002), were

positive for tetramer staining (Sze *et al* 2003). Therefore, it is unlikely that the CD8+ clonal T cell expansions in MM patients are caused by persistent viral infections like CMV. Furthermore, there has not been a correlation between viral serology and the presence of CD8+ clonal T cell expansions in MM patients.

Cytotoxic T cells are the predominant effector cell involved in cancer immune destruction. The T cell clones in MM patients can constitute up to 50% of all blood lymphocytes and are hypo-responsive *in vitro* (Li *et al* 2010) but, despite this, their presence, is related to better survival (Brown *et al* 1997, Sze *et al* 2001, Joshua *et al* 2003, Raitakari *et al* 2003, Brown *et al* 2009). Overcoming the hypo-responsiveness of these cells, regardless of their specificity, could unlock their killing potential as tumour induced cytotoxic T cells. This has implications for a novel cell therapy based on the restoration of the host's immune response. The proliferation of T cell clones that are identified in an updated cohort of MM patients should also be investigated to determine if they behave in the same manner as the T cell clones from historical MM cohorts.

Table 3.1 Incidence of Vβ expansion in different historical multiple myeloma cohorts at our institution

Dates	Cohort	Incidence of T cell clones (%)	N	Detection method	Reference
1994-1997	Pre-IMiDs	32	119	SB	(Brown *et al* 1997)
1998-2004	Pre-IMiDs	54	144	FC	(Joshua *et al* 2003)
1999	Pre-IMiDs	79	38	FC	(Raitakari *et al* 2000)
2001	Pre-IMiDs	63	16	FC	(Sze *et al* 2001)
2002-2005	MM6 Pre-transplant	48	104	FC	(Brown *et al* 2009)
2003-2005	MM6 Thal maintenance	76	61	FC	
2003-2005	MM6 control maintenance	47	57	FC	

FC: flow cytometry; IMiDs: immunomodulatory drugs; SB: Southern blotting; Thal: thalidomide

3.1.3 Long term survivors of MM

Prior to the introduction of IMiDs and proteasome inhibitors, less than 5% of MM patients survived for more than 10 years (Alexanian *et al* 2012). Factors that contributed to longer survival included presentation with low or intermediate risk disease and response to therapy (Alexanian 1985, Antipova and Andreeva 1985, Tsuchiya *et al* 1994). It has been determined that a minimum of 10 years is required to demonstrate whether a patient has attained progression-free survival (Barlogie *et al* 2014). Despite the introduction of novel therapies, MM is still considered an incurable disease and patients eventually relapse. It is believed that disease control is attainable in MM, although many have challenged this statement. Allogeneic transplantation (Gahrton *et al* 1991) and donor lymphocyte infusions (Salama *et al* 2000), which provide patients with functional and competent immune cells, supports the potential for immune-mediated control in MM. It is likely that long-term survivors of MM have better immune competence and this may allow the attainment of better disease control, which allows them to survive for longer. The ability of the host to mount an anti-tumour response in MM is supported through the presence of pre-malignant specific effector T cells (SOX-2 specific T cells), in patients with MGUS (Spisek *et al* 2007), although these cells are lost when patients progress to MM. SOX-2 specific T cells have been detected in patients with MGUS (Dhodapkar *et al* 2003). MGUS patients with anti-SOX-2 T cells had less changes in the level of involved immunoglobulin over time, and were less likely to progress when compared to patients who did not have these cells (Spisek *et al* 2007). Furthermore, some MM patients are found in a ‘plateau phase’, where there is a detectable tumour burden but the patient remains stable and does not relapse, suggesting an element of host immune control (Joshua *et al* 1994).

T cell clones have been detected in long term survivors of MM (patients who survived for more than 10 years since original diagnosis of MM), which provides more evidence that T cell clones are beneficial in MM (Bryant *et al* 2011). Proliferative studies were undertaken in a small subset of these patients and the T cell clones were found to be proliferative upon TCR ligation, suggesting that T cell clones in long term survivors are not hypo-responsive and possibly less dysfunctional. (Bryant *et al* 2011). The clonal findings and proliferation status of

the cells need to be validated in a larger cohort of long term survivors of MM and compared to non-10 year survivors. Studying the immune profile of these patients may also reveal the key to long term survival.

3.1.4 Summary

The work presented in this chapter aimed to increase our understanding of clonal T cells in MM. The incidence and significance of T cell clones was firstly studied in a new cohort of MM patients attending RPAH and receiving current therapy. A group of 10 year survivors were also screened for the presence of T cell clones. The proliferation status of T cell clones was studied in both 10 year survivors and non-10 year survivors to determine if they were hypo-responsive or responsive. Additionally, a range of cytokines and immune modulators were utilised to determine if T cell clones could be re-stimulated to proliferate.

3.2 Materials and Methods

3.2.1 Detection of clonal T cells expansions in MM patients by flow cytometry

Using the IO test Beta Mark TCR-Vβ kit, 24 specific Vβ subfamilies can be enumerated in patients, representing approximately 70% of the full TCR repertoire in humans. The kit consists of 8 antibody cocktails, each containing three antibodies bound to three different fluorochromes, FITC, PE or a combination of FITC and PE. This allows for the detection of three different TCR-Vβ subfamilies per cocktail and therefore 24 different TCR-Vβ subfamilies per assay. The TCR-Vβ families detectable by the 8 antibody cocktails are listed in Table 3.2. The use of three fluorochromes in each antibody cocktail is possible as the Vβ subfamilies are mutually exclusive.

A normal range for these 24 Vβ families was determined locally at RPAH from 40 age-matched normal controls. Based on the data collected, a clonal T cell expansion was considered to be present when the percentage of T cells expressing a certain Vβ subfamily exceeded the upper limit of the age-matched normal control range plus 3 standard deviations. These values are also listed in Table 3.2.

To screen the TCR-Vβ repertoire, PBMC were obtained from the PB of MM patients using Ficoll-Paque density gradient centrifugation and cells were resuspended in PBS. Cells (50 µL) were added to 8 FACS test tubes labelled A-H. Each tube was stained with anti-CD3 APC and anti-CD8 PerCP-Cy5.5 to identify the T cell populations. Tubes A-H were stained with the respective Vβ antibody cocktails (labelled A-H, 5 µL). Cells were incubated at RT for 15 min and then washed to remove any unbound antibodies. Cells were resuspended in PBS for flow cytometric analysis. The proportion of CD8+ T cells expressing each Vβ family was determined and compared to the normal range to determine if a clonal T cell population was present.

3.2.2 Collection of patient information

All patient information was collected after secure login to Cerner Pathnet after appropriate training by a medical professional according to institutional requirements. Clinical information including date of diagnosis and date of death were collected for the MM patients that were screened for T cell expansions. Using this information, the survival of MM patients with and without clonal T cell expansions was estimated using Kaplan Meier curves. To correlate the presence of T cell clones to clinical characteristics, details on patient age, sex, ISS staging, disease status and treatment were also collected.

Table 3.2 TCR-Vβ families detected by the 8 antibody cocktails from the IO test BetaMark TCR-Vβ kit

Vβ Ab Cocktail	Flow scattergram Location	TCR-Vβ Subfamily	Upper limit of normal range (%CD8 T cells)
A	FITC+	3	13.83
	FITC/PE+	7.1	7.08
	PE+	5.3	2.09
B	FITC+	16	2.15
	FITC/PE+	17	10.97
	PE+	9	8.03
C	FITC+	20	6.81
	FITC/PE+	5.1	8.08
	PE+	18	1.8
D	FITC+	8	10.24
	FITC/PE+	13.6	3.7
	PE+	13.1	6.15
E	FITC+	12	2.97
	FITC/PE+	2	11.1
	PE+	5.2	2.83
F	FITC+	21.3	5.18
	FITC/PE+	1	9.1
	PE+	23	4.22
G	FITC+	14	13.39
	FITC/PE+	22	6.74
	PE+	11	2.3
H	FITC+	7.2	9.46
	FITC/PE+	4	4.3
	PE+	13.2	8.74

3.2.3 Measurement of T cell proliferation using CFSE tracking dye

Use of the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) is a well established and recognised method to measure lymphocyte proliferation (Lyons and Parish 1994, Quah *et al* 2007). CFSE is a non-fluorescent dye due to the presence of two acetate groups. When cells are incubated with CFSE, the dye passively diffuses into the cell where intracellular esterases remove the acetate groups resulting in highly fluorescent CFSE which remains within the cell. As the cell divides, the two daughter cells will each possess half the amount of CFSE dye as the parent cell (Quah *et al* 2007, BJ and CR 2010). Therefore, it is possible to track cells as they divide and observe the number of cell divisions that have occurred by detecting dilutions of CFSE fluorescence by flow cytometry.

To measure clonal T cell proliferation, PBMC were obtained from 10 year and non-10 year survivors using Ficoll density gradient centrifugation and resuspended in media for counting. $1x10^6$ cells were stained with CFSE (5 μm) and cultured with or without stimulatory beads at a 1:1 ratio with IL-2 (20 international units (U)/mL) in 96 well U-bottom plates for 4 days. The stimulatory beads are anti-biotin MACSiBead particles loaded with biotinylated antibodies against human CD2, CD3 and CD28 (hereafter anti-CD3/28 beads). After 4 days, cells were harvested, stained with antibodies for the identification of clonal T cells (anti-CD3-PE-Cy7, anti-CD8 PerCP-Cy5.5, anti-CD57 APC and anti-TCR-Vβ-PE/FITC). Cells were stained for 30 min on ice, washed and analysed on the flow cytometer. Cells cultured without anti-CD3/28 beads provided the baseline proliferation levels and were used to set the gate for undivided cells (highest level of CFSE fluorescence; Figure 3.1). Cells that divided had decreasing levels of CFSE fluorescence and were observed to the left of this undivided cell gate. Cells were expressed as % proliferated using the undivided cells as the negative gate.

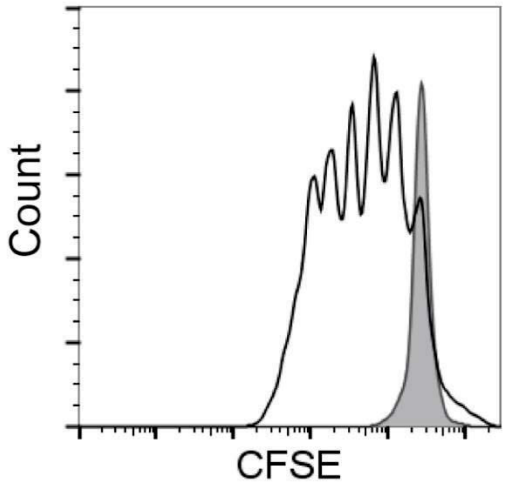


Figure 3.1 Measurement of T cell proliferation by CFSE
CD8+ T cells were labelled with CFSE proliferation tracking dye and cultured with (unshaded peak) or without (shaded peak) TCR stimulatory beads for 4 days. As the cells divide, the daughter cells inherit half the amount of CFSE possessed by the parent cell, seen as a left shift in fluorescence. Each peak corresponds to one cell division. In this example, the CD8+ T cells have divided 5 times following stimulation with TCR stimulatory beads.

3.2.4 14 day *ex vivo* expansion of clonal T cells from MM patients

Clonal T cells and non-clonal T cells were purified from PB by Ficoll density centrifugation. After cell sorting as described in section 2.9.4), cells were centrifuged for 5 min at 300 g to remove the supernatant that contained FACS flow from the cell sorter. Cells were then resuspended in expansion media containing RPMI-1640+ 10% AB serum and 20 U/mL IL-2. $1x10^5$ cells were stimulated with anti-CD3/28 beads at a ratio of 1:1 in 96 well U bottom plates for 14 days according to the Miltenyi Biotec T cell expansion protocol. On days 3, 7 and 11, cells were gently pipetted up and down to break up cell clumps. Cells were then split into two wells and fresh expansion media was added so the total well volume was 200 µL. If the cells did not divide, the culture was not split and fresh expansion was added to supplement the culture. On day 14, cells were harvested from culture and counted using a haemocytometer, to determine fold expansion.

3.2.5 Stimulation of hypo-responsive T cell clones with immune modulators

Apart from anti-CD3/28 beads and IL-2, other immune modulators were added to the culture of T cell clones in order to re-stimulate the cells to proliferate. The effect of 0.1 µg/mL IL-12, 20 ng/mL IL-15, 50 ng/mL IL-21, 50 ng/mL anti-CD137, 50 ng/mL anti-CD134 and 5 µM lenalidomide on T cell proliferation were tested. Proliferation was measured using CFSE as described in section 3.2.3 and the % cells that proliferated after the addition of these immune modulators was compared to cells stimulated with beads and IL-2 alone.

3.2.6 IFN-γ production assay

IFN-γ secretion by cytotoxic T cells occurs once antigen-specific immunity develops and therefore indicates whether the cell has been activated (Schoenborn and Wilson 2007). Therefore, to determine if the clonal T cells have been activated or are capable of being activated to produce a cytotoxic response, an assay was used to measure IFN-γ production.

The following method was adapted from Pala *et al* for the flow cytometric measurement of intracellular cytokines (Pala *et al* 2000). Intracellular IFN-γ production by T cells was stimulated using Phorbol myristic acetate (PMA) and ionomycin. The mitogen PMA, coupled with the action from the calcium ionophore, ionomycin, can stimulate T cells to produce cytokines *in vitro* (Touraine JL *et al* 1977, Irving SG *et al* 1989, Pala *et al* 2000). The use of polyclonal stimuli was necessary to stimulate these T cells since specific antigen was not present for stimulation. Brefeldin A, a fungal metabolite, was used to prevent the secretion of newly produced IFN-γ from the cell (Misumi Y *et al* 1986) by disrupting the structure and function of the Golgi apparatus to prevent protein export (Misumi Y *et al* 1986), thus allowing for investigation of intracellular cytokines such as IFN-γ by flow cytometry. All reagents were prepared at the appropriate time of culture.

PBMCs were isolated using Ficoll density gradient centrifugation. PBMC ($1x10^6$ cells) were resuspended in 1 mL of media. The cells were firstly

stimulated with PMA (50 ng/mL) and Ionomycin (500 ng/mL) in a FACS tube in a 37°C water bath (Ratek WB14). After 2 hours, brefeldin A was added to the cells and this was incubated for another 4 hours in a 37°C water bath. Cells were washed in PBS (3 mL) and centrifuged at RT for 5 min at 300 g.

Following stimulation, different CD8+ T cell subsets were identified using anti-CD3-PE-Cy7, anti-CD8-PerCP-Cy5.5, anti-Vβ PE/FITC and anti-CD57 FITC/PE. Cells were stained at RT for 15 min in the dark and washed to remove unbound antibodies. After staining, cells were washed and permeabilised using BD Cytofix/Cytoperm fixation/permeabilisation solution (250 µL) for 20 min on ice. The solution disrupts the cell membrane allowing the cytokine specific antibody to enter the cell and bind to intracellular cytokines. Cells were washed twice in BD Perm/Wash I buffer following permeabilisation. The saponin in the wash buffer maintains the cell's permeabilised state during washing. Cells were stained with anti-IFN-γ-APC or mouse IgG_1-APC (10 µL), which was used to determine background fluorescence and to ensure that non-specific binding was minimal. Cells were stained on ice for 30 min in the dark and then washed with BD Perm/wash I buffer to remove unbound antibody. Cells were resuspended in PBS for flow cytometric analysis.

3.3 Results *

3.3.1 Screening of the TCR-Vβ repertoire

MM patients were screened using flow cytometry for the presence of CD8+ clonal T cell expansions using flow cytometry. After identification of lymphocytes according to scatter properties, the CD3+ T cells were identified and divided into two subsets according to positive staining for CD8 (CD8+ T cells) or negative staining for CD8 (CD4+ cells). From the total $CD3^+$, $CD8^+$ or CD4+ T cell populations, the cells were further interrogated for any clonal expansions of Vβ families. An expanded TCRVβ+ clonal population was identified as the overexpression of that specific TCRVβ family, that as a proportion of CD8+ T cells is greater than the mean + 3 standard deviations of the normal control range. The normal control range was derived from the analysis of each TCRVβ family in 42 age-matched controls (see Table 3.2). Figure 3.2 represents a typical screening of the TCR-Vβ repertoire, illustrating the identification of three different Vβ subfamilies per tube from CD8+ cells from a representative MM patient. The expanded portion of the Vβ family is also shown on a bar graph enumerating the patient's TCR-Vβ repertoire in Figure 3.3.

* The main results of this chapter have been published. Details of the publications are:

1. Bryant, C., **Suen, H**., Brown, R., Yang, S., Favaloro, J., Aklilu, E., Gibson, J., Ho, P.J., Iland, H., Fromm, P., Woodland, N., Nassif, N., Hart, D. & Joshua, D. (2013) Long-term survival in multiple myeloma is associated with a distinct immunological profile, which includes proliferative cytotoxic T-cell clones and a favourable Treg/Th17 balance. *Blood Cancer J,* **3,** e148.

2. **Suen, H**., Brown, R., Yang, S., Weatherburn, C., Ho, P.J., Woodland, N., Nassif, N., Barbaro, P., Bryant, C., Hart, D., Gibson, J. & Joshua, D. (2016) Multiple myeloma causes clonal T cell immunosenescence: Identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade. *Leukemia***,** epub ahead of print.

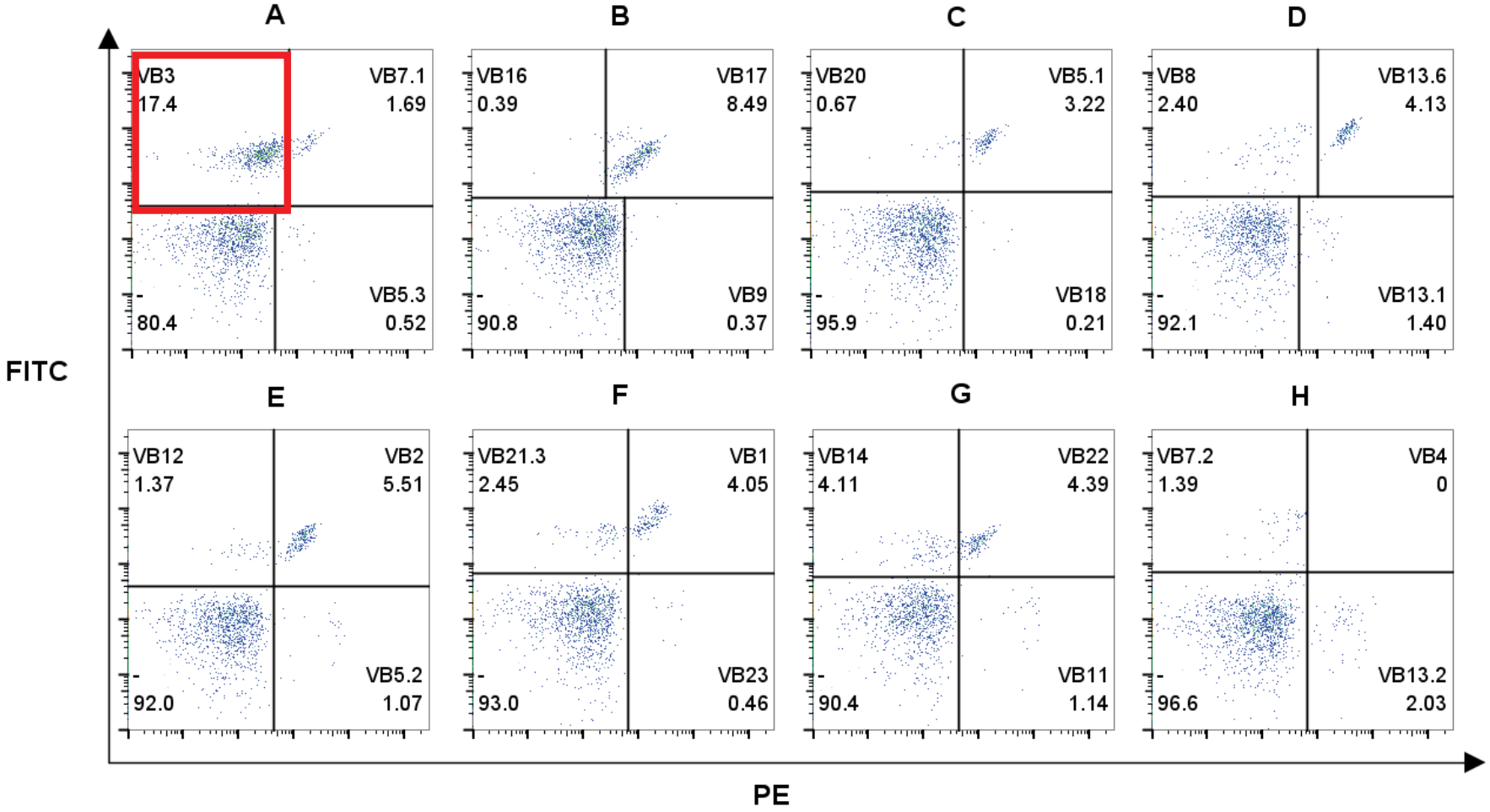


Figure 3.2 Flow scattergrams of a TCR-Vβ repertoire screen of CD3+CD8+ T cells from a representative MM patient with an expanded Vβ3 population

(A-H) Flow scattergrams illustrating the identification of the 24 TCR-Vβ families, which are detected using the 8 antibody cocktails from the IO test Beta Mark TCR-Vβ kit. **(A)** This patient had an expanded Vβ3 population (outlined in red) that accounted for 17.4% of CD8+T cells, which exceeds the 13.83% upper limit of the normal range.

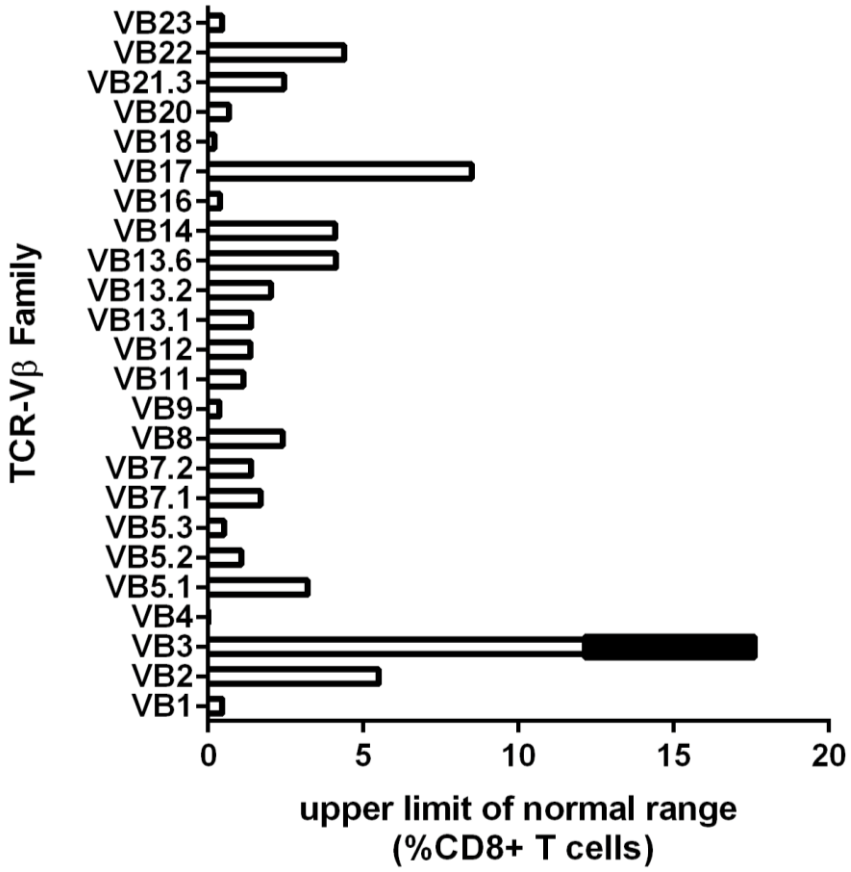


Figure 3.3 TCR-Vβ repertoire of a MM patient with a clonal expansion of the Vβ3 family

Bar graph enumerating the TCR-Vβ repertoire of a MM patient which was screened using the IO test Beta Mark TCR-Vβ kit (flow data shown in Figure 3.2). Open bars represent the expression of the 24 TCR-Vβ families, which fall within the normal range. The solid bar represents the abnormal Vβ3 expansion, the proportion of which exceeds the age-matched normal control range (open bar for Vβ3).

3.3.2 Clonal T cell expansions are detected in MM patients and have an increased incidence after IMiD therapy

A cohort of MM patients (n=103) from 2009-2014 were screened for Vβ expansions by flow cytometry. Clonal T cell expansions were detected in 75% of MM patients and more than 90% were CD8+. There was no correlation between presence of T cell clones and disease status, nor with ISS stage, previous treatment or current treatment (Table 3.3). Exposure to thalidomide induced additional T cell clones in patients and has been shown to raise T cell frequency from approximately 50% to 76% in a previous study (Brown *et al* 2009), which was similar to the incidence of 75% identified in this study.

3.3.3 MM patients do not show preferential expansion of any particular Vβ family

There are 26 different Vβ families found in humans, 24 of which can be tested for using the IO Test Beckman kit (see section 3.2.1). Apart from the Vβ18 subfamily, all other TCR-Vβ families were detected in the cohort of MM patients studied, indicating that there was no preferential expansion of any particular Vβ family (Figure 3.4). This suggests that a variety of different antigens may be responsible for stimulating the expansion of T cell clones.

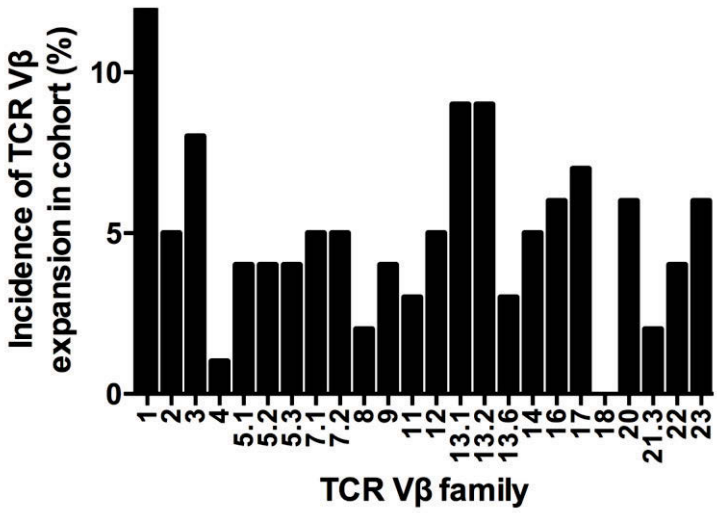


Figure 3.4 Incidence of TCR-Vβ families in MM patients
Bar graph showing the incidence of the 24 different Vβ families in MM patients with detectable T cell clones.

3.3.4 T cell clones in MM are associated with improved survival

Previous observations have demonstrated that T cell clones are prognostically significant (Brown *et al* 1997). This observation was validated in the current study as the presence of T cell clones was found to be associated with an improved survival ($\chi 2=21.01$; $p<0.0001$; Figure 3.5). The median overall survival for patients with detectable T cell clones (n=76) was more than 250 months, compared to a median survival of 55 months for patients without T cell clones (n=27).

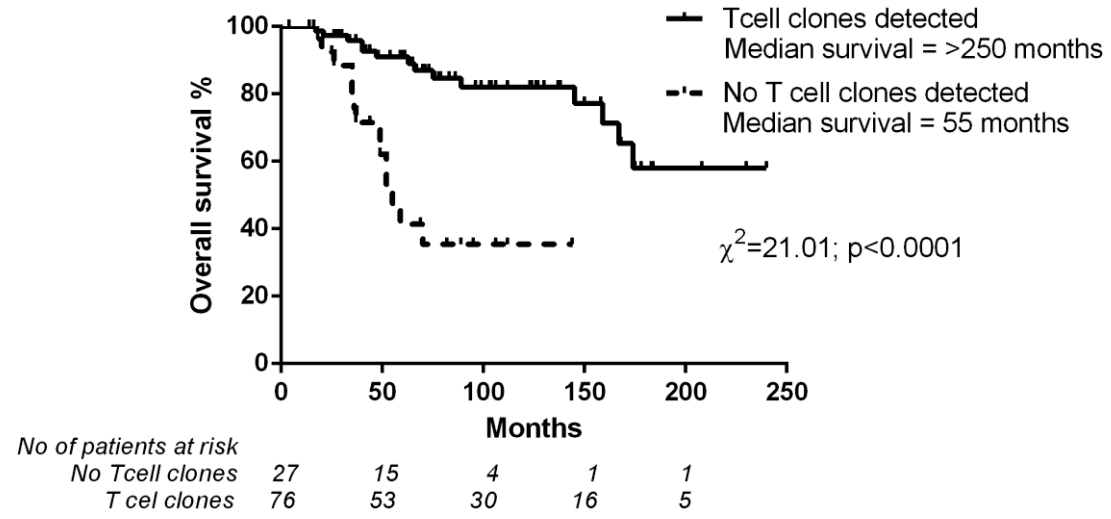


Figure 3.5 Overall survival of MM patients with T cell clones
Kaplan-Meier curves comparing overall survival of MM patients with T cell clones (solid line) to MM patients without T cell clones (dashed line). $\chi 2= 21.01$; $p<0.0001$.

3.3.5 Prognostic significance of large clonal T cell expansions

Patients were grouped according to the size of their clonal T cell expansion. Patients were considered to have large clonal T cell expansions if the expanded clone accounted for more than 20% of CD8+ T cells or a moderate clonal T cell expansion if the expanded clone constituted less than 20% of CD8+ T cells, but was greater than the normal range. The size of the T cell clone was not related to survival as patients with large clones (n= 66) did not survive longer than patients with smaller clones (n=38) ($\chi 2= 0.11$; p=NS; Figure 3.6). This may be attributed to the difference in normal ranges of the different TCR-Vβ families. A

clonal expansion of less than 20% could still be considered large if the normal range for that Vβ family is low. (e.g. the upper limit normal range for Vβ11 is 2.3% of clonal T cells). Therefore, an arbitrary value of 20% may not be suitable to determine 'size' of clonal expansions and a value that is relative to the upper limit of each Vβ family should be considered.

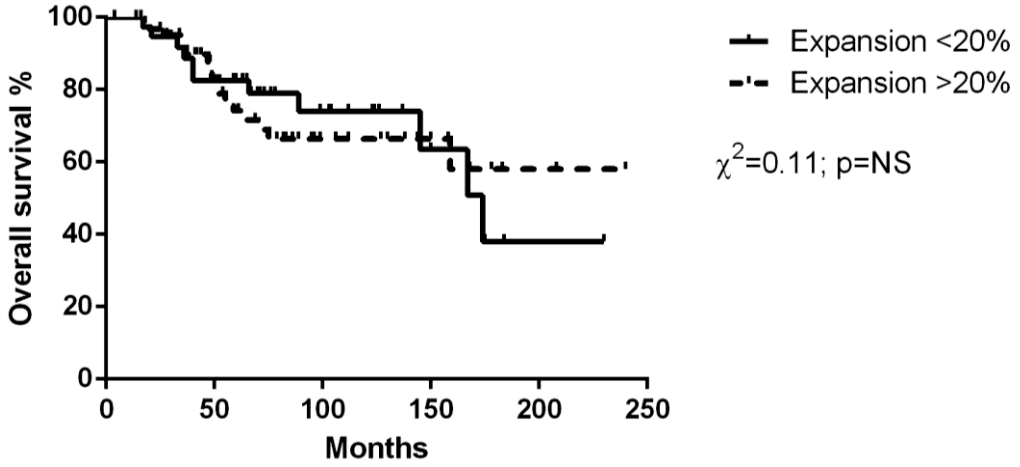


Figure 3.6 Overall survival of MM patients with large or small clonal T cell expansions
Kaplan-Meier curves comparing overall survival of MM patients with large (>20% of CD8+ T cells) or moderate clonal T cell expansions (<20% of CD8+ T cells). χ2= 0.11; p=NS.

3.3.6 T cell clones are not related to ISS stage, treatment or disease status

To determine if the presence of T cell clones was related to clinical characteristics of patients, the 103 patients screened for T cell clones were split into two groups: 1) MM patients with clones and 2) MM patients without clones. A detailed account of age, sex, months since diagnosis, ISS staging at diagnosis and at testing, previous exposure to treatment, treatment at time of testing and disease status at testing were collected for each patient and are summarised in Table 3.3. The unpaired t-test and Chi-squared test were applied to determine statistical significance. The mean age of 62 years for MM patients with clones was not statistically significant from the mean age of 59 years for MM patients without clones (t=1.8; p=NS). There was also no difference between the months since diagnosis at time of testing between the two groups (mean=58 vs 37 months; t=1.90; p=NS). There was a higher proportion of patients in ISS stage I than the other stages but all stages were represented across the two patient groups at both diagnosis (χ2=3.22; p=NS) and time of

testing ($\chi 2=1.83$; p=NS). In both groups, patients were exposed to conventional therapies, IMiDs and proteasome inhibitors, while some were not currently being treated and others had never been treated ($\chi 2=1.91$; p=NS). Treatment at time of testing was also not related to the presence of T cell clones ($\chi 2=1.03$; p=NS). Interestingly, there were more patients in the group with clones who did not require treatment. There was no difference in the disease status, when patients with and without clones were compared ($\chi 2=5.68$; p=NS). Therefore, the presence of T cell clones was not related to ISS stage, treatment or disease status of patients. Although it is to be noted, that this large cohort is a hetereogeneous cohort so this lack of correlation could be attributed to patient heterogeneity.

3.3.7 Clonal T cell expansions are a universal feature of 10 year survivors of MM

The 10 year survivor cohort for this study was selected from patients attending RPAH and included symptomatic MM patients (n=20) who were alive greater than 10 years since their original diagnosis of MM, and required treatment. Of these, 19 patients were available for testing. The TCR-Vβ repertoire was screened in all 19 patients using the IO test BetaMark Kit as described in Section 3.2.1 to confirm the correlation between the presence of T cell clones and long-term survival (Bryant *et al* 2011). The presence of T cell clones was demonstrated in 100% of 10 year survivors (n=19/19) (Table 3.4). The representation of Vβ family expansions in 10 year survivors is shown in Figure 3.7 and there were no preferential expansions of any one Vβ family.

3.3.8 Longitudinal analysis of clonal T cell expansions in 10 year survivors

T cell clones have been reported to persist for long periods of time with remarkable stability so it would be of interest to determine if the same Vβ-restricted T cell clones persisted in 10 year survivors. There were eight 10 year survivors who had serial cryopreserved blood samples over a period of more than 10 years. These serial samples were screened for Vβ expansions to determine if the same Vβ persisted in these patients. The same Vβ expansion was detected in 7/8 of 10 year survivors screened and are listed in Table 3.5.

Table 3.3 Clinical characteristics of MM patients screened for T cell clones

	Presence of clones (n=103)		
	YES n=76	**NO** n=27	**p-value**
Age (median, range)	62, 27-86	59, 40-82	0.07
Sex, n (%)			0.37
Male	37 (49)	10 (37)	
Female	39 (51)	17 (63)	
Months since diagnosis (median, range)	40, 1-261	31, 0-99	0.06
ISS at diagnosis, n (%)			0.36
I	42 (55)	10 (37)	
II	10 (13)	5 (19)	
III	11 (14)	7 (26)	
unknown	13 (17)	5 (19)	
ISS at time of testing, n (%)			0.61
I	31 (41)	11 (41)	
II	11 (14)	3 (11)	
III	5 (7)	4 (15)	
unknown	29 (38)	9 (33)	
Previous exposure to treatment, n(%)			0.75
Conventional therapy	19 (25)	12 (44)	
IMiDs	48 (63)	20 (74)	
Proteasome inhibitors	30 (39)	15 (56)	
Autologous	37 (49)	16 (59)	
Allogeneic	2 (3)	0 (0)	
Treatment at time of testing, n (%)			0.79
Conventional therapy	3 (4)	1 (4)	
IMiDs	23 (30)	12 (44)	
Proteasome inhibitors	11 (14)	5 (19)	
Not currently on treatment	26 (34)	8 (30)	
Untreated	8 (11)	0 (0)	
Disease status at time of testing, n (%)			0.22
CR/VGPR	30 (39)	8 (30)	
PR	9 (12)	3 (11)	
Stable Disease	19 (25)	7 (26)	
Progressive disease/Diagnosis	12 (16)	9 (33)	
Smouldering	6 (8)	0 (0)	

Table 3.4 List of TCRVβ expansions identified in 10 year MM survivors and their proportions of CD3+ T cells

Patient ID	TCRVβ family	TCRVβ as % of CD3 cells
10MM-1	2	15.3
10MM-2	16	7.1
10MM-3	5.1, 23	23, 43.9
10MM-4	13.1	11.8
10MM-5	23	8.9
10MM-6	3	14.3
10MM-7	1, 23	25.7,12.7
10MM-8	13.1	3.2
10MM-9	3	11.8
10MM-10	5.1	9.7
10MM-11	5.3	5.1
10MM-12	5.1, 23	12.8, 4.3
10MM-13	7.1	7.5
10MM-14	22	14
10MM-15	3	23.5
10MM-16	21.3	23.1
10MM17	13.6	6.5
10MM-18	14	14.5
10MM-19	NT	NT
10MM-20	1	8.6

NT: Not available for testing

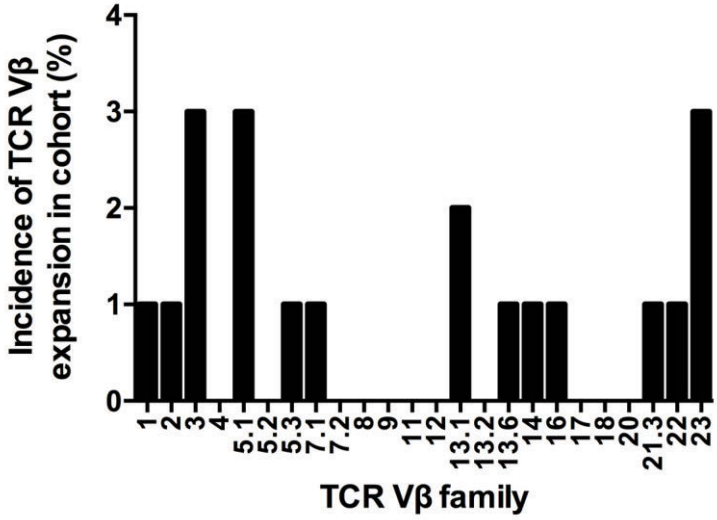


Figure 3.7 Incidence of TCR-Vβ families in 10 year survivors
Bar graph showing the incidence of Vβ families that were expanded in the 10 year survivor MM group.

Table 3.5 Longitudinal analysis of clonal T cell expansions in 10 year survivors

Patient	Vβ family	1st date tested	2nd date tested	Expansion present?	No. of years with expansion
10MM-1	2	12/02/99	17/05/10	No	N/A
10MM-3	5.1	15/09/03	19/08/14	Yes	11
10MM-5	23	11/02/00	14/6/11	Yes	11
10MM-6	3	13/12/97	17/7/12	Yes	15
10MM-9	3	25/06/02	22/6/12	Yes	10
10MM-15	3	18/06/02	13/8/12	Yes	10
10MM-16	21.3	05/07/02	06/07/12	Yes	10
10MM-17	13.6	21/06/02	25/02/13	Yes	11

N/A: Not applicable

3.3.9 Proliferative capacity of 10 year survivor T cell clones and T cell clones from other MM patients

After identification of T cell expansions, the proliferative capacity of the cells was determined through 4 day CFSE proliferation assays. The proliferation of T cell clones from non-10 year and 10 year survivors were compared. As an internal control, the proliferation of T cell clones was compared to non-clonal T cells (non-clones; CD3+CD8+Vβ+CD57-) found within the same patient. T cell clones from 10 year survivors could be stimulated to proliferate (median proliferation=60%) as did their non-clonal counterparts (median proliferation=82%) and are therefore not hypo-responsive T cells. In contrast, T cell clones from non-10 year survivors did not proliferate (median proliferation=12%), whereas the non-clonal counterparts were proliferative (median proliferation=72%) (Figure 3.8). Thus the presence of hypo-responsive T cell clones in non-10 year survivors, and responsive T cell clones in 10 year survivors of MM has been confirmed. The proliferation data were published in an online journal of the Nature group as part of an immune biomarker study on 10 year survivors (Bryant *et al* 2013).

T cell clones from 10 year survivors were expanded *ex vivo* in 14 day cultures with anti-CD3/28 bead stimulation to determine if large cell numbers of these cells could be obtained for downstream functional experiments. After 14 day *ex vivo* expansions, flow sorted clonal T cells from 6 of 8 10-year MM survivors could be expanded (median fold expansion=19.5) whereas 6 of 7 non-10-year survivors did not expand (median fold expansion=1.0) (Figure 3.9).

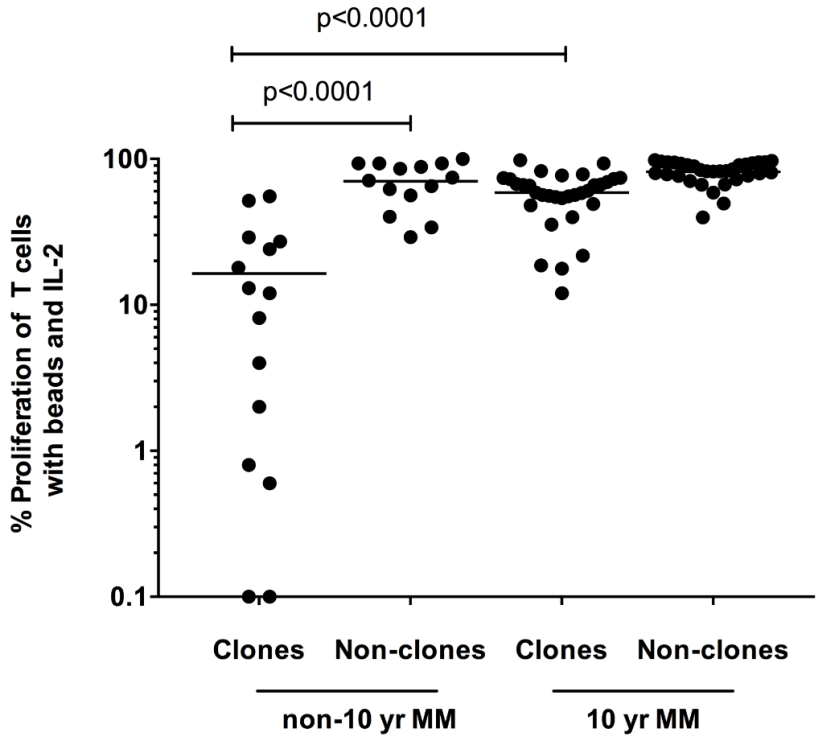


Figure 3.8 Clonal T cell proliferation in non-10 year and 10 year survivors of MM

T cell clones and non-clones (Vβ+CD57-) from 10 year survivors (10 yr MM) and non-10 year survivors (non-10 yr MM) were labelled with CFSE tracking dye and cultured with anti-CD3/CD28 beads and IL-2 for 4 days. The percentage of T cells that had undergone more than one round of proliferation as measured by CFSE fluorescence was analysed. Data are presented as % of T cells that had proliferated. (Published in Bryant et al 2013).

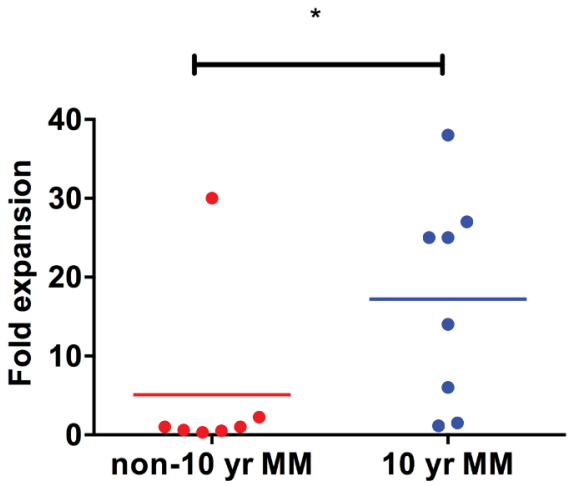


Figure 3.9 14 day *ex vivo* expansions of T cell clones from non-10 year and 10 year survivors of MM

T cell clones were flow sorted from non-10 year survivors (non-10 yr MM; n=7), and 10 year survivors (10 yr MM; n=8) and expanded for 14 days in complete media with anti-CD3/28 beads at a 1:1 ratio and 20 U/mL IL-2. Fresh media containing IL-2 was added on Days 3, 7 and 11 and cells were split when wells became over-populated. On day 14, cells were harvested and counted to determine fold expansion of T cell clones.

3.3.10 Immune modulators failed to stimulate the proliferation of hypo-responsive T cell clones and did not augment proliferation of responsive T cell clones from 10 year survivors

Anti-CD3/28 beads and IL-2 alone were insufficient to induce proliferation of hypo-responsive T cell clones from non-10 year survivors, as shown in section 3.3.9. Therefore, immune modulators IL-12, IL-15, IL-21 and ligation of CD134 and CD137 were tested to determine if these cells could be stimulated to proliferate with other means. The addition of potential immune modulators, in addition to anti-CD3/28 beads and IL-2 failed to increase the proliferation of the T cell clones *in vitro* (n=16; Figure 3.10). The effect of these immune modulators on responsive T cell clones from a 10 year survivor were also tested, however, they did not augment proliferation in comparison to stimulation with anti-CD3/28 beads and IL-2 (Figure 3.11).

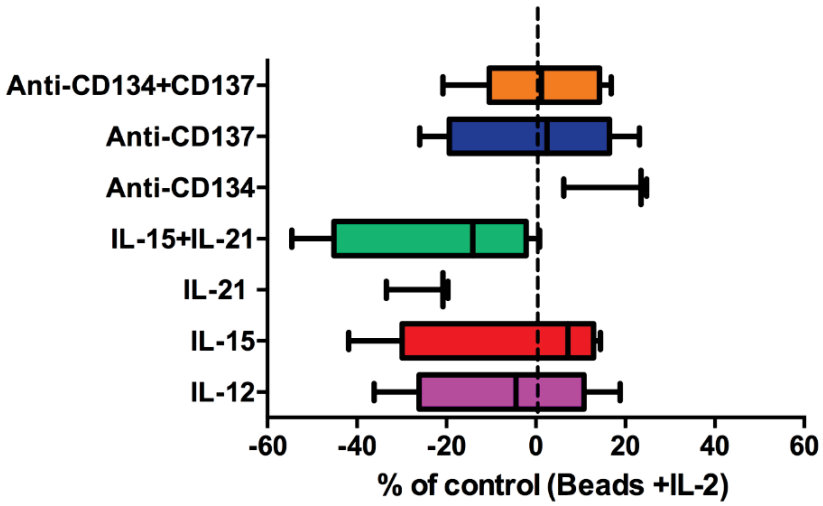


Figure 3.10 Effect of immune modulators on the proliferation of T cell clones
The effect of different immune modulators: IL-12, IL-15, IL-21, anti-CD134 and anti-CD137 on clonal T cell proliferation was tested. Data are expressed as the difference in proliferation observed in comparison to clonal T cell proliferation induced by anti-CD3/28 beads and IL-2 stimulation only (Published in Suen et al 2016).

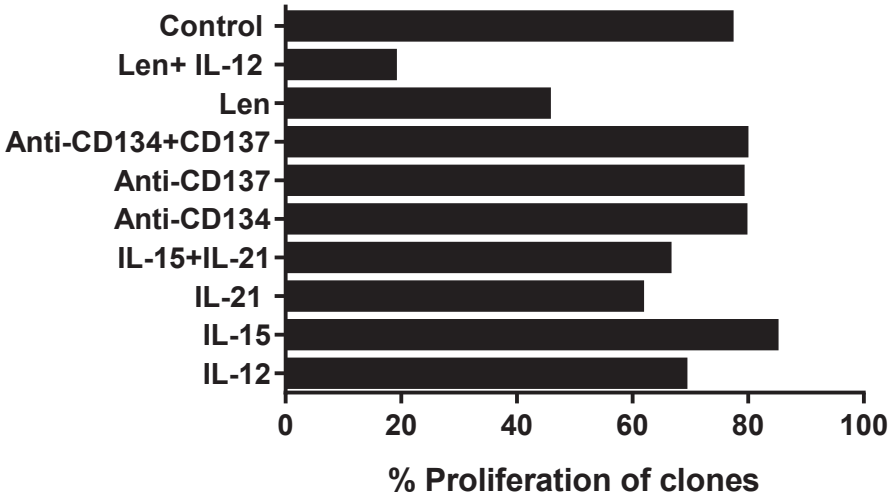


Figure 3.11 Effect of immune modulators on the proliferation of T cell clones from a 10 year survivor
The effect of different immune modulators: IL-12, IL-15, IL-21, anti-CD134 and anti-CD137 on the proliferation of T cell clones from a 10 year survivor. Data are expressed as % of cells that proliferated according to CFSE tracking. Len: Lenalidomide; IL: Interleukin. (Published in Suen et al 2016).

3.3.11 T cell clones retain the ability to produce IFN-γ

IFN-γ was measured in T cell clones by intracellular cytokine staining. The T cell clones from MM patients were able to produce IFN-γ and at similar levels (median=62% of T cell clones) to non-clonal subsets (Vβ+CD57- cells; median=69.05%). Although the T cell clones are hypo-responsive, they retain the ability to produce cytokines. Similarly, the T cell clones from 10 year survivors produced IFN-γ (median 62.30%) which was not different from their respective non-clones (Vβ+CD57-; median=70.05%).

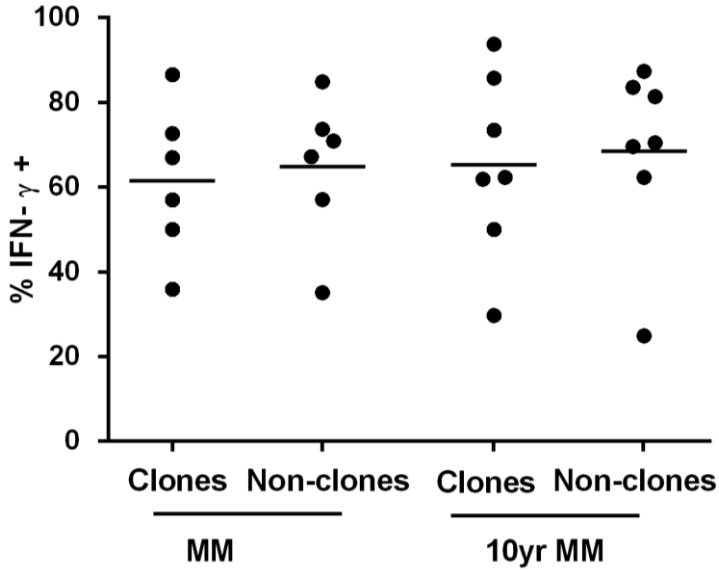


Figure 3.12 IFN-γ production by T cell clones from non-10 year and 10 year survivors of MM
IFN-γ was measured by intracellular cytokine staining. PBMC from non-10 year survivors (n=6; MM) and 10 year survivors (n=7; 10yr MM) were stimulated with PMA, ionomycin for 2 hours and then Brefeldin A was added for an additional 4 hours at 37°C. Cells were then stained with cell surface antibodies against clonal T cell markers and then fixed and permeabilised with BD Cytofix/cytoperm prior to staining for intracellular IFN-γ. Data are presented as the percentage of cells expressing IFN-γ. p=NS (Published in Bryant *et al* 2013).

3.4 Discussion

In this study, CD8+ clonal T cell expansions were studied in a new MM patient cohort receiving current therapies. T cell clones were detected in 75% of these patients and there was no preferential expansion of any particular Vβ family. The association between the presence of T cell clones, at all stages of the disease, with a favourable prognosis was validated in this cohort. The presence of T cell clones was not related to ISS stage, treatment or disease status. However, MM T cell clones failed to proliferate *in vitro* after the addition of anti-CD3/28 beads, a range of cytokines and the ligation of CD134 and CD137. Although these cells proliferated poorly *in vitro*, they were able to produce cytokines (Suen *et al* 2016). Interestingly, T cell clones were found universally in a group of patients who had survived MM long term. Furthermore, T cell clones in these patients remained proliferative and could be expanded *ex vivo* after 14 days. This provides an intriguing link between the presence of T cell

clones, their function, and long term survival (Bryant *et al* 2013). This suggests that some patients have fewer dysfunctional T cell clones than others and in these patients natural anti-MM immunity is not as heavily suppressed.

T cell clones have been detected in a number of MM patient cohorts (Table 3.1). By Southern blotting, 38% of patients were found to have T cell clones and between 48-79% when flow cytometry was used. (Brown *et al* 1997, Raitakari *et al* 2000, Sze *et al* 2001, Raitakari *et al* 2003, Brown *et al* 2009) The lower incidence detected by Southern blotting is presumably related to the lower level of detection limit of this technique, which is 5% of total CD3+ T cells (Sze *et al* 2001), hence some smaller clonal T cell expansions may have been missed by the Southern blotting studies. The incidence of T cell clones determined from the current study was 75% and the incidence is remarkably similar to the incidence of 76% found in the Australian national MM6 trial, which compared thalidomide and prednisolone maintenance following transplant in MM patients (Brown *et al* 2009). In this trial, 48% of patients were found to have T cell clones prior to transplant. After transplant, for the group that received control maintenance with prednisolone, the T cell clone incidence remained at 47%. However, an increased T cell clone incidence of 76% was seen in patients who received thalidomide maintenance, suggesting that thalidomide could stimulate the formation of additional T cell clones (Brown *et al* 2009). Many of the patients in this current study had either been treated previously, or were currently being treated with thalidomide, and also its analogues, lenalidomide and pomalidomide. As this was not a prospective study or clinical trial, the cohort is a heterogeneous mixture of patients at various stages of disease that were either untreated or had been treated with one or more lines of therapy. Therefore, it is not known whether other forms of MM treatment, like steroids or proteasome inhibitors are able to stimulate additional T cell clones, compared to patients who did not receive those treatments. A controlled study, similar to the MM6 clinical trial described above, would be required to test if particular treatments had an effect on creating additional T cell clones.

This study demonstrated that T cell clones were present at all stages of disease and that there was no correlation between the presence of T cell clones and

ISS staging, treatment or disease stage. These findings are in line with the Australian MM6 study that identified T cell clones as an independent prognostic factor in a multi-variate analysis (Brown *et al* 2009) and Raitakari *et al*. similarly showed that the presence of expanded Vβ T cells was not related to age or time since diagnosis (Raitakari *et al* 2000). Brown *et al* found a lower incidence of T cell clones in samples taken at diagnosis and stable disease, compared to progressive disease and there was a significantly higher incidence of T cell clones detected in progressive compared to stable disease (Brown *et al* 1997). The converse of this is seen in the current study as more T cell clones were detected in stable disease compared to progressive disease. This difference may be attributed to the difference in the era of testing, as the former study was performed almost 20 years ago, with a different detection method that had a lower level of sensitivity than the flow cytometric method in this study. In addition, the introduction of novel agents since the former study, may allow for more patients to gain disease control and account for the higher numbers of patients with T cell clones in stable disease and lower numbers in progressive disease. Raitakari *et al*. found the highest number of Vβ expansions in MM patients in Stage I who had received chemotherapy, and a lower incidence in patients with stage II and III (Raitakari *et al* 2000), which correlates with the findings from this study. ISS stage I patients tend to survive for longer and this increases the chance of sampling.

Historically, T cell clones have been associated with an improved survival in MM. MM patients who had T cell expansions, detected at any time, had better overall survival than patients who did not have T cell expansions. This has been demonstrated in a few different cohorts of MM patients (Brown *et al* 1997, Raitakari *et al* 2003, Brown *et al* 2009). Furthermore, patients with T cell expansions at diagnosis survived longer than patients without T cell clones detected at diagnosis and patients with detectable clones during progressive disease had improved survival when compared to patients who did not have detectable clones during disease progression (Brown *et al* 1997). Interestingly, patients with T cell clones who also received thalidomide had the best overall survival but patients with T cell clones had better survival than patients without T cell clones who did receive thalidomide (Brown *et al* 2009). This study

validated the prognostic significance of T cell clones in a more recent cohort of MM patients, receiving current therapy and was not related to any disease state. Interestingly, patients with clones required less treatment as indicated by the higher number of patients with detectable clones who did not require treatment. The data suggest that the presence of T cell clones is prognostically significant in MM in today's era of treatment and that patients with T cell clones have lower ISS staging, less disease and tend to require less treatment. Furthermore, T cell clones were detected in 100% of 10 year survivors of MM, providing a further link between the presence of these cells and prolongation of survival. Expanded cytotoxic CD8+ T cells were also identified in a previous study of MM patients who had achieved long term disease control. However, the CD8+ T cell expansions in this study simply referred to an increase in the number of T cells and an analysis of the TCR-Vβ repertoire was not conducted (Pessoa de Magalhães *et al* 2013).

There was no preferential expansion of a single Vβ family as all families were represented in the cohort except for Vβ18. This is in agreement with previous reports that demonstrate coverage of the overall Vβ repertoire in terms of expansions in MM patients (Raitakari *et al* 2000, Sze *et al* 2001). There was also no preferential expansion in the 10 year survivor group, although there were lower numbers of patients in this cohort, which accounts for a decreased incidence of Vβ families observed. This suggests that T cell clones do not arise in response to antigenic stimulation of a single antigen. T cell clones are most probably induced in response to specific patient tumour antigens, which gives rise to a wide variety of TCR-Vβ restricted TCRs.

Although T cell clones remained prognostically significant, they were non-proliferative after TCR ligation with anti-CD3/28 beads, except in the case of long term survivors. The T cell clones from long term survivors were proliferative and produced cytokines and could be expanded many fold in culture. In addition to proliferative T cell clones, the Treg/Th17 ratio in these patients are not increased, whereas in non-10 year survivors, the ratio is increased, representing a more suppressive state (Bryant *et al* 2013). These

differences suggest that there is a distinct immunological profile in 10 year survivors, whereby there is less immune suppression, that may translate to better long term survival. As there may be a spectrum of clonal T cell dysfunction, presumably related to levels of tumour-induced suppression, based on disease severity, it stands to reason that T cell dysfunction can be reversed if the cells can be freed from suppression.

Hypo-responsive T cell clones have similarly been seen in patients with WM and have been described as anergic T cells (Li *et al* 2010). Anergic cells are said to exist in a hypo-responsive state with low IL-2 production and are rendered so due to incomplete T cell activation during the presence of low co-stimulatory signals or high co-inhibitory signalling (Crespo *et al* 2013). MM T cell clones were once termed 'anergic' due to their hypo-responsive nature upon stimulation (Bryant *et al* 2011), which was thought to be due to activation of the T cell, in the presence of low co-stimulatory signals, and CD28 is lacking on these cells. Another phenomenon known as split anergy, describes T cells that are anergic but retain their ability to secrete cytokines. Anergy may be induced when Tregs suppress cytotoxic T cell function but does not inhibit IFN-γ production, through the action of TGF-β signalling (Chen *et al* 2005). As T cell clones retain the ability to secrete IFN-γ despite being hypo-responsive, they were said to exist in a state of split anergy (Brown *et al* 2012c). Gene set enrichment analysis after microarray of WM clonal T cells compared to non-clonal T cells revealed the upregulation of a number of signalling pathways that contributed to induction of anergy (Li *et al* 2010). MM T cell clones may also be classified as anergic T cells due to their lack of CD28 expression and hypo-responsiveness to IL-2 stimulation. These dysregulated pathways should also be studied to determine if they are responsible for causing MM T cell clone dysfunction. This will be explored in detail in Chapter 4.

Similar hypo-responsive T cells have been identified that also express CD57, the marker of T cell replicative senescence or clonal exhaustion (Brenchley *et al* 2003, Wood *et al* 2009). They display limited proliferative ability or failure to proliferate under stimulation despite their retained ability to secrete cytokines (Arosa 2002, Betts *et al* 2003, Weng *et al* 2009a, 2010, Focosi *et al* 2010).

Despite their proclaimed senescent nature, some groups have been able to restimulate T cell clones to proliferate again, with the use of irradiated PBMC rather than anti-CD3 stimulation (Plunkett *et al* 2007), and with a range of cytokines including IL-2, IL-12 (Broderick *et al* 2006), IL-15 (Chiu *et al* 2006), IL-21 or cross linking of ligands CD134 and CD137 (Kober *et al* 2008). This raises the question of whether these CD8+ CD57+ cells can be termed truly senescent as the definition of replicative senescence is the permanent and irrevocable arrest of the cell cycle and hence lack of proliferative capacity (Wood *et al* 2009). This study attempted to use these methods to re-stimulate T cell clones to proliferate, but was not met with success. It is noteworthy that the re-stimulation experiments mentioned above were performed on total CD8+CD57+ cells from healthy individuals and may not be representative of the same cells found in infections and malignancies which often are TCR-Vβ-restricted. Furthermore, cytokines alone may not be sufficient and other strategies are required such as in combination with DC stimulation, which can stimulate T cells to proliferate. IMiDs such as thalidomide are able to increase T cell proliferation once CD3 has been activated (LeBlanc *et al* 2004). Immune checkpoints such as lymphocyte activation gene-3 (LAG-3), CTLA-4 and PD-1 are frequently expressed on the surface of anergic or exhausted cells and blockade may reverse their dysfunction (Pardoll 2012). The conflicting reports of clonal T cells as anergic, exhausted and senescent cells necessitates a more complete characterisation of these cells in MM, which would provide more information on the most appropriate method of overturning dysfunction. This will be explored in detail in Chapter 5 of this thesis.

In summary, the incidence of clonal T cell expansions was determined in a recent cohort of MM patients, which reflects historical cohorts. In an era of novel therapies, T cell clones remained a prognostic factor for this disease and was not related to ISS stage, treatment or disease status of patients, suggesting that they play an immunogenic role in MM. The confirmation that T cell clones are a universal feature of 10 year survivors solidifies the argument that T cell clones are associated with a survival advantage. As these patients survive for longer, their disease may be less severe and may be related to the presence of these protective cells. With the exception of 10 year survivors, these cells failed to

proliferate upon stimulation with anti-CD3/28 beads and a range of cytokines and immune modulators, confirming their hypo-responsive nature. In contrast, T cell clones from 10 year survivors were proliferative and able to be expanded many fold in *ex vivo* expansions for 14 days. This has implications for the use of these cells in functional assays which require larger cell numbers. This may allow for the understanding of why these cells differ from T cell clones found in non-10 year survivors. While these cells are protective in MM, in that they confer a survival advantage, it is possible they are not functioning in their full capacity since the tumour is not completely eradicated. This may also explain how patients in plateau phase have a detectable tumour burden but do not progress (Joshua *et al* 1994). They are likely MM tumour specific T cells but, due to immune suppression, are unable to exert their full range of cytotoxic functions. It is possible that, once freed from tumour-induced suppression, these cells will be able to regain their cytotoxic functions and may form the basis for personalised immune therapy. This chapter has confirmed the presence of prognostically significant T cell clones in MM patients and identified their dysfunction. The mechanism of their dysfunction will be explored further in the following chapters to discover potential novel targets for the reversal of clonal T cell dysfunction.

CHAPTER 4 ANALYSIS OF SIGNALLING PATHWAYS IN CLONAL T CELL EXPANSIONS

4.1 Introduction

In Chapter 3, it was shown that whilst MM T cell clones are able to produce cytokines, they proliferate poorly *in vitro* to TCR stimulation and are therefore hypo-responsive. Despite this, these cells are related to an improved survival. Interestingly, these T cell clones were found universally in a group of patients who had survived MM for more than 10 years. Interestingly, in these patients the cells remained proliferative and are therefore not hypo-responsive cells. This provides an interesting association not only between their presence, but also their function, and long term survival. The paradoxical role that T cell clones play in extending survival of patients, despite being dysfunctional in non-10 year survivors, warrants further investigation. Reversal of dysfunction of protective, tumour-induced cytotoxic T cell clones may be crucial for maintaining long term survival in MM patients, but the underlying cellular or molecular mechanisms that induce dysfunction need to be firstly characterised.

4.1.1 Dysregulated pathways associated with anergy are found in T cell clones of WM patients

Dysfunctional T cell clones that do not proliferate have also been identified in patients with WM, which is also B cell malignancy. A gene set enrichment analysis that analyses abnormal expression of genes in entire signalling pathways has been used to study clonal and non-clonal T cells in WM patients. Apart from upregulated cytotoxic pathways, the dysfunctional T cell clones had upregulated pathways associated with anti-proliferation, T cell inactivation, cell cycle arrest and anti-apoptosis, providing evidence that these cells are anergic (Figure 4.1) (Li *et al* 2010). As MM is another B cell malignancy, it is possible that these same signalling pathways may be responsible for inducing dysfunction of MM T cell clones and are potential targets to be studied. If specific pathways are found to be abnormal, small molecule inhibitors could potentially be used to neutralise abnormal checkpoints. As described in chapter 3, T cell clones in long term survivors are not hypo-responsive. Determination of any differences in signalling pathways between responsive T cell clones of 10 year survivors and hypo-responsive T cell clones of non-10 year survivors may

also reveal the reason for the differences in proliferation observed between the two groups.

The specific pathways identified to be dysregulated in WM T cell clones were: (1) an upregulated Ras pathway, where the genes *RAS, BCL2L1 (BCL-XL)* and *CHUK (IKK)* confer apoptotic resistance to expanded T cells, allowing long term survival (Romashkova and Makarov 1999, Makino *et al* 2004, Fiebig *et al* 2006); (2) an upregulated CSK pathway, where *CREBBP* is recruited to activate *CSK* resulting in T cell inactivation (Kawabuchi *et al* 2000, Takeuchi *et al* 2000); (3) an upregulated TOB pathway, where TGF-β stimulates *SMADs* to interact with *Tob* to sustain an inactivated cell state (Tzachanis *et al* 2001, Tzachanis and Boussiotis 2009); (4) increased binding of TGF-β to its receptor and to *CDKN2A (P16)*, which causes a decrease in *CCND2* and *CDK6* in the G1/S transition, leading to arrest of the cell cycle in the G1/S phase (Yamato *et al* 1997, Bouchard *et al* 1999); and (5) a downregulated ERK pathway, as a result of increased *PTPN7,* which dephosphorylates and inactivates *ERK* and thereby inhibits proliferation (Saxena *et al* 1998, Pettiford and Herbst 2000, Nika *et al* 2004).

As MM T cell clones display similar properties to the T cell clones described in WM patients, it is highly possible that these pathways may be similarly dysregulated in MM patients. The signalling pathways described are mainly those involved in negatively regulating apoptosis (intrinsic and extrinsic), in promoting TGF-β-mediated negative effects including T cell inactivation and anti-proliferative effects. These signalling pathways provide a starting point for the investigation of cellular mechanisms that may be responsible for inducing the dysfunction of T cell clones in MM. These pathways will be explored in more detail below.

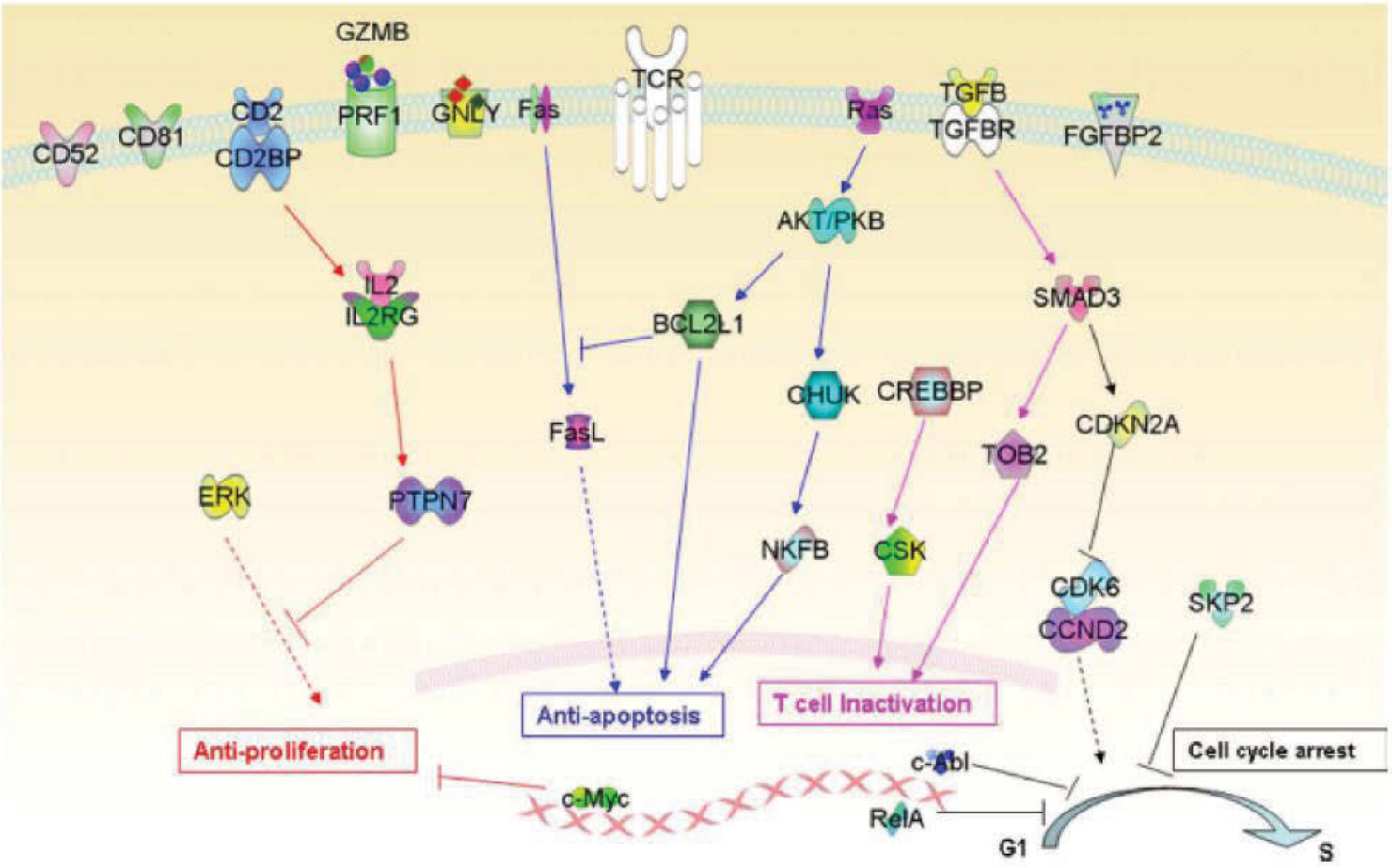


Figure 4.1 Dysfunctional pathways identified in T cell clones from patients with Waldenström's Macroglobulinaemia
Several dysfunctional pathways have been identified in the T cell clones of patients with WM. These included upregulation of the pathways associated with anti-proliferation, T cell inactivation, cell cycle arrest and anti-apoptosis (Li *et al* 2010).

4.1.2 Apoptotic pathways

Apoptosis can occur through an extrinsic pathway (involving death receptors and ligands) or an intrinsic pathway (involving mitochondrial damage and release of cytochrome C). Both pathways converge at the activation of caspases, which ultimately lead to cell apoptosis (Xu and Shi 2007).

In the extrinsic pathway, binding of Fas ligand (CD178) to cell surface receptor Fas (CD95) results in apoptosis through caspase activation (Nagata and Golstein 1995). Fas can be expressed on a number of immune and non-immune cells (Watanabe-Fukunaga *et al* 1992), whereas Fas-ligand is usually expressed on activated cells (Waring and Mullbacher 1999). Fas/Fas ligand mediated apoptosis is one pathway by which cytotoxic T cells mediate killing of foreign antigens. After TCR ligation, cytotoxic T cells upregulate Fas-ligand which allows killing of cells expressing Fas. As shown in Figure 4.2, Fas ligand causes trimerisation of the Fas receptor leading to activation of caspase 8 through the action of the adaptor protein Fas associated death domain (FADD). Activation of caspase-8 mediates downstream activation of other caspases including caspase 3, which results in apoptosis (Nagata and Golstein 1995, Waring and Mullbacher 1999).

The intrinsic pathway of apoptosis is dependent on the release of proteins from the mitochondria due to permeabilisation of the membrane, which triggers the caspase cascade and results in apoptosis (Figure 4.2). Anti-apoptotic proteins including B cell lymphoma (Bcl)-2 and Bcl-xL, prevent this release whilst pro-apoptotic proteins such as Bcl-2 associated X (Bax) and Bcl-2-antagonist/killer (Bak) promote mitochondrial permeabilisation for release of proteins from the mitochondria, resulting in apoptosis (Danial and Korsmeyer 2004, Xu and Shi 2007).

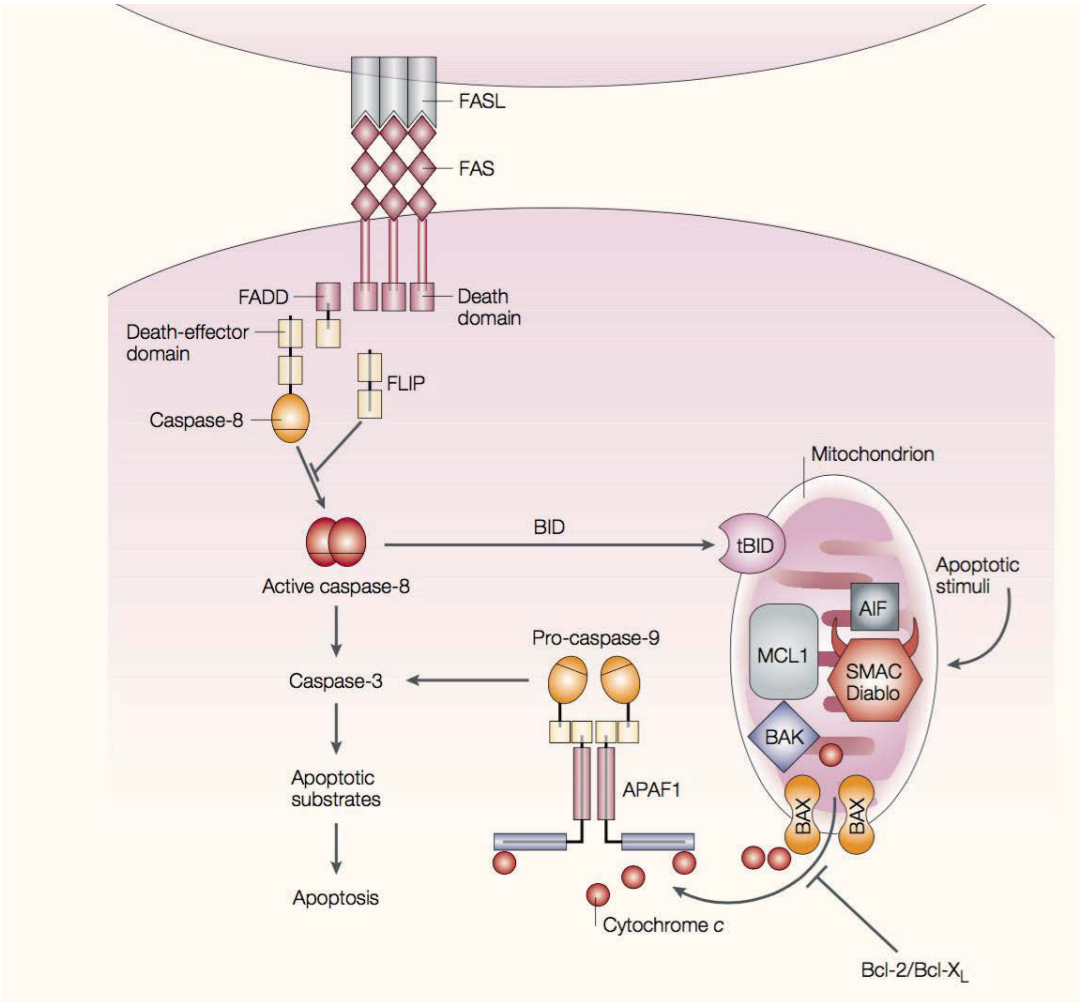


Figure 4.2 Schematic diagram of the extrinsic and intrinsic pathways of apoptosis

Diagram depicting the two different apoptotic pathways: 1) Extrinsic pathway (death receptor pathway): the binding of Fas ligand to Fas results in trimerisation of the Fas receptor, which leads to activation of caspase-8 through FADD. Activated caspase-8 can directly activate caspase 3 and lead to apoptosis or apoptosis may be mediated by BID due to mitochondrial damage. 2) Intrinsic pathway: (mitochondrial pathway) Disruption to the mitochondrial such as through DNA damage results in a loss of mitochondrial transmembrane potential, thereby releasing cytochrome C into the cytoplasm. In the presence of APAF1 and ATP, cytochrome c activates caspase 9, leading to the activation of caspase 3. The anti-apoptotic proteins Bcl-2 and Bcl-xL prevent loss of mitochondrial transmembrane potential whilst the pro-apoptotic proteins BAX and BAK promote loss of mitochondrial transmembrane potential.

AIF: apoptosis-inducing factor; APAF1: apoptotic protease-activating factor 1; BAK: Bcl-2-antagonist/killer; BAX: Bcl-2-associated X protein; BID: BH3-interacting death-domain agonist; FADD: FAS-associated death-domain protein; FASL: FAS ligand; FLIP: FADD-like IL-1β-converting enzyme-inhibitory protein; MCL1: myeloid-cell leukaemia sequence 1; SMAC: second mitochondria-derived activator of caspase.
(Pope 2002)

The expanded T cell clones of patients with MM remain remarkably stable and have been shown to persist for long periods of time. Longitudinal studies of the TCR-Vβ repertoire in a single MM patient showed remarkable stability over an 18 month period (Figure 4.3) (Raitakari *et al* 2000). In addition, 82% of repeat samples from MM patients revealed the same Vβ expansion as the original test sample (Brown *et al* 1997). Persistence of CD8+ T cell expansions has also been detected in healthy individuals and in viral infections (e.g. CMV, HIV) due to chronic viral antigen stimulation (Morley *et al* 1995, Mugnaini *et al* 1999). The underlying reasons for the prolonged survival of T cell clones in MM remain unclear.

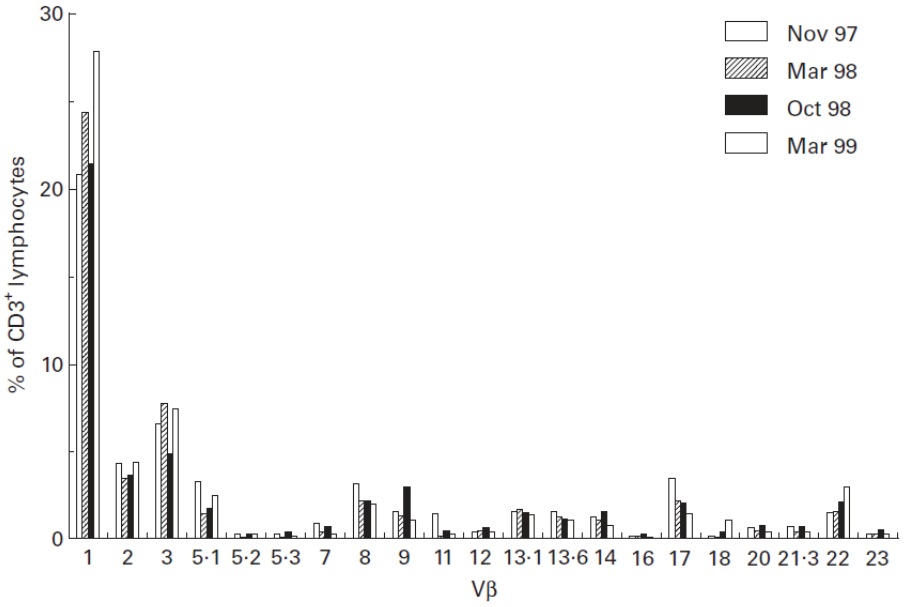


Figure 4.3 Follow-up of the TCR-Vβ repertoire in a single MM patient over 18 months

Sequential analyses of the TCR-Vβ repertoire of a single MM patient over a period of 18 months demonstrated the stability of the TCR-Vβ repertoire and the persistence of expanded Vβ1+ T cell clones. (Adapted from Raitakari *et al* 2001).

A possible explanation for their continued persistence is resistance to apoptosis. This could be associated with an up-regulation of the (phosphoinositide 3-kinase) PI3K pathway, which confers resistance to Fas induced apoptosis, constitutive caspase activation, increased expression of anti-apoptotic factors such as Bcl-2 and Bcl-xL, along with reduced expression of pro-apoptotic markers such as Bak and Bad (Strauss *et al* 2003, Wood *et al* 2010). All these aspects of apoptotic resistance have yet to

be studied in the context of MM and hence further research is required. Interestingly, Bcl-xL was also identified as an upregulated protein in WM T cell clones. As part of a pathway that is responsible for apoptotic resistance in WM T cell clones, Bcl-xL is therefore a potential target to be investigated in MM.

In patients with MM, the T cell clones tended to express lower proportions of the apoptotic marker Fas than their non-clonal counterparts (Figure 4.4) (Raitakari *et al* 2000, Sze *et al* 2001). Fas ligand levels were low on both subsets and no difference was detected. These results are not in agreement with other studies on T cell expansions in other diseases. In a study conducted on HIV infected individuals, Fas expression was detected on nearly all CD8+CD57+ and CD8+CD57- cells in the blood. Fas ligand levels were detected in both subsets and were even higher in CD8+ CD57+ cells. Interestingly, there was a significant increase in Fas ligand expression in the CD8+CD57+ T cells in the lung during late stage disease and these cells were undergoing significantly less apoptosis than normal CD8+CD57+ cells, as measured by caspase 3 detection. This suggested that in advanced disease, the CD57+ expanded cells lose the ability to undergo normal apoptosis (Wood *et al* 2005). On the other hand, T cell clones in patients with large granular lymphocytic leukaemia (LGL) display resistance to apoptosis but ambiguously have constitutively high levels of Fas and Fas ligand expression (Lamy *et al* 1998). It was found that LGL T cells were actually resistant to Fas induced apoptosis regardless of increased levels due to upregulated PI3K and signal transducer and activator of transcription (STAT3) pathways (Epling-Burnette *et al* 2001, Schade *et al* 2006). As there seems to be conflicting data regarding Fas and Fas ligand expression levels in MM, studies are needed to confirm the expression of these markers on T cell clones and also determine if the same signaling pathways play a role in regulating apoptosis in these cells.

An imbalance between the levels of anti-apoptotic and pro-apoptotic proteins causes dysregulation of apoptosis. Thus far, only the anti-apoptotic protein Bcl-2 has been studied on MM T cell clones (Figure 4.4) and there was no

detectable difference between clonal and non-clonal T cells, implying T cell clones are not less susceptible to apoptosis (Raitakari *et al* 2000). In contrast to this finding, untreated patients with haematological malignancies (4/54 patients had MM) did show an increased tendency to undergo apoptosis, albeit this was measured with Annexin V staining and after cells were cultured (Van den Hove *et al* 1998). The Bcl-2 enumeration in the Raitakari study was conducted on fresh, unactivated and uncultured cells so it may be necessary to culture MM T cell clones and then measure anti-apoptotic protein levels. The finding that the clones persist for long periods of time naturally suggests a resistance to cell death and so additional studies investigating other anti-apoptotic proteins are required.

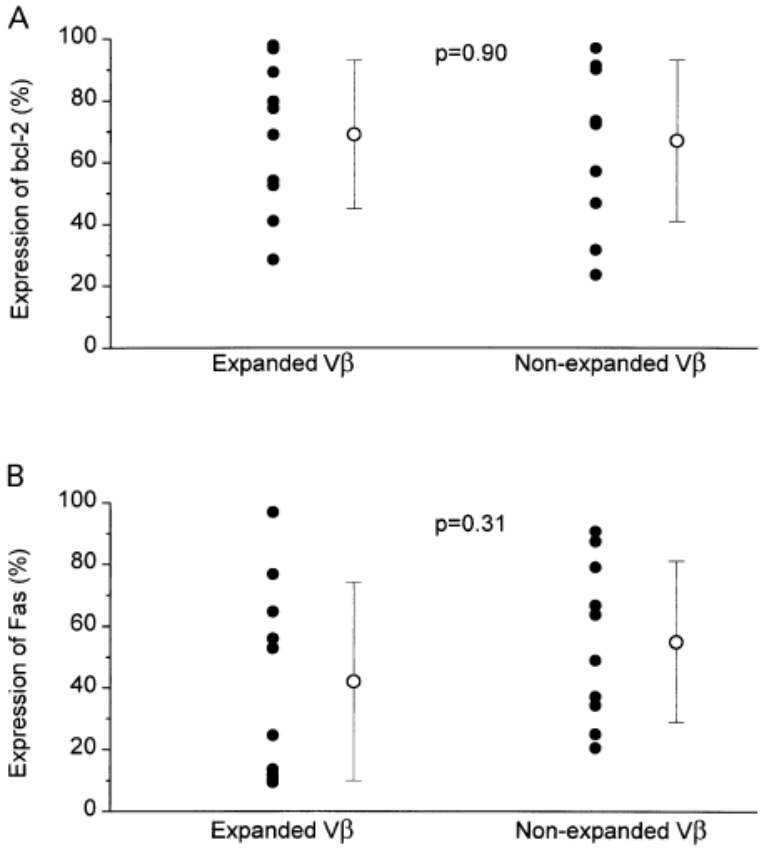


Figure 4.4 Bcl-2 and Fas expression on MM T cells
There were no detectable differences in either (A) Bcl-2 or (B) Fas expression between expanded and non-expanded T cells. (Adapted from Raitakari et al 2000).

4.1.3 TGF-β pathway

TGF-β is responsible for the regulation of cell growth and proliferation and is often involved in the inhibition of cell growth for many cell types. The TGF-β receptor consists of a type I and type II receptor, both of which are serine threonine protein kinases. Binding of TGF-β ligands to the type II receptor results in phosphorylation of the type I receptor, which then binds and phosphorylates SMAD family proteins. The name SMAD is derived from the *Drosophilia* protein, mothers against decapentaplegic (MAD) and the *Caenorhabditis elegans* protein (SMA), as SMAD proteins are homologs of these two proteins. TGF-β ligands activin and nodals result in the phosphorylation of SMAD2 and 3 whereas bone morphogenetic protein ligands activate SMAD 1, 5 and 9. Once activated, these SMADS partner with the common signalling transducer SMAD4 and translocate to the nucleus where they can modulate the transcription of TGF-β target genes (Figure 4.5) (Abdollah *et al* 1997, Massagué 2012).

SMAD3 can interact with Tob to maintain T cell inactivation (Tzachanis *et al* 2001, Tzachanis and Boussiotis 2009). Tob can also interact with cyclin-dependent kinase inhibitor (CDKI) p27kip1 causing cell cycle arrest (Tzachanis *et al* 2001). Binding of TGF-β to its receptor and to p16 causes a decrease in CDKI in the G1/S transition phase of the cell cycle, leading to G1/S phase cell cycle arrest (Yamato *et al* 1997, Bouchard *et al* 1999). SMAD3 was identified as part of the upregulated TGF-β pathway in the WM T cell clones study and the TGF-β receptor was also found to be elevated (Li *et al* 2010). Vast amounts of TGF-β are secreted by the malignant plasma cells in MM (Matthes *et al* 1995) and TGF-β has been shown to induce dendritic cell dysfunction leading to poor antigen presentation to T cells (Brown *et al* 2001). It also has the ability to arrest the differentiation and cytotoxic abilities of CD27+ CD57+/- subsets at the CD27+ stage where perforin expression remains low (Wu *et al* 2012). Thus upregulation or high endogenous levels of TGF-β leading to cell cycle arrest could potentially be a mechanism by which MM T cell clones remain hypo-responsive but persist for long periods of time. TGF-β effects on SMAD proteins have not been

investigated in MM T cell clones and hence the TGF-β signalling pathway is a target of importance to be be explored.

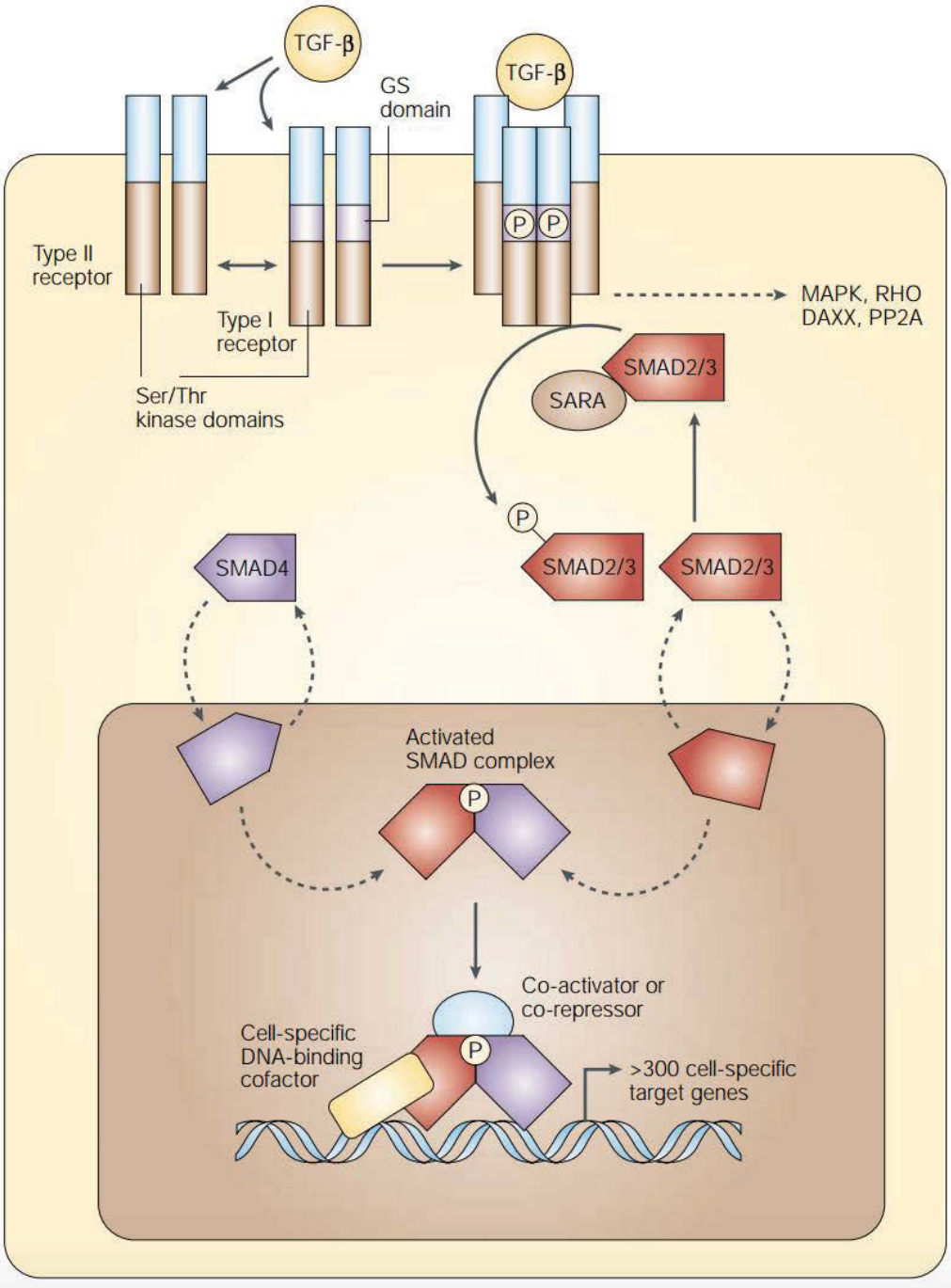


Figure 4.5 The TGF-β signalling pathway
Binding of TGF-β to the type II receptor leads to phosphorylation and the formation of a complex with the type I receptor. The type I receptor then phosphorylates SMAD2 and SMAD3, which releases them from the cytoplasmic anchoring protein, SMAD anchor for receptor activation (SARA). The phosphorylated SMAD2/3 complex forms dimers or trimers with SMAD4 and translocates to the nucleus where it regulates transcription of TGF-β target genes (Siegel and Massague 2003).

4.1.4 Proliferation pathway

The mitogen activated protein kinases/extracellular signal-related kinase (MAPK/ERK) signalling cascade is involved in the regulation of cell proliferation, differentiation, survival and death (Figure 4.6). The signalling cascade consists of a G protein working upstream of three protein kinases that are in turn activated by phosphorylation: a MAPK kinase kinase (MAPKKK), which phosphorylates a MAPK kinase (MAPKK), which then phosphorylates MAPK. These terminal MAPKs are serine/threonine kinases and include ERK 1/2, c-Jun N terminal kinases (JNK) 1, 2, 3 and 4, p38 kinases and ERK5. Growth factors are responsible for the activation of the ERK pathway, whereas stress and growth factors are responsible for the activation of the other three pathways. The substrates for MAPKs are usually transcription factors that regulate cell division, proliferation and survival (Johnson and Lapadat 2002, Roberts and Der 2007).

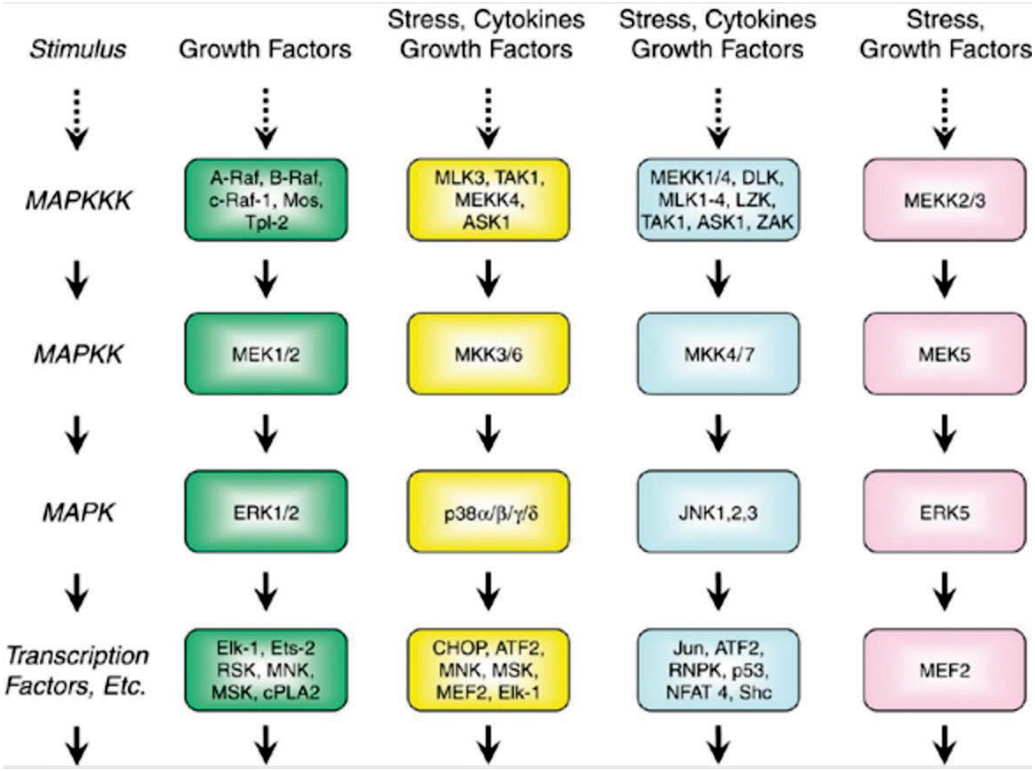


Figure 4.6 The MAPK signalling cascade
The MAPK signalling cascade is comprises of three protein kinases that phosphorylate and activate downstream molecules: MAPKKK, MAPKK and a MAPK. The terminal MAPKs include ERK1/2, p38, JNK1,2,3 and ERK5, which then activate transcription factors involved in regulating cell division, proliferation and survival (Roberts and Der 2007).

MM T cell clones are hypo-responsive as measured by *in vitro* proliferation assays (Bryant *et al* 2013). Upon stimulation of the TCR, the cells were unable to proliferate, suggesting that there may be a block in the proliferation signalling pathway. The hypo-responsiveness of anergic T cells hass been shown to be related to defective activation of ERK and c-Jun N terminal kinases (JNK), upon TCR activation (Li *et al* 1996). In hypo-responsive WM T cell clones, the ERK pathway was found to be downregulated. This was due to increased haematopoietic protein tyrosine phosphatase (HePTP), also known as protein tyrosine phosphatase non-receptor type 7 (PTPN7), which dephosphorylates ERK 1/2 (Figure 4.7) (Pettiford and Herbst 2000). Therefore, phosphorylated ERK 1/2 levels in MM should be investigated to determine if a defect in this proliferation pathway is responsible for inducing the observed hypo-responsiveness.

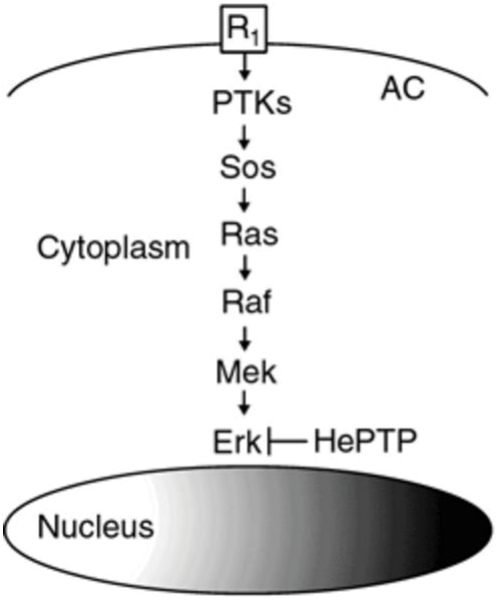


Figure 4.7 Inhibition of the ERK pathway by HePTP
The ERK pathway can be inhibited by HePTP dephosphorylation of ERK (Saxena *et al* 1999).

4.1.5 TCR signalling pathway

Immune dysfunction in cancer may also be mediated by downregulation of the expression of TCR related signaling proteins (Mizoguchi *et al* 1992). The TCR signalling pathway is depicted in Figure 4.8. In T cells, binding of cognate peptide-MHC complexes to the TCR triggers signal transduction. This results in the recruitment of lymphocyte specific protein tyrosine kinase (LCK) to the TCR-CD3 complex, which phosphorylates immuno-receptor tyrosine-based activation motifs (ITAMS) of the CD3 γ, δ, ε and ζ-chains (Guy and Vignali 2009). Phosphorylated ITAMS recruit ζ-chain associated protein kinase of 70kDa (ZAP-70), which becomes phosphorylated and activated through the action of LCK. ZAP-70 then phosphorylates four tyrosine residues on linker for activation of T cells (LAT) which leads to the recruitment of signalling molecules that form a multi-protein complex (LAT signalosome) (Au-Yeung *et al* 2009). The LAT signalosome allows for signalling through one of three major signalling pathways: 1) Ca^{2+} signalling pathways, 2) MAPK signalling pathways and the 3) NF-κB signalling pathways, which result in the activation of transcription factors that modulate expression of genes involved in T cell regulation (Brownlie and Zamoyska 2013). As immune dysfunction may result from the downregulation of TCR-related signalling proteins, components of the TCR: CD3-ζ chain and ZAP-70 were studied to determine if there were any defects in TCR-signalling in MM T cell clones. SHP-2, an Src homology 2 (SH2) domain containing protein tyrosine phosphatase (PTP) can dephosphorylate signaling molecules and this can downregulate T cell signaling (Qu 2000). This protein should also be measured in MM T cell clones to determine if it may be responsible for any negative effects on TCR signalling that induce hypo-responsiveness.

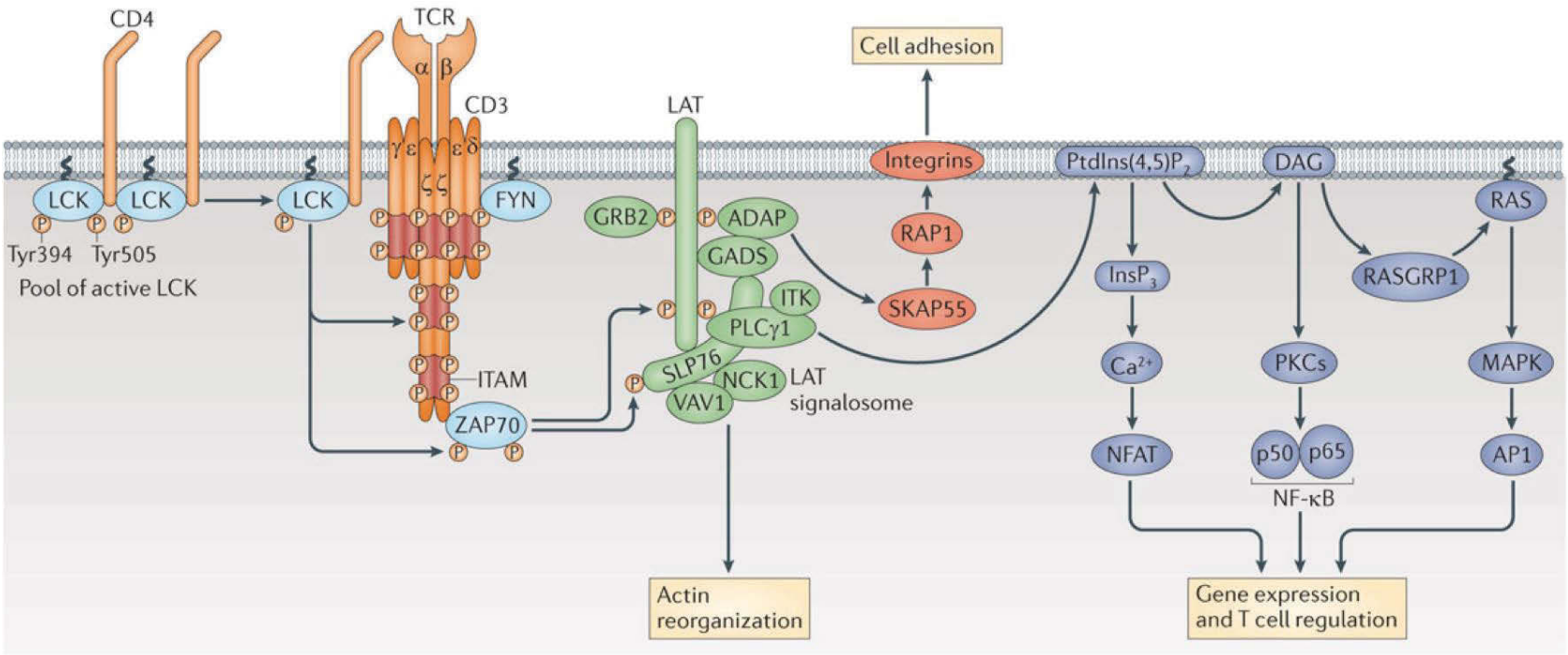


Figure 4.8 The TCR signalling pathway

Binding of cognate peptide-MHC complexes to the TCR triggers signal transduction. This results in the recruitment of LCK to the TCR-CD3 complex, which phosphorylates immuno-receptor tyrosine-based activation motifs (ITAMS) of the CD3 γ, δ, ε and ζ-chains. Phosphorylated ITAMS recruit ζ-chain associated protein kinase of 70kDa (ZAP-70) which becomes phosphorylated and activated through LCK. ZAP-70 then phosphorylates four tyrosine residues on linker for activation of T cells (LAT) which leads to the recruitment of signalling molecules that form a multi-protein complex (LAT signalosome). The LAT signalosome allows for signalling through one of three major signalling pathways: 1) Ca^{2+}, 2) mitogen activated protein kinase (MAPK) and the 3) NF-Kβ signalling pathways, which result in the activation of transcription factors involved in gene expression and T cell regulation (Brownlie and Zamoyska 2013).

4.1.6 Phospho-flow cytometry

Alterations in signalling pathways may play a role in inducing T cell dysfunction, however, there have been limited studies aimed at characterising the involvement of specific signaling pathways in MM. Traditional methods used to study changes in signalling proteins, such as western blotting, either require large numbers of cells or pure cell populations and this is a problem when analysing rare cells or mixed cell populations (Krutzik and Nolan 2003). In MM, T cell clones only comprise up to 50% of the total CD3+ population, so large amounts of blood would be required to purify and obtain cell numbers sufficient for the analysis of multiple signalling proteins by western blotting. Recent work in the area of phospho-flow may offer opportunities to study signaling pathways in MM T cell clones.

Phospho-flow allows for the detection of phosphorylated intracellular cell signalling proteins using flow cytometry. This relatively new technique is attractive as it allows the simultaneous detection of multiple phosphorylated intracellular proteins across different cell subsets and the ability to examine rare cells exhibiting a specific cell surface phenotype amongst a heterogeneous population (Perez 2002, Krutzik and Nolan 2003, Krutzik *et al* 2004, Krutzik *et al* 2005b). This technique now provides new opportunities for flow cytometry laboratories to identify functional differences at a single cell level (Irish *et al* 2004, Krutzik *et al* 2004, Krutzik *et al* 2005b). Phospho-flow allows for the measurement of two types of phosphorylated proteins: constitutive expression, which refers to the basal level of protein phosphorylation of the cell in its physiological state, or induced expression, where cells are stimulated with cytokines to uncover the signalling potential of a protein within that cell (Irish *et al* 2004). The comparison of diseased cells and healthy cells could reveal aberrant levels of protein phosphorylation, which may provide insights to the disease process. Furthermore, the elucidation of cancer cell signalling profiles could also identify new markers for diagnosis, monitoring and offer opportunities for novel therapies (Krutzik *et al* 2004, Krutzik *et al* 2005b). The general phospho-flow method is shown in Figure 4.9 and involves 4 major steps. Cells are firstly stimulated or not stimulated with cytokines and then fixed

with formaldehyde to preserve the phosphorylated state of the proteins. Methanol is used to permeabilise the cells, to allow entry of phospho-protein specific antibodies into the nucleus, and cells are simultaneously stained with cell surface markers. Slight modifications to this generic method may be necessary as fixation and permeabilisation methods can destroy epitopes, preventing antibody recognition, or it may diminish or remove the fluorescence of certain fluorochromes that are conjugated to antibodies, thus reducing the ability to identify cell populations of interest. If there is difficulty detecting expression of any surface markers, a modified technique called sequential staining can be employed to rescue cell surface staining. Sequential staining involves staining with problematic cell surface markers either prior to fixation and/or permeabilisation, however this will also be dependent on the fluorochrome that is conjugated to the antibodies being used (Krutzik *et al* 2005a). Therefore, whilst this technique is attractive for single cell analysis and the analysis of rare cell populations, optimisation for the specific antibody panel for the identification of the cell populations of interest is crucial for analysis.

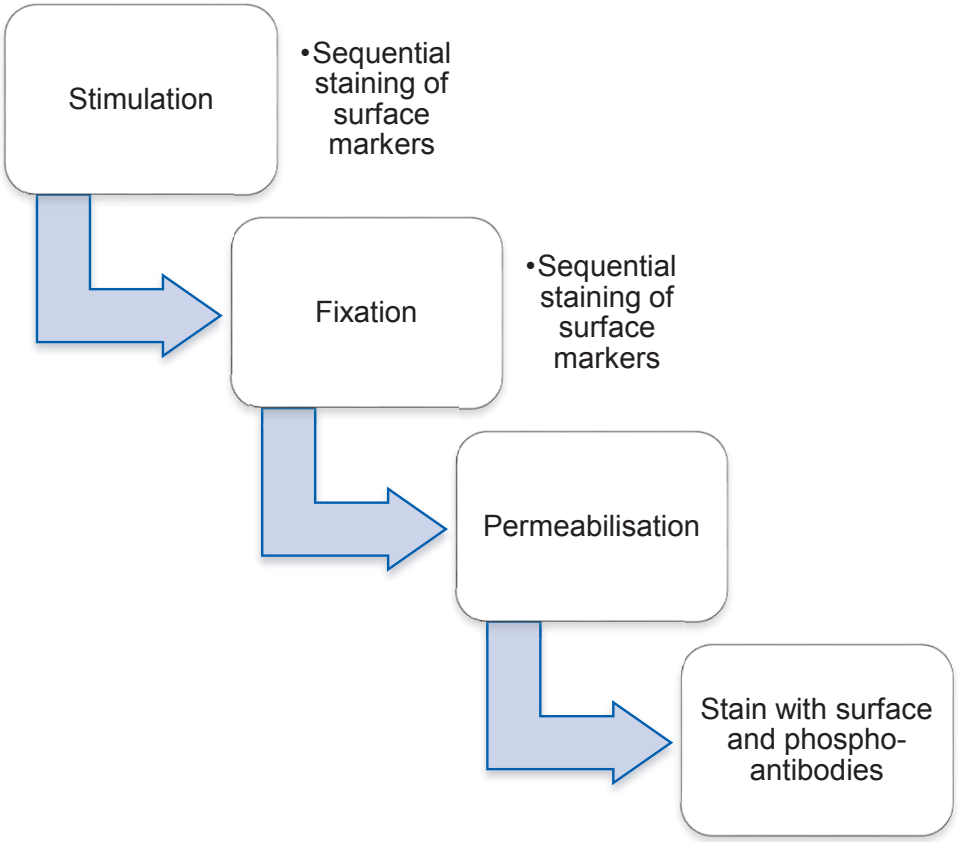


Figure 4.9 Outline of the generic generic phospho-flow technique
Phosphorylated signalling proteins can be detected using flow cytometry. Cells are initially stimulated with cytokines to induce phosphorylation of signalling proteins. Cells are then fixed with formaldehyde, which maintains the phosphorylation of proteins. Permeabilisation of the cell with methanol allows the entry of phospho-protein antibodies to enter the cytoplasm and nucleus and cells are stained simultaneously with cell surface marker antibodies. Sequential staining is a slight modification in the technique that is used when there is difficulty obtaining resolution of cell surface markers.

4.1.7 Summary

This chapter firstly outlines the steps taken to optimise the Phospho-flow protocol for analysis of specific signalling proteins in MM T cell clones. A number of other intracellular staining methods using different fixation and permeabilisation buffers for the identification of intracellular proteins are also explored. The optimised phospho-flow assay and intracellular staining methods were used to analyse targets from the dysfunctional signalling pathways identified in WM T cell clones (Li *et al* 2010). Whether these pathways are dysregulated in MM T cell clones is unknown. The targets included Fas and Fas ligand (involved in apoptosis), Bcl-xL (an anti-apoptotic protein), SMAD2/3 from the TGF-β pathway (involved in T cell inactivation) and ERK (involved in cell proliferation). Signalling profiles of responsive T cell clones from 10 year survivors were compared to the hypo-responsive T cell clones from non-10 year survivors to determine any differences in pathways that may account for the functional differences observed between these two groups. A significant difference in p-SMAD levels, associated with T cell inactivation was detected between the two groups. There was also an impaired ability of hypo-responsive T cell clones from non-10 year survivors to upregulate p-ERK following PMA stimulation, illustrating that this pathway may not be responsive to cytokine control and is dysregulated *in vivo*. These results have provided insights into possible novel targets for reversing clonal T cell dysfunction in MM. Studies of the TCR revealed that there were no abnormalities in TCR signalling proteins.

4.2 Materials and Methods

4.2.1 Fixation and Permeabilisation Methods

Different fixation and permeabilisation methods were used to measure intracellular antigens and phosphorylated signalling proteins in clonal and non-clonal T cells in MM patients and normal controls. The method was dependent on the location of the target. Harsher methods of permeabilisation are required in order for the antibody to enter into the cell nucleus to stain its target, compared to targets in the cytoplasm. The different methods are outlined below. Table 4.1 lists the different fixation and permeabilisation methods used for the detection of various intracellular and signalling proteins and also the stimulation methods for induction of protein expression.

Table 4.1 Fixation and permeabilisation methods for the detection of surface and intracellular proteins of interest

Pathway	Protein	Technique	Stimulation
Apoptosis	Fas	Surface stain only	No stimulation
	Fas ligand	Surface stain only	Anti-CD3/28 beads for 2 days
Anti-apoptotic	Bcl-xL	Leucoperm kit	Anti-CD3/28 beads for 2 days
T cell inactivation & cell-cycle arrest	p-SMAD	Phosflow (BD lyse/fix)	PMA and TGF-β
Anti-proliferation	p-ERK	Beckman Perfix-P Kit	PMA
TCR-signalling	CD3-ζ	BD Cytofix/Cytoperm	No stimulation
	ZAP-70	BD Cytofix/Cytoperm	Hydrogen peroxide
	SHP-2	Phosflow (BD Cytofix)	Anti-CD3/28 beads

4.2.1.1 Leucoperm

The Leucoperm kit is a permeabilisation method for the detection of intracellular antigens. It has a reduced preparation time compared to other

fixation and permeabilisation methods as it allows for the simultaneous addition of permeabilisation medium and staining with intracellular antibody. The kit consists of two reagents, reagent A, a formaldehyde and methanol containing fixative, and reagent B, which is a permeabilisation medium. The leuocoperm method was used to measure Bcl-xL following 2 day stimulations with anti-CD3/28 beads in culture. PBMC from MM patients were harvested from 2 day bead stimulation cultures and stained with clonal T cell surface markers. After 30 min incubation on ice, cells were washed and then fixed in 100 μL of leucoperm reagent A for 15 min at RT. Cells were washed in 3 mL PBS at 300 g for 5 minutes. The supernatant was discarded and the cells were vortexed gently. 100 μL of leucoperm reagent B was added to the cells and simultaneously, the anti-Bcl-xL-PE antibody was added for a staining time of 30 min at RT. Following this, unbound antibody was washed away with PBS and cells were resuspended in PBS for flow cytometric analysis.

4.2.1.2 BD Cytofix/cytoperm

The BD Cytofix/cytoperm permeabilisation solution allows for simultaneous fixation and permeabilisation of cells. This method utilises saponin-mediated permeabilisation of cells, so it is a gentle and reversible method of permeabilisation. As it is reversible, BD Perm wash buffer (1X), which contains saponin, is used as an antibody diluent and wash buffer to ensure the cells remained permeabilised during washing and staining. This gentle method is suitable for intracellular antigens of cytosolic or cytoplasmic location. PBMC from MM patients were firstly stained with clonal T cell surface markers for 30 min on ice and then washed to remove unbound antibody. After gentle vortexing, the BD Cytofix/cytoperm permeabilisation solution (250 μL) was added to cells and cells were incubated on ice for 20 min. Cells were washed twice in 1 mL perm wash buffer and resuspended in 100 μL of perm wash I buffer for staining with intracellular antibody for 30 min at RT. Following incubation, cells were washed twice in 1 mL perm wash I buffer and then resuspended in PBS for flow cytometric analysis.

4.2.1.3 Whole blood detection of phosphorylated signalling proteins

Phosphorylated proteins can also be detected in whole blood using the Beckman Coulter Perfix P Cell Preparation kit. This system is useful for batch analysis of fresh blood and also reduces preparation time as mononuclear cells do not need to be firstly obtained through Ficoll density gradient centrifugation. The kit contains a fixation buffer, a lysis/permeabilisation buffer and a resuspension buffer.

Whole blood (100 μL) from EDTA anti-coagulated blood was firstly incubated at 37°C for 10 min to reduce basal levels of phosphorylation. Cells were then stimulated with PMA (150 nM) for 10 min at 37°C to induce phosphorylation of ERK. Unstimulated cells were used as a negative control. Cells were immediately fixed after stimulation with the fixation buffer (65 μL) for 10 min at RT. After fixation, red cells were lysed and cells were permeabilised with the lysis buffer (1 mL) at 37°C for 15 min. Following lysis, cells were washed twice in 2 mL ice cold stain buffer (PBS + 2% FCS) and then stained in 100 μL stain buffer containing an antibody cocktail of anti-p-ERK and clonal T cell surface markers (anti-CD3-PE-Cy7, anti-CD8 PerCP-Cy5.5, anti-CD57 efluor-450 and anti-TCR-Vβ-PE/FITC) for 30 min at RT. Cells were then washed in 2 mL ice cold stain buffer to remove unbound antibodies and resuspended in 200 μL of resuspension buffer prior to flow cytometric analysis.

4.2.1.4 BD Phosflow method

For the detection of phospho-epitopes of nuclear location a harsher method of permeabilisation is required to allow antibodies to enter the nucleus and bind to its specific phosphorylated epitopes. The BD Phosflow protocol was used for the detection of these phospho-epitopes. PBMC ($1x10^6$/mL) were resuspended in 250 μL of RPMI-10. Cells were then simultaneously stained with surface marker anti-CD8-PE-Cy7 and fixed with 250 μL BD Cytofix buffer (containing 4% formaldehyde) for 10 min at 37°C. After fixation, cells were centrifuged at 600 g for 6 min and the supernatant was decanted. Cells were vortexed to loosen the cell pellet, prior to permeabilisation with 1 mL ice

cold BD Perm III buffer (containing 87.68% methanol). Cells were permeabilised for 30 min on ice and then washed three times in 3 mL stain buffer (PBS + 2% FBS) at 600g for 6 min. It was important to ensure as much supernatant was removed as possible and that cells were vortexed to loosen the cell pellet, prior to further wash steps. After washing, cells were resuspended in 100 µL stain buffer containing antibodies to phospho-epitopes and clonal T cell surface markers (anti-CD3-PerCP-Cy5.5, anti-CD57 efluor450 and anti-TCR-Vβ-FITC/PE). Cells were stained with antibodies for 1 h at RT and then washed with stain buffer. Cells were resuspended in PBS prior to flow cytometric analysis.

4.2.1.5 Analysis of p-SMAD

p-SMAD2 (pS465/pS467)/ p-SMAD3 (pS423/pS425) (p-SMAD) was analysed using the BD Phosflow method. Cells were firstly serum starved overnight in RPMI-1640 containing 0.1% FCS. Serum starvation is required because FCS contains TGF-β and TGF-β induced p-SMAD was an endpoint for this analysis. Cells were stimulated with either PMA (150 nM) or TGF-β (10 ng/mL) for 30 min at 37°C. Cells were then fixed with the BD Phosflow lyse/fix buffer (containing 7.15% methanol, 20.35% formaldehyde and 15.65% diethylene glycol) instead of the BD Cytofix buffer. This protocol is recommended by the manufacturers as it has been shown that there is less background staining with the lyse/fix buffer as compared to the cytfix buffer. Cells were then permeabilised and stained with antibodies as described in Section 4.2.1.4.

4.2.1.6 Sequential staining

For surface markers that were difficult to resolve following fixation and permeabilisation, a slightly modified method called ‘sequential staining’ was used. This involved staining the cells for the problematic surface antibody prior to fixation and/or permeabilisation. As CD8 PE-Cy7 was a problematic surface antibody, the cells were sequentially stained with this antibody during the stimulation period, prior to fixation and permeabilisation, and this was sufficient to discern positive and negative populations for CD8.

4.2.2 Controls

Sample cells were either stimulated or not stimulated with cytokines or anti-CD3/28 beads to induce phosphorylation of signalling proteins. A fluorescence minus one (FMO) negative control, whereby an unstimulated sample was stained with surface markers and not the phosphorylated protein, was used to define positive expression limits or constitutive expression of the phospho-protein of interest. The MFI ratio, defined as the ratio of stimulated to constitutive level of target protein phosphorylation was calculated by dividing the MFI of stimulated sample by the MFI of the unstimulated sample. This was used to measure the level of induced phosphorylation in stimulated samples.

The primary internal biological control or comparator was the non-clonal Vβ-CD57+ subset as these are CD8+ T cells that express CD57+ but do not express a restricted TCR-Vβ and are therefore not expanded T cells. However, the Vβ+CD57- and Vβ-CD57- subsets are also included for completion and for interest. Age-matched normal controls were also studied and the CD8+CD57+ subset was used as the comparator.

4.3 Results

PART 1: OPTIMISATION OF PHOSPHO-FLOW PROTOCOL[†]

The BD Phosflow method was used to investigate various cell signalling proteins in clonal T cells from MM patients. Whilst this method was sufficient to detect constitutive and induced levels of protein phosphorylation, detection of cell surface expression of some molecules were not optimal as a result of fixation and permeabilisation of the cells. Of particular importance was the inability to distinguish between negative and positive CD8 populations, which hinders the identification of clonal T cells. The fixative used in the BD Phosflow method contained formaldehyde and the permeabilisation buffer

[†] The optimised phospho-flow method was published in Brown, R., Yang, S., Weatherburn, C., Gibson, J., Ho, P.J., Suen, H., Hart, D. & Joshua, D. (2015) Phospho-flow detection of constitutive and cytokine-induced pSTAT3/5, pAKT and pERK expression highlights novel prognostic biomarkers for patients with multiple myeloma. *Leukemia,* **29,** 483-490.

contained methanol, both of which can affect surface marker characteristics. Their effects can include changes to, or destruction of, epitopes, so monoclonal antibodies can no longer bind; and also destruction of antigen-antibody and/or fluorochrome complexes, which means cell surface expression is not detectable by flow cytometry. As a result, it may be necessary to stain the cells with antibodies at a different time point in the assay and this method is known as sequential staining. Therefore, it was necessary to understand the effect of fixation and permeabilisation on T cell markers and on the fluorochromes. This method was originally optimised for an assay measuring phosphorylated (p)-STAT3 proteins in CD8+ T cells, therefore the following optimisation experiments utilise T cell and pSTAT3 antibodies.

4.3.1 Effect of fixation and permeabilisation on surface marker detection

To investigate the effect of formaldehyde fixation (BD Cytofix buffer) and methanol permeabilisation (BD perm III buffer) on fluorochromes and to elucidate whether the loss of surface marker signal was due to epitope destruction or the choice of fluorochrome, Jurkat cells were stained with CD3 antibodies conjugated to APC, FITC, PE, PE-Cy7 and PerCP-Cy5.5 (Figure 4.10). As Jurkat cells are a T cell line, the entire population should stain positively for CD3. Hence, any loss of CD3 staining would indicate the method has affected the ability to detect CD3 positivity.

Figure 4.10 shows the detection of CD3 positivity by measuring MFI on flow histograms according to the staining of Jurkat cells with CD3 antibodies conjugated to 5 different fluorochromes at different points in the phospho-flow protocol. As a control, cells were not fixed nor permeabilised and a strong CD3+ peak was detected, irrespective of the CD3 antibody that was added (Figure 4.10, *1st column*). When cells were fixed prior to staining (Figure 4.10, *2nd column*), similar positive results were observed. This indicates formaldehyde fixation did not severely affect the staining characteristics of the tested antibodies, although slight decreases are observed for anti-CD3 PerCP-Cy5.5. The general phospho-flow method was used, which included fixation followed by permeabilisation and then staining

of anti-CD3 (repeated in duplicate Figure 4.10, *fourth and fifth column*). A noticeable effect of permeabilisation on staining characteristics with the general method is the constriction of positive CD3 peaks for all tested antibodies and also a drop in MFI. An alternate method involving sequential staining where cells were stained with anti-CD3 after fixation but prior to permeabilisation (Figure 4.10, third *column*) was also tested. Interestingly, when cells were sequentially stained with anti-CD3 PE-Cy7 and anti-CD3 PerCP-Cy5.5 after fixation but prior to permeabilisation (Figure 4.10M,W), the CD3 signal was abrogated. Cells stained with anti-CD3-APC (Figure 4.10C) and CD3-PE (Figure 4.10R) were affected to a lesser extent, however, a logarithmic decrease in staining was still observed. Anti-CD3-FITC stained cells (Figure 4.10H) appeared to be the only antibody unaffected by this change in method. As shown in the second column of Figure 4.10, detection of CD3 when staining occurred after fixation, was unaltered so these results indicate that methanol permeabilisation is most likely responsible for destroying fluorochromes and, in particular, for the APC, PE, and tandem dyes. These results confirm that fixation and methanol can have detrimental effects on the detection of high density antigens on populations that are uniform in expression. Therefore, it will be crucial to optimise all antibodies, especially low density antigens that may become lost after treatment. Furthermore, manipulation of cells with fixation and permeabilisation will have varying effects on individual antibodies so these will need to be individually optimised.

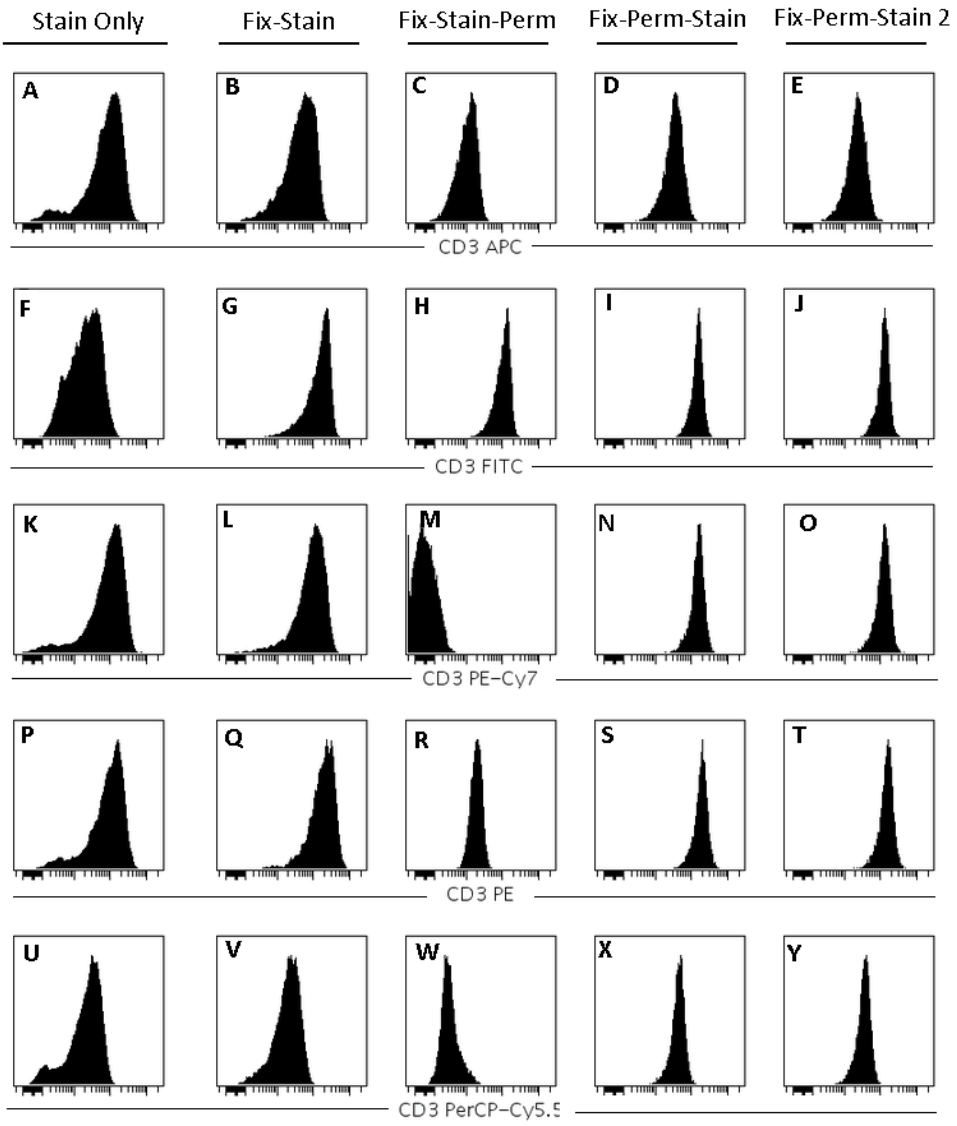


Figure 4.10 Comparison of the effects of traditional and sequential staining protocols on anti-CD3 antibodies conjugated to different fluorochromes

Jurkat cells were stained with CD3 antibodies conjugated to APC, FITC, PE-Cy7, PE and PerCP-Cy5.5 either after fixation and before permeabilisation (third column) or after fixation and permeabilisation (duplicated; fourth and fifth column). For comparison, cells were stained with antibody alone (first column) or fixed and then stained (second column).

4.3.2 Dilution of permeabilisation buffer to improve cell surface marker resolution

Section 4.3.1 demonstrated that methanol permeabilisation compromised cell surface marker resolution, permeabilising with varying concentrations of methanol were tested to determine if the use of lower concentrations could prevent the loss of cell surface markers. PBMCs from a healthy donor were fixed, then permeabilised with neat, or different dilutions (1/2, 1/5 and 1/10) of BD Perm III buffer and stained with anti-CD3-PerCP-Cy5.5 and anti-CD8 PE-Cy7. The ability to identify CD8+ and CD8- T cell populations after treatment with the different concentrations of permeabilisation buffer was examined in Figure 4.11. Cells were either stimulated or not stimulated with IL-6 for detection of phosphorylated (p)-STAT3 to investigate whether changes in cell surface phenotype were related to cytokine stimulation.

With a neat concentration of permeabilisation buffer, it is evident that methanol affects the anti-CD8-PE-Cy7 tested, with no clear distinction between the positive and negative CD8 populations. When a 1/2 dilution of permeabilisation buffer was used, the resolution of the surface markers was restored and clear CD8+ and CD8- T cell populations are observed. Further dilutions of 1/5 and 1/10 reveal the same results and do not provide an improvement in cell surface resolution as compared to the 1/2 dilution.

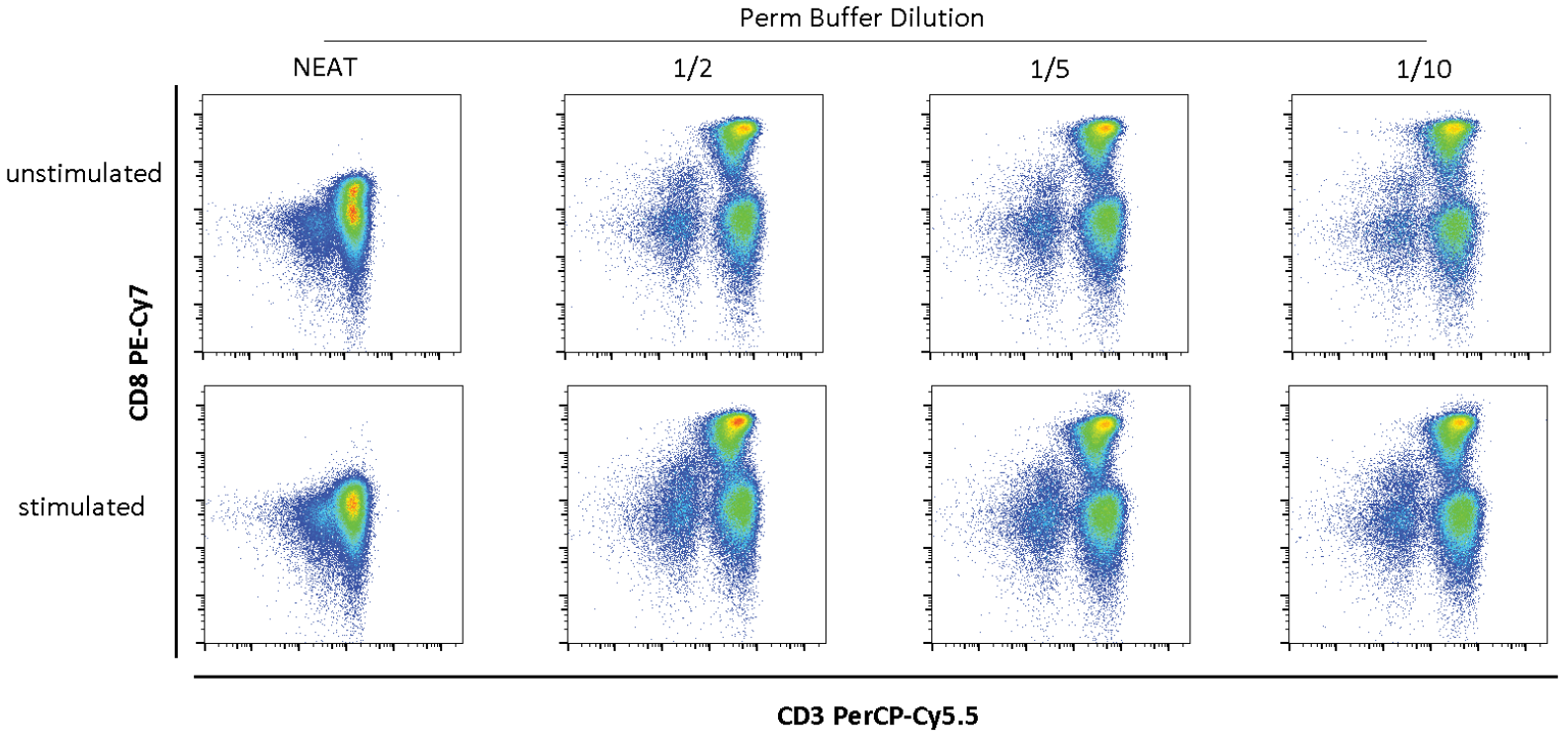


Figure 4.11 Effect of permeabilisation buffer on the detection of surface antibodies
Permeabilisation buffer was diluted to determine if it would improve the detection of problematic surface marker anti-CD8-PE-Cy7. PBMC from a healthy control were either stimulated or not stimulated with IL-6, fixed and permeabilised with varying concentrations (neat, 1:1, 1:2, 1:5 and 1:10 dilution in PBS) of BD Phosflow Perm III buffer. Cells were stained with CD3 and CD8 antibodies to identify the CD8+ T cell population and also p-STAT3 to measure constitutive and induced phosphorylation levels. When neat concentrations of perm buffer were used, positive and negative CD8+ populations were not discernible. Dilutions of the perm buffer resulted in clear distinctions of CD8 positive and negative populations.

4.3.3 Effect of diluted permeabilisation buffer on phospho-protein detection

The dilution of the permeabilisation buffer allowed clear distinction of the CD8+ and CD8- T cell populations in section . However, the effect of diluting the permeabilisation buffer on the ability of cells to be permeabilised sufficiently for the phospho-specific antibody to enter the cell is not known. pSTAT3 was chosen as the phosphorylated protein target as this method was being developed and optimised for IL-6 induced STAT signalling in T cells. As seen in Figure 4.12, PBMCs from a healthy donor were either stimulated or not stimulated with IL-6, fixed and then permeabilised with neat or diluted (1/2, 1/5 and 1/10) permeabilisation buffer before staining with anti-p-STAT3 for 1 h at RT. As a control, cells were not permeabilised. Cells were analysed by flow cytometry and p-STAT3 was measured on CD8+ T cells. With neat concentrations of permeabilisation buffer, detection of CD8+ T cells was significantly impaired but was gated as best as possible for the measurement of pSTAT3 levels after sufficient permeabilisation. After IL-6 stimulation, 68.2% of CD8+ T cells expressed p-STAT3. When the permeabilisation buffer was diluted, this detection of p-STAT3 was negated. These results indicate the permeabilisation buffer cannot be diluted, as this compromises detection of phospho-proteins, even though it improves surface marker detection. Hence, a different method needs to be applied to improve surface marker resolution.

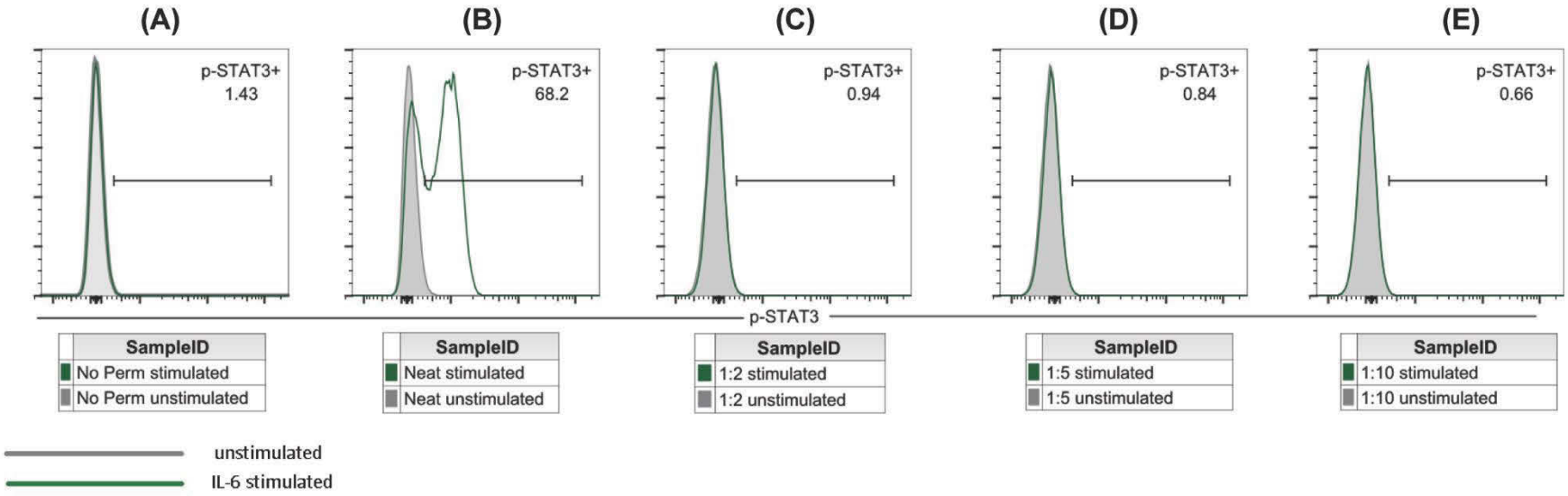


Figure 4.12 Dilution of permeabilisation buffer compromises the ability to detect intracellular phospho-proteins
The ability of diluted permeabilisation buffer to detect p-STAT3 was tested with fresh PBMCs. PBMCs from healthy controls were either stimulated or not stimulated with IL-6, fixed and permeabilised with varying concentrations (neat, 1:1, 1:2, 1:5 and 1:10 dilution in PBS) of BD Phosflow Perm III buffer. Cells were stained with CD3 and CD8 antibodies to identify the CD8+ T cell population and also p-STAT3 to measure constitutive and induced phosphorylation levels. **(A)** As a control, cells were not permeabilised. **(B)** Using neat concentrations of permeabilisation buffer, pSTAT-3 was detectable in 68.2% of cells. **(C-E)** The ability to detect pSTAT3 was abrogated upon dilution of the permeabilisation buffer.

4.3.4 Effect of sequential staining on the detection of CD8+ T cells

Sequential staining has been documented as an alternate method for resolving problematic surface antigens affected by methanol permeabilisation (Krutzik *et al* 2005a). Cells are stained with the antibodies to difficult surface antigens either before or after fixation and prior to subsequent permeabilisation. Sequential staining of anti-CD3 PE-Cy7 and anti-CD3 PerCP-Cy5.5 in section 4.3.1 was performed after fixation but before permeabilisation, however, this modification did not restore surface marker resolution. It appears that these antibodies may be sensitive to both fixation and permeabilisation manipulation and hence, another variation involving staining problematic antibodies prior to fixation and permeabilisation should be trialled. To conserve time, staining of these problematic antibodies can be conducted simultaneously with cytokine stimulation.

Fresh PBMCs from a healthy donor were stained with problematic anti-CD8 PE-Cy7 and simultaneously stimulated or not stimulated with IL-6, followed by fixation, permeabilisation and staining with anti-STAT3. For comparison, cells were treated with the generic phosflow method and anti-CD8 PE-Cy7 was added after permeabilisation. Using the generic phosflow method, resolution of CD8 positive and negative populations was unsuccessful irrespective of stimulation (Figure 4.13A). When cells were sequentially stained for CD8 prior to fixation and permeabilisation, the ability to resolve CD8 populations was rescued (Figure 4.13B).The phosflow method with sequential staining is optimal for the detection of clonal T cell surface markers and phospho-specific markers in T cells and is illustrated in Figure 4.14. This optimised phospho-flow method, as well as other fixation and permeabilisation methods were used to study protein targets in the following signalling pathways in Part 2 of this Results section.

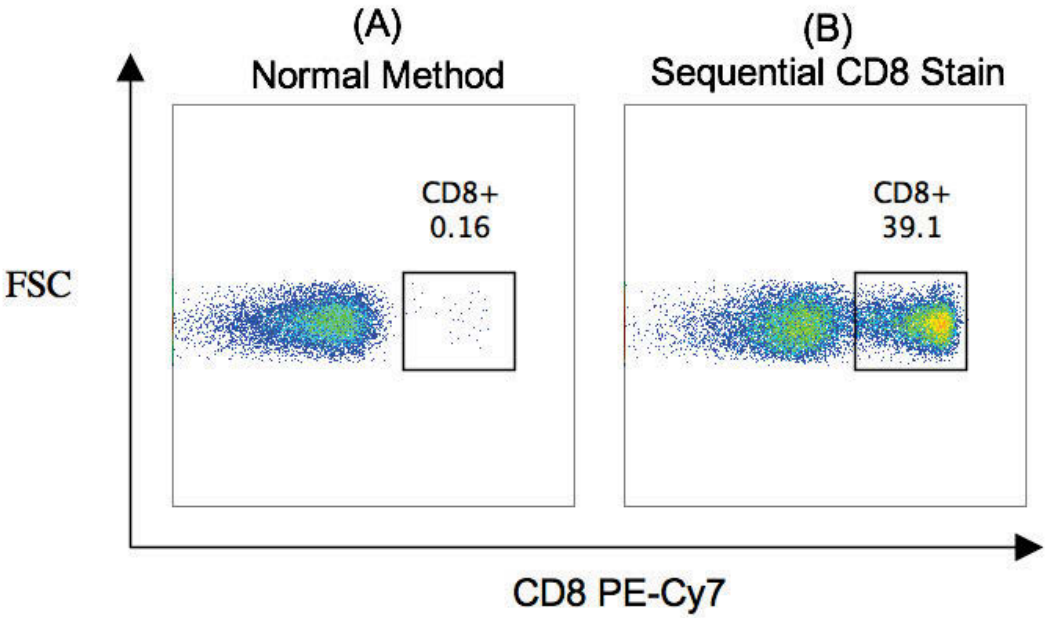


Figure 4.13 The effect of sequential staining on the detection of problematic surface antibodies
PBMC from healthy controls were **(A)** treated with the normal phosflow method and cells were stained with anti-CD8-PE-Cy7 following fixation and permeabilsation. **(B)** Sequential staining of cells with anti-CD8-PE-Cy7, prior to fixation and permeabilisation significantly improves the resolution of this cell surface marker. *FSC: forward scatter*

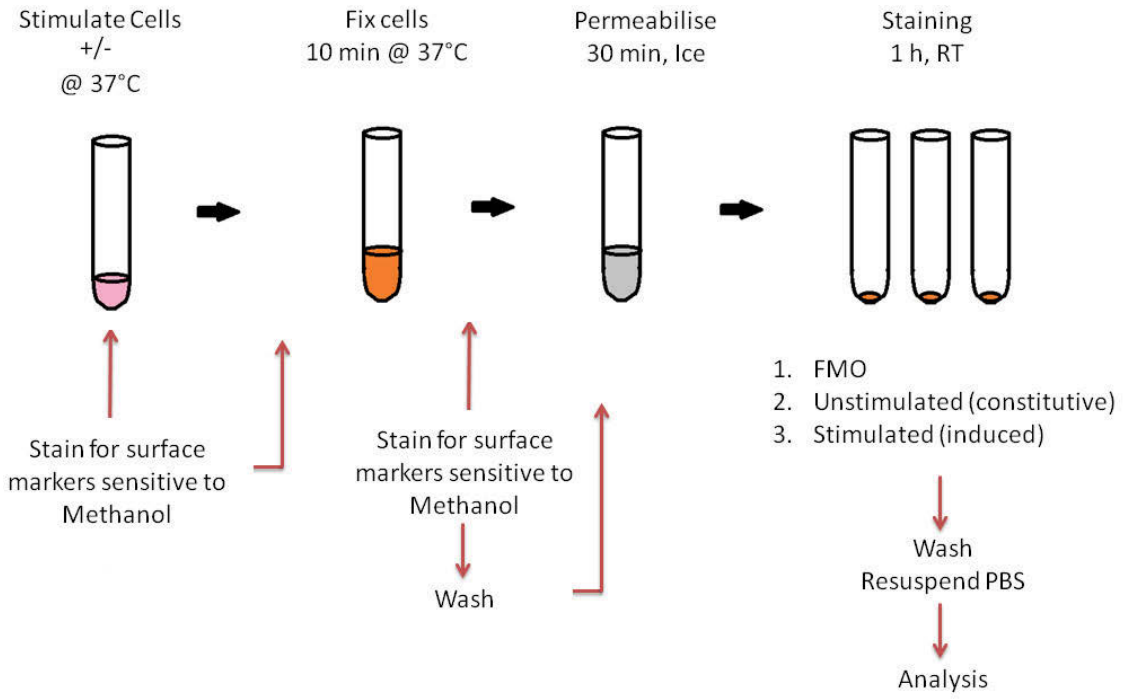


Figure 4.14 Flow diagram of the optimised phospho-flow cytometry method used to detect phosphorylated proteins
PBMC are firstly obtained from whole blood through density gradient centrifugation. The general protocol for detecting intracellular phosphorylated proteins involves firstly stimulating cells at 37°C with cytokines to initiate the signaling cascade. Cells are fixed in BD Cytofix buffer for 10 min at 37°C to capture the phosphorylated state of the cell. Cells are permeabilised with BD Perm III buffer to allow for penetration of phospho-specific antibodies to detect phosphorylated proteins located in the nucleus of the cell. Staining with phospho-specific and other surface marker antibodies requires a 1 h incubation at room temperature. Following 3 washes in staining buffer, cells are resuspended and analysed on the flow cytometer. Variations from this general method are necessary when surface markers are unable to be resolved and cells are sequentially stained with the antibodies to these difficult surface antigens either prior to both fixation and permeabilisation, or after fixation and before subsequent permeabilisation.

PART 2: ANALYSIS OF SIGNALLING PATHWAYS BY PHOSPHO-FLOW‡

4.3.5 Investigation of apoptotic pathways

MM T cell clones persist for long periods of time and this suggests a resistance to apoptosis. The unstimulated and stimulated levels of proteins involved in apoptosis were studied. These included Fas, Fas ligand and Bcl-xL.

4.3.5.1 Optimisation of testing time

To determine the optimal time of testing for proteins related to apoptosis (Fas, Fas-ligand and Bcl-xL), PBMC from a healthy donor were stimulated or not stimulated with anti-CD3/28 beads at a 1:1 ratio in a 37°C + 5% CO_2 incubator for up to 4 days. The levels of these proteins on CD8+CD57+ T cells were measured following 1, 2, 3 and 4 days of culture with beads. Baseline levels of these proteins were also measured on the day of blood collection from patients, immediately after isolation of PBMC by Ficoll (Day 0). Fas levels of stimulated cells remained consistent over the first 2 days, and were not different from baseline levels and then declined when cells were cultured for more than 3 days (Figure 4.15A). As the results for the first 2 days are similar to baseline levels, Fas expression can be detected on day 0, without the need for cell culture. Low levels of Fas-ligand were detected on cells but this level peaked on the second day in both unstimulated and stimulated cells, and then declined (Figure 4.15B). Similar patterns of expression were also observed for Bcl-xL expression (Figure 4.15C). Based on these results, 2 days of culture with anti-CD3/28 beads was chosen as the optimal time of testing for Fas-ligand and Bcl-xL.

‡ The main results of this chapter were previously published in **Suen, H**., Brown, R., Yang, S., Weatherburn, C., Ho, P.J., Woodland, N., Nassif, N., Barbaro, P., Bryant, C., Hart, D., Gibson, J. & Joshua, D. (2016) Multiple myeloma causes clonal T cell immunosenescence: Identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade. *Leukemia***,** epub ahead of print.

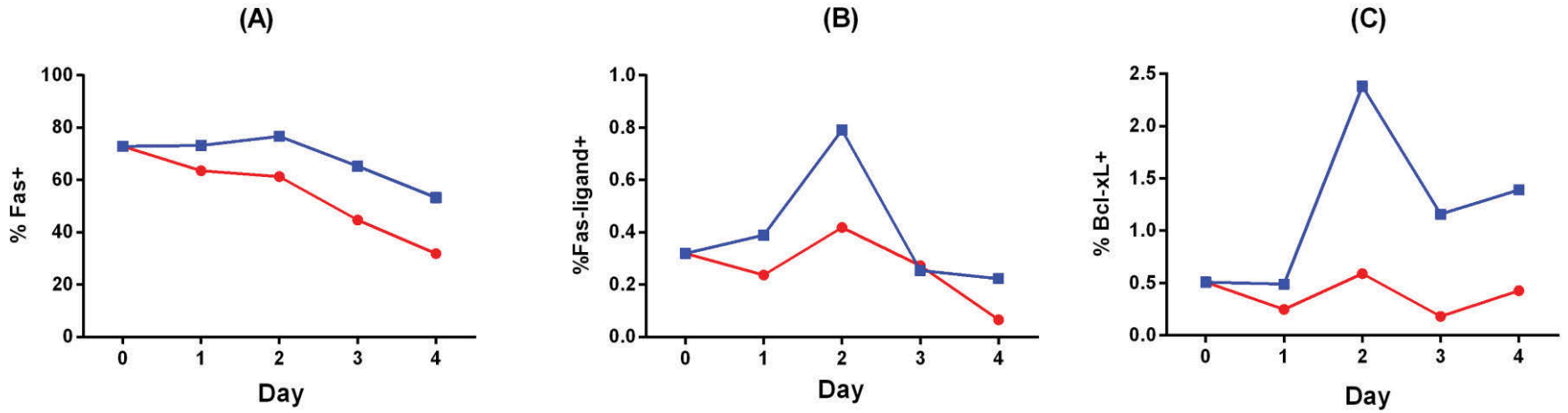


Figure 4.15 Detection of apoptotic proteins Fas, Fas-ligand and Bcl-xL after bead stimulations for up to 4 days
To determine the optimal time of testing for the apoptotic proteins of interest, PBMC from a healthy donor were stimulated (blue) or not stimulated (red) with anti-CD3/28 beads at a 1:1 ratio for up to 4 days. The expression of apoptotic proteins was measured on CD8+CD57+ T cells. Cells were stained with **(A)** anti-Fas-APC and **(B)** anti-Fas-ligand-PE for the detection of surface expression. **(C)** For the detection of Bcl-xL, cells were treated with leucoperm before intracellular staining with anti-BCL-xL-PE. Data are presented as % of cells expressing the protein.

4.3.5.2 Fas/Fas ligand levels are not upregulated in MM T cell clones

Fas was expressed at high levels on MM T cell clones and these levels were not different from non-clonal T cells nor other T cell subsets (Figure 4.16). There was no significant difference in unstimulated or stimulated levels of Fas ligand (2 days stimulation with anti-CD3/28 beads) between T cell clones and non-clonal T cells (Figure 4.17). As the levels of Fas and Fas ligand are not different from that of other CD8+ T cells in MM patients, it is unlikely that the Fas/Fas ligand pathway is responsible for their resistance to apoptosis and the long-term persistence of these cells.

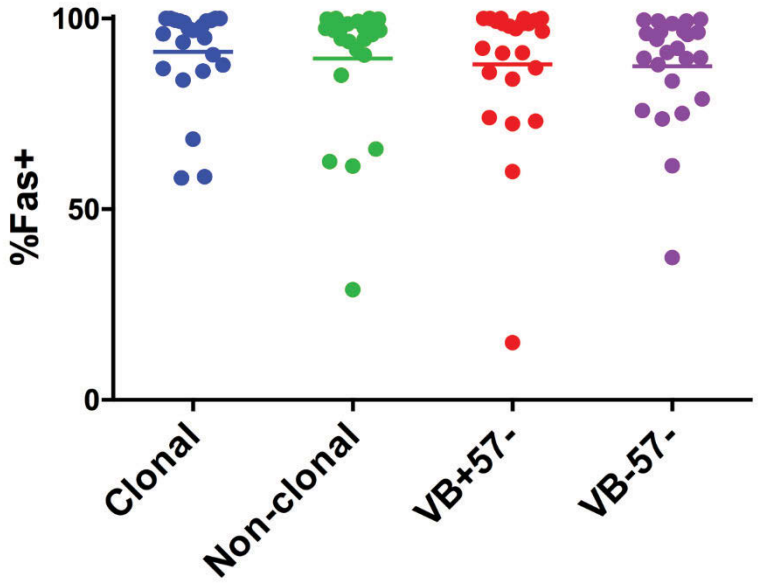


Figure 4.16 Fas expression on unstimulated T cells from MM patients
PBMC were obtained from patients with MM (n=24) and Fas expression was measured on untreated CD8+ T cell subsets by flow cytometry. Data are presented as % of cells expressing Fas. p=NS.

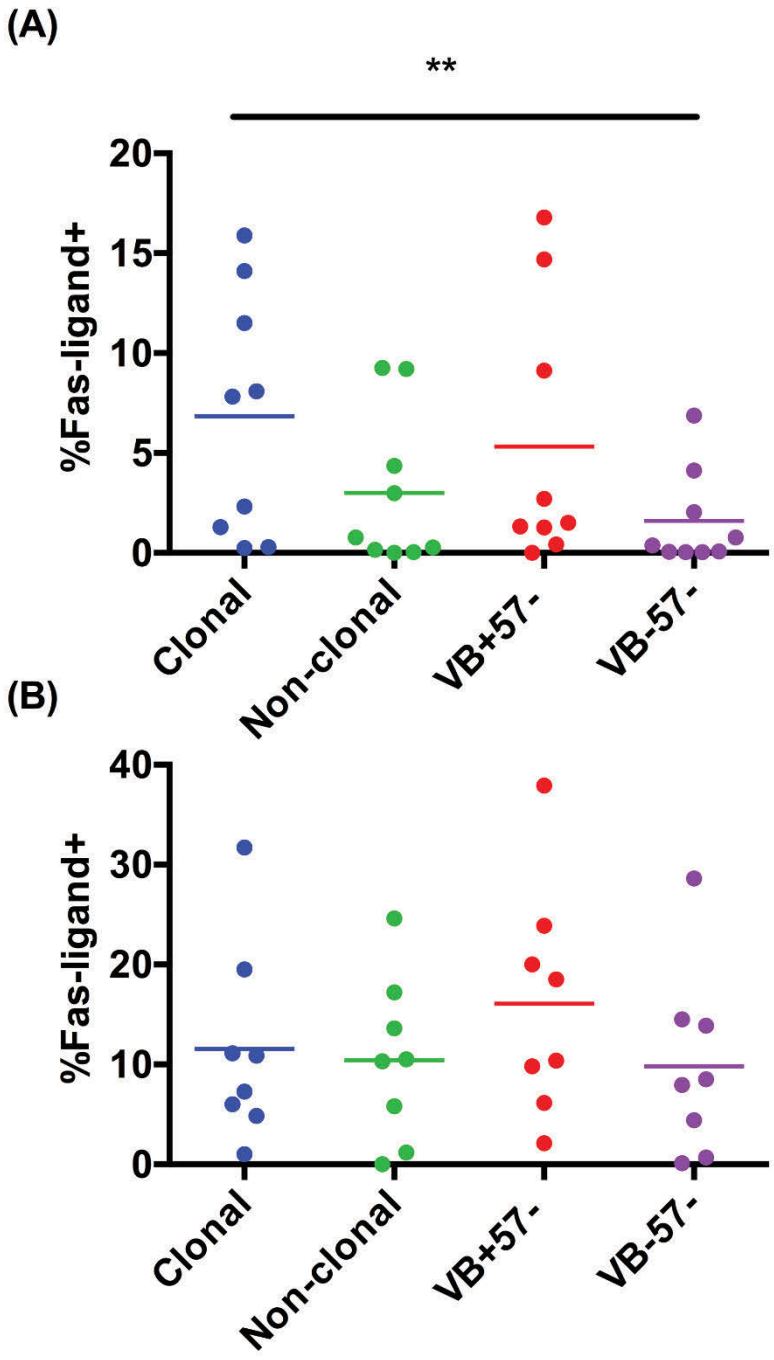


Figure 4.17 Fas ligand expression on T cells after 2 day bead stimulations

PBMC were obtained from patients with MM (n=9) and either cultured with **(A)** IL-2 alone or stimulated with **(B)** IL-2 and anti-CD3/28 beads at a 1:1 ratio for 2 days. Cells were harvested following 2 days of culture and Fas-ligand expression was measured on CD8+ T cell subsets by flow cytometry. Data are presented as % of cells expressing Fas-ligand. **p<0.01.

4.3.5.3 Anti-apoptotic protein Bcl-xL is not highly expressed in T cell clones

Bcl-xL is an anti-apoptotic protein from the Bcl-2 family of anti-apoptotic proteins (Boise *et al* 1993). Both constitutive and stimulated Bcl-xL levels on T cell clones were not different from other non-clonal T cells (Figure 4.18). There was a trend for Bcl-xL levels to be higher in T cell clones from 10 year and non-10 year MM survivors when compared to CD8+CD57+ T cells from age matched normal controls but this was not statistically significant. (Figure 4.19). Therefore, the anti-apoptotic protein Bcl-xL was not aberrantly expressed and is most likely not responsible for apoptotic resistance in MM T cell clones.

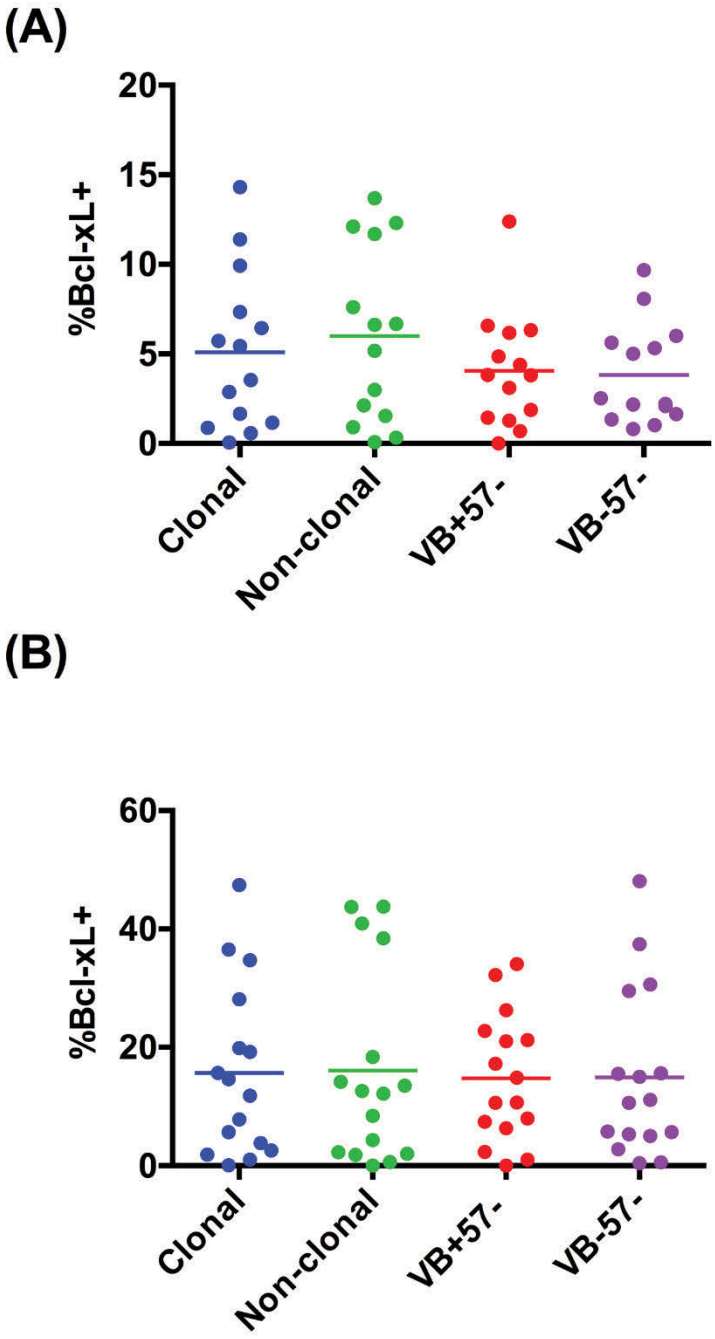


Figure 4.18 Bcl-xL expression on CD8+ T cells after 2 day bead stimulations

PBMC were obtained from patients with MM (n=14) and either cultured with **(A)** IL-2 alone or stimulated with **(B)** IL-2 and anti-CD3/28 beads at a 1:1 ratio for 2 days. Cells were harvested following 2 days of culture and then fixed and permeabilised with leucoperm. Bcl-xL expression was measured on CD8+ T cell subsets by flow cytometry. Data are presented as % of cells expressing Bcl-xL.

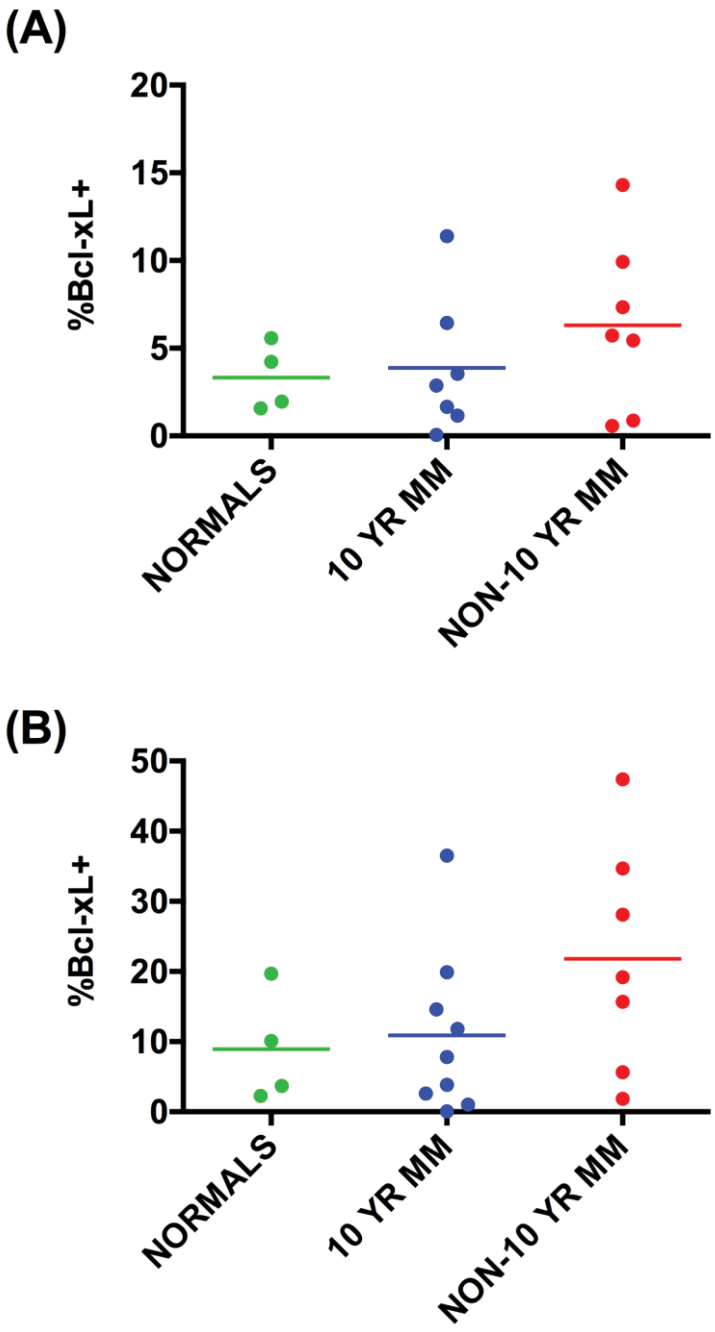


Figure 4.19 Bcl-xL expression on T cell clones from 10 year and non-10 year survivors of MM

PBMC were obtained from patients and either cultured with **(A)** IL-2 alone (constitutive expression) or stimulated with **(B)** IL-2 and anti-CD3/28 beads at a 1:1 ratio for 2 days. Cells were harvested following 2 days of culture and then fixed and permeabilised with leucoperm. Bcl-xL expression was measured on T cell clones from 10 year (n=7) and non-10 year (n=7) MM survivors and compared to age-matched normal controls (n=4). Data are presented as % of cells expressing Bcl-xL. p=NS

4.3.6 Investigation of the TGF-β-SMAD signalling pathway

To investigate whether TGF-β induced phosphorylation of SMAD was involved in inducing T cell inactivation, constitutive and induced (PMA and TGF-β stimulation) pSMAD levels were measured in MM T cell clones.

4.3.6.1 MM T cell clones have a defective p-SMAD pathway that blocks cell cycle progression

Expression of p-SMAD did not differ between the different CD8+ T cell subsets when all MM patients were analysed together, irrespective of no stimulation (constitutive expression) or stimulation with PMA or TGF-β (Figure 4.20). However, significantly higher levels of constitutive p-SMAD were found in T cell clones of non-10-year survivors, which have previously been shown to be non-proliferative, when compared to T cell clones of 10-year MM survivors that are proliferative (U=7; p=0.01; Figure 4.21A). T cell clones from 10 year survivors were better able to upregulate p-SMAD after both PMA and TGF-β stimulation, suggesting that this pathway is still responsive to stimuli, unlike the T cell clones of non-10 year survivors who may have a constitutively activated pathway, incapable of being activated further (Figure 4.21B,C). This suggests that p-SMAD may be a potential novel target involved in regulating cell proliferation of T cell clones and is a possible candidate for reversing clonal T cell dysfunction.

4.3.6.2 Patient demographics for the p-SMAD cohort

The clinical characteristics of patients were collected to determine if higher p-SMAD levels detected in hypo-responsive T cell clones of non-10 year survivors was related to treatment or disease status (Table 4.2). There were patients actively receiving treatment that included conventional therapy, IMiDs and proteasome inhibitors, whilst others were not receiving treatment. 13/14 patients had received prior IMiDs therapy at some stage of their disease. There were patients with stable disease, progressive disease or in remission. High or low pSMAD levels were not related to disease or therapy. However, due to the heterogeneity of this cohort and the small sample cohort, statistical analysis was not conducted.

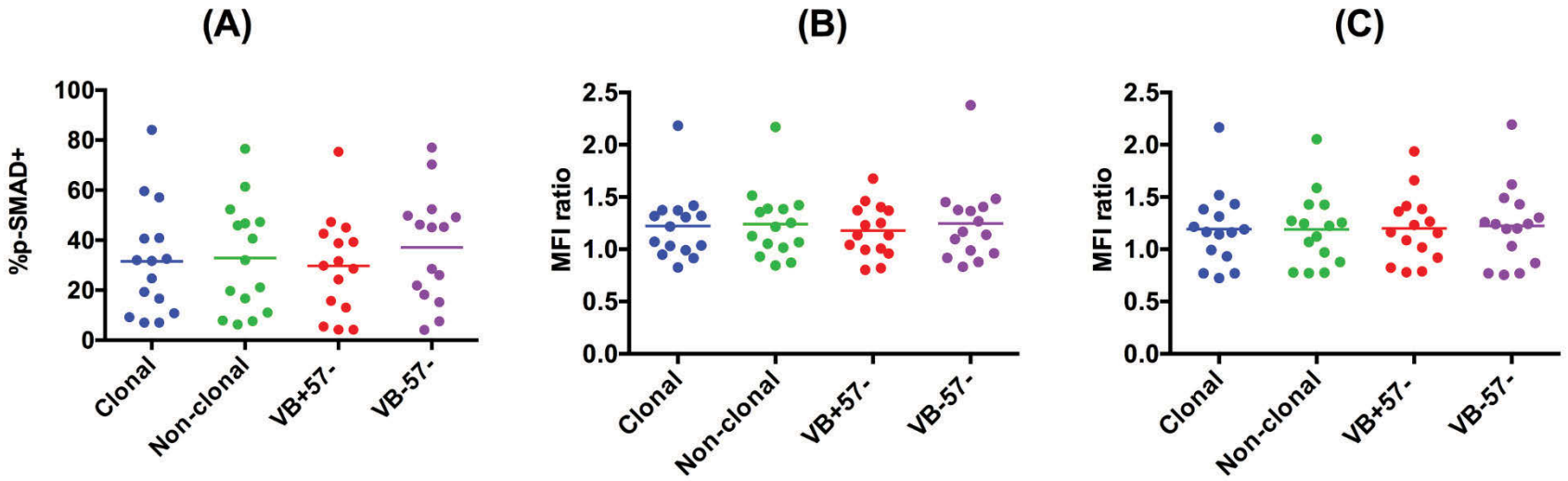


Figure 4.20 p-SMAD expression on MM T cell clones

PBMC from MM patients (n=10) were serum starved overnight by culturing in RPMI + 0.1% FCS. Cells were **(A)** not stimulated (constitutive) or stimulated with **(B)** PMA (150 nM) or **(C)** TGF-β (100 ng/mL) for 30 min before fixation and permeabilisation with the BD Phosflow method. p-SMAD expression was measured on CD8+ T cell subsets by phospho-flow cytometry. Data are presented as % of cells expressing p-SMAD for constitutive expression and as MFI ratio (MFI of stimulated/MFI of unstimulated) for PMA and TGF-β stimulated samples for induced expression. p=NS.

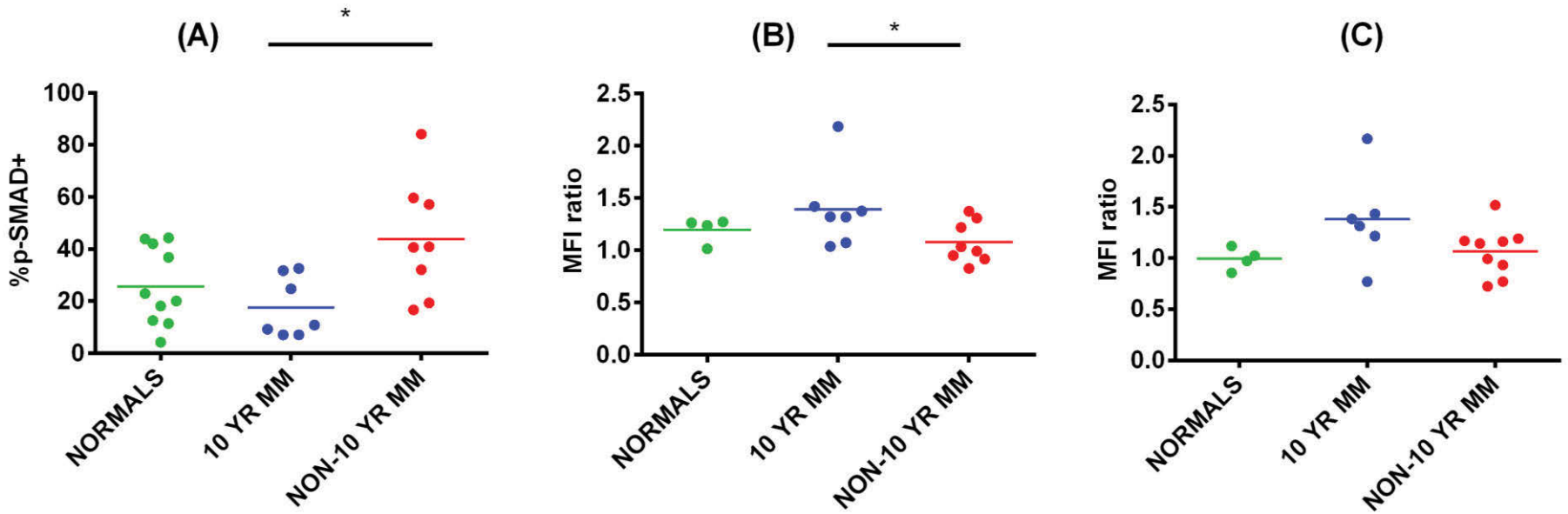


Figure 4.21 p-SMAD expression on MM T cell clones from 10 year and non-10 year survivors of MM
PBMC from MM patients were serum starved overnight by culturing in RPMI + 0.1% FCS. Cells were **(A)** not stimulated (constitutive) or stimulated with **(B)** PMA (150 nM) or **(C)** TGF-β (100 ng/mL) for 30 min before fixation and permeabilisation with the BD Phosflow method. p-SMAD expression was measured on T cell clones from 10 year (n=7) and non-10 year MM survivors (n=9) and CD8+CD57+ T cells from normal controls (constitutive: n=10; stimulated: n=4) by phospho-flow cytometry. Data are presented as % of cells expressing p-SMAD for constitutive expression and as MFI ratio (MFI of stimulated sample/MFI of unstimulated sample) for PMA or TGF-β stimulated cells for induced expression. *$p<0.05$.

Table 4.2 Demographics of MM patients studied for p-SMAD expression

Patient	10 year survivor	Paraprotein	ISS (at diagnosis)	Prior Transplant	Active treatment at time of testing	Disease state
1*	No	IgG K	I	Auto	D-PACE	Relapse
2	No	LLC	unknown	Auto, Allo	Vel/Dex	Relapse
3	No	LLC	I	Auto	Len/Dex	Partial response
4	No	IgA L	unknown	Auto	Car/Dex	Partial response
5	No	IgA/G K	I	Auto	None	Complete remission
6	No	IgG	I	Auto	Len/Dex	Stable disease
7	No	IgG K	I	Auto	Thal	Complete remission
8	No	IgG K	unknown	Auto	PCAB	Relapse
9	Yes	IgA K	I	NIL	None	Relapse
10	Yes	IgG K	I	Auto x2	Pom	Partial response
11	Yes	IgG L	I	NIL	Thal	Partial response
12	Yes	IgG K	I	NIL	Len	Complete remission
13	Yes	IgA L	I	Auto, Allo	None	Complete remission
14	Yes	IgG K	I	Auto	None	Relapse

Allo: allogeneic stem cell transplant; Auto: autologous stem cell transplant; Car: carfilzomib; CyBORD: cyclophosphamide, bortezomib and dexamethasone; Dex: dexamethasone; D-PACE: dexamethasone, cisplatin, doxorubicin, cyclophosphamide and etoposide; Ig: immunoglobulin; ISS; International Staging System; K: kappa; L: lambda; LC: light chain; Len: lenalidomide; PCAB: prednisone, cyclophosphamide, BCNU, doxorubicin; Pom: pomalidomide; Vel: velcade; *patient had 2 different clonal expansions. Adapted from Suen *et al* 2016.

4.3.7 Investigation of the ERK Proliferation pathway

p-ERK levels are normally responsible for inducing proliferation of cells and can be dephosphorylated by HePTP or PTPN7, resulting in an anti-proliferative state. To determine if non-proliferative T cell clones had a blocked ERK pathway, constitutive and PMA-induced p-ERK levels were measured by phospho-flow cytometry.

4.3.7.1 Optimisation of PMA concentration for T cell stimulation and detection of p-ERK

To determine the optimal PMA concentration for the stimulation of whole blood samples to induce p-ERK, whole blood from a healthy control was stimulated with varying concentrations of PMA for 10 minutes at 37°C. The p-ERK levels detected on CD4+ and CD8+ T cells are shown in Figure 4.22. The optimal PMA concentration was 150 nm as this concentration allowed for upregulation of p-ERK as compared to an unstimulated sample but was not the maximum level that could be achieved, allowing us to discern differences in p-ERK levels between different T cell subsets.

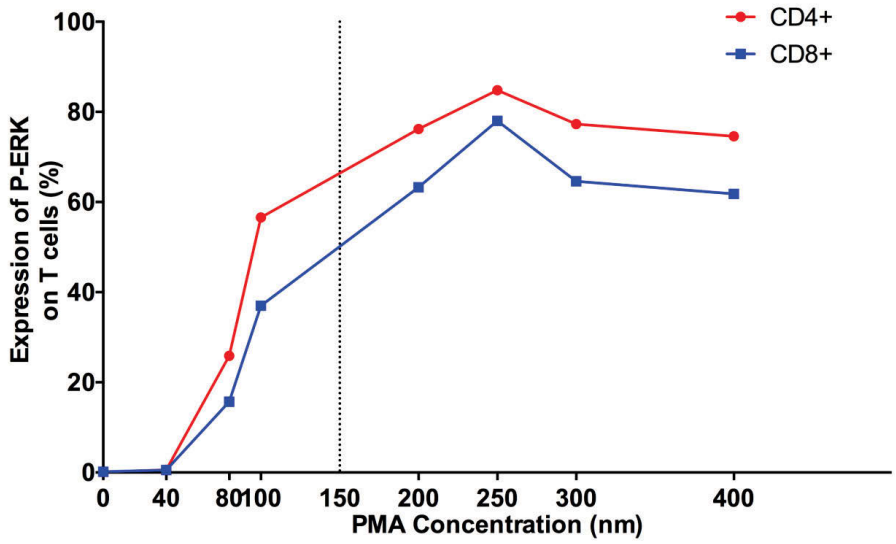


Figure 4.22 Optimisation of PMA concentration for the detection of p-ERK in T cells from a healthy control
Whole blood (100 µL) was stimulated with varying concentrations of PMA (40, 80, 100, 200, 250, 300, 400 nM) for 10 minutes at 37°C. Cells were then fixed and permeabilised using the Perfix-P kit. CD4+ and CD8+ T cells were analysed for p-ERK expression. Controls included non-stimulated samples and were used to determine positive expression limits for p-ERK. Data are represented as % of T cells expressing p-ERK.

4.3.7.2 p-ERK expression on MM T cells

The expression of constitutive and stimulated p-ERK levels (after 150 nM PMA, 10 min stimulation @ 37°C) was measured on MM patients with T cell clones and normal controls. There was no significant difference in both constitutive and PMA induced p-ERK expression observed between T cell clones and other CD8 subsets (Figure 4.23). When T cell clones were split into 10 year and non-10 year groups, there were no differences in constitutive p-ERK levels between the two groups and also when compared to age-matched normal controls. Whilst there was also no difference in induced p-ERK levels between 10 year and non-10 year groups, the ability of T cell clones to up-regulate p-ERK after PMA stimulation was impaired in both groups in comparison to normal controls (U= 16; p=0.03; U=7; p=0.03, respectively) and more so in the non-10 year group (Figure 4.24). This suggests that the ERK proliferation pathway is inhibited in MM patients, and as the pathway appears to be inhibited at higher levels in the non-10 year MM group, this may in part be responsible for the hypo-responsiveness that is observed in this group.

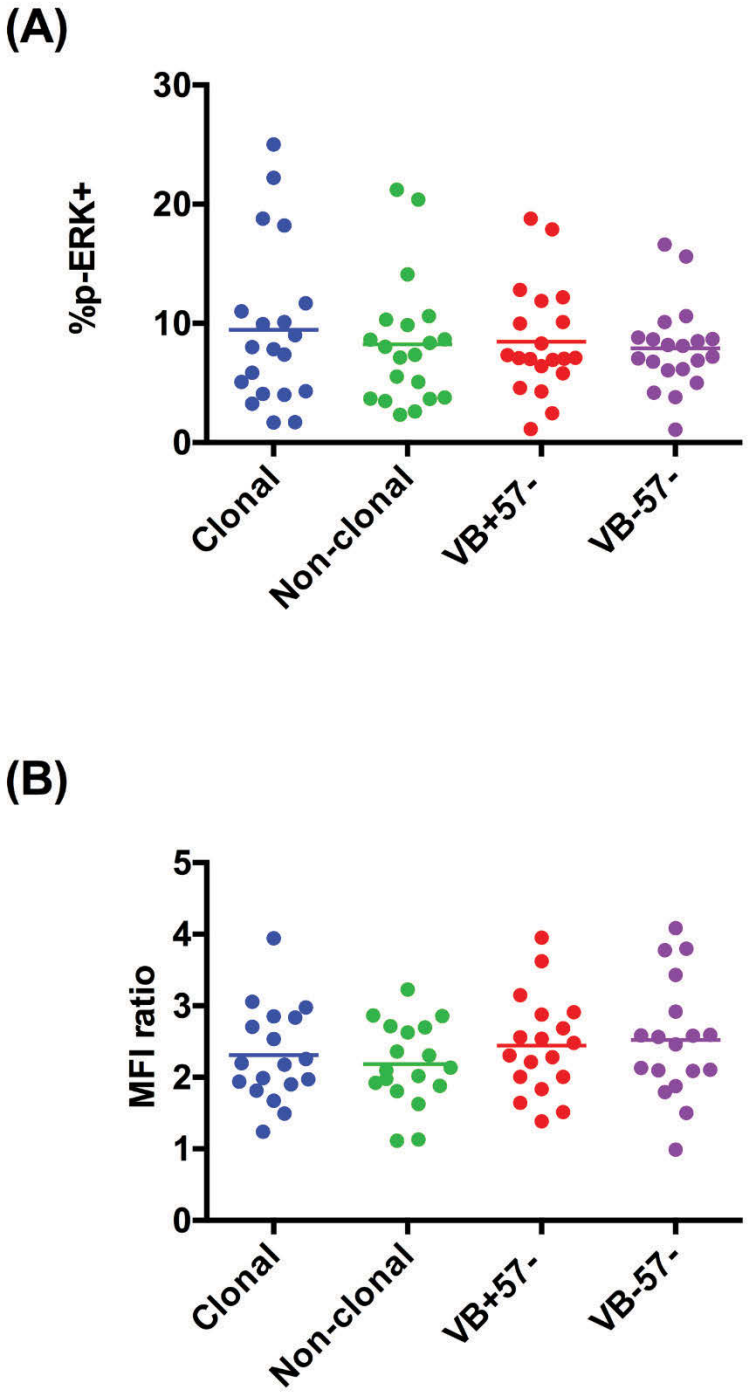


Figure 4.23 p-ERK expression on MM T cell clones
PBMC from MM patients (n=20) were either stimulated or not stimulated with PMA (150 nM) for 10 minutes before fixation and permeabilisation using the Beckman Perfix-P kit. **(A)** Constitutive p-ERK expression and **(B)** MFI ratio (MFI stimulated/MFI unstimulated) was measured on CD8+ T cell subsets. Data are presented as % cells expressing p-ERK for constitutive expression and as MFI ratio (MFI of stimulated/MFI of unstimulated) for induced expression. p=NS.

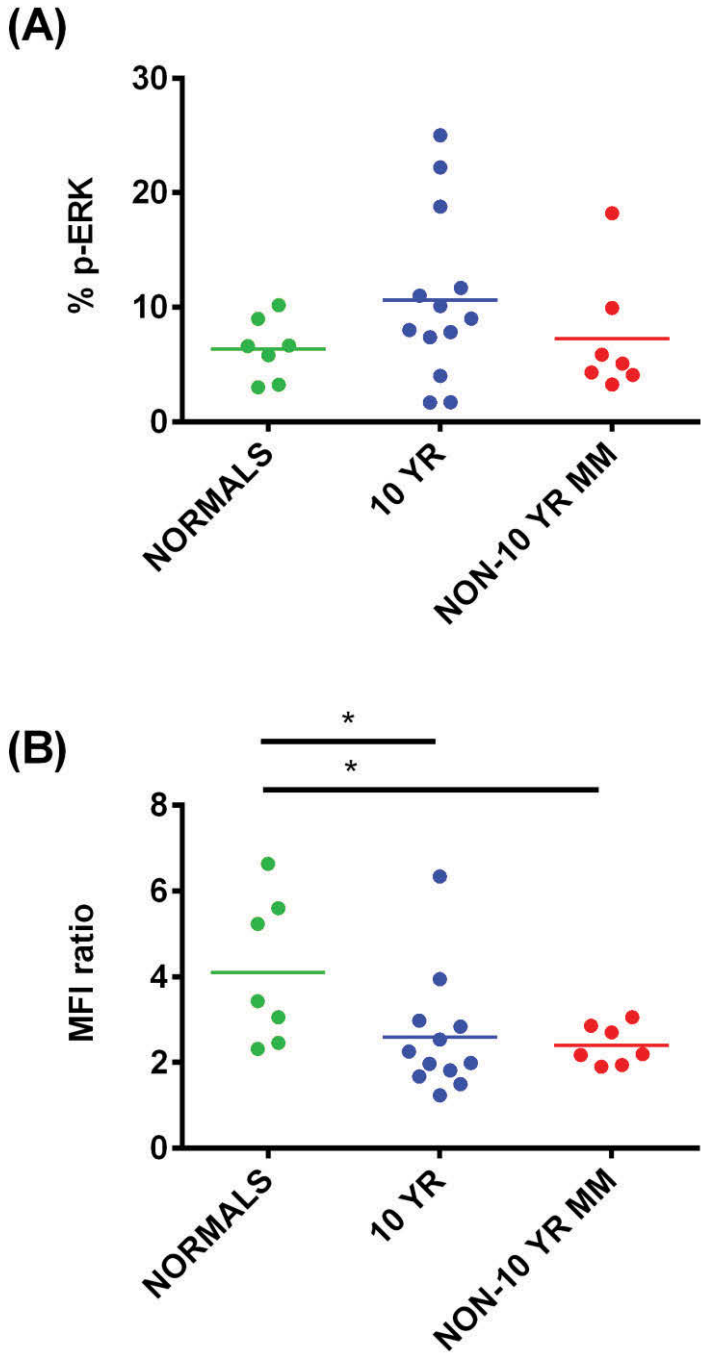


Figure 4.24 p-ERK expression on MM T cell clones from 10 year and non-10 year MM survivors

PBMC from MM patients were either stimulated or not stimulated with PMA (150 nM) for 10 minutes before fixation and permeabilisation using the Beckman Perfix-P kit. **(A)** Constitutive p-ERK expression and **(B)** MFI ratio (MFI stimulated/MFI unstimulated) was measured on T cell clones from 10 year (n=10) and non-10 year (n=7) MM survivors and compared to age-matched normal controls (n=7). Data are presented as % cells expressing p-ERK for constitutive expression and as MFI ratio (MFI of stimulated/MFI of unstimulated) for induced expression. *p<0.05.

4.3.8 Investigation of the TCR-signalling pathway

Immune dysfunction in cancer may also be attributed to down-regulation of TCR signalling proteins (Mizoguchi *et al* 1992) resulting in defective TCR activation and downstream effects. To determine if hypo-responsiveness of T cell clones was related to defective TCR signalling, the levels of the proteins CD3-ζ chain and ZAP-70 were quantified. These two proteins are crucial for initial activation of the TCR (Au-Yeung *et al* 2009, Guy and Vignali 2009). SHP-2, which has an inhibitory effect on TCR signalling, was also measured (Qu 2000).

4.3.8.1 CD3-ζ chain expression on MM T cell clones

CD3-ζ chain expression was significantly higher on clonal T cells compared to non-clonal T cells (t=2.36; p<0.02) and Vβ-CD57- cells (t=2.32; p=0.03) but was not significantly different from normal controls, suggesting that CD3-ζ chain levels were adequate in the T cell clones (Figure 4.25).

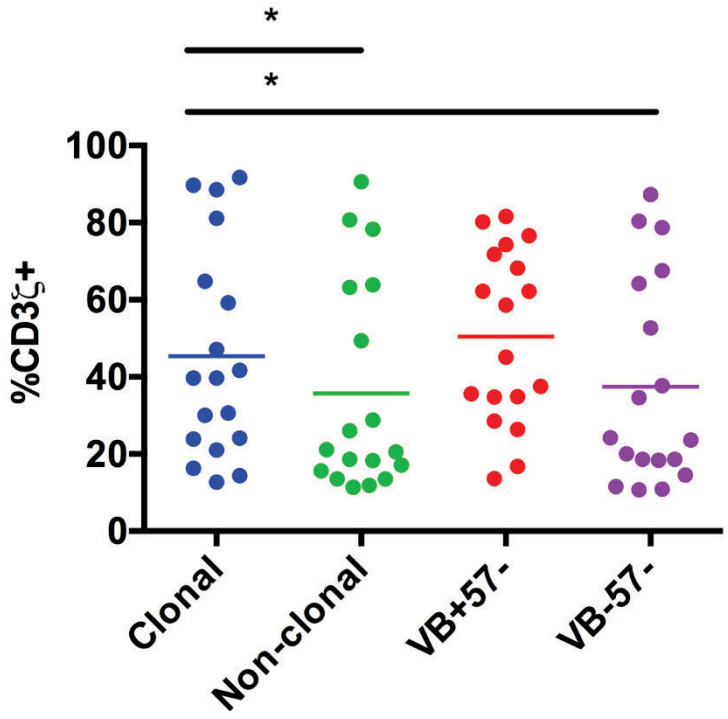


Figure 4.25 CD3- ζ chain expression on MM T cells and T cells from normal controls
PBMC were fixed and pemeabilised using the Cytofix/cytoperm method. CD3-ζ chain expression was measured on CD8+ T cell subsets from MM patients (n=19) and age-matched normal controls (n=18). Data are presented as cells expressing CD3-ζ chain. *p<0.05.

4.3.8.2 ZAP-70 expression on MM T cell clones

Both constitutive and stimulated p-ZAP-70 expression (after 5 mM hydrogen peroxide stimulation) were present at similar levels in MM T cell clones compared to other CD8+ T cell subsets (Figure 4.26), suggesting that ZAP-70 is not deficient in T cell clones.

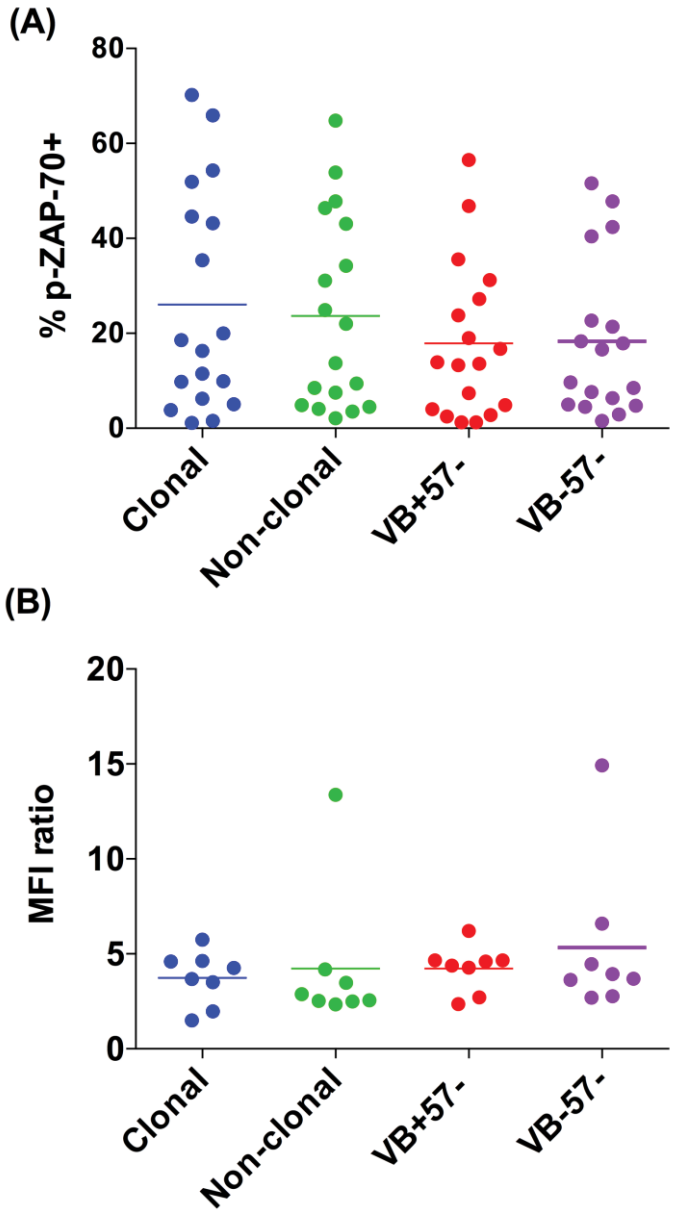


Figure 4.26 p-ZAP-70 expression on MM T cells and T cells from normal controls

PBMC from MM patients (n=20) were either stimulated or not stimulated with 5 mM hydrogen peroxide before fixation and permeabilisation with the BD cytofix/cytoperm method. **(A)** Constitutive p-ZAP-70 expression and **(B)** MFI ratio (MFI stimulated/MFI unstimulated) was measured on CD8+ T cell subsets. Data are presented as % cells expressing p-ZAP=70 for constitutive expression and as MFI ratio (MFI of stimulated/MFI of unstimulated) for induced expression. p=NS.

4.3.8.3 Optimisation of bead concentration for the detection of induced SHP-2 expression

To measure the induced SHP-2 expression in T cells, cells from a MM patient were stimulated with anti-CD3/28 beads to mimic TCR ligation. The bead to cell ratios: 1:1, 1:5, 1:20, 1:50 or 1:100 were used to stimulate cells and p-SHP-2 expression was measured in T cell clones and other CD8+ T cell subsets. As shown in Figure 4.27, there was an initial up-regulation in p-SHP-2 expression when 1:1 beads were used before a plateau in expression, followed by a second up-regulation when 1:20 beads were used, which also resulted in maximal p-SHP-2 expression for all subsets. Further increases in bead:cell ratios did not augment p-SHP2 levels. There were differences in the 4 subsets at all bead concentrations tested and in order to determine the maximal p-SHP-2 expression possible, the 1:20 bead to cell ratio was chosen as the optimal concentration for testing.

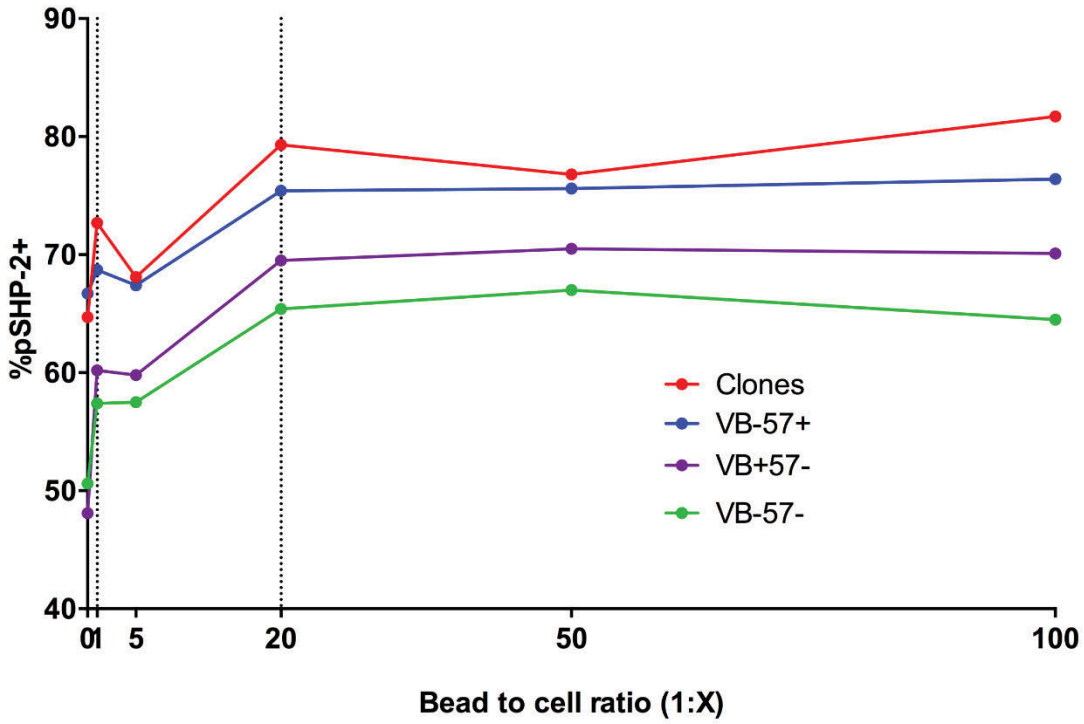


Figure 4.27 Determination of optimal bead concentration for the induction of p-SHP-2 expression in CD8+ T cell subsets
PBMC from a heathy control were stimulated with anti-CD3/28 beads at a bead to cell ratio of 1:1, 1:5, 1:20, 1:50 and 1:100 before fixation and permeabilisation using the BD Phosflow method. p-SHP-2 expression was measured in T cell clones and other CD8+ T cell subsets. Data are presented as % of cells expressing p-SHP-2 after stimulation.

4.3.8.4 SHP-2

As seen in Figure 4.28, constitutive p-SHP-2 levels in T cell clones were significantly higher than that of the Vβ+CD57- subset (t=2.23; p=0.04) and the Vβ-CD57- subset (t=3.47; p=0.003) but was not significantly different from the Vβ-CD57+ non-clonal comparison subset (t=1.89; p=NS). Following stimulation, T cell clones upregulated p-SHP-2 levels at similar levels to other CD8+ T cell subsets. This suggests p-SHP-2 mediated down-regulation of T cell signaling is not present in activated T cell clones.

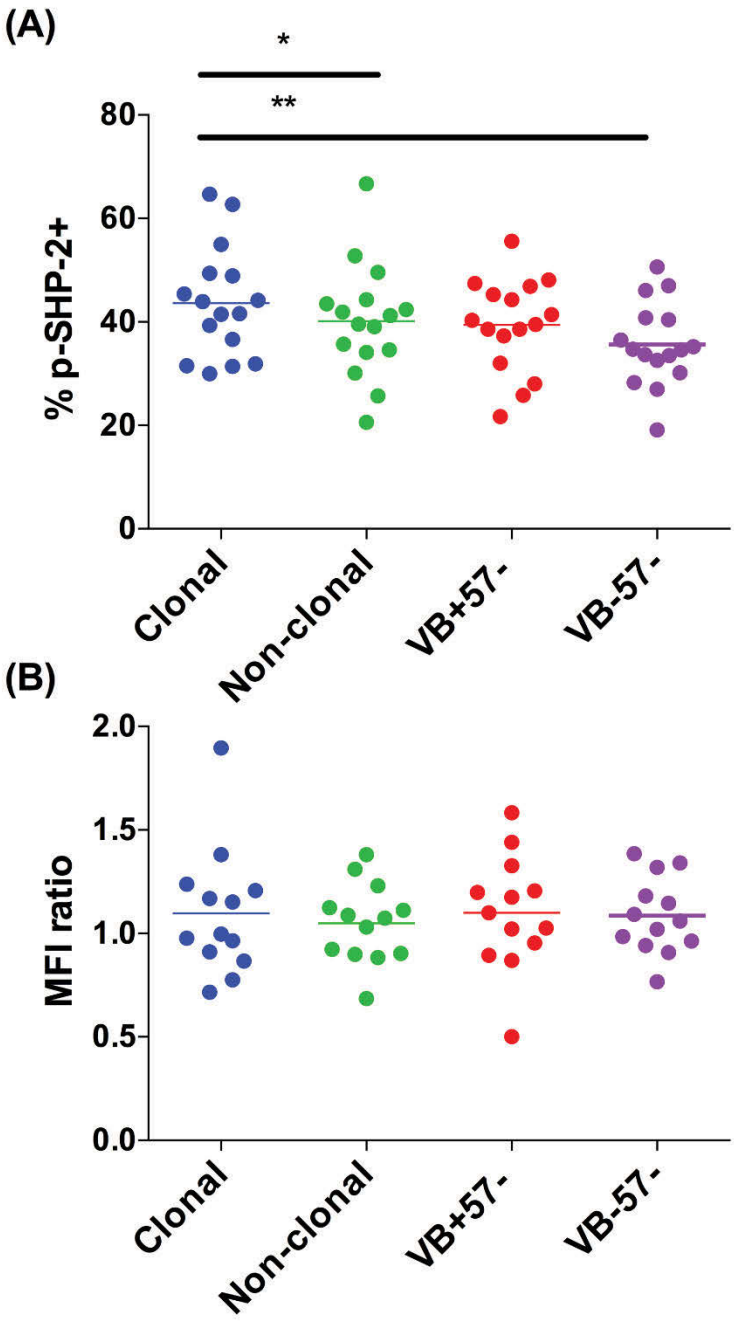


Figure 4.28 p-SHP-2 expression on MM T cells and normal controls
PBMC from MM patients (n=16) were either stimulated or not stimulated with anti-CD3/28 beads at a bead to cell ratio of 1:20 before fixation and permeabilisation with the BD Phosflow method. **(A)** Constitutive p-SHP-2 expression and **(B)** MFI ratio (MFI stimulated/MFI unstimulated) were measured on CD8+ T cell subsets. Data are presented as % cells expressing p-SHP-2 for constitutive expression and as MFI ratio (MFI of stimulated/MFI of unstimulated) for induced expression. *p<0.05, **p<0.01.

4.4 Discussion

Dysregulated signal transduction is a hallmark of malignant cells and plays a role in the uncontrolled proliferation observed in malignant cells. Signal transduction of immune cells may also be dysregulated as a result of tumour suppressive mechanisms that can cause cytotoxic T cells to lose their function. Previous work from our laboratory demonstrated dysregulated signalling pathways in the T cell clones of WM patients and suggested T cell clones displayed anergic properties. (Li *et al* 2010) These observations led to the investigation of these same signalling pathways in MM T cell clones in this study. A number of intracellular staining methods, including a newly developed and optimised phospho-flow assay for detection of intracellular signalling proteins, were used. Using these methods, apoptotic, TGF-β and proliferation pathways were examined to determine if hypo-responsive T cell clones exhibited anergic properties like the T cell clones of WM patients. In addition, the TCR signalling pathway was examined to determine if T cell clones had any defects in TCR signalling. Comparison of target proteins in these pathways between clonal and non-clonal T cells did not reveal aberrant expression levels of Fas, Fas-ligand, Bcl-xL, p-SMAD, p-ERK, CD3-ζ, ZAP-70 and SHP-2 proteins. However, upon comparison of 10 year and non-10 year survivors of MM, significantly higher levels of p-SMAD were detected in hypo-responsive T cell clones of non-10 year survivors when compared to the responsive T cell clones of 10 year survivors. In addition, hypo-responsive T cell clones from non-10 year survivors had an impaired ability to upregulate ERK after mitogen stimulation, when compared to 10 year survivors and normal controls, suggesting that this pathway is defective. This is the first study to successfully identify aberrant or impaired signalling pathways that may explain how T cell clones are inhibited. Therefore, SMAD and ERK are potential novel targets for reversing clonal T cell dysfunction.

MM T cell clones are long-lived cells but do not proliferate *in vitro,* so this naturally suggests a resistance to apoptosis. This led to the investigation of the apoptotic pathways including Fas and Fas ligand and the anti-apoptotic protein, Bcl-xL. These pathways were also found to be upregulated in WM T cell clones

(Li *et al* 2010). This chapter shows ubiquitous expression of Fas expression on clonal T cells and other CD8+ T cell subsets in MM patients. In another study, higher Fas levels were reported on T cells from MM patients compared to controls, however this was examined on total CD3+ T cells and did not examine individual CD4+ or CD8+ T cell subsets (Massaia *et al* 1995). The numerical levels reported (mean=63% of T cells) were also lower than those detected in the current study (mean=91% of T cell clones), which may be attributed to the fact that the current study is interrogating a more specific and smaller T cell subset. The elevated Fas levels in the earlier study also caused the cells to be more sensitive to apoptosis but this was reversible with the addition of IL-2 (Massaia *et al* 1995). However, these are unlikely to be the same as MM T cell clones, which persist for long periods of time and display anti-apoptotic features, but are not IL-2 responsive. This was demonstrated by the lack of proliferation in response to IL-2 in Chapter 3 of this thesis.

Fas ligand is not normally expressed on resting, unstimulated cells and activation of T cells results in the upregulation of Fas-ligand. Re-stimulation of T cells through the TCR can induce a phenomenon known as activation induced cell death (AICD), which is mediated through Fas and Fas ligand interactions. This occurs on pre-activated T cells, where re-stimulation leads to the expression of Fas-ligand, that can bind to Fas receptors on the same cell or neighboring cells, resulting in AICD (Alderson *et al* 1995). AICD is also used to stop T cell expansion following elimination of foreign antigens. After TCR ligation, cytotoxic T cells can also upregulate Fas-ligand to kill Fas expressing cells. MM T cell did not upregulate Fas ligand and levels were found to be similar to unstimulated cells. As T cell clones are still detectable following 2 days of culture, this would rule out the possibility that high expressing Fas-ligand T cell clones after activation have undergone AICD and are therefore not measured.

Only one other study has specifically examined Fas and Fas ligand levels on T cell clones from MM patients and was an earlier study performed by our laboratory. The results from this current study are in agreement with this earlier study as similar levels of Fas on both clonal and non-clonal T cells (Raitakari *et*

al 2000), and low levels of Fas ligand on both clonal and non-clonal T cells were detected. Fas levels have also been measured in large granular lymphocytic (LGL) leukemia. LGL is a chronic disease characterised by an expanded population of CD3+CD8+CD57+ T cells (LGL cells), which can also express TCR-Vβ restriction (Zambello *et al* 1995). Constitutive expression of Fas ligand was found on the surface of LGL cells and the levels observed exceeded that of activated T cells from healthy individuals. Fas was also co-expressed on the LGL cells. Surprisingly, the LGL cells were resistant to apoptosis even though they expressed high levels of Fas ligand, suggesting that this pathway may be dysregulated in LGL leukemia (Lamy *et al* 1998). This finding also suggests that MM T cell clones are different to LGL cells, even though their phenotypes are the same.

It would be interesting to measure Fas levels on T cells from BM and the corresponding Fas ligand levels on autologous plasma cells, which would provide insight to the mechanics of Fas ligand mediated cytotoxicity by T cells against MM plasma cells. As Fas and Fas-ligand levels are not abnormally expressed in T cell clones, in comparison to non-clonal T cells, it is not likely to be an involved pathway in the induction of hypo-responsiveness.

Bcl-2, another anti-apoptotic protein has also been investigated on MM T cell clones but was found at similar levels to non-clonal T cells (Raitakari *et al* 2000). This study measured the levels of another anti-apoptotic protein Bcl-xL, which also had biological relevance in dysfunctional WM T cell clones. Bcl-2 expression levels in the Raitakari study were examined on resting state fresh PBMC only as it has been demonstrated that Bcl-2 protein levels are the same in resting and activated cells. In contrast, Bcl-xL protein can be induced by stimulation with anti-CD3/28 beads and thus levels can be different between the resting and activated state. Bcl-xL levels can also be augmented by exogenous IL-2 and CD2 stimulation (Boise *et al* 1995). Therefore, stimulation with anti-CD3/28 beads in culture with IL-2 may provide more biological relevance. To this end, Bcl-xL expression was measured on unstimulated and stimulated cells but there were no differences detected between clonal and other non-clonal T cells. Stimulation of T cell clones did increase levels of Bcl-xL, however this also

occurred in the case of non-clonal T cells in MM. There was also a trend for higher Bcl-xL levels in T cell clones in MM patients, irrespective of survival status, when compared to age-matched normal controls suggesting there may be increased Bcl-xL levels in MM. However, it is to be noted that this study only attempted to measure levels of a single anti-apoptotic protein involved in apoptotic resistance and this may be a limitation of this study. Perhaps it is necessary to investigate the balance between anti- and pro-apoptotic proteins to provide insight into how apoptosis operates in these T cells.

TGF-β stimulates p-SMAD, which can interact with Tob to maintain T cell inactivation (Li *et al* 2010). Tob can also interact with CDKI p27kip1 causing cell cycle arrest (Tzachanis *et al* 2001). This pathway was identified in the microarray analysis of WM T cell clones and may explain why the cells are anergic. These interactions result in an inactive T cell in cell cycle arrest. Studies of this protein in MM T cell clones revealed that constitutive p-SMAD expression did not differ between the different CD8+ T cell subsets studied when the MM patients were analysed as a whole group. However, significantly higher constitutive levels of this protein were found in non-proliferative T cell clones of non-10-year survivors compared to proliferative T cell clones of 10-year MM survivors. As p-SMAD can mediate T cell inactivation and cause cell cycle arrest, this suggests that hypo-responsiveness of non-proliferative T cell clones may relate to p-SMAD-induced downstream effects. Furthermore, these cells also had a diminished ability to upregulate p-SMAD in response to PMA and TGF-β stimulation, which suggests that this pathway is dysregulated and no longer under cytokine control. This concept of resistance and sensitivity to cytokine stimulation has been demonstrated in a study of STAT signalling pathways in MM plasma cells, whereby patients who have pathways resistant to cytokine stimulation have a worse prognosis than patients with pathways that are still sensitive (Brown *et al* 2015). This concept may similarly be applied to the clonal T cell situation. Clones that have pathways that remain sensitive to mitogen and cytokine upregulation of p-SMAD maintain control of this pathway, whereas clones with pathways that are resistant to mitogen and cytokine control suggest a dysregulation of the pathway.

TGF-β-dependent p-SMAD activation has been shown to inhibit CD3/28 mediated T cell proliferation in mouse models (McKarns *et al* 2004). Significantly higher levels of this protein in the T cell clones of non-10-year survivors, which are non-proliferative after CD3/28 activation, suggesting that SMAD is a possible target to reverse hypo-responsiveness. As there were observed differences between 10 year survivors and non-10 year survivors, clinical characteristics of this patient group were collected to see if they impacted the findings. Both cohorts included patients actively receiving treatment that included conventional therapy, IMiDs and proteasome inhibitors, whilst others were not receiving treatment. 13/14 patients had received prior IMiDs therapy at some stage of their disease. There were patients with stable disease, progressive disease or in remission. High or low p-SMAD levels were not related to disease or therapy. As the sample cohort was small and heterogenous, statistical analysis was not performed and is a limitation of the study. More patients are required to be studied to determine the relationship between p-SMAD levels and disease or therapy.

This study provides further evidence that aberrant constitutive p-SMAD levels are related to suppression of tumour-induced T cells. Targeting of the TGF-β induced pSMAD with small molecule inhibitors may return SMAD expression to normal and restore clonal T cell function. The TGF-β receptor type I/II inhibitor LY2109761 has been shown to suppress pSMAD expression. A study utilised this inhibitor to block TGF-β mediated phosphorylation of SMAD, which was responsible for maintaining resistance to apoptosis in hepatocellular carcinoma cells (Wang *et al* 2014). Another study used the same inhibitor to similarly reverse anti-apoptosis effects of TGF-β on myelo-monocytic leukaemic cells, which was also mediated by pSMAD (Xu *et al* 2008). Therefore, the use of a TGF-β inhibitor that represses pSMAD expression may reverse SMAD mediated inactivation effects on MM T cell clones.

Lack of proliferation is a hallmark of hypo-responsive cells and, therefore, pathways involved in regulating cell proliferation are likely to be dysregulated. One such pathway is the ERK 1/2 pathway where the localisation of ERK upon

activation can lead to different cellular responses including proliferation, differentiation or cell death (Murphy and Blenis 2006). Because of the crucial role that this pathway plays in maintaining cell proliferation and survival it is tightly regulated by protein tyrosine phosphatases such as HePTP, which can dephosphorylate ERK2 to negatively regulate T cells (Saxena *et al* 1998, Francis *et al* 2011). This mechanism of inducing an anti-proliferative state in T cells was demonstrated in hypo-responsive WM T cell clones. As a result, this pathway was also investigated in MM T cell clones. Constitutive and induced pERK levels were not lower in T cell clones compared to other non-clonal T cells. However, there was an impairment of MM T cell clones to upregulate ERK upon mitogen stimulation, and more so in non-10 year survivors when compared to age-matched normal controls. It was demonstrated in Chapter 3 that non-10 year survivors had non-proliferative T cell clones, whereas 10 year survivors had proliferative T cell clones. Although the clones from 10 year survivors were able to proliferate, the levels of proliferation were slightly inferior to other non-clonal T cells found within the same patient. This supports the notion of tumour-induced suppression of T cells but also that there may be varying levels of suppression in patients with MM that are dependent on the disease bulk. These data implicate suppression of ERK as a mediator in blocking T cell proliferation. As was mentioned previously, HePTP is responsible for dephosphorylation of ERK, leading to an anti-proliferative state (Pettiford and Herbst 2000) and so levels of this protein in MM T cell clones is worth investigating, to determine if it is responsible for repressing ERK levels. If it is the major player in ERK dephosphorylation, small molecules that inhibitors HePTP would augment ERK activation to restore proliferation (Sergienko *et al* 2012) and to reverse this dysfunction in clonal T cells.

Another mechanism of immune dysfunction in cancer may involve the down-regulation of TCR signalling proteins (Mizoguchi *et al* 1992). As a result, this study aimed to quantify the levels of the proteins CD3-ζ chain and ZAP-70, that are both crucial for initial activation of the TCR. A previous study of MM patients demonstrated marginally lower levels of CD3-ζ chain and total ZAP-70 expression in unstimulated CD4+ and CD8+ T cells when compared to healthy controls. Upon *in vitro* activation with super antigen staphylococcal enterotoxin

B, both these proteins were significantly downregulated. This observation was more marked in patients with stage III MM (Mozaffari *et al* 2004). In contrast to this study, these two TCR-signaling proteins were not significantly downregulated in MM T cell clones and levels were not different from non-clonal T cells. This disparity may be explained by the measurement of the functionally relevant ZAP-70 in the phosphorylated state (pY292) in this study, whereas the former MM study measured total ZAP-70 protein (Mozaffari *et al* 2004). After hydrogen peroxide stimulation, induced pZAP-70 levels were also not significantly different between clonal and non-clonal T cells. Perhaps a more physiological stimulation, like TCR ligation through anti-CD3/28 bead stimulation would be more suitable in this case.

The TCR signalling pathway can also be suppressed by PD-1 directed recruitment of SHP-2 to directly inactivate T cells. In aging T cells that demonstrate replicative senescence, there is an imbalance of positive and negative TCR signaling, with elevated signalling through SHP1/2 (Moro-Garcia *et al* 2012). However, this study shows that even after TCR ligation with anti-CD3/28 beads, both constitutive and stimulated pSHP-2 levels were observed at similar levels in clonal and non-clonal T cells, suggesting that SHP-2 does not play an inhibitory role in T cell signalling in MM T cell clones. As will be shown in Chapter 5, PD-1 expression is low on these T cell clones, suggesting that there would be minimal SHP-2 recruitment to these cells. As the TCR signalling protein levels in T cell clones are not aberrantly expressed, this suggests that there is no defect in TCR signaling in the MM T cell clones.

This chapter has encompassed an extensive study of the signalling pathways in T cell clones in order to identify the mechanisms that induce dysfunction in these cells. There were no defects in the TCR signalling pathways or apoptotic pathways but the ERK and SMAD pathways were found to be dysregulated. MM T cell clones had a defective ability in the upregulation of ERK in response to PMA stimulation and this may explain their decreased proliferative ability. SMAD was aberrantly expressed in hypo-responsive T cell clones and this pathway was more resistant to mitogen and cytokine stimulation than T cells

from normal controls, suggesting dysregulation. Therefore, this study is the first to identify potential novel targets for reversing clonal T cell dysfunction.

A study demonstrated that senescent cells had impaired ERK activation and growth arrest is a hallmark of senescence (Zhang *et al* 2015). Hypo-responsive T cell clones also had elevated levels of SMAD and this is associated with an inactivated T cell in cell cycle arrest. These observations raise the possibility that T cell clones may be senescent T cells that do not proliferate due to a block in the cell cycle. Replicative senescence is associated with the normal aging of cells. As cells undergo many rounds of proliferation, they eventually reach a terminal state associated with dysfunction, shortened telomeres and increased production of effector cytokines (Chou and Effros 2013). The next chapter will explore clonal T cell dysfunction by investigating the phenotype and signaling pathway program of the cells to classify T cell clones as either anergic, exhausted or senescent cells. Pinpointing the type of dysfunction that is exhibited by clonal T cells is of importance as there are different ways to reverse dysfunction depending on the phenotype of the dysfunctional T cell.

CHAPTER 5 UNDERSTANDING TUMOUR INDUCED T CELL DYSFUNCTION

5.1 Introduction

Tumours are able to evade the host immune system through a combination of molecular and cellular mechanisms that incapacitate effector T cells and allow tumour escape. Recent evidence points to the role of multiple inhibitory cell subsets that form suppressive networks that can inhibit T cell function (Brown *et al* 2012b). T cells that are introduced as adoptive immunotherapy can also be suppressed by these immunosuppressive mechanisms (Kofler *et al* 2011, Motz and Coukos 2013). Immune dysfunction in patients with multiple myeloma (MM) is multi-factorial and includes TGF-β induced dendritic cell dysfunction (Brown *et al* 2001), Treg/Th17 cell imbalance (Bryant *et al* 2013, Favaloro *et al* 2014a), generation of acquired Tregs by trogocytosis (Brown *et al* 2012a) and increased numbers of MDSCs (Favaloro *et al* 2014b). Tumor-induced immune dysfunction is also greater in patients with MM compared to other B cell malignancies such as WM and B-CLL (Brown *et al* 2012a, Yang *et al* 2012, Favaloro *et al* 2014a, Favaloro *et al* 2014b, Brown RD and Joshua DE 2012).

In order to restore T cell function and allow for effective killing of tumour cells, the underlying mechanism of T cell dysfunction must be fully characterised. T cell dysfunction has been classified into different categories based on phenotype and functionality, however the mutually non-exclusive nature of the phenotypic markers of these subsets does not simplify our approach to understanding these cells. In cancer, dysfunctional T cells can be classified into either of 4 main types, namely anergic, exhausted, senescent or stem-like T cells (Figure 5.1) (Crespo *et al* 2013).

5.1.1 Anergic T cells

Anergic T cells are hypo-responsive cells induced when there is inadequate co-stimulation provided by CD28 and its corresponding ligands on the APC, or in the presence of high co-inhibitory signalling involving molecules such as CTLA-4. These T cells are unable to produce IL-2 which is required for T cell proliferation (Schwartz RH 2003). Hypo-responsiveness of these cells can be reversed with the addition of certain cytokines including IL-2, IL-12 (Broderick *et al* 2006), IL-15 (Chiu *et al* 2006), IL-21 or cross linking of ligands CD134 (Redmond *et al* 2009) and CD137 (Wilcox *et al* 2004) (Kober *et al* 2008).

5.1.2 Exhausted T cells

T cells become exhausted after chronic antigen over-stimulation, such as in the case of viral infections where there is a high viral load or in cancers where there is high tumour antigen load. Exhausted T cells upregulate multiple inhibitory receptors including PD-1, CTLA-4, CD160, Tim-3 and LAG-3 and are unable to secrete IFN-γ (Blackburn *et al* 2009, Fourcade *et al* 2012, Woo *et al* 2012, Crespo *et al* 2013). The over-expression of these negative checkpoint targets has been exploited for therapy through immune checkpoint blockade. The use of antibodies that specifically inhibit checkpoints like PD-1 (Figure 5.2) has been successful in melanoma (Topalian *et al* 2012) and haematological malignancies such as Hodgkin lymphoma (Ansell *et al* 2015). Checkpoint inhibitors block the tumour-mediated interactions that suppress effector T cells, thereby allowing T cells to exert their functional activity against tumour cells. In order for immune checkpoint blockade to be effective in MM, there needs to be specific recognition of malignant plasma cells by tumour-specific T cells that are able to be re-activated. The T cells would therefore need to exhibit an exhausted phenotype as it is the reversal of exhaustion, and not the generation of new tumour-specific T cells that is required for this type of immune response (Suen *et al* 2015). If MM T cells are exhausted and express these inhibitory receptors, then immune checkpoint blockade is a possible way to reverse dysfunction.

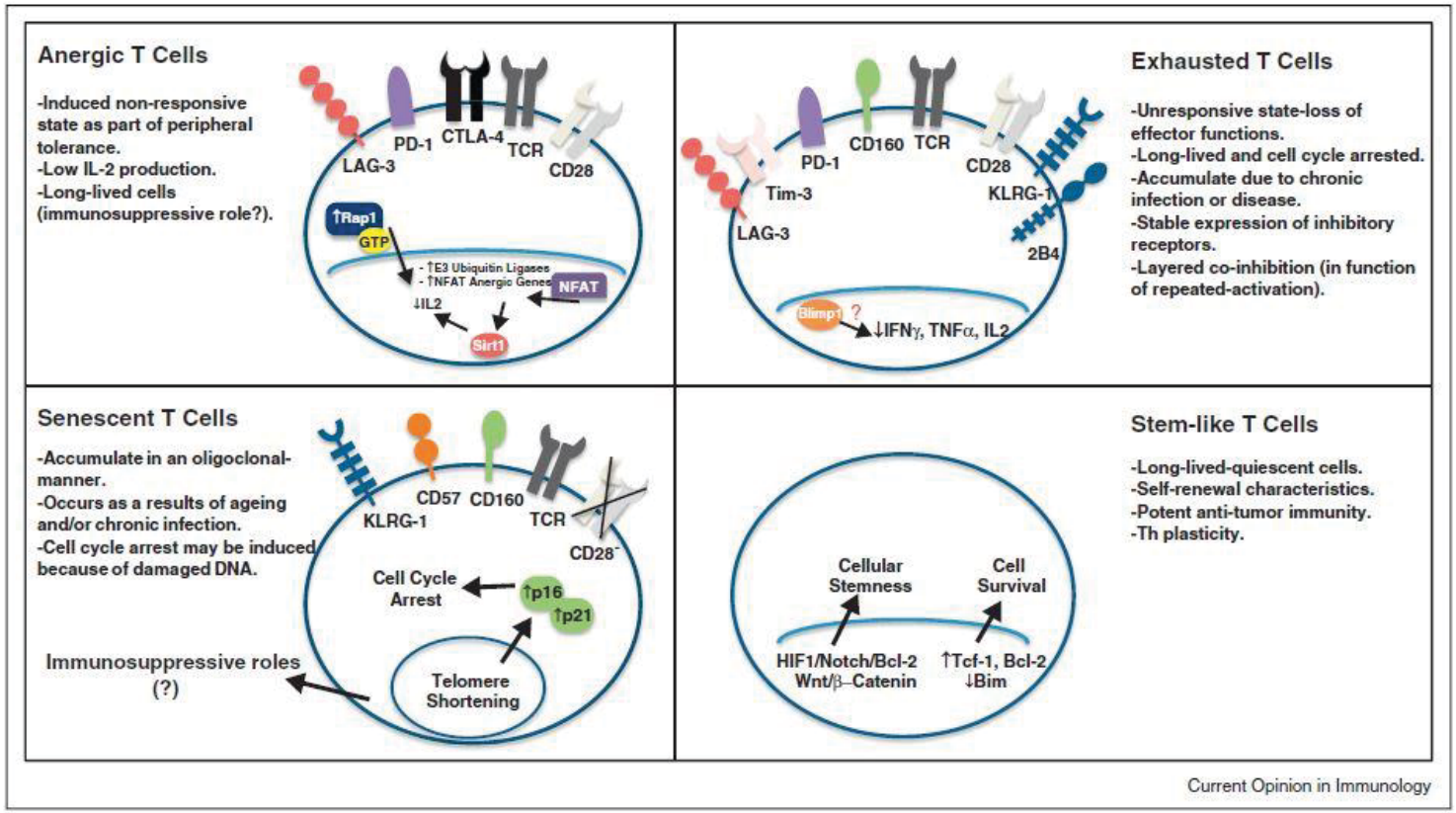


Figure 5.1 Characteristics of anergic, senescent, exhausted and stem-like T cells found in the tumour microenvironment. Anergic T cells are induced during sub-optimal stimulation such as in the presence of low co-stimulatory or high inhibitory signals. These cells survive for long periods of time and produce low levels of IL-2. Exhausted T cells are also long-lived and accumulate due to chronic infection or disease. As a result of repeated chronic antigen stimulation, these effector T cells lose their effector function, express a number of regulatory receptors and cannot secrete effector cytokines. Senescent T cells are terminally differentiated cells that occur as a result of ageing and/or chronic infection. The cells are in cell cycle arrest and have shortened telomeres. Stem-like T cells can have a naïve or memory phenotype. These cells have the ability to renew themselves, have enhanced anti-tumour responses and survive for long periods of time (Crespo *et al* 2013).

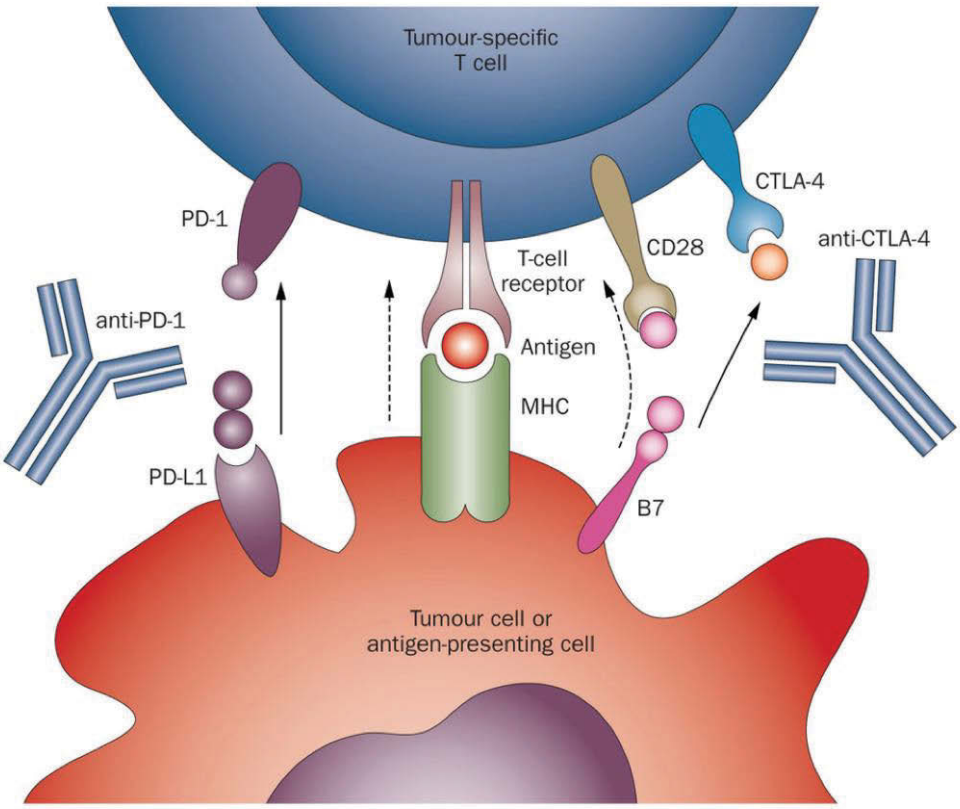


Figure 5.2 Immune checkpoint blockade
Tumour cells suppress T cells by binding to negative receptors like PD-1 and CTLA-4. Immune checkpoint blockade uses antibodies targeted against PD-1 or CTLA-4 to directly block tumour-T cell interactions, thus releasing cytotoxic T cells from tumour-suppression, allowing them to perform their function in targeting and killing tumour cells (Drake *et al* 2014).

5.1.3 Senescent T cells

Senescent T cells are late differentiated memory T cells generally associated with ageing. They have also been described in younger individuals with chronic viral infections and in different types of cancers (Effros *et al* 2005, Montes *et al* 2008, Chen *et al* 2009, Weng *et al* 2009b). These cells lack CD28, express CD57 and regulatory receptors like CD160 and killer-cell lectin like receptor (KLRG-1), are in cell cycle arrest and have enhanced secretion of inflammatory cytokines (Dock and Effros 2011, Crespo *et al* 2013). Other features of the senescent phenotype include altered gene expression and apoptotic resistance (Campisi and d'Adda di Fagagna 2007). Cellular senescence may be induced by telomere shortening, oncogenic stimuli, DNA damage or a variety of stress signals. Cells may undergo senescence of two types: telomere dependent and telomere independent senescence. Telomere-dependent senescence (replicative senescence) results from telomere attrition triggered by DNA damage, that is mediated by the p53 protein or CDKI p21CIP1/WAF1 (p21) pathways (Herbig *et al* 2004). Telomere-independent senescence is induced by stress and an altered signal transduction pathway generally involving the CDKI p16INK4a (p16) and the retinoblastoma tumor suppressor (pRb) pathways (Campisi and d'Adda di Fagagna 2007). NF-κB signaling has also been implicated in the induction of a phenomenon known as senescence-associated secretory phenotype (SASP). These senescent cells have the ability to secrete inflammatory cytokines and chemokines (Salminen *et al* 2012).

Thus far there is no single marker or feature of senescence that is unique to the senescent state and not all senescent cells will exhibit all of the potential markers associated with senescence (Rodier and Campisi 2011). If MM T cell clones are senescent cells, successful reversal of senescence is only possible if they are telomere-independent senescent cells. It is not possible to rescue cells that display telomere-dependent senescence or replicative senescence as they have shortened telomeres and exhibit irreversible cell-cycle arrest. Telomere-dependent senescence is regulated by signaling through ataxia telangiectasia mutated (ATM) to p53, leading to the upregulation of p21 and therefore cell-cycle arrest (Herbig *et al* 2004).

Telomere independent senescence generally involves the p16, rather than p21 pathway. Cells with low levels of p16 at senescence are able to proliferate again after p53 inactivation, whereas cells with high p16 are unable to proliferate after p53 inactivation (Beausejour *et al* 2003). Recently, blockade of p38-MAPK signaling has reversed telomere-independent senescence in CD4+CD45RA+CD27- T cells (Di Mitri *et al* 2011) and in CD8+ effector memory T cells that express CD45RA (EMRA) (Henson *et al* 2014, Henson *et al* 2015). These T cells had either high constitutive p38-MAPK expression, or upon PMA activation, were unable to upregulate p38-MAPK expression at the levels of non-senescent T cells. Identification of the changes in the molecular programming of cells that result in senescence induction would provide the key to reversing this phenomenon.

5.1.4 Stem-like T cells

Stem-like T cells have the capability for self-renewal and can develop into differentiated memory T cells (Fearon *et al* 2001) which may be related to Wnt signaling (Gattinoni *et al* 2009). As MM T cell clones are not stem cells, this type of dysfunctional T cell is beyond the scope of this thesis and was not studied in this project.

5.1.5 MM T cell clones have been described as three types of dysfunctional T cells that are found in cancer

The clonal nature of the cells is also highly suggestive of an antigen-specific event that, once initiated and sustained continued proliferation, (Morley *et al* 1995) possibly resulting in loss of CD28 and accumulation as a highly antigen experienced, terminally differentiated CD57+ T cell. This suggests the T cell clones are exposed to chronic antigenic stimulation (Brenchley *et al* 2003, Le Priol *et al* 2006, Vivar *et al* 2008). Chronic antigen stimulation may result in the build up of late differentiated, highly antigen-specific, oligoclonal T cells which are often located within the $CD8^+$ T cell compartment (Strioga *et al* 2011). A study following the Vβ repertoire of a MM patient over 18 months showed remarkable stability in the Vβ repertoire over this time (Raitakari *et al* 2000). CD8+ T cells recognise antigens presented by APC in the context of MHC class I (Parkin and Cohen 2001) and these are most often tumour antigens or viral antigens. Some of these antigens actually persist for long periods of time and this also infers that chronic antigen stimulation may be responsible for persistence of clonal T cell expansions (Mugnaini *et al* 1999). Chronic viral antigen stimulation is most often thought to be responsible for inducing clonal CD8+ T cell expansions as individuals with such expansions frequently test positive for persistent viruses such as CMV (Khan *et al* 2002, Focosi *et al* 2010). To rule out viral antigens as stimulants of CD8+ T cell expansions in MM, common viral serology and tetramers specific for an immuno-dominant peptide from CMV lower matrix protein pp65 were performed (Sze *et al* 2003). Less than 10% of the number of cells within the expanded 'late' differentiated CD8+ CD28- CD27- compartment, where CMV-specific CD8+ T cells are usually detected (Appay *et al* 2002), were positive for tetramer staining. Therefore, it is unlikely that the CD8+ clonal T cell expansions in MM patients are caused by persistent viral infections like CMV. Furthermore, there has not been a correlation between viral serology and the presence of CD8+ clonal T cell expansions in MM patients. Chronic antigenic stimulation can induce functionally exhausted cells (exhaustion) that lose their function and also cells that have impaired replicative ability (senescence). These two terms are often used interchangeably when describing dysfunctional T cells that are exposed to repeated antigenic

stimulation, which causes confusion but it is evident that these are two delineated subsets (Akbar and Henson 2011).

CD8+CD57+ T cell expansions often lack the co-stimulatory molecule CD28 and this has been well documented in elderly individuals as part of the ageing process (Merino *et al* 1998). The existing paradigm involves a reciprocal relationship whereby as CD28 decreases, CD57 increases (Merino *et al* 1998). Progressive loss of CD28 can occur as a result of chronic antigenic stimulation (Strioga *et al* 2011), as at birth essentially all T cells express CD28 (Weng *et al* 2009a). With persistent stimulation and many activation cycles, the T cells begin to irreversibly down-regulate CD28 expression and accumulate as an expanded population of CD8+CD57+CD28- highly antigen experienced, but chronically exhausted T cells. The T cell clones in MM predominantly do not express CD28 (Sze *et al* 2001) which is consistent with the literature where untreated patients with haematological malignancies demonstrate the reciprocal relationship of CD28 and CD57 (Van den Hove *et al* 1998). The lack of CD28 on MM T cell clones and their hypo-responsive nature has led many to describe them as anergic T cells. However, this is not completely accurate as anergy is reversible with the addition of cytokines such as IL-2 or IL-12 and the hypo-responsiveness of MM T cell clones was not corrected with these cytokines (shown in section 3.3.10).

PD-1 is a marker found on exhausted or anergic CD8 T cells (Sharpe *et al* 2007) with reports that blocking of PD-1 can re-establish proliferation (Day *et al* 2006, Trautmann *et al* 2006). PD-1 expression on MM T cell clones is unknown and so it needs to be investigated as one of the possible mechanisms responsible for inducing dysfunction. If elevated levels of PD-1 are detected on MM T cell clones, PD-1 blocking antibodies (checkpoint immune blockade) may be an alternative method for inducing proliferation and overcoming clonal T cell dysfunction. Such methods are currently being investigated in a number of haematological malignancies, including MM (Lesokhin *et al* 2014).

CD57+CD28- T cells have also been described as aged or senescent T cells. Some studies in the literature have also described these T cells as exhausted/senescent as it has been difficult to distinguish between the two (Chung *et al* 2016). MM T cell clones express both these markers but other markers of senescence like CD160 and KLRG-1 and signalling pathways involved in senescence induction (Crespo *et al* 2013) have not been investigated in the context of MM T cell clones. Furthermore, it is not known whether the telomere lengths of MM T cell clones are normal or shortened.

Therefore, there are conflicting reports that describe MM T cell clones as anergic, exhausted and senescent. Furthermore, there has previously been no systematic study to elucidate the nature and mechanism of the T cell dysfunction. Based on the described features of dysfunctional T cells in cancer (Crespo *et al* 2013), different phenotypic markers should be studied to classify MM T cell clones as either anergic, exhausted or senescent T cells. It is already known that T cell clones of MM patients are CD57+ and do not express the co-stimulatory molecule CD28 (Sze *et al* 2001). However, the other surface markers expressed by the T cell subsets and signalling pathways pictured in Figure 5.1 have been studied in MM T cell clones.

5.1.6 Summary

Based on the findings of this study, MM T cell clones were not anergic or exhausted as they did not express the markers PD-1, LAG-3, TIM-3 or CTLA-4. They expressed all the senescent markers: CD57, CD160 and KLRG-1 and lacked CD28. The telomere length of these cells was measured using two techniques: quantitative polymerase chain reaction (qPCR) and flow-fluorescence *in situ* hybridisation (FISH) and was found to be normal length for patient age. Therefore, MM T cell clones display telomere-independent senescence. Telomerase activity was measured by detecting human telomerase reverse transcriptase (hTERT), the catalytic subunit of human telomerase in T cell clones using a flow cytometric method and elevated levels of telomerase were detected. Whilst none of the signalling pathways examined seemed to be responsible for inducing T cell dysfunction, elevated levels of telomerase may explain how senescent T cells maintain normal

telomere lengths. Based on the surface phenotype of the cells and the fact that they have normal-for-age telomere lengths and elevated telomerase activity, T cell clones in MM are telomere-independent senescent T cells.

5.2 Methods

5.2.1 Investigating cell surface phenotype by flow cytometry

The surface phenotype was firstly studied to classify the T cell clones as anergic, exhausted or senescent. The surface markers that were chosen are shown in Figure 5.3 and include PD-1, LAG-3, Tim-3, CD160 and KLRG-1. CD27 and CD28 expression were also studied for the classification of T cells into early, intermediate or late stage phenotype. These markers were used in combination with the clonal T cell panel of antibodies and the different antibody panels are listed in Table 5.1.

Table 5.1 Antibody panels for investigating cell surface phenotype of T cell clones

Panel 1	Panel 2	Panel 3	Panel 4
CD3 V500	CD3 V500	CD3 V500	CD3 PE-Cy7
CD8 APC-H7	CD8 APC-H7	CD8 APC-H7	CD8 APC-H7
CD57 Pacific Blue	CD57 Pacific Blue	CD57 Pacific Blue	CD57 APC
Vβ FITC/PE	Vβ FITC/PE	Vβ FITC/PE	Vβ FITC/PE
PD-1 PE-Cy7	LAG-3 APC	KLRG-1 APC	CD27 PE/FITC
CD160 Alexa Fluor 647	TIM-3 PE-Cy7		CD28 PerCP-CY5.5

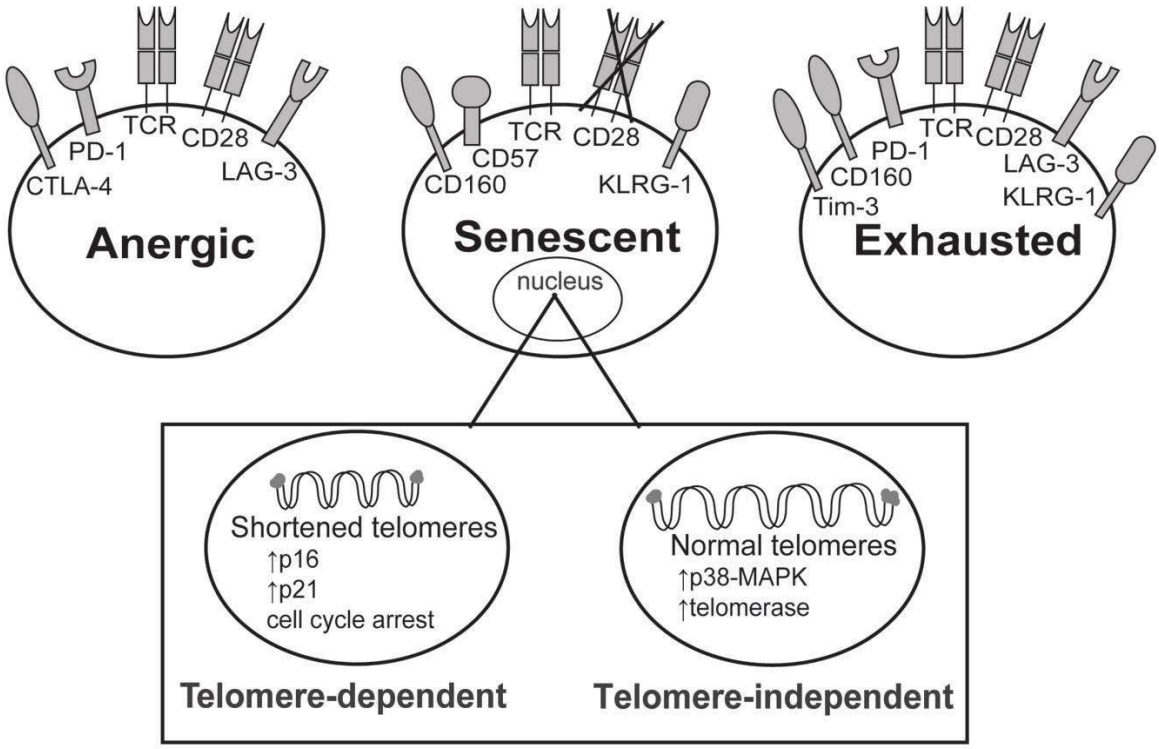


Figure 5.3 Phenotypic features of dysfunctional T cells in cancer investigated in this study

Dysfunctional T cells can be classified as anergic, exhausted or senescent based on the expression of different phenotypic markers or the activation of specific signaling pathways. Anergic T cells are induced when there is incomplete co-stimulation provided by CD28 during TCR ligation or in the presence of high-inhibitory signaling by CTLA-4. Exhausted T cells are induced from chronic antigen stimulation, which results in the overexpression of multiple inhibitory markers like PD-1, CTLA-4, CD160, Tim-3 and LAG-3. Senescent T cells are associated with ageing and the natural life span of cells and express markers CD57, CD160, KLRG-1 and lack CD28. Telomere-dependent senescent cells are characterised by shortened telomeres and cell-cycle arrest and upregulated p16 and p21 pathways (replicative senescence). Telomere-independent senescent cells have normal telomere length, possibly maintained with upregulated telomerase activity and senescence can be induced by an upregulated p38-MAPK pathway. Published in Suen *et al* 2016.

5.2.2 Measurement of telomere length by q-PCR

Purified T cell clones (CD3+CD8+Vβ+CD57+) and a comparison non-clonal T cell subset (CD3+CD8+Vβ-CD57+) were firstly obtained by cell sorting as described in Section 2.9.4. DNA was extracted from purified clonal and non-clonal T cells using the Maxwell® 16 cell low elution volume DNA purification kit on the automated Maxwell® 16 instrument (Promega, Wisconsin, USA) and cryopreserved at -20°C for PCR at a later date.

For PCR, the samples were firstly diluted in Tris-EDTA to achieve a 1 ng/μL concentration and telomere length measurement was performed according to a published method (Cawthon 2002) and telomeric primers. (Aviv *et al* 2011) Two PCR reactions were run in parallel, the first using telomeric primers (*tel1b* 5'-CGGTTT(GTTTGG)^{5}GTT-3', and *tel2b*, 5'-GGCTTG(CCTTAC)^{5}CCT-3') at a final concentration of 100 nM and the second with primers for the single copy gene, beta globin (hbg1, 5' GCTTCTGACACAACTGTGTTCACTAGC-3', and hbg2, 5'-CACCAACTTCATCCACGTTCACC-3') at a final concentration of 300 nM. 5 ng of DNA was added to each reaction, along with Rotorgene SYBR Green Master Mix to a final volume of 25 μl, and all samples were analysed in triplicate within each run. PCR reactions were carried out on the Rotor-Gene Q. Serial dilutions of commercial normal DNA were used as reference standards and the quantity of telomeric and single copy gene DNA were extrapolated from these standards. Relative telomere length (T/S ratio) was calculated by dividing the telomeric DNA quantity (T) by the single copy gene DNA quantity (S) identified in each sample and is proportional to the average telomere length per cell compared with the commercial normal DNA. T/S ratio for each sample was obtained 3 times, and the two closest T/S values were averaged.

5.2.3 Measurement of telomere length by Flow-FISH

Telomere length of clonal and non-clonal T cells was also measured by the DAKO Telomere PNA kit/FITC for flow cytometry. This technique is a combination of FISH and flow cytometry. It involves the use of a peptide nucleic acid (PNA) probes that hybridise and specifically bind to telomeric

sequences. PNA is a DNA/RNA analog that is able to bind to specific sequences. The telomere-PNA probe is conjugated to a fluorochrome, FITC, which allows fluorescence to be detected by flow cytometry. The fluorescence detected therefore correlates directly to the number of bound telomere-PNA probes and can be used to calculate telomere length in cells. The kit was used according to the manufacturer's protocol and contains four bottles: hybridisation solution without telomere probe, hybridisation solution with probe, wash solution and DNA staining solution containing PI. Relative telomere length was calculated by comparing the telomere length of patient cells to a control cell line (CCRF-CEM; kindly provided by Dr. Tatjana Kilo) that has long telomeres.

Purified T cell clones (CD3+CD8+Vβ+CD57+) and a comparison non-clonal T cell subset (CD3+CD8+Vβ-CD57+) were firstly obtained by cell sorting as described in Section 2.9.4. Patient cells were washed in PBS and mixed with equal numbers of control cells (CCRF-CEM cell line) in eppendorf tubes. The sample was divided into two and resuspended in hybridisation solution either with or without FITC-conjugated telomere probe. The sample that was incubated with the hybridization solution without PNA probe-FITC, was used to determine background fluorescence. Cells were incubated at 82°C for 10 min and left overnight to allow for hybridisation of telomere PNA probe to telomeric sequences. The following day, cells were washed twice with wash solution before staining with DNA staining solution containing propidium iodide and RNase for 2 hours, in the dark at 2-8°C. Cells were analysed on a BD FACS Canto II flow cytometer and the MFI of FITC in the test and control samples were used to calculate the relative telomere length (RTL) using the following formula, as per kit instructions:

$$\text{Relative telomere length} = \frac{\text{MFI test cells with probe - MFI test cells without probe x DNA index (control cells) x 100}}{\text{MFI control cells with probe – MFI control cells without probe) x DNA index of test cells}}$$

5.2.4 Signalling pathways involved in the induction of senescence

The p16, p21 and p38-MAPK pathways were investigated to determine if they were responsible for inducing clonal T cell senescence in MM. The Cytofix/cytoperm method described in 4.2.1.2 was used to measure constitutive expression levels of p16 and p21. Constitutive and PMA-induced p38-MAPK expression were detected using the BD Phosflow I method as follows. PBMC were sequentially stained with anti-CD8-PE-Cy7 and then stimulated or not stimulated with PMA (150 nm) for 10 min at 37°C. Cells were then fixed with BD Cytofix buffer (250 μL) for 10 min at RT and centrifuged at 600g for 6 min before permeabilisation with BD Perm/Wash I buffer for 30 min at RT. Cells were washed twice with 1 mL BD Perm/Wash I buffer and then stained with the remaining clonal T cell surface markers (anti-CD3 APC-H7, CD57 efluor450, anti-TCR-Vβ-FITC/PE and anti-p-p38-MAPK-PerCP-Cy5.5 for 30 min at RT. After incubation, cells were washed in BD Perm/Wash I buffer and then resuspended in PBS for flow cytometric analysis.

5.2.5 Measurement of telomerase by flow cytometry

To measure telomerase levels, human telomerase reverse transcriptase (hTERT), the catalytic subunit of human telomerase was measured by flow cytometry. PBMC were fixed and permeabilised using the Leucoperm kit (as described in section 4.2.1.1 and then labelled with purified anti-hTERT (1:200; Clone 2C4) or purified mouse anti-IgM κ isotype control (1:100) for 40 min at RT. Cells were washed before labelling with secondary goat anti-mouse Ig-APC (1:100) for 30 min at RT. Following another two washes, cells were labelled with clonal T cell surface antibodies for 30 min at RT. Cells were washed and then resuspended in PBS for flow cytometric analysis. The telomere levels were expressed as a change in MFI (ΔMFI), which was calculated by subtracting the MFI of the isotype from the mean fluorescence of the stained sample.

5.2.6 Flow detection of immune checkpoint proteins and other signalling pathways

Immune checkpoint targets PD-1 and CTLA-4 were studied on T cells from PB and BM of MM patients by flow cytometry and then compared to age-matched normal controls. PBMC were obtained by Ficoll separation and then stained with clonal T cell antibodies and antibodies against the two checkpoint targets (anti-PD-1 PE-Cy7 and anti-CTLA-4-APC). Another inhibitory receptor, BTLA was also investigated on the cell surface of T cells by flow cytometry (anti-BTLA-APC). The BD Phosflow method described in 4.2.1.4 was used to measure p-Akt and T-bet using phospho-flow antibodies anti-pAkt (Ser473)-PE and anti-T-bet

5.2.7 Controls

Fluorescence minus one controls or isotype controls were used to establish positive expression limits and determine protein expression. The primary internal biological control or comparator was the non-clonal Vβ-CD57+ subset as these are CD8+ T cells that express CD57+ but do not have a restricted TCR-Vβ and are therefore not expanded T cells. However, the Vβ+CD57- and Vβ-CD57- subsets are also included for completion and for interest. CD8+CD57+ T cells from age-matched healthy controls were used to determine normal expression of markers.

5.3 Results[§]

5.3.1 Introduction

To determine the phenotype of dysfunctional clonal T cells, the phenotypic markers PD-1, LAG-3, Tim-3, CD160 and KLRG-1 were firstly studied by flow cytometry (see Figure 5.3) to help classify T cell clones as either anergic,

[§]The main results of this chapter were previously published in **Suen, H**., Brown, R., Yang, S., Weatherburn, C., Ho, P.J., Woodland, N., Nassif, N., Barbaro, P., Bryant, C., Hart, D., Gibson, J. & Joshua, D. (2016) Multiple myeloma causes clonal T cell immunosenescence: Identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade. *Leukemia***,** epub ahead of print.

exhausted or senescent cells. An example of flow histogram gating for all the phenotypic markers studied is shown in Figure 5.4.

5.3.2 T cell clones are neither exhausted nor anergic

Anergic T cells express the inhibitory receptors PD-1, LAG-3 and CTLA-4 and also express CD28. Exhausted T cells commonly express the inhibitory receptors PD-1, LAG-3, TIM-3 and CTLA-4. As seen in Figure 5.5, T cell clones expressed low levels of PD-1 and at significantly lower levels than non-clonal ($p=0.004$), Vβ-CD57- cells ($t=10.08$, $p<0.0001$) and CD8+CD57+ from normal controls ($U=0$, $p=0.001$). T cell clones also expressed very low levels of LAG-3 but these levels were not different from non-clonal T cells, the two other CD8+ T cell subsets or normal controls (Figure 5.6). TIM-3 expression was similarly low on T cell clones and was not significantly different from non-clonal T cells nor CD8+CD57+ T cells from normal controls. Interestingly, significantly higher levels of TIM-3 were found on the Vβ-CD57- T cells subset (Figure 5.7). Interestingly, PD-1 was also expressed at higher levels on this subset and suggest a dual checkpoint expressing T cell. CTLA-4 expression was found to be significantly lower in T cell clones compared to non-clonal T cells ($p=0.005$) and Vβ-CD57- cells ($p=0.001$) but were not different from CD8+CD57+ T cells from normal controls ($p=0.03$) (Figure 5.8). Therefore, MM T cell clones expressed low levels of PD-1, LAG-3, TIM-3 and CTLA-4. The cells were also negative for CD28 (Figure 5.9). Based on the exclusion of these phenotypic markers, MM T cell clones are not anergic or exhausted cells.

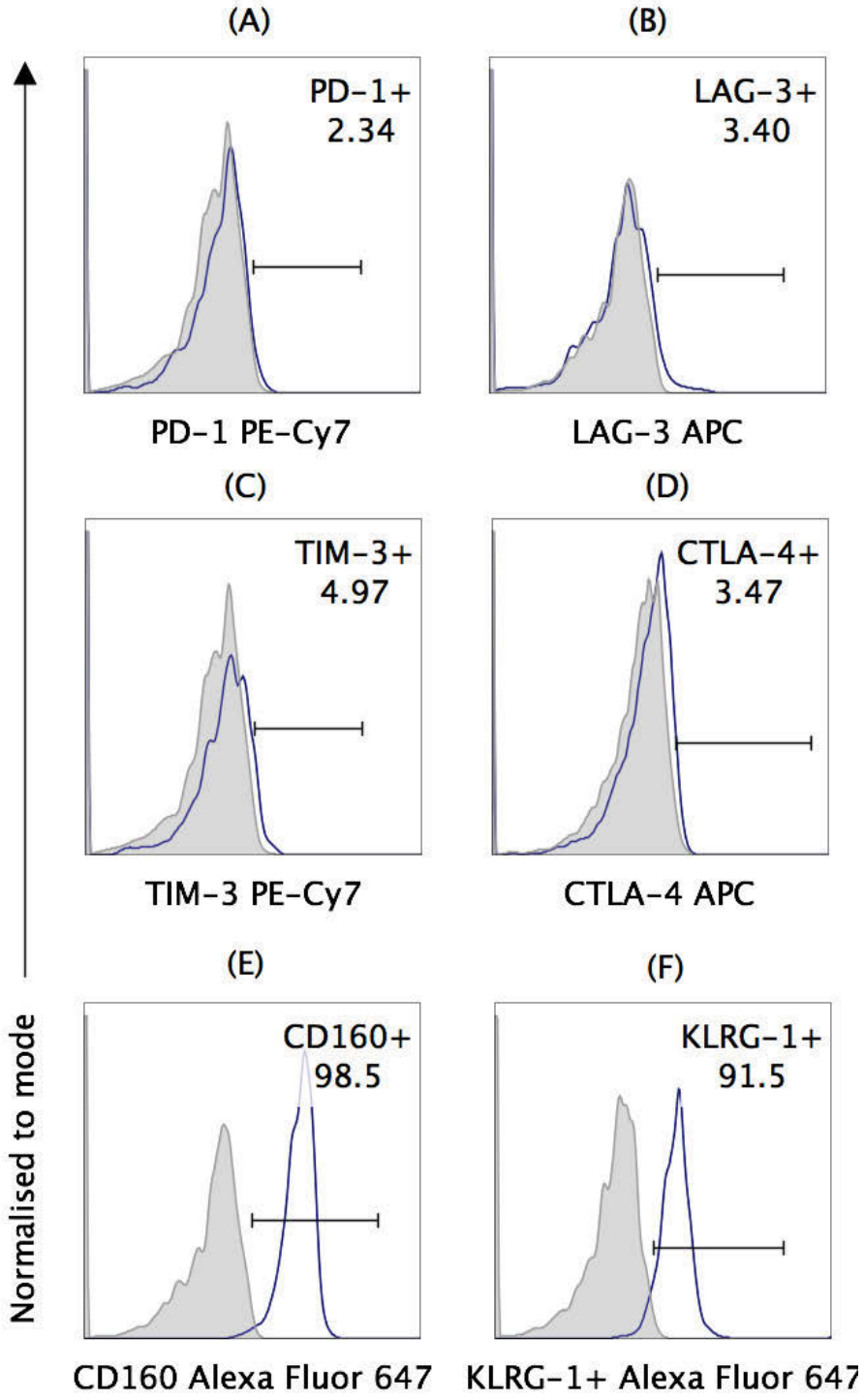


Figure 5.4 Representative flow histogram gating for determination of cell surface phenotype of T cell clones

PBMC were obtained by Ficoll from patients with MM. Clonal T cells were firstly identified by the flow gating strategy described in section 2.9.3. The phenotypic markers **(A)** PD-1, **(B)** LAG-3, **(C)** TIM-3 and **(D)** CTLA-4 were analysed on T cell clones to investigate the anergic and exhausted phenotype. **(E)** CD160 and **(F)** KLRG-1 were used to investigate the senescent phenotype. Isotype controls (grey tinted histograms) were used to identify positive expression limits for each of the phenotypic markers (blue untinted histograms).

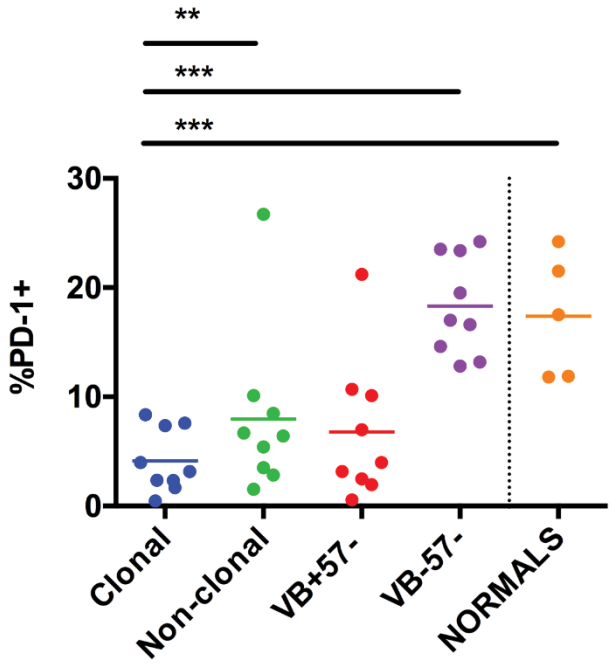


Figure 5.5 PD-1 expression on T cells from MM patients and normal controls

PBMC were obtained from MM patients (n=9) and PD-1 expression was studied on T cell clones by flow cytometry and compared to other CD8+ T cell subsets and CD8+CD57+ T cells from age-matched normal controls (n=5). Data are presented as % of cells expressing PD-1. **p<0.01, ***p<0.0001.

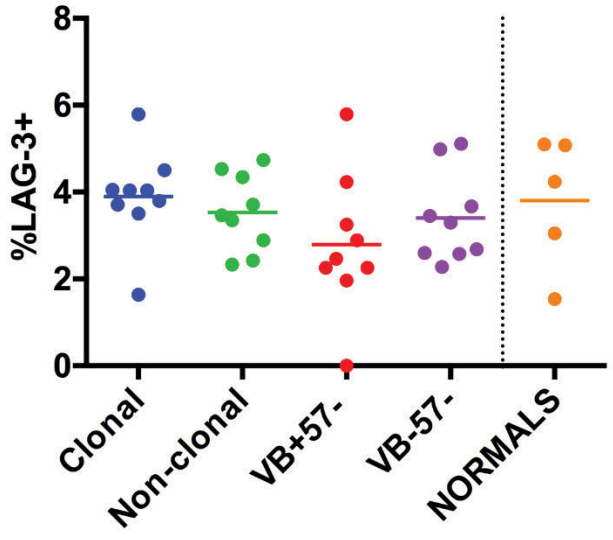


Figure 5.6 LAG-3 expression on T cells from MM patients and normal controls

PBMC were obtained from MM patients (n=9) and LAG-3 expression was studied on T cell clones by flow cytometry and compared to other CD8+ T cell subsets and CD8+CD57+ T cells from age-matched normal controls (n=5). Data are presented as % of cells expressing LAG-3. p=NS.

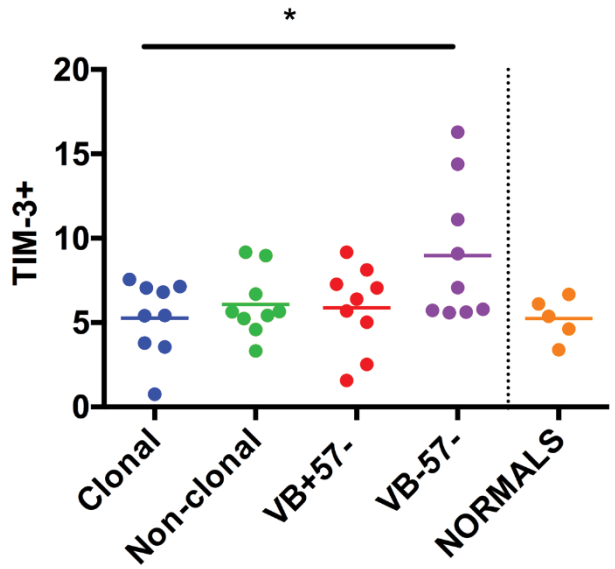


Figure 5.7 TIM-3 expression on T cells from MM patients and normal controls

PBMC were obtained from MM patients (n=9) and TIM-3 expression was studied on T cell clones by flow cytometry and compared to other CD8+ T cell subsets and CD8+CD57+ T cells from age-matched normal controls (n=5). Data are presented as % of cells expressing TIM-3. *p<0.05.

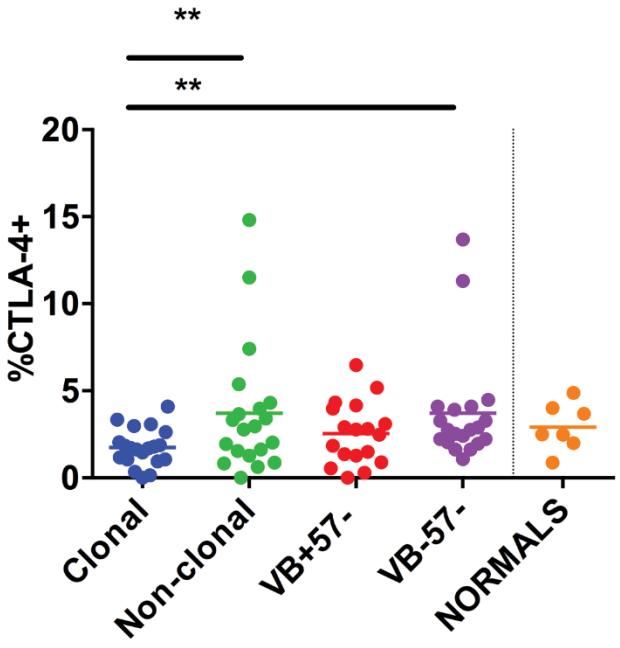


Figure 5.8 CTLA-4 expression on T cells from MM patients and normal controls

PBMC were obtained from MM patients (n=20) and CTLA-4 expression was studied on T cell clones by flow cytometry and compared to other CD8+ T cell subsets and CD8+CD57+ T cells from age-matched normal controls (n=7). Data are presented as % of cells expressing CTLA-4. **p<0.01.

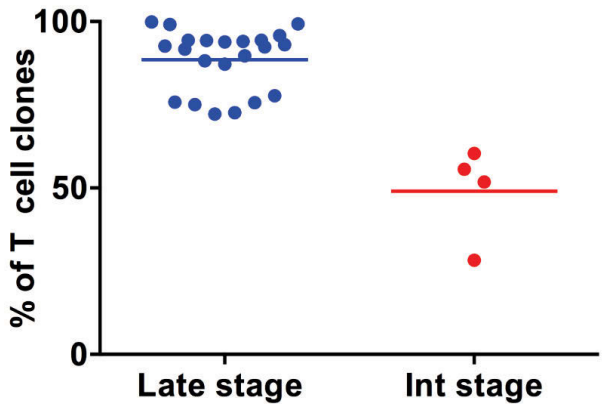


Figure 5.9 T cell clones in MM are mostly of the late differentiated stage
Classification of T cell clones from MM patients into late stage phenotype (CD27-CD28-) or intermediate (int) stage phenotype (CD27+CD28-). There were no T cell clones detected with an early stage phenotype (CD27+CD28+).

5.3.3 T cell clones display the phenotype of senescent T cells

It is already known that T cell clones express two features of senescent T cells: they express CD57 and lack CD28 (Figure 5.9). Previously, the senescent markers, CD160 and KLRG-1 have not been studied on T cell clones. CD160 expression was found to be expressed at mostly high levels on T cell clones and was significantly higher than CD8+CD57+ T cells from normal controls ($t=2.59$, $p=0.03$). However, it was not significantly different from non-clonal and other CD8+ T cell subsets from MM patients, suggesting that high CD160 expression may be associated with the MM disease state (Figure 5.10). KLRG-1 expression on T cell clones was significantly higher than non-clonal T cells ($t=4.60$; $p=0.003$) and the Vβ-CD57- subset ($t=4.95$; $p=0.002$) but was significantly lower than CD8+CD57+ T cells from normal controls ($U=0$; $p=0.0007$) (Figure 5.11). Therefore, MM T cell clones expressed all the phenotypic markers of senescence: CD28-, CD57+, CD160+, KLRG-1+ and are most likely senescent T cells.

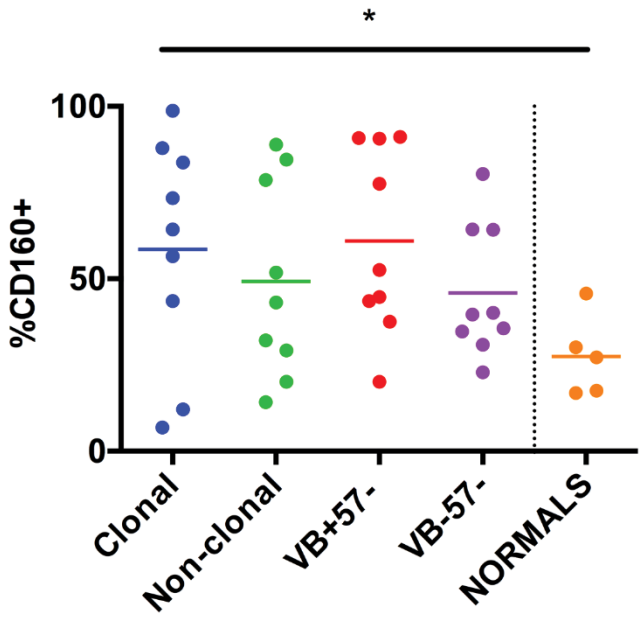


Figure 5.10 CD160 expression on T cells from MM patients and normal controls
PBMC were obtained from MM patients (n=9) and CD160 expression was studied on T cell clones by flow cytometry and compared to other CD8+ T cell subsets and CD8+CD57+ T cells from age-matched normal controls (n=5). Data are presented as % of cells expressing CD160. *p<0.05.

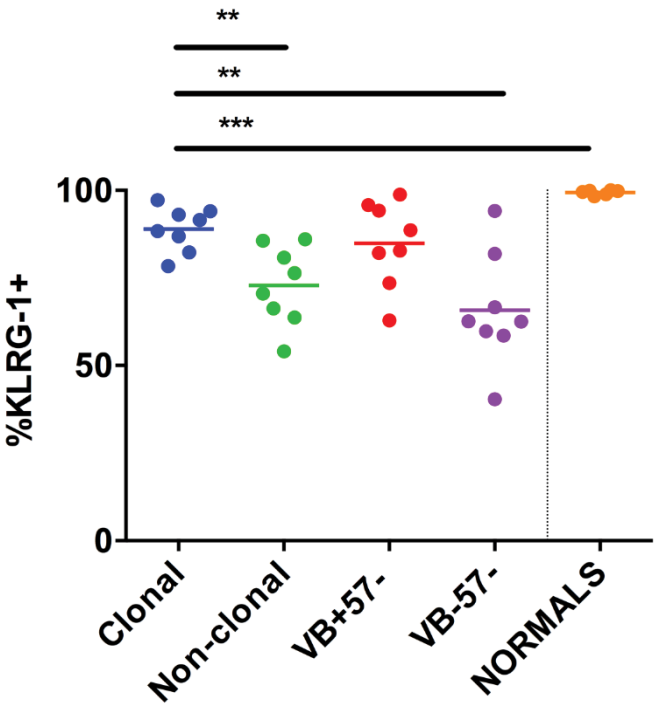


Figure 5.11 KLRG-1 expression on T cells from MM patients and normal controls
PBMC were obtained from MM patients (n=8) and KLRG-1 expression was studied on T cell clones by flow cytometry and compared to other CD8+ T cell subsets and CD8+CD57+ T cells from age-matched normal controls (n=5). Data are presented as % of cells expressing KLRG-1. **p<0.01; ***p<0.001.

5.3.4 MM T cell clones have normal for age telomere lengths

As MM T cell clones expressed the phenotypic markers of senescence, the telomere length was determined, in order to further classify the cells into the two subtypes of senescence. As shown in Figure 5.3, telomere-dependent senescent cells have shortened telomeres whilst telomere-independent senescent cells do not. The telomere length of MM T cell clones was measured by qPCR and flow-FISH.

Using the qPCR method, the T/S ratio was calculated by dividing the telomeric DNA quantity by the single copy gene DNA quantity. The T/S ratio for MM T cell clones and their non-clonal counterparts from the same patient are plotted on a graph, which also shows the healthy control range for T/S ratios according to the patient age (Figure 5.12). The T/S ratio for the T cell clones from the 4 MM patients studied fell within the normal range of T/S ratio for age indicating that MM patients had normal for age telomere lengths and were not shortened. There was also no difference between clonal T cells and their respective non-clonal T counterparts in the MM patients (Figure 5.13).

Using the flow-FISH technique, telomere length was represented as a proportion of the telomere length of a control cell line with long telomeres (RTL). An example of flow gating and calculation of the RTL from a representative MM patient is shown in Figure 5.14. RTL of MM T cell clones was not significantly different from their non-clonal counterparts ($p=0.63$) nor CD8+CD57+ T cells from age-matched normal controls ($p=0.81$) (Figure 5.15). These results are in agreement with the telomere lengths measured using the qPCR technique. As telomeres are not shortened, MM T cell clones, display features of telomere-independent senescence.

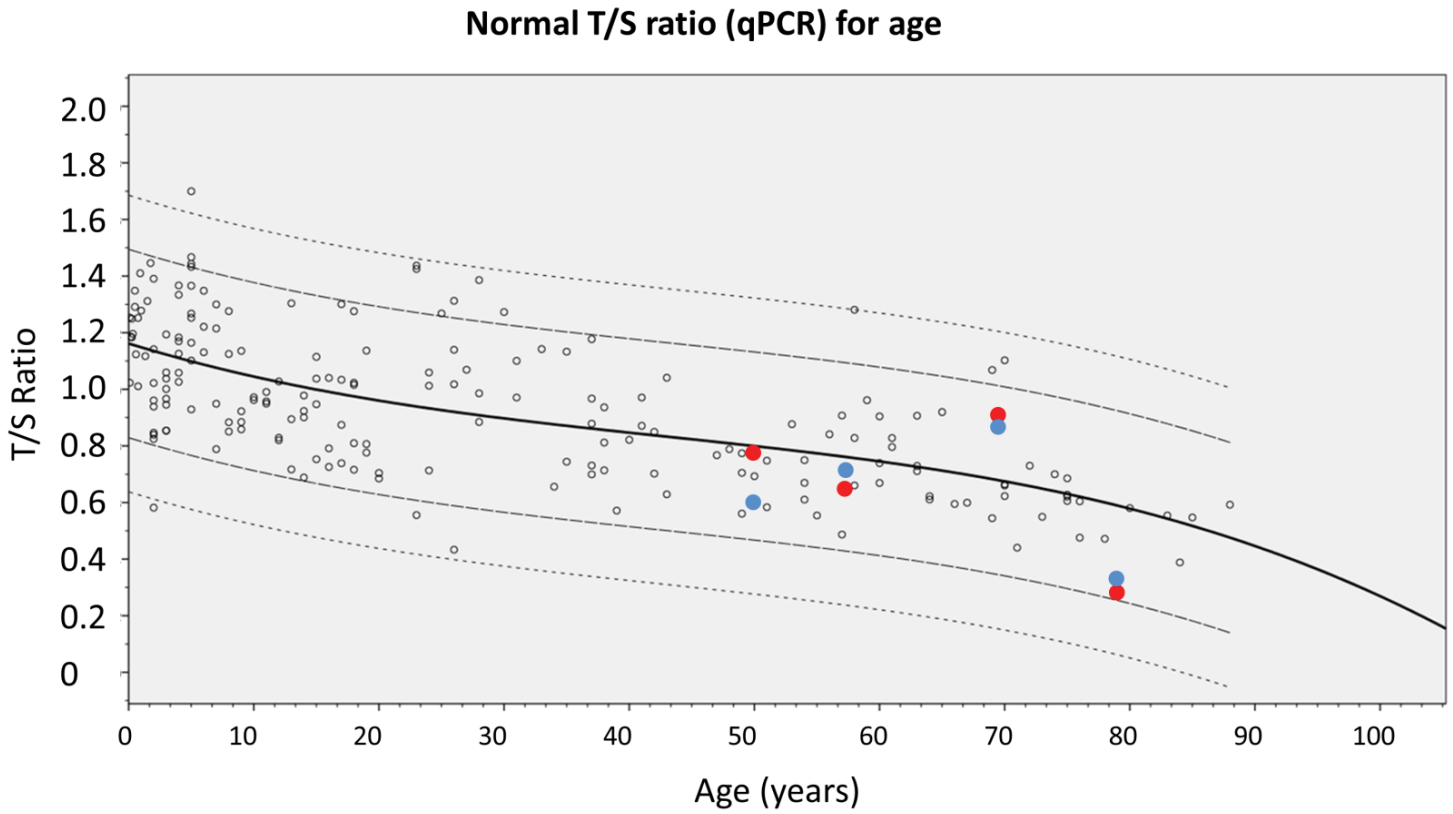


Figure 5.12 Telomere length of MM T cell clones compared to the telomere lengths of PBMC from healthy individuals according to age

DNA was extracted from PBMC obtained from heathy controls of different ages (performed by Dr. P. Barbaro, Children's Medical Research Institute, Westmead, NSW, Australia) for telomere length measurement and to create a normal T/S ratio range according to patient age. Clonal and non-clonal T cells from MM patients (n=4) were purified by flow sorting and DNA was extracted using the Maxwell kit. Two PCR reactions were run using a telomeric primer and a single copy gene, beta globin primer. The T/S ratio was calculated by dividing the telomeric DNA quantity by the single copy gene DNA quantity for each patient, which included normal controls (open circles), MM T cell clones (red circles) and the corresponding non-clonal T cells from the same patient (blue circles).

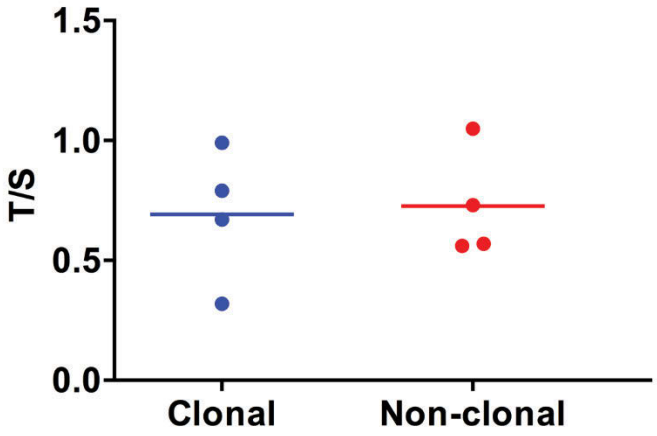


Figure 5.13 Telomere length of MM clonal and non-clonal T cells measured by qpCR
Clonal and non-clonal T cells from MM patients (n=4) were purified by flow sorting and DNA was extracted using the Maxwell kit. Two PCR reactions were run using a telomeric primer and a single copy gene, beta globin primer. Data are presented as a T/S ratio, which was calculated by dividing the telomeric DNA quantity by the single copy gene DNA quantity. p=NS.

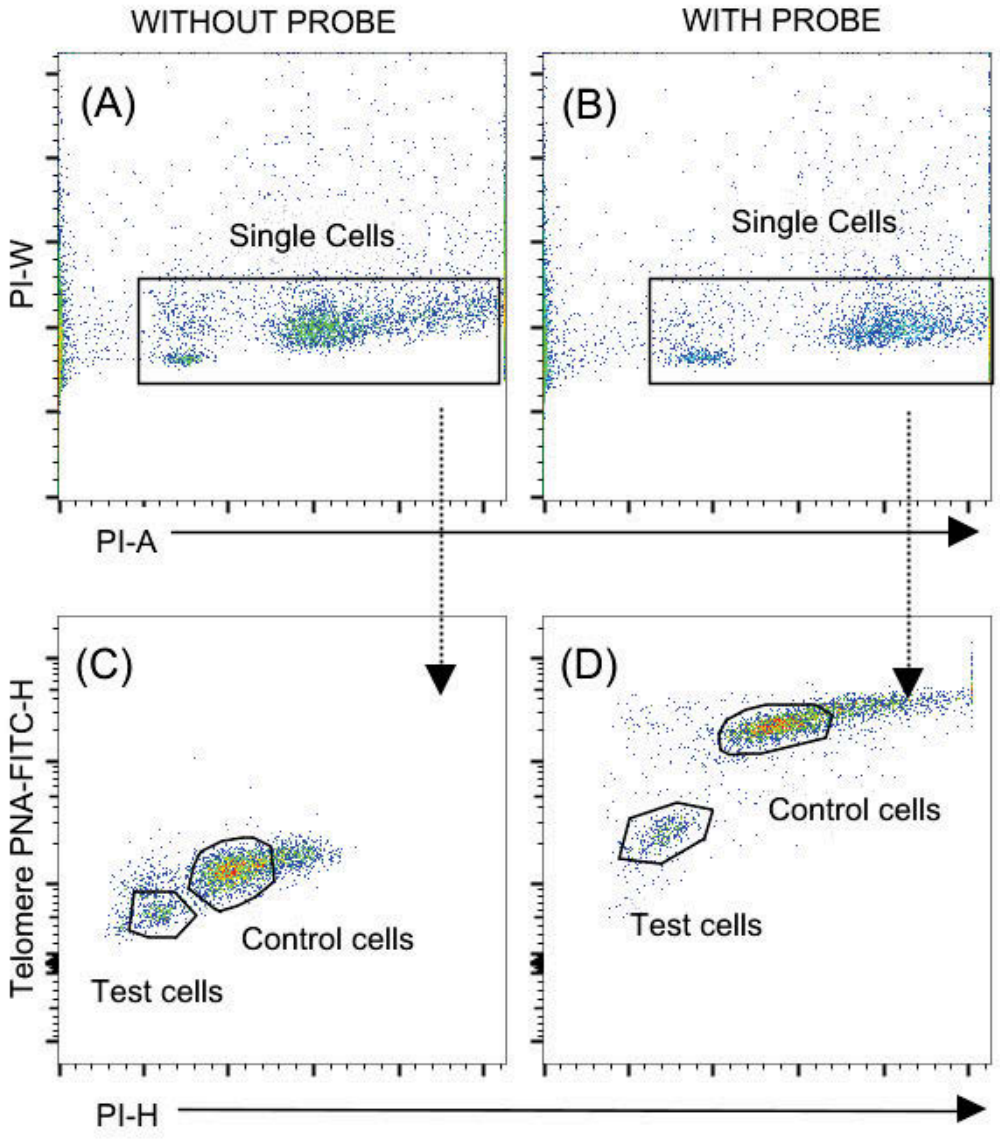


Figure 5.14 Representative flow histograms for Flow-FISH determination of telomere length of MM clonal T cells

Purified clonal T cells were treated with the DAKO Telomere PNA kit/FITC according to the manufacturer's protocol for measurement of telomere length by flow-FISH. Two tubes were run in duplicate, one without telomeric probe **(A,C)**, which indicates background fluorescence and one with telomeric probe **(B, D)**. Single cells were firstly identified according to the width and area of cells positive for DNA stain PI (PI-W vs. PI-A). From the single cell population, cells in $G_{0/1}$ phase were selected (PI intensity indicates amount of DNA present) as the cell has one copy of the genome and cells in S or G2/M phase were excluded. The tetraploid CEM control cell line (control cells) had higher levels of DNA than the patient clonal T cells (test cells), as indicated by PI intensity. The MFI values for both cell populations in both tubes were measured and used to calculate the RTL as described section 5.2.3.

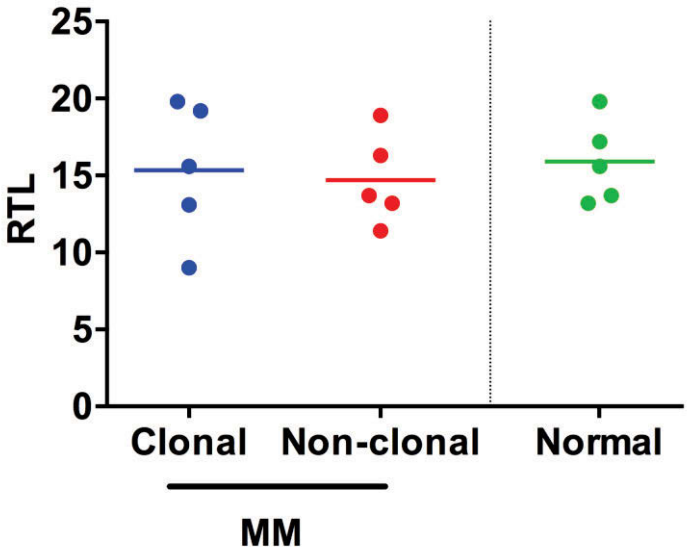


Figure 5.15 Telomere length of MM clonal and non-clonal T cells measured by flow-FISH
Purified clonal and non-clonal T cells from MM patients (n=5) and PBMC from normal controls (n=5) were treated with the DAKO Telomere PNA kit/FITC according to manufacturer's protocol for measurement of telomere length by flow-FISH. Data are presented as a relative telomere length (RTL) which is calculated by the formula outlined in section 5.2.3. p=NS.

5.3.5 Senescent T cells are not related to patient demographics

MM patients (n=15) were tested for cell surface phenotype, telomere length and telomerase. To determine if the T cell senescence phenomenon was related to treatment or disease status, clinical details for all 15 MM patients were accessed and are listed in

Table 5.2. There were patients actively receiving treatment that included conventional therapy, IMiDs and proteasome inhibitors, whilst others were not receiving treatment. 14/16 patients had received prior IMiDs therapy at some stage of their disease. All stages of disease were represented with patients with stable disease, progressive disease or in remission. Therefore, the T cell senescence phenomenon was not induced by any specific treatment regimen and was not associated with a specific disease state.

Table 5.2 Demographics for MM patients studied for T cell senescence phenotype and telomere length

Patient	Paraprotein	ISS (at diagnosis)	Transplant	Active treatment at time of testing	Disease State
1	LLC	I	Auto	Len/Dex	Partial remission
2	IgG	I	Auto	Len/Dex	Stable disease
3	IgG L	I	NIL	Thal	Partial remission
4	IgG K	II	NIL	Len/Pred	Stable disease
5	IgG	I	NIL	None	Stable disease
6	IgA K	unknown	NIL	None	Stable disease
7	IgG K	II	NIL	Len/Dex	Complete remission
8	IgA K	II	Auto	Vel/Dex	Relapse
9	IgG K	I	Auto	Len/Dex	Relapse
10	IgG K	I	NIL	Len	Complete remission
11	KLC	I	Auto	CyBORD	Relapse
12	IgG K	I	Auto	Pom	Complete remission
13	IgG L	I	Auto	Pom	Stable disease
14	KLC	I	Auto	Len/Pred	Stable disease
15	IgA K	I	NIL	None	Relapse

Allo: allogeneic stem cell transplant; Auto: autologous stem cell transplant; CyBORD: Cyclophosphamide, bortezomib and Dexamethasone; Dex: dexamethasone; Ig: immunoglublin; ISS; International Staging System; K: kappa; L: lambda; LC: light chain; Len: lenalidomide; Pred: prednisone; Pom: pomalidomide, Thal: thalidomide; Vel: velcade.

5.3.6 p16 and p21 levels are not upregulated in MM T cell clones

Different signalling pathways are responsible for inducing senescence in T cells. In general, telomere-dependent senescence (replicative senescence) involving shortened telomeres has upregulated p53 or p21 pathways (Herbig *et al* 2004); and telomere-independent senescence, which is induced by stress and altered signal transduction pathways, generally involving the upregulation of p16 and pRB pathways (Campisi and d'Adda di Fagagna 2007), however other signalling pathways may also be involved. Both constitutive p16 (Figure 5.16) and p21 expression (Figure 5.17) was studied on T cell clones from MM patients and their expression levels were found to

be similar to non-clonal and other CD8+ T cell subsets. Therefore, the p16 and p21 pathways are not upregulated in T cell clones and are not responsible for inducing senescence of MM T cell clones.

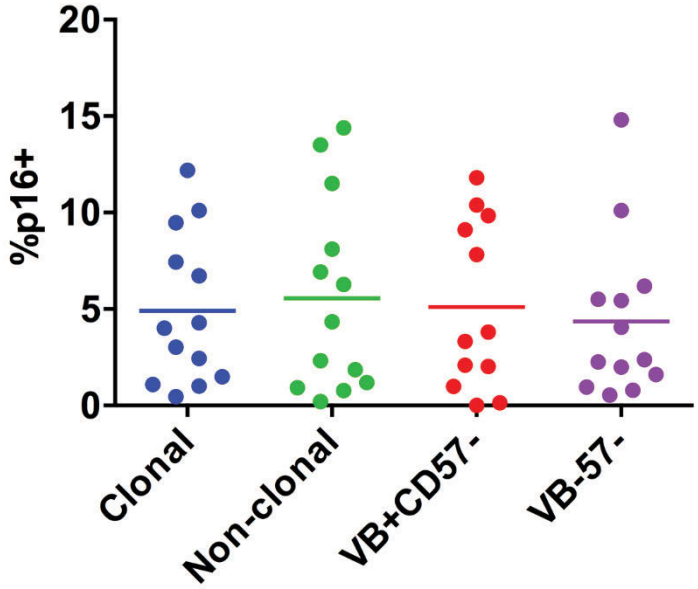


Figure 5.16 p16 expression on T cells from MM patients
PBMC from MM patients were fixed and permeabilised using the BD Cytofix/cytoperm method for the detection of constitutive p16 expression on CD8+ T cell subsets from MM patients (n=13). Data are presented as cells expressing p16. p=NS.

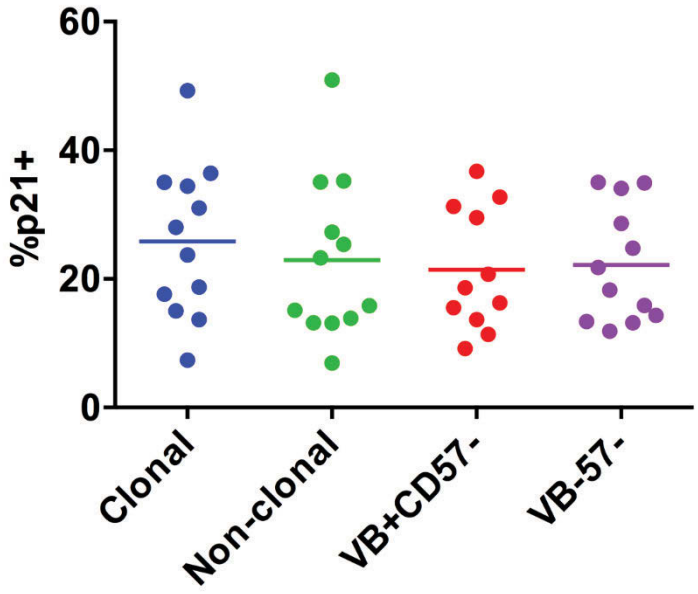


Figure 5.17 p21 expression on T cells from MM patients
PBMC were fixed and permeabilised using the BD Cytofix/cytoperm method for the detection of constitutive p21 levels on CD8+ T cell subsets from MM patients (n=12). Data are presented as cells expressing p21. p=NS.

5.3.7 The p38-MAPK pathway is not responsible for inducing MM clonal T cell senescence

The p38-MAPK pathway has been implicated in the induction of reversible senescence. The senescent T cells either expressed high levels of constitutive p-p38-MAPK or had impaired up-regulation of p-p38-MAPK upon PMA stimulation (Henson *et al* 2014, Henson *et al* 2015). Therefore constitutive and PMA induced p-p38-MAPK expression was studied in MM T cell clones. Constitutive p-p38 expression in MM T cell clones did not differ from non-clonal T cells or CD8+ T cell subsets (Figure 5.18A) indicating that constitutive levels were not elevated. Whilst PMA stimulation did not augment p38 levels in T cell clones, there was a slight impairment in T cell clone upregulation of p38 in comparison to Vβ+CD57- cells but this was not significantly different from the non-clonal T cell comparator subset (Figure 5.18B) indicating that this pathway was not responsible for inducing senescence.

5.3.8 Summary of phenotypic features in MM T cell clones

Table 5.3 summarises the phenotypic features of MM T cell clones and shows how they compare to the features of anergic, exhausted and senescent (telomere-dependent and telomere-independent) T cells in the literature.

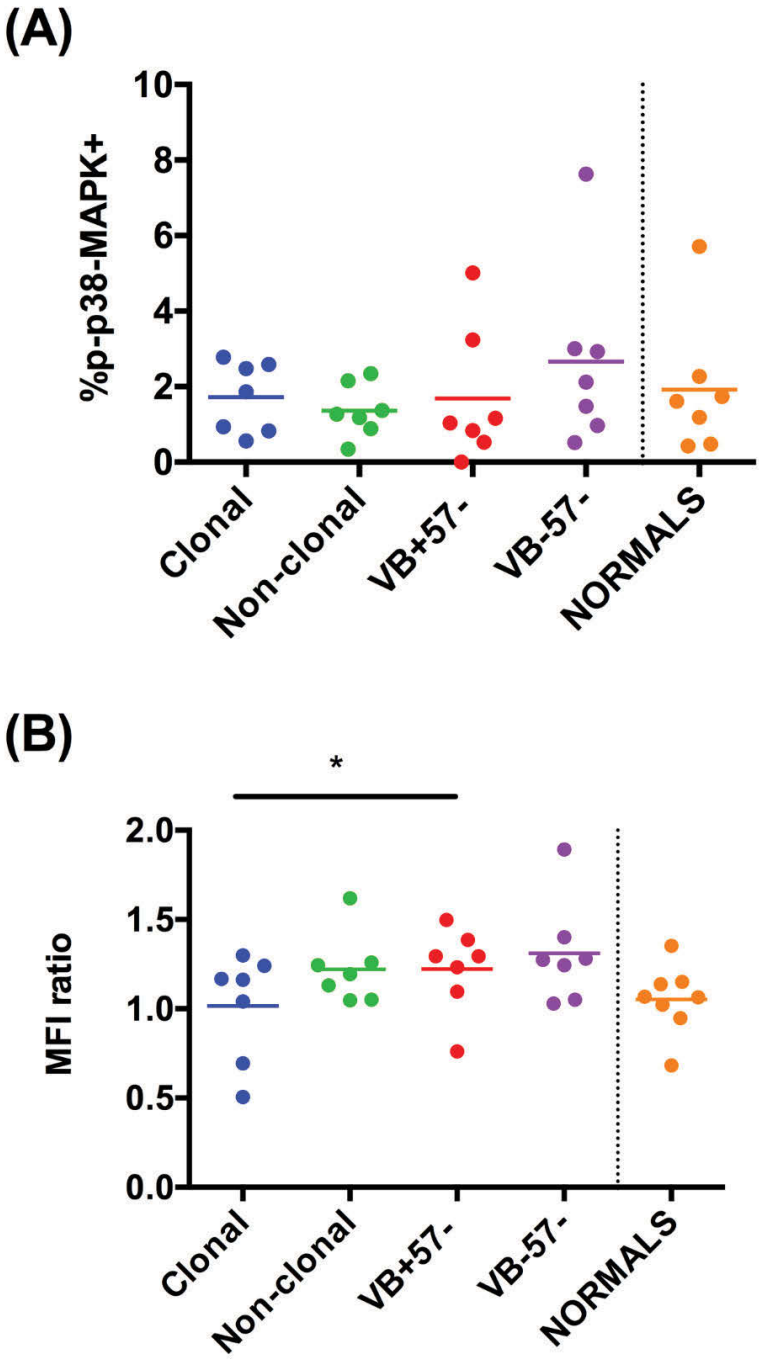


Figure 5.18 p-p38-MAPK expression on T cells from MM patients and normal controls

PBMC from MM patients (n=7) were either stimulated or not stimulated with 100 nM PMA before fixation and permeabilisation with the BD Phosflow I method **(A)** Constitutive p-p38-MAPK expression and **(B)** MFI ratio (MFI stimulated/MFI unstimulated) was measured on CD8+ T cell subsets. For constitutive expression, data are presented as cells expressing p-p38-MAPK and for induced expression, the MFI ratio of stimulated to unstimulated cells was used.*$p<0.05$

Table 5.3 Identification and categorisation of dysfunctional T cells by phenotype and signalling pathways

	Anergic	Exhausted	Senescent-telomere dependent	Senescent-telomere independent	References	MM T cell clones (current study)
CD28	+	+	-	-	(Crespo *et al* 2013)	-
CD57	+	-	+	+	(Crespo *et al* 2013)	+
PD-1	+	+	-	-	(Crespo *et al* 2013)	-
CTLA-4	+	+/-	-	-	(Crespo *et al* 2013)	-
LAG-3	+	+	-	-	(Crespo *et al* 2013)	-
Tim-3	-	+	+	-	(Crespo *et al* 2013)	-
CD160	-	+	+	+	(Crespo *et al* 2013)	+
KLRG-1	-	-	+	+	(Crespo *et al* 2013)	+
p16	?	?	↑	- /low	(Beausejour *et al* 2003, Herbig *et al* 2004, Campisi and d'Adda di Fagagna 2007)	- /low
p21	?	?	↑	-	(Herbig *et al* 2004)	Normal
p38-MAPK	↑	?	↑	↑	(Henson *et al* 2014, Henson *et al* 2015)	Normal
IFN-γ	+	-	+	+	(Crespo *et al* 2013)	+
Telomere length	Normal	Shortened	Shortened	Normal	(Beausejour *et al* 2003, Herbig *et al* 2004, Campisi and d'Adda di Fagagna 2007)	Normal

Abbreviations: CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; IFN, interferon; KLRG-1, killer cell lectin-like receptor subfamily G member 1; LAG-3, lymphocyte activation gene-3; MAPK, mitogen-activated protein kinase; MM, multiple myeloma; PD-1, programmed death 1; Tim-3, T-cell immunoglobulin and mucin protein-3. +: positive; −: negative. Adapted from Suen et al 2016.

5.3.9 MM T cell clones exhibit elevated levels of telomerase

A plausible explanation for the presence of senescent T cells that do not have shortened telomeres is the upregulation of telomerase to maintain telomere length during long term persistence, despite being senescent. Telomerase maintains telomere length in human cells by adding TTAGGG repeats onto telomeres with its internal RNA as a template for reverse transcription (Yu *et al* 1990, Feng *et al* 1995).

Telomerase levels, as measured by hTERT expression by flow cytometry (Handa *et al* 2010) (Figure 5.19A) revealed that T cell clones had significantly higher levels of hTERT than non-clonal T cells ($t=2.35$; $p<0.04$). (Figure 5.20). Therefore, T cell clones have upregulated telomerase levels that may help to maintain their telomere lengths.

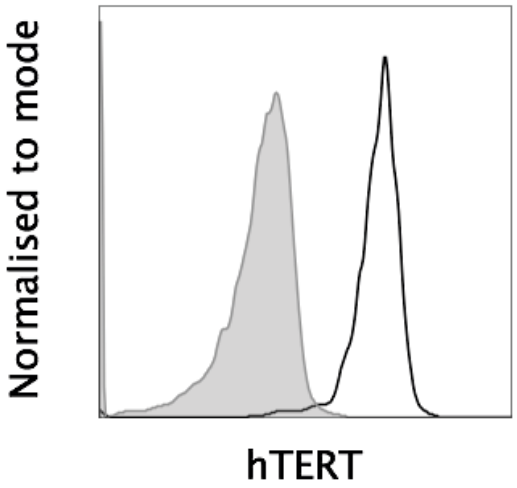


Figure 5.19 Representative histogram for measurement of hTERT by flow cytometry
Representative flow histogram demonstrating cell gating for measurement of the mean fluorescence intensity of hTERT. PBMC were fixed and permeabilised using the Leucoperm kit and then labelled with purified anti-hTERT or purified mouse anti-IgM κ isotype control. Cells were washed and labelled with secondary goat anti-mouse Ig-APC and then washed twice before labelling with clonal T cell surface antibodies. The change in MFI (ΔMFI) of each sample was calculated by subtracting the MFI of the isotype control (filled histogram) from the MFI of the samples stained with anti-hTERT (unfilled histogram).

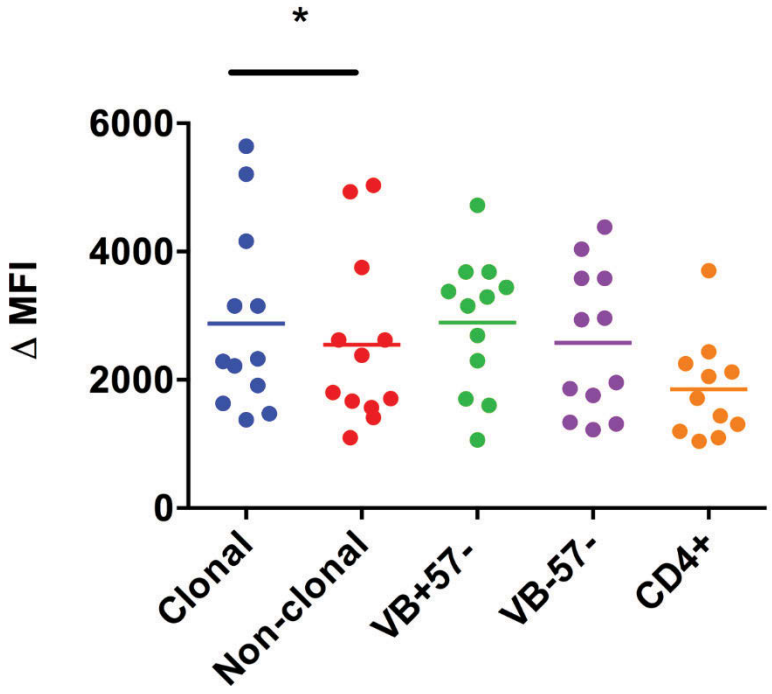


Figure 5.20 hTERT expression on T cells from MM patients
PBMC were fixed and permeabilised using the Leucoperm kit and then labelled with purified anti-hTERT or purified mouse anti-IgM κ isotype control. Cells were washed and labelled with secondary goat anti-mouse Ig-APC and then washed twice before labelling with clonal T cell surface antibodies. The change in MFI (ΔMFI) of each sample was calculated by subtracting the MFI of the isotype control (filled histogram) from the MFI of the samples stained with anti-hTERT (unfilled histogram). Data are presented as ΔMFI, which represents the level of hTERT in clonal, non-clonal, other CD8+ T cells and CD4 T cells in MM patients. *p<0.05.

5.3.10 p-Akt expression is elevated in MM T cells but is not related to telomerase activity

p-Akt has been linked to telomerase levels. The expression of p-AKT can upregulate telomerase activity by phosphorylation of hTERT (Kang *et al* 1999). Whilst p-Akt levels in T cell clones were significantly higher than that of CD8+CD57+ T cells from normal controls (U=0; p<001), it was similar to the levels found in other CD8+ T cells subsets in MM patients (Figure 5.21) suggesting that elevated pAkt expression may be a feature of the MM disease state.

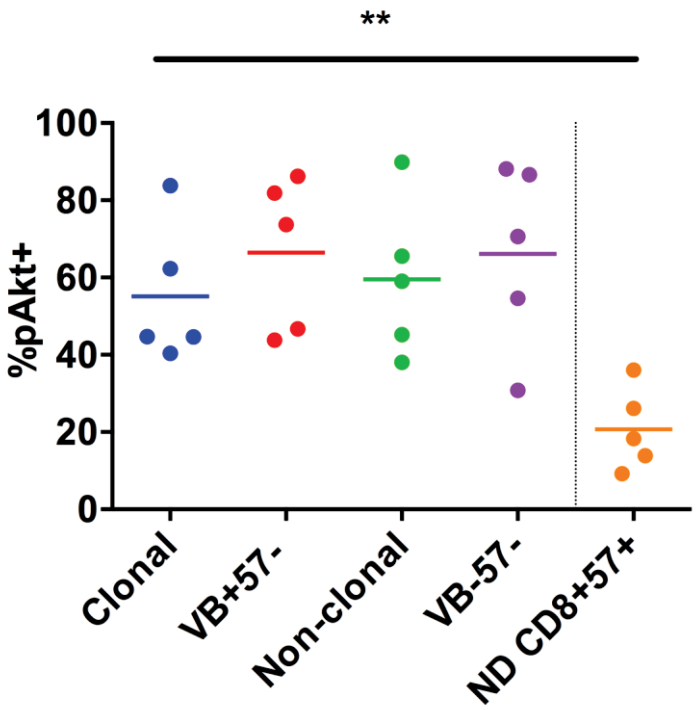


Figure 5.21 pAkt expression on T cells from MM patients
PBMC were obtained from MM patients (n=5) and fixed and permeabilised according to the BD Phosflow method for the detection of pAkt. Data are presented as cells expressing pAkt. **p<0.01.

5.3.11 Checkpoint expression in MM[5]

For immune checkpoint blockade to be successful in MM, it is essential that antigen-specific effector CD8+ T cells are present and that these cells are able to recognise myeloma-specific targets and that they can be re-activated. As clonal T cells in MM are possibly tumour-specific T cells, negative checkpoints CTLA-4 and PD-1 expression on PB and BM T cells from MM patients and age-matched controls were examined. The T cell clones expressed significantly lower levels of PD-1 than the non-clonal T cells in both PB ($p=0.004$; Figure 5.5) and BM ($p=0.02$; Figure 5.22) and also in comparison to CD8+CD57+ T cells from age-matched normal controls ($U=11$; $p=0.01$). Similarly, CTLA-4 expression was significantly lower on T cell clones found in the PB compared to non-clonal cells ($p=0.005$; Figure 5.8) but this was not significantly different in the BM (Figure 5.23). Low PD-1 and CTLA-4 expression detected on T cell clones infers that these cells are not exhausted T cells and suggests that there would be a sub-optimal response to immune checkpoint blockade in MM.

[5]The data presented in this section on PD-1 expression on MM BM T cell clones has been published in **Suen, H**., Brown, R., Yang, S., Ho, P.J., Gibson, J. & Joshua, D. (2015) The failure of immune checkpoint blockade in multiple myeloma with PD-1 inhibitors in a phase 1 study. *Leukemia,* **29,** 1621-1622.

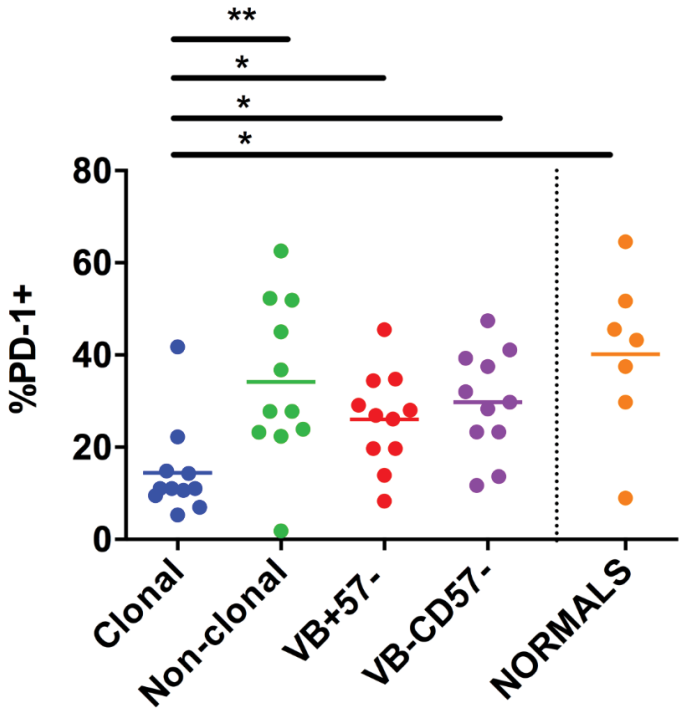


Figure 5.22 PD-1 expression on BM T cells from MM patients and normal controls
BMMC were obtained from patients with MM (n=13) and age matched normal controls (n=7) and PD-1 expression was measured on CD8+ T cell subsets by flow cytometry. Data are presented as % of cells expressing PD-1. *p<0.05, **p<0.01.

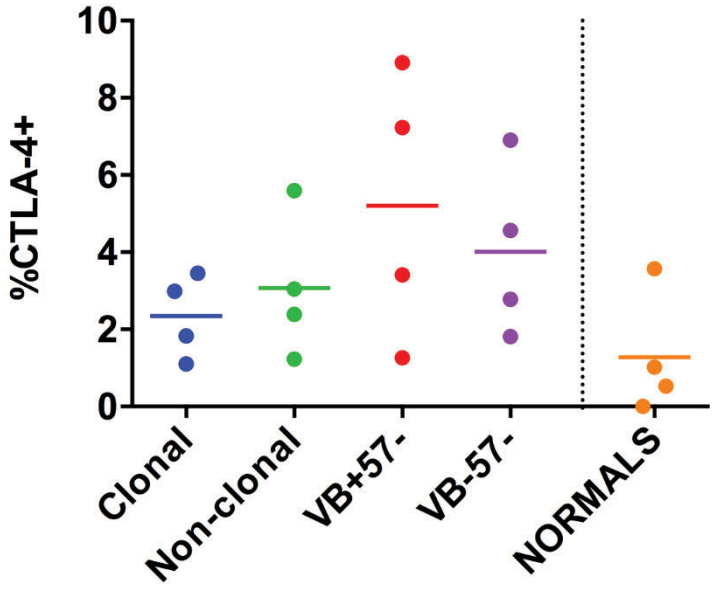


Figure 5.23 CTLA-4 expression on BM T cells from MM patients and normal controls
BMMC were obtained from patients with MM (n=4) and age matched normal controls (n=4) and CTLA-4 expression was measured on CD8+ T cell subsets by flow cytometry. Data are presented as % of cells expressing CTLA-4. p=NS.

BTLA, an inhibitory receptor similar to PD-1 and CTLA-4 (Watanabe *et al* 2003) that has been shown to inhibit proliferation and cytokine production in tumour-specific CD8+ T cells (Derre *et al* 2010), was also studied (Figure 5.24). There were no differences in BTLA expression between clonal and non-clonal T cells, other CD8+ T cell subsets or CD8+CD57+ T cells from normal controls, suggesting that BTLA does not play a role in T cell dysfunction.

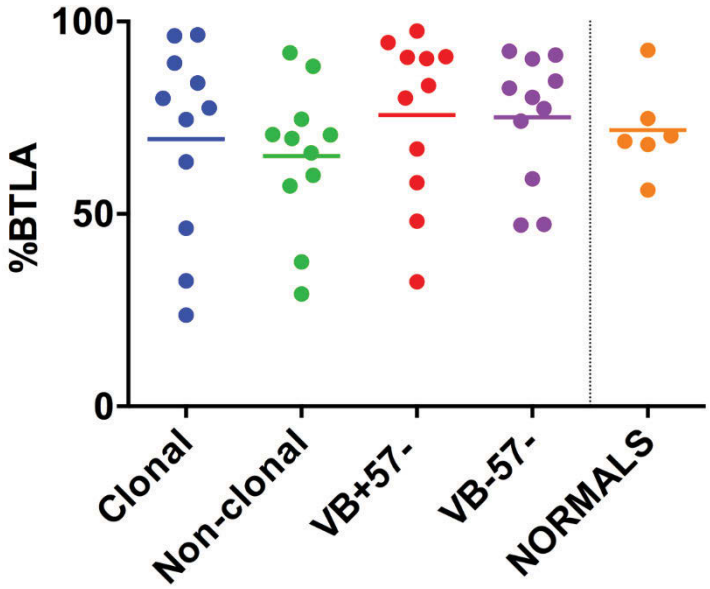


Figure 5.24 BTLA expression on T cells from MM patients and normal controls
PBMC were obtained from patients with MM (n=11) and age matched normal controls (n=6). BTLA expression was measured on CD8+ T cell subsets by flow cytometry. Data are presented as % of cells expressing BTLA. p=NS

5.3.12 T-bet

T cell clones expressed significantly lower levels of PD-1 compared to other MM CD8+ T cells and normal controls (see section 5.3.2 and 5.3.11). To investigate the mechanism by which T cell clones downregulate PD-1 in comparison to other CD8+ T cells, the expression of master transcription factor T-bet was investigated. It has been shown that the level of T-bet is inversely proportional to its target PD-1 (Kao *et al* 2011b).

T-bet was present at very high levels in T cell clones but this was similar to the levels observed in the non-clonal T cells. CD8+ T cells from MM patients that were negative for CD57 had slightly lower T-bet levels (Figure 5.25), which correlates with higher PD-1 expression that is observed in these cells (Figure 5.5 and Figure 5.22). This suggests that T-bet may play a role in regulating PD-1 expression in MM T cells.

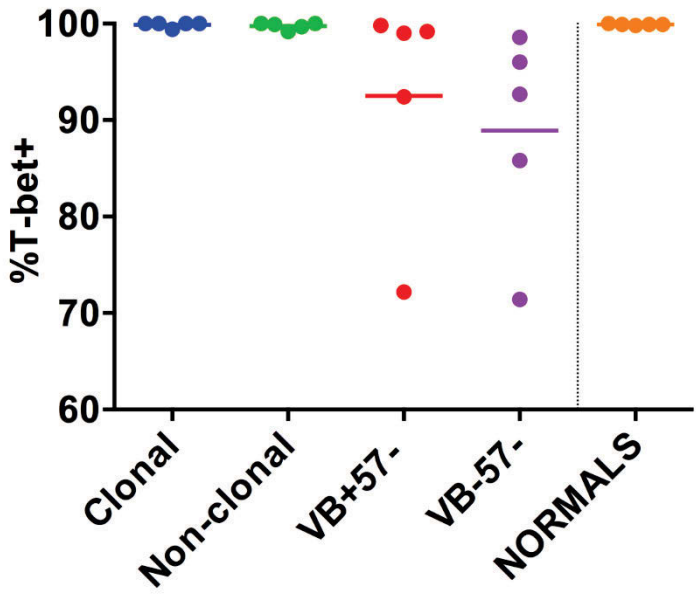


Figure 5.25 T-bet expression on T cells from MM patients and normal controls
PBMC were obtained from MM patients (n=5) and age-matched normal controls (n=5) and fixed and permeabilised according to the BD Phosflow method for the detection of T-bet. Data are presented as cells expressing T-bet. p=NS

5.4 Discussion

In order to understand the basis of the hypo-responsiveness of MM T cell clones, and determine if this was reversible, it was necessary to classify MM T cell clones as anergic, exhausted or senescent. There has been no clear definition of MM T cell clones in terms of these subtypes, with confounding evidence identifying these cells as all three subtypes. This is the first study to successfully characterise the phenotype of these cells and this discovery will assist in the reversal of clonal T cell dysfunction. By examining the cell surface phenotype, telomere length and signalling pathways, an important new observation was made. The T cell clones displayed features of senescent T cells: i.e. CD57+, CD28-, CD160+ and KLRG-1+ cell surface phenotype. A key finding of this study is that these clonal T cells have normal-for age telomere lengths that were not shorter than non-clonal T cells from the same patient or CD8+ CD57+ T cells from normal controls. As telomeres are not critically shortened, this suggests that cellular senescence is telomere-independent and therefore potentially reversible. The senescence was not related to upregulated p16, p21 or p38-MAPK pathways, suggesting that other signalling pathways are responsible for inducing senescence. However, the T cell clones had elevated levels of telomerase, which may explain how normal telomere lengths are maintained in senescent T cells. Low PD-1 and CTLA-4 expression detected on T cell clones infers that these cells are not exhausted but suggests that there would be a sub-optimal response to immune checkpoint blockade in MM as these negative immune interactions are not present.

The clonal nature of the cells suggests exposure to cognate antigen, resulting in proliferation (Morley *et al* 1995). The fact that these cells remain stable and endure for long periods suggests the cells are constantly exposed to antigenic stimulation. This results in a continuous stimulus for proliferation and expansion. As T cells go through multiple rounds of proliferation, they begin to downregulate and eventually lose CD28 and accumulate as a highly antigen experienced, terminally differentiated CD57+CD28- T cell. MM T cell clones are CD57+CD28- cells and high levels of paraprotein detected in patients with MM would indicate large amounts of circulating tumour antigens that could

constantly stimulate these cells. The T cell clones also express high levels of CD160. CD160 expression increases on CD8+ T cells during life, and predominantly on the CD28- subset (Cai and Freeman 2009) and has been linked to the dysfunction of CD8+ T cells. Crosslinking of CD160 on CD8+ T cells inhibits T cell proliferation after CD3/28 stimulation (Cai and Freeman 2009). It can become upregulated when CD8+CD28+ T cells are exposed to repetitive *in vitro* antigenic stimulation (Merino *et al* 2007), which is why CD160 can be a marker of T cell exhaustion and senescence (Crespo *et al* 2013). T cell clones also expressed high levels of KLRG-1, which is expressed on NK cells and antigen-experienced T cells and is a marker of senescence (Henson and Akbar 2009). It has been shown that KLRG-1 expression correlates with age and highest levels of KLRG-1 are shown in the CD27-CD28- T cells from elderly healthy donors (Henson *et al* 2009). KLRG-1 also plays a role in the inhibition of T cell proliferation, but only when MHC/antigen and KLRG-1 ligands are expressed by the same cell that interacts with the T cells (Rosshart *et al* 2008). One of the natural ligands of KLRG-1 is E-cadherin which is responsible for mediating epithelial cell-to-cell adhesion (Rosshart *et al* 2008). E-cadherin can be expressed on a variety of APC and MM plasma cells, and higher levels of soluble E-cadherin have been found in MM patients compared to healthy volunteers, suggesting a potential role for MM induced T cell dysfunction through KLRG-1 (Syrigos *et al* 2004). The data from this study suggests that T cell clones are highly differentiated, antigen-experienced senescent T cells that express inhibitory receptors that can contribute to inhibition of proliferation.

To characterise the mechanism of senescence induction in T cell clones, pathways that are associated with telomere-dependent and independent senescence were investigated. Telomere-independent senescence is induced in response to cellular stress or disruptions to normal cell signalling, such as upregulation of p16 and Rb, leading to an anti-proliferative state (Beausejour *et al* 2003). Elevated levels of p16 were not detected in the T cell clones of MM patients and levels were not different from non-clonal T cells, indicating that the p16 pathway is not responsible for inducing clonal T cell senescence. This correlates with a study that also showed senescent cells with average TL had low levels of p16 (Migliaccio *et al* 2005). Cells with low levels of p16 at

senescence are able to proliferate again after p53 inactivation, whereas cells with high p16 are unable to proliferate after p53 inactivation (Beausejour *et al* 2003), suggesting that it may be possible to reactivate the MM T cell clones. Senescence triggered by shortened telomeres (telomere-dependent senescence) is regulated by signaling through ATM to p53, leading to the upregulation of p21 and therefore cell-cycle arrest (Herbig *et al* 2004). As expected, MM T cell clones did not have upregulated p21 expression and this confirms that the cells do not have telomere-dependent senescence.

The p38-MAPK pathway has been implicated in senescence and blocking of this pathway has successfully reversed senescence in CD4+CD27-CD28- T cells (Di Mitri *et al* 2011) and CD8+ EMRA T cells (Henson *et al* 2014, Henson *et al* 2015). The senescent T cells had either high constitutive p38-MAPK expression or upon PMA activation, were unable to upregulate p38-MAPK expression to the levels of non-senescent T cells. The data from this study indicate constitutive p38-MAPK levels are not elevated in clonal T cells and upon PMA activation, there was no impairment of p38-MAPK upregulation when compared to non-clonal T cells. These results suggest that this pathway does not play a role in inducing senescence in MM T cell clones.

Other cell signaling molecules may be responsible for inducing senescence in MM T cell clones. NF-κβ signaling is required for the maintenance of SASP (Salminen *et al* 2012) and inhibition of this pathway can bypass growth arrest and reverse senescence (Rovillain *et al* 2011). IL-6, a cytokine present at high levels in the MM microenvironment, has been described as a key player in oncogene-induced senescence (Kuilman *et al* 2008) and can up-regulate NF-κβ signaling, whilst preventing apoptosis to maintain cell senescence (Salminen *et al* 2012). MM T cell clones retain their ability to produce IFN-γ (Bryant *et al* 2013) and display the characteristics of SASP so the NF-κβ pathway should be investigated. Overexpression of c-myc can also cause cell senescence as part of a cancer defence mechanism (Grandori *et al* 2003). Microarray data from WM T cell clones indicated that c-myc and NF-κβ were upregulated in T cell clones (Li *et al* 2010) and the relationship between these proteins and clonal T cell responses need to be explored in the MM setting. In mouse models,

KLRG-1+/CD8+ T cell senescence was related to p15 INK4b expression. The human equivalent of this protein is P14 ARF (Grange *et al* 2015). Further investigation of the levels of these other cell-cycle regulatory proteins is required to elucidate the mechanism of senescence induction.

The finding that these MM T cell clones are senescent rather than anergic or exhausted is significant. T cells in the cancer setting are usually considered to be exhausted, similar to the T cells observed in patients with chronic viral infection due to the constant exposure to high levels of tumor antigens and an immunosuppressive microenvironment. (Schietinger and Greenberg 2014) Exhausted T cells commonly express the inhibitory receptors PD-1, LAG-3, TIM-3 and CTLA-4 and have dysregulated signaling pathways (Fourcade *et al* 2012, Woo *et al* 2012). The MM T cell clones expressed low levels of these inhibitory receptors. They are also unlike the exhausted T cells found in melanoma patients, a highly antigenic tumor, which have high levels of PD-1 and therefore are susceptible to PD-1 blockade. Anergic T cells are induced during inadequate TCR co-stimulation or in the presence of high inhibitory signaling, which may be present in the cancer microenvironment. The hypo-responsiveness of the MM T cell clones however cannot be reversed with endogenous IL-2 and inhibitory receptors like CTLA-4 and PD-1 are not highly expressed. MM T cell clones are also unlike an aged T cell (replicative senescence) as they do not have shortened telomeres exhibited by T cells that have gone through multiple rounds of proliferation. The MM T cell clones are held in a suspended non-proliferative state, and persist for long periods of time due to inhibition of apoptosis. T cell clones in MM are also not expansions of large granular lymphocytes. They exhibit the same phenotype (CD3+CD8+CD57+) but do not have increased STAT3 expression (data not shown) that is associated with large granular lymphocytic leukaemia.(Teramo *et al* 2013)

A plausible explanation for the presence of senescent T cells that do not have shortened telomeres, is the upregulation of telomerase to maintain TL during long term persistence. T cell clones had significantly higher levels of hTERT than non-clonal T cells and this may be responsible for circumventing the

senescence barrier. Senescent HIV-specific CD8+ T cell clones are also remarkably similar to MM T cell clones as they expressed hTERT, which was linked to decreases in the levels of p16 and p21 cell cycle inhibitors, demonstrating that stable hTERT expression play a role in overcoming senescence induced effects (Dagarag *et al* 2004). The T cell clones in MM are highly differentiated effector memory T cells that re-express CD45RA (EMRA) and studies have shown that whilst EMRA T cells do have shorter telomeres than naïve CD8 T cells, they have longer telomeres than effector memory T cells that express CD45RO (Henson *et al* 2014). Other phenotypic markers of T cell clones such as expression of CD57, CCR7, KLRG-1 and lack of CD28, PD-1 also demonstrate T cell clones fit into the EMRA T cell subset (Larbi and Fulop 2014).

In this study, pAkt expression was found to be elevated in T cell clones compared to healthy controls, although, elevated pAkt levels was not exclusive to T cell clones and was found in all MM CD8+ T cells. pAkt expression can increase telomerase activity by phosphorylation of hTERT (Kang *et al* 1999) and elevated hTERT levels were also detected in T cell clones, suggesting that pAkt induced phosphorylation of hTERT may be responsible for upregulating telomerase in senescent T cells. Cytokines like IL-6 and insulin growth factor-1 can increase telomerase levels through the involvement of the PI3k/Akt/NF-κβ signalling (Akiyama *et al* 2002). IL-6 and NF-κβ are also implicated in oncogene-induce senescence (Kuilman *et al* 2008) so the NF-κβ pathway is a potential pathway to be explored in the context of MM T cell clones.

This study documents low levels of PD-1 and CTLA-4 detected on T cell clones which may explain why Phase 1 studies of PD-1 inhibitor Nivolumab failed to provide a meaningful clinical response in 100% of MM patients (Lesokhin *et al* 2014, Suen *et al* 2015). The reason for down-regulated PD-1 expression in T cell clones in MM is unknown. PD-1 expression in CD8+ T cells is inversely regulated at the transcription level by transcription repressors like T-bet and B lymphocyte–induced maturation protein 1 (Blimp-1) (Kao *et al* 2011b, Lu *et al* 2014). T-bet represses PD-1 expression and is necessary for the persistence of antigen-specific CD8+ T cells during chronic antigen exposure (Kao *et al*

2011a). Interestingly, it has also been reported that T-bet controls cytotoxic and cytokine secretory functions of CD8+ effector T cells and also the sensitivity of cells to senescence (Intlekofer *et al* 2007). All clonal and non-clonal T cells in MM patients were found to have extremely high levels of T-bet, which correlates with a study demonstrating that T-bet is increased in CD8+ T cells from elderly healthy individuals and correlates with CD57 and KLRG-1 expression (Dolfi *et al* 2013). Therefore, T-bet expression may be a potential explanation for the repression of PD-1 in MM T cell clones. High T-bet expression was similarly found in non-clonal T cells in MM patients but these cells have higher PD-1 expression than clones. T-bet induced repression may not be the complete explanation and it has been shown that when expressed at high levels, T-bet's repressive effect on PD-1 is not strong and other transcription factors may be responsible for suppressing PD-1 expression in CD8+ T cells (Lu *et al* 2014). Blimp-1 is a potential candidate to be studied as it becomes activated during later stages of CD8+ T cell activation (Kallies *et al* 2009).

Based on these findings, it is likely that PD-1 and CTLA-4 checkpoint immune blockade would only yield sub-optimal results in MM as the potentially tumour-specific T cell clones do not over-express these negative checkpoint receptors. Furthermore, MM plasma cells do not express high levels of PD-L1, one of the ligands for PD-1 (Paiva *et al* 2015). If the PD-1/PD-L1 axis does not play a crucial role as a master checkpoint regulator in MM, this may explain why patients have not displayed a clinical benefit with PD-1 blockade, at least when used as a monotherapy. It has recently been demonstrated that lenalidomide can enhance immune checkpoint blockade-induced immune responses in MM (Görgün *et al* 2015). Other preliminary trials with pembrolizumab, another PD-1 monoclonal antibody, in combination with either lenalidomide and dexamethasone (Mateos *et al* 2015) or with pomalidomide (San Miguel *et al* 2015) have also induced good clinical responses in MM patients. Therefore, it may be worthwhile to continue investigation of immune checkpoint blockade as part of combination therapy in multiple myeloma.

In conclusion, the T cell clones in MM have a distinct molecular signature of telomere-independent senescence rather than anergy or exhaustion. As MM T

cell clone immunosenescence is not related to shortened telomeres, it is potentially reversible. An improved understanding of the mechanisms involved in the induction of these senescent clonal T cells and how their cellular functions are affected will provide potential targets to restore clonal T cell function. If it is related to dysfunctional signalling pathways, it may be possible to re-modulate the pathways with small molecule inhibitors or drugs such as IMiDs or HDAC inhibitors. This may present a unique opportunity to enhance tumour immunity in MM.

CHAPTER 6 FINAL DISCUSSION AND FUTURE DIRECTIONS

6.1 Key Findings from this work

Cancer is associated with a failure of immune surveillance. Tumour-induced mechanisms incapacitate immune cells, including tumour-specific cytotoxic T cells, thus permitting tumour evasion and consequent progression (Kim *et al* 2007). Cytotoxic T cells are the predominant type of effector cell involved in immune-mediated cancer destruction. Therefore, reversing tumour-induced T cell dysfunction is an important consideration for cancer immunotherapies. Expanded clones of cytotoxic T cells have been detected in patients with MM (Brown *et al* 1997, Joshua *et al* 2003, Raitakari *et al* 2003) and can constitute up to 50% of all blood lymphocytes. However, these cells have been shown to be hypo-responsive *in vitro* (Li *et al* 2010, Bryant *et al* 2013) but, despite this, their presence is associated with improved survival (Brown *et al* 1997, Sze *et al* 2001, Joshua *et al* 2003, Raitakari *et al* 2003, Brown *et al* 2009, Suen *et al* 2016). The exact function of these cells and the mechanism(s) for their persistence for long periods of time in the host to provide protection and improve survival have yet to be elucidated. In order to achieve a greater understanding of the role of these cells in MM, a full characterisation of these cells was necessary.

The primary aim of this thesis was to characterise hypo-responsive T cell clones in patients with MM, in order to gain insight into the molecular mechanisms inducing their dysfunction and thus provide novel and specific targets to restore the immune response. The major findings and conclusions from the work described in this thesis are:

1. T cell clones were detected in 75% of a new cohort of MM patients and, despite being hypo-responsive *in vitro*, their presence was related to improved survival
2. T cell clones were a universal feature of 10 year survivors of MM and the cells of these survivors retained their ability to proliferate *in vitro*, providing a further link between the presence of these cells and long term survival in MM

3. Dysfunctional signalling pathways that contributed to clonal T cell dysfunction were identified including the SMAD and ERK pathway
4. For the first time it has been demonstrated that T cell clones displayed features of telomere-independent senescence. They are unlike aging T cell clones, and therefore senescence is a tumour related phenomenon. As their telomere lengths were normal, this suggests that the observed senescence is potentially reversible.

These novel findings contribute greatly to the current understanding of clonal T cell biology in MM. This is the first study to successfully identify the nature of the dysfunction of these protective cells and conclude that this dysfunction is potentially reversible. This has important implications for future studies that may allow for experimental reversal of dysfunction, determination of the specificity of these cells and understanding their cytotoxic and/or other functions. This would lead to the ultimate aim of freeing T cell clones from tumour suppression so that they may target and kill malignant plasma cells. These novel findings support the concept of specifically targeting a patient's own tumour cells and could form the basis of a personalised therapeutic strategy for patients with MM.

T cell clones have been identified in a number of historical MM cohorts and the cells were associated with an improved survival (Brown *et al* 1997, Raitakari *et al* 2000, Sze *et al* 2001, Sze *et al* 2003). To gain an understanding of the relevance of these cells to survival in the age of current disease and therapy, the incidence of T cell clones was measured in a new cohort of MM patients, as described in Chapter 3 of this thesis. T cell clones were detected in 75% of MM patients and these results are in agreement with the findings from a previous cohort of MM patients, of which 76% had been found to harbour T cell clones (Brown *et al* 2009). Both cohorts also consisted of patients who had received IMiDs therapy. The prognostic significance of these cells was re-affirmed in the cohort of this thesis, confirming previous findings that T cell clones are associated with an improved survival in patients with MM (Brown *et al* 1997, Brown *et al* 2009). Furthermore, this information is important, as it shows that T cell clones are able to prolong survival, even in the age of new therapies that have already extended the median survival of patients (Kumar *et al* 2008). This

positive prognostic association was present despite the fact that the cells were unable to proliferate *in vitro*, as demonstrated through CFSE proliferation assays described in Chapter 3. This suggests that although tumour related immune suppression has limited the function of these cells, they are still able to confer a small degree of protection and prolong survival in patients who possess these cells. T cell clones were also found to be universally present in a group of 10 year MM survivors. This finding is of great interest and further confirms previous findings that T cell clones are found in long term survivors (Bryant *et al* 2011). This finding also provides a crucial link not only between the presence of these cells, but also their function, and potential contribution to long term survival in MM.

Another significant finding highlighted in Chapter 3 was the difference in the proliferative capacity of T cell clones according to patient survivor status. Upon TCR activation and culture with IL-2, T cell clones from long term survivors were proliferative and differed from the T cell clones of non-10 year survivors that were non-proliferative. As the T cell clones in long term survivors remain responsive to stimulus and proliferate, this provides further evidence that T cell clones contribute to long term survival. This finding agrees with an earlier study that found T cell clones to be proliferative in 10 year survivors (Bryant *et al* 2011). It is therefore interesting to speculate that these cells are not as heavily suppressed by the inherent tumour cells and that tumour-induced dysfunction of T cells is reversible.

The next step was to gain a clear understanding of the mechanisms by which T cell clonal dysfunction is induced. In order to do this, the signalling pathways of T cell clones were studied by intracellular and phospho-flow cytometry, as detailed in Chapter 4. Pathways chosen for analysis were based on a study of T cell clones from WM patients (Li *et al* 2010) and included pathways relating to 1) anti-apoptosis, which result in long term persistence of cells; 2) T cell inactivation by TGF-β induced pSMAD-mediated effects and 3) anti-proliferation, which may explain the hypo-responsiveness of T cell clones. Furthermore, the levels of target signalling proteins within these pathways were compared between the T cells of 10 year survivors and non-10 year survivors to

determine what may contribute to the differences in proliferation that were observed in Chapter 3. In doing so, two novel mechanisms of clonal T cell dysfunction were identified: an upregulation of the SMAD pathway and a defective ERK pathway. These two pathways were also similarly dysregulated in T cell clones from WM patients (Li *et al* 2010). This suggests a potential link between dysregulated clonal T cell populations in two related B cell malignancies.

TGF-β induced phosphorylation of SMAD can lead to the interaction with the Tob protein that, in turn, interacts with CDKI p27kip1 to induce cell cycle arrest (Tzachanis *et al* 2001). Interestingly, it has also been shown that TGF-β-dependent p-SMAD activation can inhibit CD3/28 mediated T cell proliferation in mouse models (McKarns *et al* 2004). Elevated levels of pSMAD were detected in the T cell clones of non-10 year survivors (Chapter 4), which had been shown to be hypo-responsive in Chapter 3. Therefore, elevated SMAD levels in hypo-responsive T cell clones of non-10 year survivors may be responsible for inactivating the T cells through inhibition of proliferation and/or induction of cell cycle arrest. Another novel dysfunctional mechanism identified in MM T cell clones is a dysregulated ERK pathway. ERK plays a crucial role in controlling proliferation of cells (Johnson and Lapadat 2002). T cell clones from MM patients had an impaired ability to augment phosphorylation of ERK after mitogen stimulation in comparison to age-matched normal controls, suggesting that this pathway is dysregulated or inhibited by other proteins such as HePTP, and therefore proliferation is blocked.

Chapter 5 aimed to understand the nature of the dysfunction in these cells. Different types of dysfunctional T cells have been documented in cancer and can include anergic, exhausted or senescent T cells (Crespo *et al* 2013). Determining whether the protective T cell clones from MM patients are anergic, exhausted or senescent would provide insights into the nature of the hypo-responsiveness and help determine if it is reversible. This knowledge is of great importance as there are specific means to overcome the different types of dysfunction. Anergy can be be reversed by the addition of cytokines to overcome incomplete co-stimulation or high inhibitory signalling. Checkpoint

inhibition of over-expressed receptors such as PD-1 or CTLA-4 on exhausted T cells may reverse T cell exhaustion. Telomere-dependent senescence is irreversible due to the presence of critically shortened telomeres, whilst telomere independent senescence can be reversed with blockade of up-regulated pathways responsible for inducing senescence (Crespo *et al* 2013). There is also considerable confusion between these three subsets of T cells as these terms are sometimes used interchangeably in the literature (Akbar and Henson 2011) and some of the phenotypic markers are expressed on more then one type of dysfunctional T cell (Crespo *et al* 2013). By examining the phenotypic characteristics, signalling pathways, telomere length and telomerase levels in hypo-responsive MM T cell clones, an important new observation has been made. This work has shown that these cells are senescent T cells with normal telomere lengths according to age and exhibit elevated telomerase levels, suggesting that they are telomere-independent senescent T cells. This is a crucial finding because it suggests that senescence can potentially be reversed since the telomeres in these cells are not critically shortened. These highly novel observations are currently in press for publication in the journal Leukemia (Suen *et al* 2016). This adds knowledge to the limited body of literature on clonal T cell dysfunction in MM.

Having shown that T cell clones displayed telomere independent senescence, the next step was to understand the precise signalling mechanisms that mediate senescence. As described in Chapter 5, the p16, p21 and p38-MAPK pathways, associated with senescence induction (Herbig *et al* 2004, Campisi and d'Adda di Fagagna 2007, Di Mitri *et al* 2011, Henson *et al* 2014, Henson *et al* 2015), were investigated. Phospho-flow studies revealed low to normal levels of p16, p21 and p38-MAPK proteins in T cell clones and levels were not different from those of non-clonal T cells. The p16 levels detected in this study correlate with another study that documents low levels of p16 in senescent cells that do not have shortened telomeres (Migliaccio *et al* 2005). This finding supports the notion that this senescence phenotype is reversible as it has been shown that cells with low levels of p16 are able to proliferate again after p53 inactivation (Beausejour *et al* 2003). Full activation of pRb by p16 can actually lead to irreversible senescence (Takahashi *et al* 2007) so the low p16 levels in

MM T cell clones also support that this phenotype is reversible. Telomerase levels, as measured by hTERT activity by flow cytometry, were found to be significantly higher in the T cell clones when compared to non-clonal T cells. This provides a potential explanation for the maintenance of normal telomere lengths in senescent cells, as the telomerase can elongate telomeres through the addition of TTAGGG repeats.

In Chapter 5, PD-1 and CTLA-4 expression were studied as part of the aim to describe the dysfunctional phenotype of T cell clones. Blocking of negative receptors like PD-1 and CTLA-4, referred to as immune checkpoint blockade, can release T cells from tumour-mediated suppression (Pardoll 2012). This type of immunotherapy has been effective in the treatment of malignancies such as melanoma (Topalian *et al* 2012) and Hodgkin lymphoma (Ansell *et al* 2015). However, results have been disappointing in patients with MM (Lesokhin *et al* 2014). For immune checkpoint blockade to be successful, T cells need to over express negative checkpoint receptors and be of an exhausted phenotype. Once the cells are freed from inhibition, there needs to be immune recognition of tumour cells by tumour-specific T cells (Suen *et al* 2015). Prior trials of PD-1 inhibitors only utilised pooled CD8+ T cells that may not have been tumour-specific (Lesokhin *et al* 2014). Chapter 5 demonstrated low PD-1 and CTLA-4 expression on T cell clones from MM patients. This infers that these cells are not exhausted cells. The data therefore disagrees with the use of immune checkpoint blockade in MM and provides a potential explanation as to why PD-1 immune checkpoint blockade has been unsuccessful as monotherapy in MM (Suen *et al* 2015). This has important implications for the development of this therapy in the field of MM. Expression of one of the PD-1 ligands, PD-L1, is not elevated on plasma cells from MM patients when compared to normal plasma cells from healthy donors (Paiva *et al* 2015). This raises the question of whether or not the PD-1/PD-L1 axis is an immune evasion mechanism utilised by MM tumour cells. If this axis does not play a role in tumour induced suppression of T cells, it may be necessary to determine more suitable targets for immune checkpoint blockade, combine multiple checkpoint inhibitors or combine with other types of therapies. There is interesting new data on the combination of checkpoint blockade and targeted therapy in solid tumour malignancy settings

(John *et al* 2013a, John *et al* 2013b). Data on the clinical efficacy of CTLA-4 checkpoint blockade in MM are yet to be published as phase I trials are still currently recruiting patients (Clinical trial number: NCT01592370). This thesis has demonstrated low levels of CTLA-4 expression on T cell clones from MM patients. This suggests that CTLA-4 checkpoint blockade in myeloma is unlikely to be as effective as has been seen in patients with melanoma (Hodi *et al* 2010).

MM is an immunogenic disease and the immune system has the ability to play an active role in controlling malignant plasma cells. Evidence for this includes 1) the clinical state of MGUS patients and the detection of pre-malignancy specific T cells, suggesting the ability of the host immune system to control disease (Dhodapkar *et al* 2003, Spisek *et al* 2007); 2) the phenomenon of plateau phase (despite quantifiable disease), where patients remain stable, suggesting an element of host immune control to maintain this equilibrium (Joshua *et al* 1994); 3) the effectiveness of allogeneic stem cell transplant in MM, which suggests that an adequate immune system has the potential to eradicate MM (Giaccone *et al* 2011, Rutella and Locatelli 2012) and 4) the presence of cytotoxic T cell clones that are related to improved survival (Brown *et al* 1997, Brown *et al* 2009, Suen *et al* 2016). However, tumour-T cell interactions result in suppression of the cytotoxic T cell response against MM plasma cells (Rutella and Locatelli 2012).

A model has been proposed that outlines three phases that occur during interactions between host immune cells and tumour cells in cancer (Kim *et al* 2007). Termed the ‘three E’s’, the phases include elimination, equilibrium and escape and these phases are also evident in the immune landscape of MM (Joshua *et al* 2016). MM plasma cells employ mechanisms to evade the host immune system, including the action of cytotoxic T cells. This new model of ‘immunoediting’ in MM adds to the knowledge of tumour surveillance in MM and is illustrated in Figure 6.1. In the **elimination** phase, there is efficient antigen presentation by mature DCs to T cells, which leads to the clonal expansion of cytotoxic T cells that recognise and eliminate plasma cells. This process is inhibited by tumour suppressive mechanisms that induce senescence, leading

to an immunological *status quo* or **equilibrium.** The tumour-induced senescent T cell clones are unable to exert their full cytotoxic role and instead exhibit a SASP, which only confers a degree of control, as described by this thesis. This phase appears to last the longest and T cells and cytokines play an important role in applying immune pressure on tumour clones. In the final phase, **escape** from immunological equilibrium results in progressive disease as the tumour cells surpass all host immune defences, due to factors including, but not limited to, drug resistance or clonal evolution. This thesis describes the second phase of this model of immune surveillance from immunoediting to immune escape. Restoring the function of these protective cytotoxic T cell clones will allow restoration of the elimination phase whereby T cell clones target and kill malignant plasma cells.

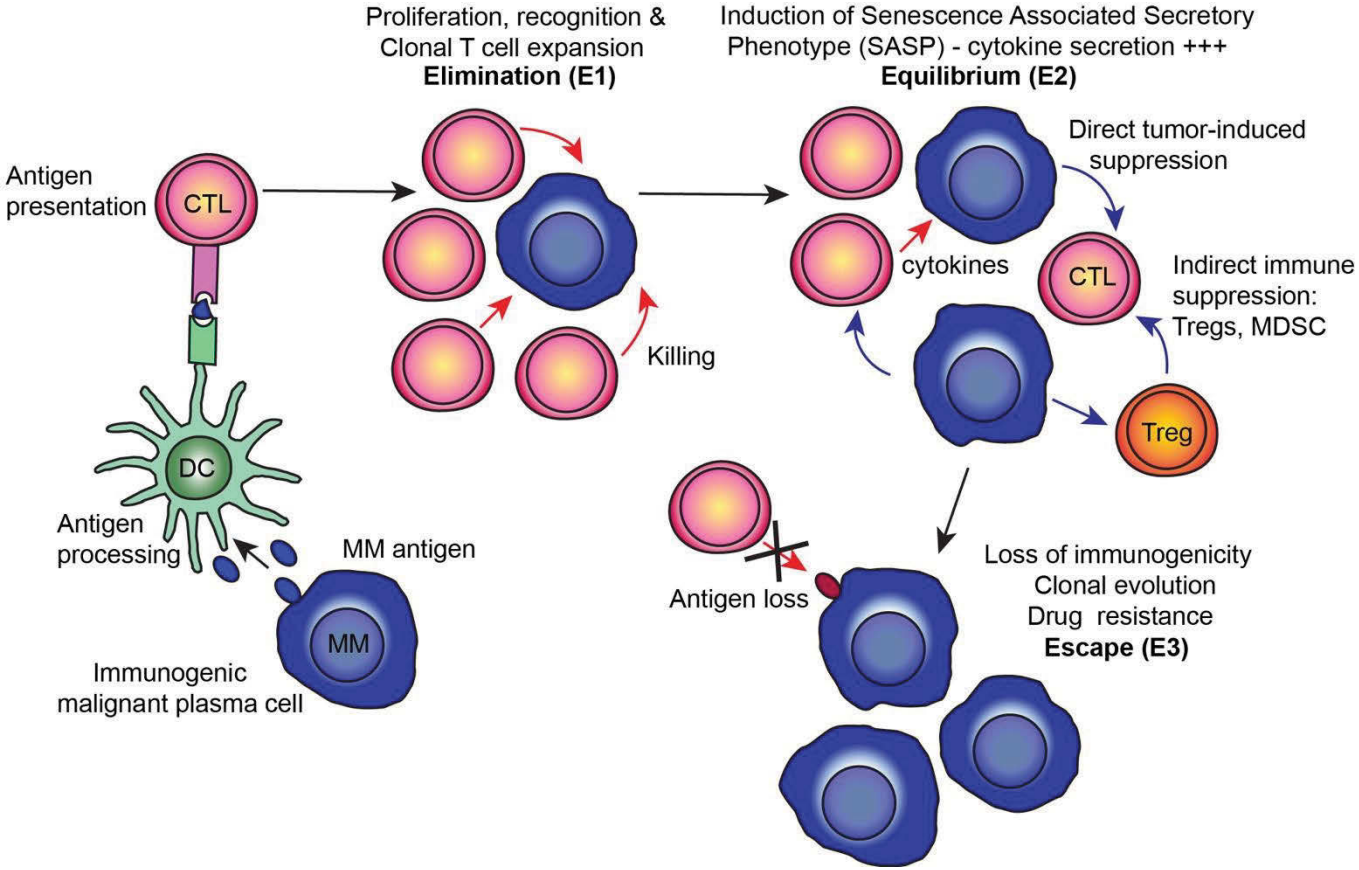


Figure 6.1 The process of cancer immunoediting from immune surveillance to tumour escape in MM

MM associated antigens are presented to MM-specific cytotoxic CD8+ T cells, leading to proliferation and expansion of T cell clones. As MM immunogenicity is variable, a clonal T cell response may not necessarily be initiated in all patients. T cell clones are detected in up to 75% of MM patients and elimination of immunogenic MM plasma cell clones may result. (E1). However, MM tumour cells employ tumour suppressive mechanisms that induce senescence of T cell clones, converting them to a senescence associated secretory phenotype (SASP). These cells retain the ability to secrete high levels of cytokines and play a protective prognostic role in the equilibrium phase (E2). When there is loss of immunogenicity and clonal evolution of plasma cells, this results in tumour escape from immunological control and this leads to relapse and progressive disease (E3). Adapted from Joshua *et al* 2016.

6.2 Future Directions

The findings presented in this thesis progress our understanding of the role of T cell clones in MM. Although the nature of their dysfunction has been described in this thesis as telomere-independent senescence, further work is required to identify the mechanisms by which senescence is induced. In addition, there are many avenues for future research into the role and function of these cells in MM.

In this thesis, the SMAD and ERK pathways were identified as dysregulated in MM T cell clones. Therefore, targeting of these pathways with small molecule inhibitors may allow for the restoration of clonal T cell function in MM. Small molecules that inhibit these two proteins should be tested in future studies. A TGF-β inhibitor, LY2109761, that represses pSMAD2 expression, is a potential candidate and has been shown to be effective in reversing TGF-β induced SMAD-mediated anti-apoptotic effects in myelo-monocytic leukaemic cells (Xu *et al* 2008). HePTP inhibitors that allow for augmentation of ERK activation could also be another approach (Sergienko *et al* 2012). It would also be of interest to further explore the differences in the ERK pathway between 10 year and non-10 year survivors as sample size was limited in this study. Due to the heterogeneous nature of the patient cohort, further studies on correlations between disease state and therapy and these signalling pathways would be of clinical interest.

This was the first study to identify the phenotype of dysfunctional T cell clones as telomere-independent senescence. This type of dysfunction is reversible but the exact mechanisms that induce senescence in T cell clones was not identified. A more detailed investigation of signalling pathways that induce senescence is required. Whilst the p16, p21 and p38-MAPK pathways were found not to be responsible for the senescent nature of MM T cell clones, another pathway worth investigating is the NF-κB pathway. NF-κB signalling has been implicated in the induction of a phenomenon known as the SASP and these cells are able to secrete inflammatory cytokines and chemokines

(Salminen *et al* 2012). MM T cell clones are also able to secrete cytokines including IFN-γ (Chapter 3) (Bryant *et al* 2013). IL-6, a cytokine present at high levels in patients with MM, and a pro-survival factor for plasma cells, has been implicated as a major player in oncogene induced senescence through upregulation of the NF-κB pathway to maintain apoptotic resistance and induce cell senescence (Kuilman *et al* 2008). NF-κB is also central to MM pathogenesis. Mutations in MM plasma cells lead to chronic activation of the NF-κB pathway, which contributes to pro-survival effects in plasma cells (Annunziata *et al* 2007, Demchenko and Kuehl 2010). Therefore, inhibition of this pathway could potentially be two-fold in controlling MM. Inactivation of p53 is another potential candidate for future investigations to reverse telomere-independent senescence. Senescent cells with low expression of p16 have been shown to proliferate following p53 inactivation (Beausejour *et al* 2003), whereas cells with high levels of p16, that fully activate pRb, undergo irreversible senescence (Takahashi *et al* 2007). As MM T cell clones expressed low levels of p16, the senescence is reversible and inactivation of p53 may allow for proliferation of these cells.

Another opportunity for further investigation is to determine whether T cell clones from long term survivors of MM display features of senescence. Traditionally, the hallmark of senescence is irreversible cell cycle arrest so the cells are unable to proliferate (Effros *et al* 2005). However, it was shown in Chapter 3 of this thesis that T cell clones from 10 year survivors do proliferate. Upon examination of the clonal T cell phenotype selectively from 10 year survivor patients, which formed part of the patient group that was studied in Chapter 5 of this thesis, it was found that the T cell clones of 10 year survivors also show the hallmarks of telomere-independent senescence. The cells lacked PD-1, LAG-3, TIM-3 and CTLA-4, and are therefore not anergic or exhausted cells (Figure 6.2). Similar results were also seen with the T cell clones of non-10 year survivors. These cells expressed the senescence markers CD160 and KLRG-1. Data from qPCR and flow-FISH techniques, albeit small in number, suggest that 10 year survivors' T cell clones have longer telomere lengths than non-10 year survivors (Figure 6.3). This finding was not related to the age of patients. A limitation of this study was insufficient time to collate data on 10 year

survivors in terms of their dysfunctional phenotype and telomere lengths. This is because 10 year survivors are a rare population of patients and also infrequently visit the clinic for blood sampling due to the stability of their disease. Therefore, further studies with more patients are required before statistical calculations and to make conclusive statements about the T cell clones in this group of highly distinct individuals and whether they differ in telomere lengths and telomerase activity compared to other MM patients.

It is interesting to speculate that perhaps the T cell clones of 10 year survivors are functionally different from the T cell clones of non-10 year survivors, and the key differences are responsible for conferring long term survival. CD27 can be used as a marker to determine which cells are terminally senescent. CD8+CD28-CD57+ cells that have lost CD27 expression had the shortest telomeres, decreased telomerase levels and an inability to proliferate despite stimulation, as compared to CD27+ cells (Plunkett *et al* 2007). Our laboratory has previously published that T cell clones from MM patients can have the following two phenotypes: CD28-CD27- (late stage) or CD28-CD27+ (intermediate stage) (Sze *et al* 2003). This thesis has also shown that a higher proportion of T cell clones from long term survivors expressed CD27 compared to T cell clones from non-long term survivors (Figure 5.9), which were predominantly CD27-. It is possible that T cell clones from long term survivors may be in a pre-senescent state, where they retain the potential to proliferate and have higher telomerase activity, whereas clones from non-long term survivors are more likely terminally senescent cells. Furthermore, there appeared to be higher levels of telomerase activity in T cell clones of 10 year survivors, which may also contribute to overcoming terminal senescence. Investigations into this are required to understand the spectrum of tumour suppression of T cell clones in MM.

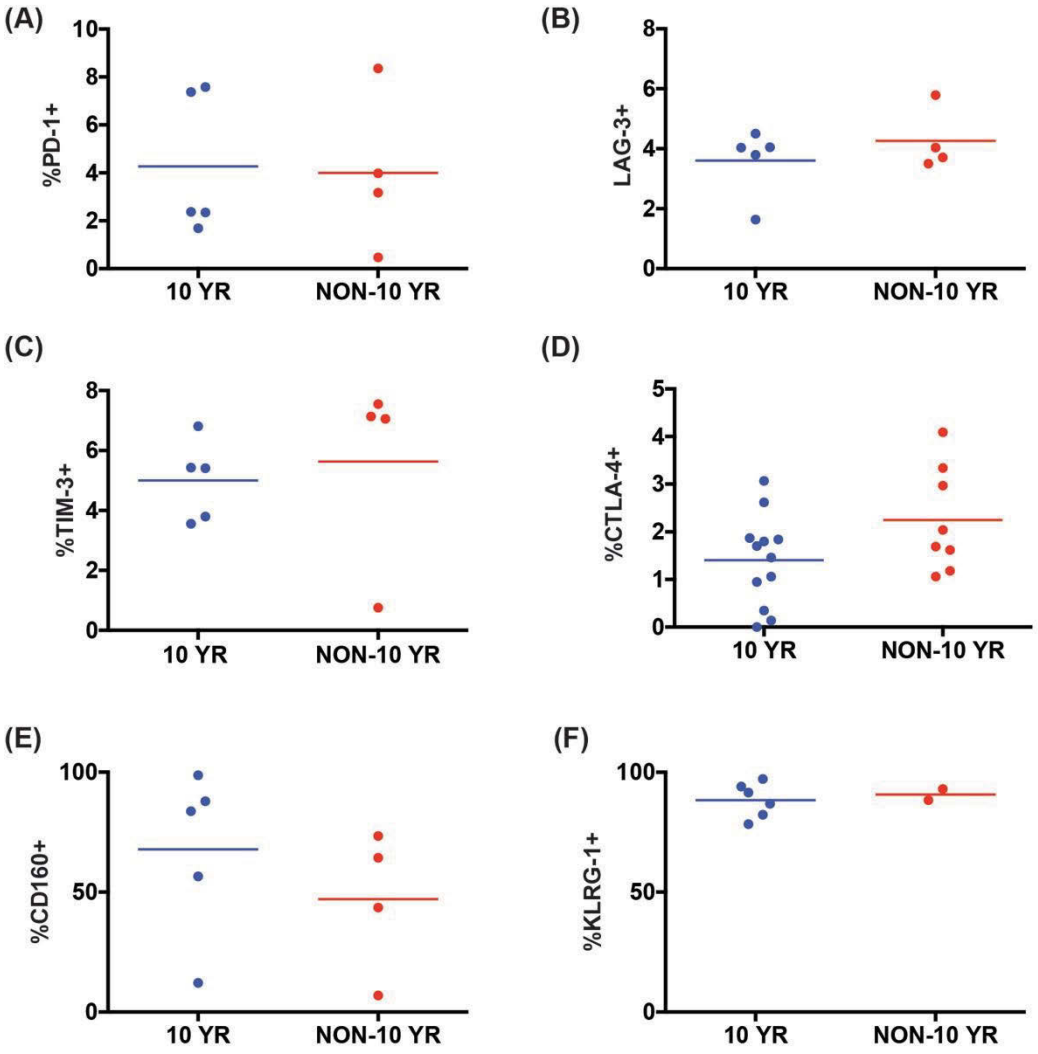


Figure 6.2 Preliminary data of the phenotype of 10 year survivor MM T cell clones

PBMC were obtained from MM patients to determine if T cell clones were anergic, exhausted or senescent T cells. The data from patients who were 10 year survivors are displayed and are presented as % of cells expressing **(A)** PD-1, **(B)** LAG-3+, **(C)** TIM-3 and **(D)** CTLA-4 to determine if the cells were anergic or exhausted; **(E)** CD160 and **(F)** KLRG-1 to determine if cells were senescent.

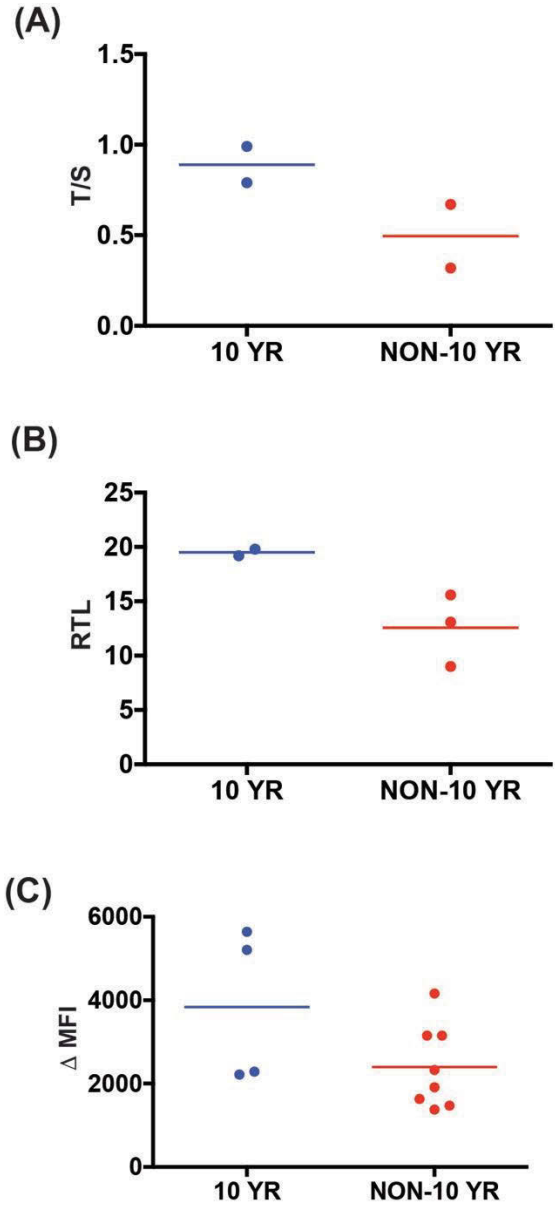


Figure 6.3 Preliminary data of telomere length and telomerase activity of 10 year survivor T cell clones

(A) Clonal and non-clonal T cells were purified from MM patients and DNA was extracted using the Maxwell kit. Two PCR reactions were conducted using a telomeric primer and primers for a single copy gene, beta globin. Data are presented as a T/S ratio, which was calculated by dividing the telomeric DNA quantity by the single copy gene DNA quantity for normal controls. **(B)** Purified clonal and non-clonal T cells from MM patients were treated with the PNA kit/FITC according to the manufacturer's protocol for measurement of telomere length by flow-FISH. Data are presented as a relative telomere length (RTL). **(C)** PBMC were fixed and permeabilised using the Leucoperm kit and then labelled with purified anti-hTERT or purified mouse anti-IgM κ isotype control. The change in MFI (ΔMFI) of each sample was calculated by subtracting the MFI of the isotype control (filled histogram) from the MFI of the samples stained with anti-hTERT (unfilled histogram). Data are presented as ΔMFI.

It is also of importance to determine the specificity of these tumour-induced T cell clones. Previously, these studies have been hindered by the lack of proliferative response of T cell clones *in vitro.* However, this study has demonstrated T cell clones in 10 year survivors are proliferative, can be expanded *ex vivo* and large fold expansions were obtainable. This opens up new avenues for investigation of the cytotoxicity and specificity of these T cell clones. Specificity of T cells against autologous tumour, following autologous stem cell transplant in MM patients, was demonstrated by the production of IFN-γ when T cells were co-cultured with tumour lysates of autologous BM plasma cells (Rosenblatt *et al* 2013). The ability to access large cell numbers means it is possible to perform experiments that test the cytotoxic potential of T cell clones. T cell clones from 10 year survivors of MM could be expanded and then co-cultured with either tumour lysates of MM cell lines or tumour lysates of autologous BM plasma cells. Production of IFN-γ by T cell clones would indicate that T cell clones have recognised the MM cell line or autologous plasma cell and therefore are MM-specific T cells.

In a recent landmark analysis of the antigenic landscape of MM plasma cells, novel antigens were determined to be displayed specifically within MHC class I of primary MM cells (Walz *et al* 2015). Pre-existing, specific T cell responses against these antigens as measured by IFN-γ production and positive staining with peptide: tetramer complexes were demonstrated exclusively in MM patients and also response-naïve patients. MM T cell clones could be screened for the recognition of the potentially immunogenic peptides identified in the aforementioned study. These peptides could be presented to T cell clones by antigen presenting cells and T cell mediated responses such as IFN-γ production could be measured. Alternatively, specific peptide: tetramer complexes can be generated, which are conjugated to a fluorochrome. If the T cell clones recognise the peptide, the tetramer fluorescence can be detected by flow cytometry.

T cell clones from 10 year survivors could also be expanded in 14 day *ex vivo* expansions for sequencing of their TCR regions. A bioinformatics approach can be used to predict peptide binding partners (MM target antigens) before

validation with engineered peptides (Szomolay *et al* 2016). Although this is outside the scope of this thesis, it would be interesting to examine in future investigations. This thesis has shown that these cells are linked with improved survival, which suggests they play a role in anti-tumour immunity. Consequently, overcoming the hypo-responsiveness of these cells, regardless of their specificity, would still provide the basis of a novel, personalised cell therapy based on the restoration of the host immune response.

The crucial role of the cytotoxic T cell in eliminating tumour cells has recently been highlighted by significant advances in cancer immunotherapy using CAR T cells and immune checkpoint blockade (Ikeda 2016). CAR T cells are genetically modified autologous T cells that recognise a specific target that is expressed by tumour cells. Infusion of these T cells aims to induce T cell recognition and killing of malignant tumour cells. The infusion of CAR-T cells engineered to recognise B cell maturation antigen in MM patients has shown preliminary success with the induction of stable responses and remissions (Ali *et al* 2015). CAR-T cells against CD19 have also demonstrated efficacy in MM despite the lack of this marker on the surface of plasma cells (Garfall *et al* 2015). Autologous transplantation and infusion of CD19 CAR-T cells led to a complete response in a MM patient who was previously refractory to nine lines of therapy. There was no detectable serum or urine monoclonal protein and the response persisted despite a disappearance of the CAR T cells. The efficacy of this specialised therapy was attributed to the CAR T cells targeting a CD19+ MM precursor plasma cell population or to the possible depletion of normal CD19+ B cells that have the potential to transform and are pro-myeloma (Garfall *et al* 2016). Immune checkpoint blockade aims to free tumour-specific T cells from tumour suppression so that cytotoxic T cells can perform their killing functions. As described previously, it has not been as successful in MM, as in other diseases (Lesokhin *et al* 2014, Suen *et al* 2015), but is still under investigation as a combination therapy (Görgün *et al* 2015).

Therefore, whilst it is evident that immunotherapies are paving the way for disease control and remission, or ultimately a cure, in many patients with haematological malignancies, their mechanisms of action and efficacy are not

completely understood. The long term persistence of these cells is uncertain and tumour escape can occur through clonal evolution (Keats *et al* 2012) and antigen down-regulation (Igney and Krammer 2002). In addition, adoptively-transferred T cells are also autologous T cells, which may be imprinted to be susceptible to tumour suppressive mechanisms (Beatty and Moon 2014). Tregs that are present in the tumour microenvironment have been shown to inhibit the action of CAR T cells against tumour cells (Lee *et al* 2011) and modifications to the early generation of CAR T cells to secrete IL-12 were required to combat the suppressive effects exerted by Tregs (Pegram *et al* 2012). Further issues include off target issues, where target antigens may be expressed on normal tissues. CD19 CAR T cells also targeted CD19+ B cells, leading to B cell aplasia (Maude *et al* 2014). Unexpected serious adverse events and fatalities toxicity against cardiac tissue were seen with the use of CAR T cells directed against cancer testis antigen MAGE-A3 as cardiac muscle titin was found to be a previously unknown alternative target for the MAGE-3 CAR T cells (Cameron *et al* 2013).

Although adoptive and antibody targeted T cell therapies are changing the therapeutic landscape in cancer, surprisingly little is known about natural anti-cancer T cell immunity. Of clinical interest is the re-activation of innate, natural tumour immunity in MM patients, which remains a prospect to eradicate residual tumour cells and produce a cure. Only allogeneic haematopoietic stem cell transplantation, the prototypic cellular immune therapy, can lead to a cure in MM. However, this form of therapy is still associated with high mortality rates (Gahrton *et al* 2001). Therefore, harnessing existing natural immunity to MM is the key to controlling the disease. The only definitive T cells that have clinical significance in MM are the cytotoxic clonal T cells (Brown *et al* 1997, Brown *et al* 2009, Suen *et al* 2016) that have been extensively studied in this thesis. T cell clones are also present in other haematological malignancies and findings from this thesis may be applicable to those diseases. Finally, future studies may further elucidate the role of T cell clones in a broad range of haematological malignancies.

6.3 Conclusions

This thesis has confirmed that natural immunity occurs in MM, in the form of T cell clones, and is relevant to clinical outcomes. In this study, T cell clones were detected in 75% of MM patients and 100% of long term survivors. This thesis has also underscored the critical role of T cell clones in maintaining long term survival in MM. It is the first study to identify the dysfunction of T cell clones as telomere-independent senescence. Furthermore, the mechanisms involved in the induction of senescence in MM clonal T cells, and the effects on their cellular function, were investigated. Although a number of signalling pathways were studied, the exact mechanism of senescence induction was not identified. However, upregulated telomerase activity was detected in T cell clones and may explain how normal telomere lengths are maintained in senescent cells. In addition, phospho-flow cytometric analysis of signalling pathways revealed defective TGF-β/SMAD and ERK pathways, which could be responsible for inducing clonal T cell dysfunction.

These results therefore contribute to an increased understanding of the complex pleiotropic effects of tumour suppression on cytotoxic T cells and suggest that multiple dysregulated pathways would need to be targeted for reversal of clonal T cell dysfunction. Restoring clonal T cell function provides a unique opportunity to enhance natural tumour immunity in MM and has potential to develop a novel cell therapy based on the restoration of the host's immune response. New immune therapies are required to eradicate residual disease present after conventional therapy and prevent disease relapse to maintain long term survival in patients with MM. Understanding the molecular mechanisms responsible for the silencing of natural T cell immunity in MM will also provide tools to improve adoptive T cell therapy. It would direct the field to more relevant cellular targets for adoptive therapies such as CAR-T cells and immune checkpoint blockade. Clearly, understanding the molecular mechanisms that induce dysfunction of clonal T cells and identifying methods to unleash cytotoxic T cell clones from tumour suppressive mechanisms has enormous clinical implications in MM and other malignancies.

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