Trogocytosis in Multiple Myeloma

Karieshma Kabani

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DECLARATION

I declare that no part of the work described in this thesis has been submitted for any other degree nor has it been submitted as part of the requirements for another degree.

As the author of this thesis, 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 has been acknowledged. All due acknowledgement has been made where appropriate.

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Karieshma Kabani

DEDICATION

This thesis is dedicated to my family whose love and support encourages me to work hard and do the best I can.

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PUBLICATIONS ARISING FROM THE WORK DESCRIBED IN THIS THESIS

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B) Conference Abstracts/Presentations

Brown R, **Kabani K**, Yang S, Favaloro J, Ho PJ, Gibson J, Fromm P, Hart D, Woodland N, Nassif N, Joshua D. (2011) HLA-G acquired by bone marrow T cells mediates tumour escape in myeloma, oral presentation to be presented at *Haematology society of Australia and New Zealand (HAA)*, *O16.*

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Brown R, Li J, Sze D, Yang S, **Kabani K**, Aklilu E, Ho PJ, Gibson J, Cowley M, Kaplan W, Loh YS, Yamagishi T, Joshua D. (2010) Flow detection and analysis of the anergic and expanded cytotoxic T cell clones in patients with plasma cell dyscrasias, poster presented at the *Australasian Flow Cytometry group (AFCG) p63.*

Yang S, Brown R, Reid S, **Kabani K**, Aklilu E, Ho PJ, Woodland N, Joshua D. (2009). Characterisation of $CD138^-$ plasma cells in multiple myeloma, poster presented at the *Australasian Flow Cytometry group (AFCG)*, *p66*.

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Yang S, Brown RD, Sze DM, **Kabani K**, Aklilu E, Ho PJ, Joshua D (2008) Detection, phenotype and prognostic significance of T cell clones in multiple myeloma. *Proc AFCG 54.*

Brown RD, **Kabani K,** Aklilu E, Sze D, Ho PJ, Gibson J, Joshua DE. (2008) Trogocytosis in Multiple Myeloma. *Blood* 112 S1: 594.

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Kabani K, Brown R, Yang S, Aklilu E, Sze D, Woodland N, Nassif N, Ho PJ, Gibson J, Joshua D. (2008) Trogocytosis in Multiple Myeloma, poster presented at *Haematology society of Australia and New Zealand (HAA),* pA355.

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Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APCs	Antigen presenting cells
APC	Allophycocyanin
ASCT	Autologous stem-cell transplantation
ATCC	American type culture collection
BCR	B cell receptor
BM	Bone marrow
CD	Cluster differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CLL	Chronic lymphocytic leukemia
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytolytic T lymphocyte-associated antigen 4
Cy5.5	Cyanine 5.5
Cy7	Cyanine 7
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
EFS	Event free survival
FACS	Fluorescent-activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FISH	Fluorescent *in situ* hybridisation
FITC	Fluorescein isothiocyanate
FLC	Free light chains
FoxP3	Forkhead transcription factor 3
FSC	Forward scatter
GM-CSF	Granulocyte macrophage – colony stimulating factor
Hb	Haemoglobin
HLA	Human leukocyte antigen
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
ILT	Immunoglobulin-like transcript receptors
IS	Immunological synapse
ISS	International staging system
KIR	Killer Ig-like receptors
M-protein	Monoclonal protein
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MM	Multiple myeloma
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NF-κB	Nuclear Factor-kappa B
NHL	Non-Hodgkin's lymphoma
NK	Natural killer
OS	Overall survival
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Plasma cell
PCLI	Plasma cell labelling index
PCR	Polymerase chain reaction
PerCP	Peridinin chlorophyll protein
PFS	Progression free survival
PHA	Phytohemagglutinin
pMHC	peptide MHC
R-PE	R-phycoerythrin
rpm	Revolutions per minute
RPMI	Rosewell Park Memorial Institute
RT	Room temperature
Rt-PCR	Reverse transcription polymerase chain reaction
SMM	Smoldering multiple myeloma
SSC	Side scatter
TCR	T cell receptor

TGF	Tumour growth factor
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
WBC	White blood cells
WM	Walsendtrom macroglobulinemia
β_2M	Beta-2-microglobulin

Abstract

The term trogocytosis is used to describe the fast, cell-to-cell contact-dependent transfer of membrane proteins between cells, with T and B-lymphocytes, natural killer (NK) cells, antigen presenting cells (APC) and tumour cells being the most widely studied. This study aimed to: (1) identify the extent of trogocytosis in patients with multiple myeloma (MM), a malignancy of bone marrow plasma cells, compared with the other B cell malignancies, (2) to identify some of the molecules involved with trogocytosis in these patients and then (3) determine if cells that had acquired molecules had altered function.

An *in vitro* model of trogocytosis was established in which plasma cell lines and flow-sorted bone marrow plasma cells ($CD38^{++}$) of patients with MM (n=11) or the malignant B cells from patients with chronic lymphocytic leukaemia (CLL) ($CD5^{+}CD19^{+}$) (n=4) and Waldenstrom macroglobulinaemia (WM) ($CD19^{+}$) were biotinylated and then cultured with either patient or normal mononuclear cells. The acquisition of biotinylated membrane proteins was determined by flow cytometry and confocal microscopy. Screening for potential molecules involved suggested that CD86 and HLA-G were likely candidates for trogocytosis. CD86 is a co-stimulatory molecule at the immune synapse and HLA-G is a non-classical MHC class I molecule which prevents antigen-specific cytolysis by cytotoxic T lymphocytes (CTLs), inhibits the function of circulating NK cells and prevents proliferation of allogeneic $CD4^{+}$ T cells. These observations have lead to the hypothesis that expression of HLA-G may aid in the escape of tumours from immune surveillance.

T cells acquired significantly more biotinylated proteins (mean=13.55%) than B cells (mean=2.43%; $t=2.80$; $p<0.05$) or NK cells (mean=3.15%; $t=2.57$; $p<0.05$). There was no significant difference between levels of biotin transferred to T cells from either plasma cell lines or primary plasma cells and acquisition was the same with autologous and allogeneic T cells. Significantly more trogocytosis was observed in myeloma patients than other B cell malignancies (n=5) as <1% T cells acquired membrane fragments when cultured with malignant B cells from patients with CLL or WM ($t=3.86$; $p<0.05$). Upon culture with biotinylated $CD3^{+}$ flow-sorted normal T

cells, approximately 2% of $CD38^{++}$ plasma cells acquired membrane fragments, suggesting that in patients with myeloma, trogocytosis was predominantly unidirectional.

Although HLA-G expression was found on 0.02 – 0.56% of normal T cells (mean =0.23%), 20% of MM patients (11 of 56) demonstrated a level of HLA-G^{+} $CD3^{+}$ T cells above the normal range. Addition of flow-sorted $CD3^{+}$ HLA-G^{pos} T cells led to a reduction in the proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labelled $CD3^{+}$ HLA-G^{neg} T cells stimulated with anti-CD3/CD28 beads and this inhibition was greater than the inhibition due to $CD38^{++}$ HLA-G^{pos} plasma cells ($t=2.64$; $p=0.046$). The $CD3^{+}$ HLA-G^{pos} T cells acquired inhibitory function but were not natural T regulatory cells as they were $CD25^{neg}$. Overall survival was significantly worse for the 11/46 patients with HLA-G^{pos} plasma cells ($\chi^2=12.4$; $p<0.0004$).

Flow cytometric analysis of $CD38^{++}$ bone marrow plasma cells from MM patients showed varied HLA-G expression ranging from 0.2% to 96% (n=46). The clinical relevance of HLA-G^{pos} plasma cells was demonstrated by a significant reduction in overall survival (n= 46; $\chi^2= 12.4$; $p<0.004$). CD86 expression on T cells of myeloma patients (n=98) ranged from 0 – 30% (normals = 0 – 2.7%; n=10). T cells from myeloma patients (n=7), when co-cultured with CD86 expressing plasma cells, were found to acquire significantly ($p<0.0001$) higher levels of CD86.

This study reports several new findings. It has shown that trogocytosis is more common in multiple myeloma than other B cell malignancies, is primarily unidirectional, HLA independent and T cells are more likely to be involved than other lymphocytes. T cells which acquire tumour antigens may have altered function and it has been demonstrated that HLA-G^{pos} T cells form a new subset of acquired regulatory T cells that inhibit the proliferation of HLA-G^{neg} T cells and therefore protect MM cells against the host's immune defences.

Chapter One: Introduction

1. Introduction

1.1 Multiple Myeloma: an Introduction

Multiple myeloma (MM) is an incurable, but treatable, progressive haematologic malignancy of bone marrow plasma cells. The World Health Organisation (WHO) classification of tumours of haematopoietic and lymphoid tissues now classifies the disease as plasma cell myeloma but it is generally referred to as myeloma (McKenna, *et al* 2008).

Myeloma accounts for approximately 10% of haematological cancers (Katzel, *et al* 2007) and is thought to evolve from two asymptomatic premalignant disorders; monoclonal gammopathy of undetermined significance (MGUS) or smoldering multiple myeloma (SMM) (Kyle and Rajkumar 2008, Weiss, *et al* 2009).

Multiple myeloma is predominately characterised by an increase in plasma cells in the bone marrow which invade adjacent bone, destroying skeletal structures and resulting in bone pain. These cells, or the proteins they produce, may also infiltrate organs, producing a diverse range of symptoms. Clonal expansions of plasma cells results in increased levels of monoclonal (M) immunoglobulin (Ig) that can cause hyperviscosity as well as increased levels of light chains (Bence-Jones proteins) that may eventually cause renal failure. Other common clinical manifestations include hypercalcaemia, anaemia, neurological symptoms and increased susceptibility to bacterial infection (Edwards, *et al* 2008, Kyle and Rajkumar 2009, Zaidi and Vesole 2001, Zhan, *et al* 2006).

The majority of MGUS and MM patients have structural or numeric chromosomal abnormalities. For example immunoglobulin switch translocations, particularly IgH translocations at the 14q32 locus, are common in patients with MM, for example t(11;14), t(4;14) and t(14;16). Other cytogenetic abnormalities involve hyperdiploidy at 6p21, partial or complete deletion of chromosome 13 or 11q abnormalities (Bommert, *et al* 2006, Ho, *et al* 2002, Kyle and Rajkumar 2009, Tricot, *et al* 1995, Zhou, *et al* 2008).

1.1.1 Plasma cells

Like all blood cells, the lymphoid cells that are destined to become immune cells arise from stem cells within the bone marrow. Lymphoid cells develop into two types of lymphocytes, B-lymphocytes (B cells) and T lymphocytes (T cells). Plasma cells are the mature effectors of the B cell lineage that secrete antibodies. When naive B cells encounter antigen in the splenic marginal zone, they proliferate and differentiate into short lived plasma cells that secrete low affinity antibody, typically IgM, which is the first antibody response to pathogens. Antigen and antigen specific T helper cells trigger naive follicular B cells in the germinal center to proliferate and undergo affinity maturation and isotype switch recombination to ultimately become high affinity secreting plasma cells. If these plasma cells receive survival signals from bone marrow stromal cells, they can survive for months (Calame*, et al* 2003, McHeyzer-Williams*, et al* 2001, Shapiro-Shelef and Calame 2004, Slifka and Ahmed 1998).

1.1.1.1 Plasma cell differentiation

Like all cells, B cells begin the cell cycle in the G0 phase and remain there until activated by antigens and antigen specific T helper cells. Whilst in the germinal centre, activated B cells continue to divide and undergo a process of class switching and hypermutation. Following this process, the B cells exit the germinal centre and enter the extra-follicular foci where they become memory B cells, differentiate into plasma cells or die. The differentiation between normal plasma cells and Ig secreting, terminally differentiated plasma cells is that end-stage plasma cells undergo cell cycle arrest in the G1 phase and normal plasma cells retain their ability to replicate and regulate the cell cycle. Plasma cells then migrate and accumulate in the bone marrow. Normal plasma cells are generally non-dividing, are arrested in the G1 phase of the cell cycle and they undergo cell death following Ig secretion. Myeloma cells, on the other hand, can secrete Ig without activation, they do not re-enter the cell cycle and there is a loss of apoptotic control (Chen-Kiang 2005, Reid*, et al* 2010).

Cell surface phenotyping has been used to charaterise the different stages of B cell development, differentiation to malignant B cells and categorise B cell tumours. All cells express cluster differentiation (CD) markers, for example normal B cells express

CD19, CD20 and CD45RA and plasma cells express CD38. There are currently three different plasma cells stages, all of which express clonal Ig light chains. Immature plasma cells are characterised as $CD38^{++}$ $CD138^{-}$ and $CD45^{++}$, whereas early plasma cells are $CD38^{++}$ $CD138^{+/-}$ and $CD45^{+}$, whilst mature plasma cells are $CD38^{++}$ $CD138^{+/-}$ and $CD45^{-}$ (Joshua, *et al* 1996, Reid, *et al* 2010).

1.1.2 Bone marrow microenvironment and angiogenesis

The bone marrow microenvironment is believed to play an essential role in tumour growth and survival. The adherence of myeloma cells to stromal cells results in the release of cytokines such as interleukin 6 (IL-6), tumour necrosis factor (TNF) and the receptors for activation of Nuclear Factor-kappa B (NF-κB) ligand, stimulating the proliferation of myeloma cells, preventing apoptosis and increase bone destruction (Bommert, *et al* 2006, Mitsiades, *et al* 2006, Tohnya and Figg 2004, Zhou, *et al* 2008).

Malignant plasma cells produce growth factors, such as vascular endothelial growth factor (VEGF), which promote angiogenesis (formation of new blood vessels). These vessels supply nutrients and oxygen that support tumour growth. This, in turn, promotes the reproduction of myeloma cells that infiltrate the bone marrow which eventually comprise more than 10% of cells present (Jakob, *et al* 2006, Menu, *et al* 2004, Tohnya and Figg 2004).

1.1.3 Epidemiology of Myeloma

The cause of MM is yet to be determined, however there are some risk factors that have been identified. These include age, with 65-70 years being the median age at diagnosis; gender, with males being more commonly affected than females (Bergsagel 2005); the presence of immune disorders, as patients with human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) have a greater incidence of MM; race, with African Americans having a rate of MM development that is twice that of Caucasians (Raab, *et al* 2009); exposure to ionising radiation (Zaidi and Vesole 2001) and environmental toxins (Caers, *et al* 2008). Median survival following conventional treatments is 3-4 years. Median survival can be

extended to 5-7 years with high-dose treatment followed by autologous stem-cell transplantation (ASCT) (Attal, *et al* 2003, Raab, *et al* 2009).

1.1.4 Aetiology of myeloma

There appears to be no symptoms in the preliminary stages of myeloma. In a few patients, myeloma is diagnosed during routine blood testing. The most common clinical characteristic of MM at diagnosis is bone pain caused by bone destruction. Other clinical manifestations include (but are not limited to) anaemia, recurrent infections, uremia, renal insufficiency, hyperviscosity, fractures, immune system deficiencies, spinal cord compression and hypercalcemia (Katzel, *et al* 2007, Tariman 2007, Winterbottom and Shaw 2009).

1.1.5 Classification of the monoclonal gammopathies and myeloma

There has been an international consensus on the classification of the monoclonal gammopathies as described in Table 1-1 (The International Myeloma Working Group 2003).

Table 1-1: Characteristics of monoclonal gammopathy of undetermined significance, smoldering multiple myeloma and multiple myeloma

Disorder	Characteristics
Monoclonal Gammopathy of Undetermined Significance (MGUS)	Serum M protein <30g/L Clonal bone marrow plasma cells <10% No related organ or tissue impairment
Asymptomatic Multiple Myeloma (or Smoldering Multiple Myeloma)	Serum M-protein ≥30g/L and/or Bone marrow clonal plasma cells ≥10% No related organ or tissue impairment
Multiple Myeloma	Clonal bone marrow plasma cells ≥ 10% Presence of serum and/or urinary M protein Evidence of end-organ damage; ***C**alcium levels increased: corrected calcium >2.75 mmol/L ***R**enal insufficiency: creatinine >173 mmol/L ***A**naemia: haemoglobin <100g/L ***B**one lesions: lytic lesions or osteoporosis with compression fractures Frequent severe infection (>2 per year) Hyperviscosity syndrome

*CRAB features (Calcium, Renal insufficiency, Anaemia and Bone lesions)

Adapted from Zaidi and Vesole (2001) and The International Myeloma Working Group (2003).

1. Monoclonal Gammopathy of Undetermined Significance (MGUS) is a more common condition than MM. Patients are asymptomatic but M proteins are present and the cause for this increase in protein is unknown. It is a benign disease but it progresses to a malignant plasma cell disorder at a rate of just over 1% per year (Katzel*, et al* 2007). The genetic aberrations present in myeloma (14q32 chromosome translocations, aneuploidy and monosomy 13) may also be present in MGUS. Patients with MGUS are usually not given any therapy but are regularly monitored (Bergsagel 2003, The International Myeloma Working Group 2003, Zaidi and Vesole 2001).

2. Patients with asymptomatic Myeloma or Smoldering Multiple Myeloma (SMM) may have decreased concentrations of normal immunoglobulins (Ig) in their serum as well as increased amounts of M-protein in the urine (Kyle and Greipp 1980). There should be no evidence of related end organ damage. They may not require immediate therapy (The International Myeloma Working Group 2003, Zaidi and Vesole 2001).

3. Patients with symptomatic myeloma generally have a monoclonal protein and more than 10% plasma cells in the bone marrow. They also present with at least one positive indicator of organ involvement – ie hyper**c**alcemia, **r**enal failure, **a**naemia and/or **b**one lesions (CRAB). These patients require immediate treatment (The International Myeloma Working Group 2003, Zaidi and Vesole 2001).

1.1.6 Diagnosis of multiple myeloma

The diagnosis of MM is not made on a single laboratory test result. There are a number of factors that need to be considered such as patient history, physical examination, symptoms and a number of laboratory results including a bone marrow biopsy smear (Figure 1-1), full blood count with differential white blood cell count, serum protein electrophoresis, immunofixation and quantitative immunoglobulin measurement (Kyle and Rajkumar 2009).

A biochemistry screen must also be performed. This includes creatinine, calcium, albumin and Beta-2-microglobulin (β_2M) levels. Serum protein electrophoresis and immunofixation (Figure 1-3) are used to determine the size and type of M protein (IgM or IgA have an increased risk as compared with an IgG protein) together with measurements of free light chains (FLC). The presence of any skeletal lytic lesions in the spine, femurs, humeri, pelvis and skull (Figure 1-2) are detected by radiological skeletal surveys (Caers*, et al* 2008, Kyle and Rajkumar 2009, Winterbottom and Shaw 2009). Additional tests can include magnetic resonance imaging (MRI) for the detection of suspected spinal cord compression, as well as to rule out systemic disease (Kyle and Rajkumar 2009, Miguel and Garcia-Sanz 2005, Tariman 2007).

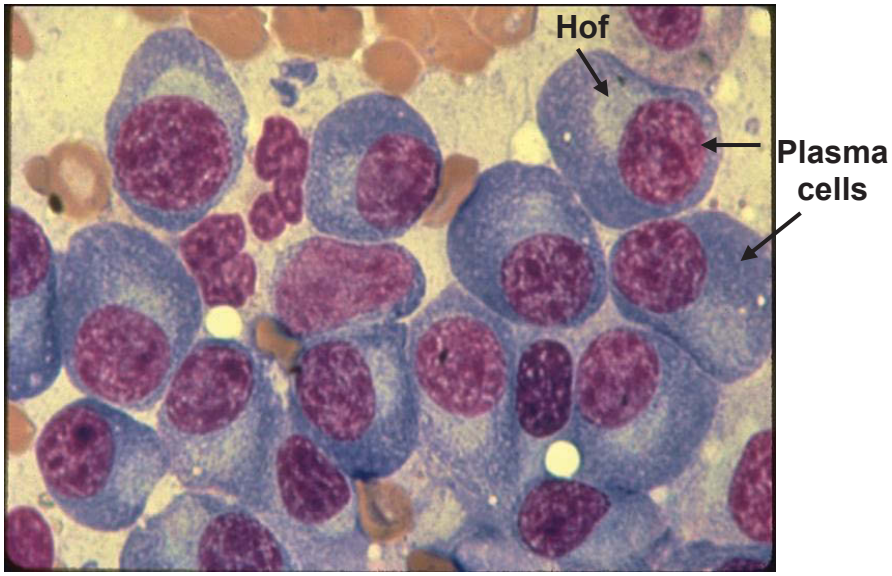
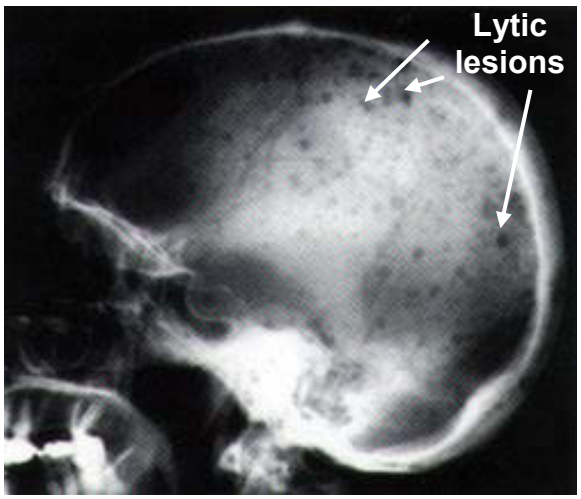


Figure 1-1: Bone marrow film from a multiple myeloma patient, showing an abundance of plasma cells.
Immature plasma cells have a small, eccentrically placed nucleus with a prominent perinuclear hof, a relatively clear area representing the golgi region, dense chromatin clumping and a well developed cytoplasm. Mature plasma cells have a larger eccentrically placed nucleus with a hof and diffuse chromatin pattern, with or without the nucleolus, and abundant cytoplasm (Courtesy of Dr Ross Brown).

A)



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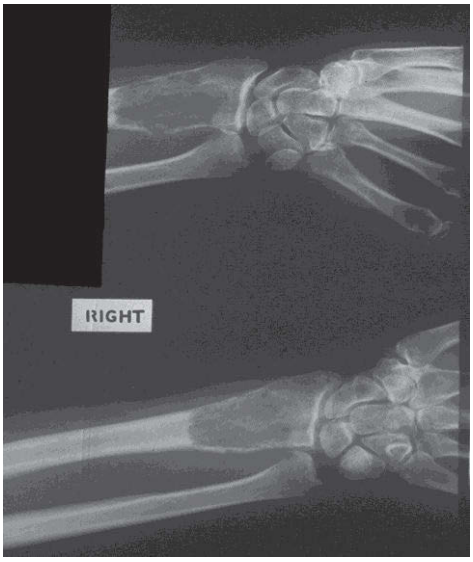

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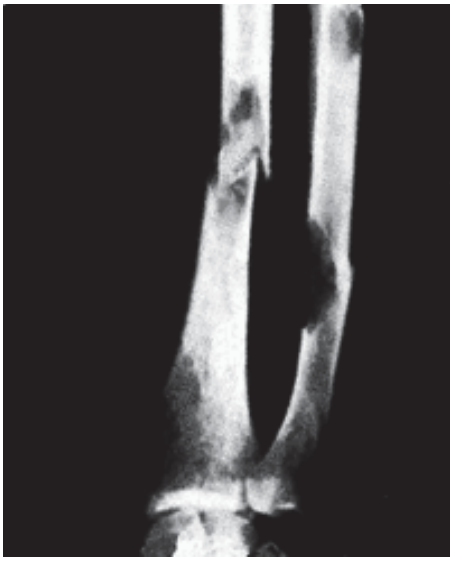
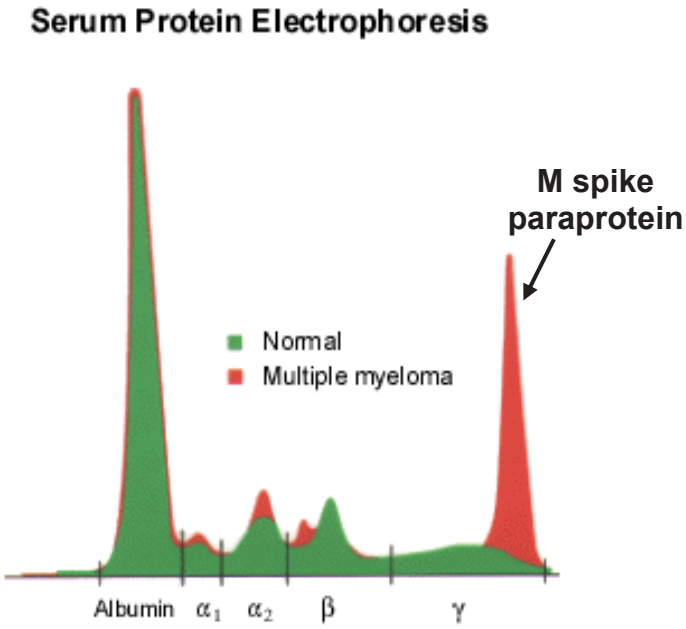


Figure 1-2: X rays of bones and skull from myeloma patients.
Multiple lytic lesions can be seen in (A) the skull of a patient. Bone destruction in (B) the radius and (C) long bones. Bone lesions are a hallmark of MM and used diagnostically (Courtesy of Dr Ross Brown).

A)



B)

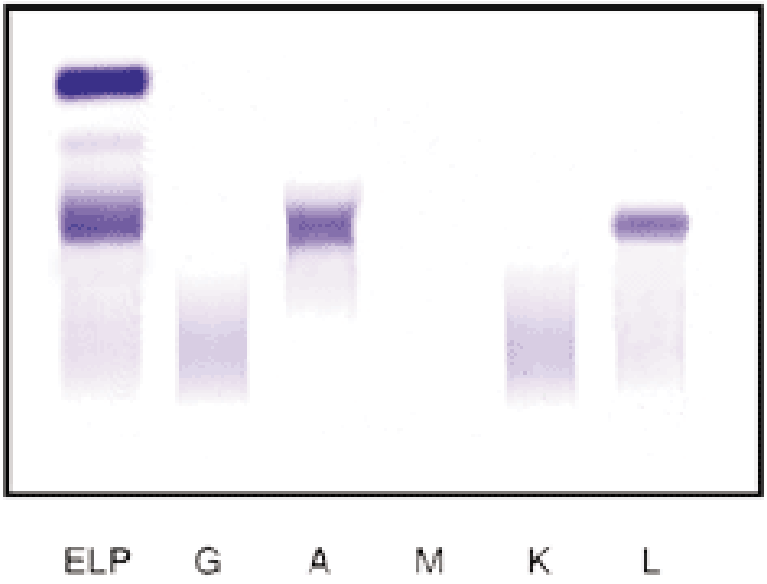


Figure 1-3: Electrophoretic analysis of monoclonal proteins from a myeloma patient.
Once a monoclonal protein has been detected, the heavy and light chain isotypes must be identified by (A) M-spike paraprotein by immunoelectrophoresis or (B) immunofixation. Both (A) and the ELP row of (B) show the routine serum protein electrophoresis with a large albumin band and an increase in the gamma region proteins. By immunofixation, the heavy banding of IgA rather than IgG or IgM indicates that the patient has an IgA paraprotein and the heavy band in the Lambda (L) row and not in the kappa (K) row indicates that the M protein has lambda light chains (Courtesy of Dr Ross Brown).

1.1.7 Staging of multiple myeloma

1.1.7.1 The Durie–Salmon staging system

Staging of myeloma is critical to developing effective treatment plans. For over 30 years MM tumour burden was assessed based on the Durie-Salmon staging system (Durie and Salmon 1975) in which patients were categorised into one of three groups, namely stage I, II or III. Clinical stage was defined by the levels of haemoglobin, serum and urine M protein, serum calcium and creatinine, as well as the number and size of bone lesions (as listed in Table 1-2). There were a few limitations to this staging system which resulted in the introduction of a newer alternative, the International Staging System (ISS) (Kyle and Rajkumar 2009, Winterbottom and Shaw 2009).

1.1.7.2 The International Staging System

The ISS is based on two simple but influential laboratory parameters. Patients are categorised as either stage I, II, or III based on two blood test results: serum β_2M and albumin as described in Table 1-3 (Kyle and Rajkumar 2009). Unlike the Durie-Salmon staging system, the ISS has been confirmed in the context of autologous stem cell transplantation. Although this system of staging has only been recently introduced, it has proven to be more sensitive in distinguishing the three clinical stages by several investigators (Bergsagel 2003, Greipp*, et al* 2005, Kyle and Rajkumar 2009).

Table 1-2: Clinical parameters for multiple myeloma staging by the Durie-Salmon staging system

Stage	Criteria
I	Low tumour mass (<$0.6x10^{12}$ cells/m^2); IgG <50g/L, IgA <30g/L; Bence Jones protein <4g/24 h Normal haemoglobin, serum calcium level Normal or solitary bone lesion
II	Values between stage I and stage III
III	High tumour mass (>1.2 $x10^{12}$ cells/m^2) IgG >70g/L; IgA >50g/L; Bence Jones protein >12 g/24 h Serum calcium level >120 mg/L; Hb <85g/L Advanced lytic bone lesions
Sub-classification	**Based on renal function**
A	Serum creatinine <20mg/L
B	Serum creatinine ≥20mg/L

Adapted from Winterbottom and Shaw (2009).

Table 1-3: Clinical parameters for multiple myeloma staging by the International Staging System

Stage	Criteria	Median Survival (months)
I	Serum β_2M <3.5 mg/L Serum albumin ≥35 g/L	62
II	Serum β_2M between 3.5 mg/L and 5.5 mg/L	44
III	Serum β_2M >5.5 mg/L	29

Adapted from Greipp *et al* (2005).

1.1.8 Prognostic indicators in multiple myeloma

Prognostic factors are those signs and symptoms which predict the time it will take for a clinically significant endpoint to occur. The major endpoints used are event free and overall survival. There are many laboratory and clinical tests that can be used to provide significant prognostic information. These indicators can then be used to group patients into those with a good prognosis and those with a poor prognostic. They may aid in determining how fast the tumour is growing and may be used to predict the likely response to therapy. If tests are performed early, they can indicate the level of disease at diagnosis and provide a baseline against which disease progression, as well as response to therapy, can be compared (Miguel and Garcia-Sanz 2005).

Some prognostic tests include a plasma cell labelling index (PCLI), which is used to identify the fraction of myeloma cells that are proliferating (Greipp*, et al* 1993), C-reactive protein levels, a marker for IL-6 (Bataille*, et al* 1992), and lactate dehydrogenase level, a measure of tumour burden. Cytogenetic and fluorescent *in situ* hybridisation (FISH) testing is used to detect chromosomal abnormalities, in particular translocations of t(4;14), the musculoaponeurotic fibrosarcoma (MAF) translocation t(14;16) and t(14;20), hyperdiploidy, deletion of chromosome 13, or aneuploidy by metaphase analysis (McKenna*, et al* 2008, Tricot*, et al* 1995).

1.1.9 Multiple myeloma treatment

The current frontline therapy for patients up to 65 years of age is high-dose chemotherapy followed by autologous stem cell transplantation (ASCT). Studies have indicated that this treatment increases the rate of complete remission, and improves event free (EFS) and overall survival (OS) of patients. Some patients fail to respond to chemotherapy and those patients who achieve remission ultimately relapse and require further treatment (Attal*, et al* 2003, Barlogie*, et al* 2004, Blade and Rosinol 2008, Tariman 2007).

Traditional chemotherapeutic agents like oral melphalan, prednisone and anthracycline-based chemotherapy combinations play an important role in myeloma therapy. While more potent steroids, like dexamethasone, are now used in place of

prednisone, there are several new agents available which target myeloma cell interactions within the BM microenvironment. These include thalidomide, bortezomib (Velcade®) and lenalidomide (Revlimid®) (Barlogie*, et al* 2004, Palumbo*, et al* 2008, Zaidi and Vesole 2001).

Novel chemotherapeutic agents like bortezomib (a proteasome inhibitor), in conjunction with other steroids like melphalan, have been developed for patients with refractory and relapsed myeloma. Bortezomib is a dipeptide boronic acid that inhibits the 26S proteasome. *In vivo* and *in vitro* studies have shown that bortezomib (alone or in conjunction with other agents) has an anti-tumour response against haematologic malignancies including lymphomas, leukemias and MM. Bortezomib is known to induce MM cell apoptosis, block angiogenesis in the bone marrow (BM) milieu, block the activation of nuclear factor-kappa B (NF-κB) and inhibit cytokine secretion (Chauhan*, et al* 2008, Tariman 2007).

Another new therapeutic agent is the immunomodulatory (IMiD) drug, lenalidomide, a potent thalidomide analog (formerly referred to as CC-5013). This drug is one of the next generation of IMiDs after thalidomide. Lenalidomide inhibits the production of tumour necrosis factor α (TNF-α), stimulates T-cell proliferation, INF-γ and IL-2 production. Both lenalidomide and bortezomib are used in conjuction with other chemotherapeutic agents and steroids (Thomas*, et al* 2007).

Like thalidomide, lenalidomide activates apoptotic pathways. It also triggers a dose-dependent decrease in secretion of IL-1β, IL-6 and TNF-α, leading to cessation of myeloma cell growth and apoptosis. There is also increased secretion of IL-10 and inhibition of MM cell proliferation by decreasing the binding of myeloma cells to bone marrow stromal cells (BMSCs). Lenalidomde also inhibits angiogenesis by inhibiting the production of cytokines, fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). Because it is more potent than thalidomide it is effective at lower doses. More recently, a third generation IMiD, pomalidomide, has become available for the treatment of MM (Tariman 2007, Thomas*, et al* 2007).

1.2 The Immune System

The immune system is a complex arrangement of tightly regulated cells, the function of which is to detect and eliminate infectious organisms like parasites, bacteria and viruses, protect from cancer, detect malignancy or non-self and immune surveillance. A series of complex cellular processes must occur for an effective immune response and lasting immunity (Janeway Jr 2001).

The first step in an immune response is the identification of an exogenous antigen, followed by processing by professional antigen presenting cells (APCs) which, in turn, activate T and B cells when in contact with these APCs. There must also be proliferation and differentiation in order to amplify the number of effector cells. In addition, memory cells must be generated to orchestrate a strong immune response to respond to future encounters with the same antigen. Homeostasis needs to be maintained when such immune responses are generated and is also required to prevent immuno-pathological or autoimmune conditions (Ahmed*, et al* 2008, Aucher*, et al* 2008).

The immune system consists of lymphoid cells (T and B lymphocytes), natural killer cells (NK cells), as well as cells from the myeloid lineage (basophils, eosinophils, neutrophils, monocytes, dendritic cells (DC) and mast cells) that work in synergy to provide an efficient system to eliminate invading pathogens whilst ensuring self-tolerance. There are two arms to the immune system: the innate immune system and the adaptive immune system.

1.2.1 The innate immune system

Innate immunity is non-specific and protects the host from injuries or infection. It includes host defenses such as barriers (skin and mucous membrane) and certain proteins (like the complement cascade and interferons). It also involves processes such as phagocytosis and inflammation. Phagocytic cells involved in the immune response include neutrophils, basophils, eosinophils, monocytes, macrophages, mast cells and DCs (Levinson 2004). Natural killer cells are an important part of the innate immune system. They remove infected cells, tumour cells, and cells in stress, after

binding to a complex array of killer-activating and killer-inhibitory receptors (Soloski 2001).

Unlike adaptive immunity, the innate immune system recognises a range of pathogens without prior exposure. It does not improve after subsequent exposure to the organism and there is no memory of the processes. The innate arm of the immune system has two major functions. In the first function, cells such as neutrophils kill invading micro-organisms. The second function is the activation of the adaptive immune processes to provide a more specific and targeted immune response (Medzhitov and Janeway 2000).

Macrophages and other APCs play a role in both arms of the immune system. When they phagocytose microbes they serve as part of the innate arm, but when they process and present antigens to T helper cells ($CD4^+$ T cells), they activate the acquired arm of the immune system which leads to the production of cytotoxic T lymphocytes (CTL) and antibody production. Initiation of adaptive immunity can only be achieved after recognition of a foreign antigen by the innate arm of the immune system (Levinson 2004).

1.2.2 The adaptive immune system

The adaptive immune system is specific and occurs after exposure to a foreign antigen. It improves with repeated exposure hence the basis for vaccination. Adaptive immunity can be passive or active and the cells involved have long-term memory for a specific antigen (Levinson 2004). The cells involved in adaptive immunity are of the lymphoid lineage and consist of T and B lymphocytes. These cells are capable of recognising a wide spectrum of antigens derived from viruses, bacteria and other pathogens. Both T and B lymphocytes require activation which is provided by highly specific antigen recognition through surface receptors (Medzhitov 2007, Parkin and Cohen 2001).

1.2.3 B cells

B cells produce antigen recognition molecules called immunoglobulins (Ig) in response to activation through the membrane-bound Ig, the B cell receptor (BCR). In response to antigen challenge, Ig of the same antigen specificity as the BCR are released by activated B cells called plasma cells (Vyas, *et al* 2008). Adaptive immunity relies on antigen recognition by B cells. Once recognised, B cells internalise the antigen, process it into peptide fragments and present them on their surface, bound to major histocompatibilty complex (MHC) class II molecules. Helper T cells then recognise these conjugated peptide-MHC (p-MHC) complexes, which stimulate B cells to produce antibodies (Ab) of high affinity for those antigens (Aucher, *et al* 2008, Lanzavecchia 1985).

1.2.4 T cells

T cells can be sub-divided into two major subsets that are characterised by their functions. Surface expressing proteins play a vital role in cellular functions and establish the basis of cellular phenotypic characterisation. For instance, expression of T cell receptor (TCR) and cluster of differentiations (CD) 3 indicates T cells. CD3 and CD4 or CD8 define $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells respectively (Ahmed, *et al* 2008, Caumartin, *et al* 2006). $CD8^+$ CTLs recognise peptides that are presented by MHC class I and kill virus infected cells whilst $CD4^+$ helper T cells are activated by antigen presentation on APCs bound to MHC class II molecules, providing additional signals to activate other immune cells (Vyas, *et al* 2008).

1.2.5 T cell activation

When a foreign antigen is endocytosed by an APC, intracellular proteolytic degradation results in the production of short peptide fragments that are presented by MHC molecules on the surface of APCs. The interaction between T cells and p-MHC on APCs results in proliferation and differentiation of naïve T cells into effector cells. For effective activation of T cells, two signals are required: signal 1 which is induced when T cells recognise p-MHC complexes and signal 2 provided by the engagement of CD28 on T cells and CD80 or CD86 on APCs. In the presence of signal 1 alone, T

cells will fail to become activated and possibly undergo peripheral tolerance or become anergic (Key 2004, Lenschow*, et al* 1996, Medzhitov 2007, Weil and Israel 2006).

1.2.6 T cell clones

The TCR of peripheral blood T cells consists of heterodimeric α and β chains. The α chain is produced by the "random rearrangement of multiple variable (V) and joining (J) gene segments and the β chain by V, diversity (D) and J gene segments" (Lim*, et al* 2001). Imprecise VDJ joining and non-coding (N) nucleotides results in a repertoire that is diverse and capable of detecting any foreign antigen. In an individual, the T cell repertoire can be influenced by environmental and genetic factors. Modification of the T cell repertoire is attained by clonal selection during maturation. Due to the lack of monoclonal antibodies to cover all the major TCR families, reverse transcription-polymerase chain reaction (RT-PCR) is the method of choice to analyse the complete T cell repertoire at a clonal level (Lim*, et al* 2001).

Several studies have reported that expanded $CD8^+$ T cell populations exist in the peripheral blood (PB) of patients with MM and SMM (Brown*, et al* 2009, Brown*, et al* 1997, Li*, et al* 2010). Expanded cell populations are detected by an increase in the number of cells that are positive for monoclonal antibodies to V regions of the human TCR subfamilies. These expanded cells are typically $CD8^+$, persist for long periods, but are not necessarily all clonal (Sze*, et al* 2001). Brown *et al.* (1997) and Raitakari *et al.* (2000) have shown that MM patients with expanded T cell clones have a better prognosis than those patients without clones. They further investigated the phenotype of expanded clonal cells and report that they express the phenotype of CTLs, that is, these cells are $CD8^+$, $CD57^+$, $CD28^-$ and $perforin^+$ (Brown*, et al* 1997, Raitakari*, et al* 2000).

Sze *et al.* (2001) demonstrated the monoclonal or oligoclonal nature of expanded $CD57^+$ $CD8^+$ $TCRV\beta^+$ cell populations in MM patients by analysing the length of the complementarity-determining region 3 (CDR3) of the variable region of TCRV genes, followed by sequencing. It was found that expanded TCRVβ subfamilies express low

levels of the apoptotic marker Fas (CD95) on $CD57^+$ $CD8^+$ cells and were proliferating compared to their $CD57^-$ $CD8^+$ counterparts. It was concluded that reduced expression of Fas, and therefore a reduced death rate of tumour cells along with persistent stimulation by tumour-associated antigens may result in an anti-tumour response with expansion and accumulation of activated clonal $CD8^+$ T cells in MM (Raitakari*, et al* 2000, Sze*, et al* 2001).

The cause and specificity of the T cell clones in the PB of patients with myeloma is still not known despite attempts with tetramers containing idiotype peptides and bioinfromatic analysis of the TCR sequence (Brown 2011, personal communication). The incidence of these clones depends on the sensitivity of the recognition method and their presence is related to prolonged survival. The specificity of the expanded T cell clones to tumour idiotype or other tumour proteins has been difficult to establish and whether their presence is a result of recent or past antigenic stimulation has not be identified (Brown*, et al* 1997, Pope*, et al* 2000).

1.2.7 Formation of an immunological synapse

When lymphocytes form a conjugate with APCs or tumour targets, numerous protein molecules on both cells slide together and form what is known as the immunological synapse (IS), and this has been observed for T, B and NK cells. Formation of an immunological synapse is related not only to MHC and TCR molecules but also to different co-stimulatory molecules such as LFA/ICAM and CD28/B7 receptor ligands (Figure 1-4). Thus the synapse includes adhesion, TCR/antigen peptide signaling and binding by co-stimulatory molecules. The IS is the seat of initiation of TCR signaling events that lead to diverse lymphocyte functions, including cytokine production and proliferation. This interaction regulates and orchestrates the cell-to-cell interaction required to elicit an immune response (Ahmed*, et al* 2008, Sabzevari*, et al* 2001).

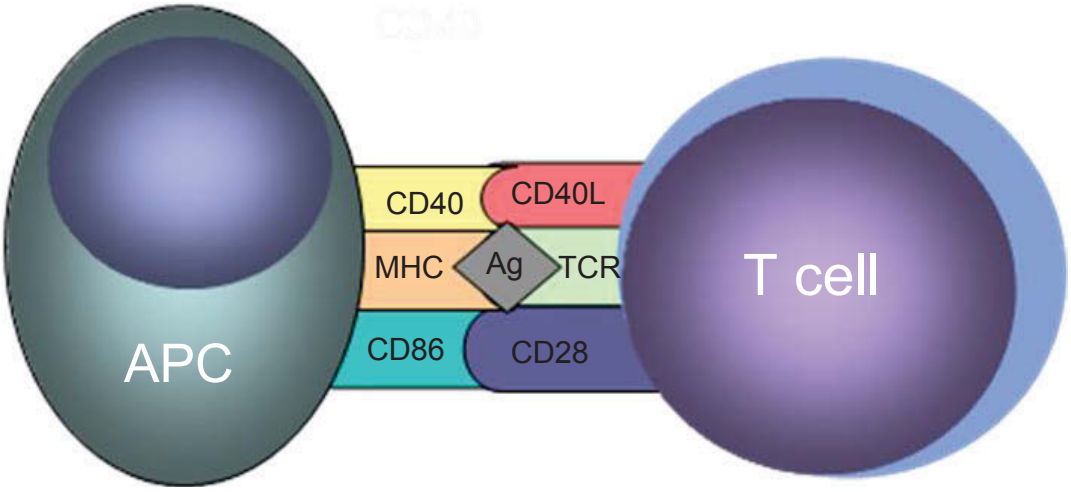


Figure 1-4: Formation of an immunological synapse.
When lymphocytes form a conjugate with antigen presenting cells, numerous protein molecules on both cells slide together and form what is known as the immunological synapse. Formation is not only related to major histocompatibility complex molecules and T cell receptor molecules but also with different co-stimulatory molecules like CD28/CD80 receptor ligands (Courtesy of Dr Ross Brown).

Following the formation of an immunological synapse, T cells acquire membrane fragments as well as associated molecules from donor cells that contain MHC molecules, co-stimulatory molecules, membrane receptors and adhesion molecules. The exact mechanism of antigen transfer between T cells and APCs is not understood. There is, however, evidence which suggests that the recipient cells acquire at least a portion of both the antigenic phenotype as well as the functional characteristics of the donor cells. Once a T cell has acquired MHC class II and co-stimulatory molecules from APCs, it has the ability to promote proliferation of bystander T cells and prime an immune response in the absence of APCs (Joly and Hudrisier 2003, Puaux*, et al* 2006, Waschbisch*, et al* 2009).

The differentiation of naïve T cells to effector cells occurs when T cells interact with MHC molecules on APCs. This interaction also leads to the formation of a tight conjugate at the contact site. When disengaged, cells have the ability to retain or donate their surface molecules. Mouse model have indicated that APC derived surface molecules adhere to T cells following APC and T cell engagement. Naïve and memory T cells acquired CD80 from APCs and were able to activate bystander T cells (Tatari-Calderone*, et al* 2002).

Formation of an IS leads to a stable attachment between interacting cells, an increase in T cell sensitivity towards peptide presented on the surface of cells, and recruitment of effector molecules to their final destination. For example, T cells encountering tumour antigens will migrate to the site of the tumour (Lotem*, et al* 2008).

A study conducted by Lotem and colleagues, reported that activated human CTLs can generate an IS-like interaction with fluorochrome-labelled melanoma cells, resulting in membrane fragment acquisition by T cells. The biological significance of membrane acquisition by T cells is poorly understood. T cells that capture p-MHCs are able to express these fragments on their own surface thereby allowing them to become secondary APCs. This process ensures enhanced antigen presentation and prolonged T cell activation (Lotem*, et al* 2008).

1.2.8 Antigen acquisition and mechanisms of molecular transfer

Molecular information can be transferred between cells through the exchange of membrane proteins from one cell to another. The latest imaging and analytical technology has followed intracellular communication resulting from the intracellular exchange of anchored membrane patches. Aucher and colleagues have shown that the transfer of membrane proteins between DCs and T cells can occur either by internalisation, uptake of exosomes and nanotube formations or the dissociation pathway (Aucher*, et al* 2008).

The exchange of membrane proteins and antigens between immune cells has been demonstrated for a long time. Aucher and colleagues were able to show, in murine models, the acquisition of membrane bound antigens on B cells and on both $CD8^+$ and $CD4^+$ T cells. Membrane exchange has also been reported for other haematopoietic cells such as NK cells, DCs, neutrophils and monocytes (Ahmed*, et al* 2008, Aucher*, et al* 2008).

Proteins are anchored to the cell surface by hydrophobic interactions and disturbance of this interaction is essential to initiate the intracellular transfer of proteins. "TCR-mediated internalisation and recycling" is the best mechanism used to describe direct cell-to-cell intracellular transfer of proteins from APCs to T cells. Initiation of a T cell response occurs when the TCR recognises p-MHC on APCs forming clusters at the contact site. TCR down-regulation follows the interactions between T cells and p-MHC complexes. Antigen presenting cell derived surface proteins then adhere to T cells after which there is internalisation of these clusters via TCR mediated endocytosis, followed by recycling and surface expression of the processed antigens (Huang*, et al* 1999).

Surface antigen receptors undergo endocytosis together with the antigens they bind. Antigens recognised by the TCR – p-MHC complex, are integral membrane proteins that do not typically pass from one cell membrane to another. The two major classes of T cells: CD4 and CD8 cells confirmed this observation. Studies have shown that B cells also acquire antigens, which are membrane bound, and are able to introduce them in the presentation pathway. Aucher and colleagues demonstrated that peptides

bound to MHC complexes transfer from APCs to T cells in fragments containing lipids and several other membrane bound proteins (Aucher*, et al* 2008).

A pre-requisite for specific and efficient acquisition of p-MHC complexes is the need for prolonged TCR signaling. Several studies have shown that lymphocytes are able to extract surface molecules from APCs through the IS (Figure 1-5). The active transfer of plasma membrane fragments from an APC to the lymphocyte is now known as trogocytosis and has been demonstrated *in vivo* and *in vitro*. Trogocytosis is thought to play an important role in the induction and regulation of the immune response. It has also been suggested that MHC ligands and proteins create a force that disrupts the "high-avidity protein-protein interactions" during TCR internalisation (Ahmed*, et al* 2008, Joly and Hudrisier 2003).

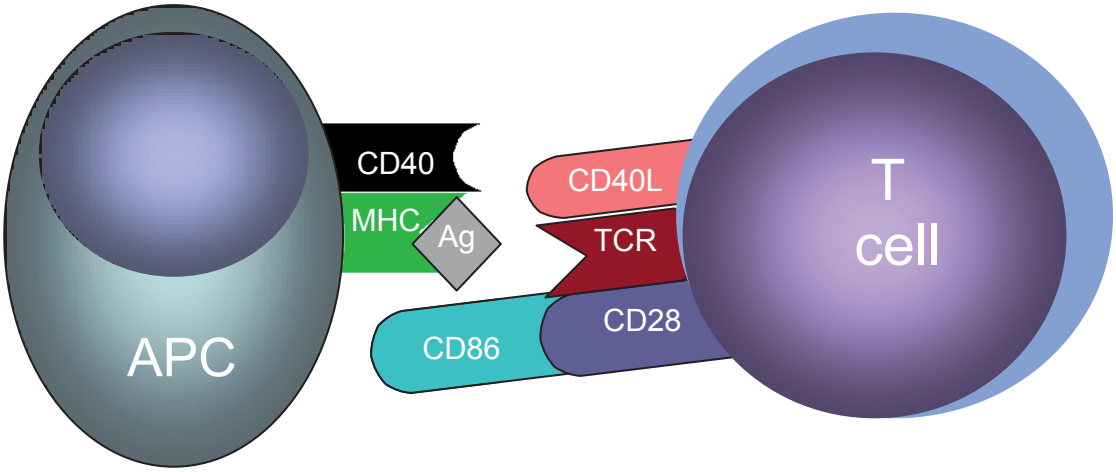


Figure 1-5: Molecular transfer between antigen presenting cells and T cells.
T cells are capable of acquiring membrane fragments following disengagement from the immunological synapse. In the figure, CD86, which was originally present on the antigen presenting cell, is now present on the surface of a T cell and attached to CD28 (Courtesy of Dr Ross Brown).

1.3 Trogocytosis

Cell-to-cell contact-dependent transfer of membrane fragments has been widely studied and documented since the 1980s. Recently, the process has been termed 'trogocytosis' (Joly and Hudrisier 2003). Trogocytosis occurs between many cells but has been best described between APCs, T, B, NK cells and tumour cells both *in vivo* and *in vitro* (Hudrisier and Bongrand 2002). The process involves the transfer of membrane patches rather than individual molecules. Studies have proposed that transfer is facilitated by engagement of the immune synapse (MHC:TCR, MHC-I:KIR, MHC-I:ILT-2, CD28:B7), and may occur in the presence or absence of antigen (Joly and Hudrisier 2003, LeMaoult*, et al* 2007b, Wiendl 2007).

Trogocytosis is usually fast, requiring less than a few minutes, and includes cell-surface, intra-membrane and adaptor molecules. Most of the work conducted on trogocytosis by T cells has been performed in the murine system in which it has been demonstrated that $CD4^+$ and $CD8^+$ T cells acquire MHC class II and MHC class I molecules respectively, from APCs in an antigen-specific manner. It has also been noted that TCR engagement is required for trogocytosis to occur (LeMaoult*, et al* 2007b, Wiendl 2007).

Trogocytosis may also be involved in lymphocyte activation by prolonging the engagement of antigen receptors after APC and T cell separation. Trogocytosis by CTLs might contribute to the selection of high-affinity T cells through the removal of antigenic complexes from the presenting DCs and to the elimination of CTLs through a process known as 'fratricide', occurring after the acquisition of p-MHC molecules. Such fratricide may not be physiological, however, it only occurs when an extremely high concentration of MHC class I−restricted antigenic peptide is added externally to the target cell (Joly and Hudrisier 2003).

It has been shown that T cells can acquire not only MHC class I and class II molecules but also co-stimulatory molecules, proteins from endothelial cells and membrane fragments from APCs. Daubeuf *et.al.* (2008) reported that trogocytosis occurs for all lymphocyte populations as well as other haematopoietic lineages, that it

is a process of fragment transfer from APCs to lymphocytes and also that trogocytosis is a uni-directional process.

However, it has been demonstrated that NK cells can acquire target cell MHC class I protein *in vivo* and *in vitro*. NK cell receptors for MHC class I can be transferred to target cells, indicating a bi-directional membrane transfer. B cells can also acquire membrane-associated antigens from target cells with the amount acquired correlated with increasing affinity of the BCR to the antigen (Ahmed*, et al* 2008).

Many dyes have been used to demonstrate trogocytosis, these include fluorescent lipophilic probes such as DiO C18, DiI C18, PKH26, and PKH67, cytosolic markers like CMTMR and biotin (Aucher*, et al* 2008, Daubeuf*, et al* 2008, Puaux*, et al* 2006). In a study by Puaux *et al.* (2006), donor cells were labelled with various markers and, after pulsing with, or without, OVA257, these cells were then exposed to CTLs from OT-I mice. It was found that the membrane markers DiL, PKH67, DiO and biotin were significantly detected on the surface of CTLs compared to the cytosolic markers Carboxyfluorescein succinimidyl ester (CFSE), CMTMR or calcein. The marker that gave the highest signal was demonstrated to be biotin. It was concluded that this may be due to the amplification achieved based on streptavidin binding as it can be conjugated to multiple fluorochromes and thus this method of detection was considered most sensitive.

Furthermore, biotin transfer from target cells to $CD4^+$ helper T cells and B cells has been investigated and it has been shown that biotin acquisition can occur on all lymphocyte populations and sub-populations. Furthermore, detecting the transfer of membrane proteins to activated T cells appears to be of specific reactivity rather than specific interaction (Puaux*, et al* 2006).

In another study conducted by Rosenitis *et al.* (2010), the acquisition of biotinylated membrane fragments from C57BL/6 mouse splenocytes by HLA matched memory P14 TCR transgenic T cells was investigated. It was reported that 40-70% of P14 T cells expressed biotinylated membrane fragments from peptide-pulsed APCs compared to the small fraction of P14 T cells expressing biotin when in contact with

control APCs. They therefore concluded that trogocytosis is an antigen-specific process.

It has been suggested that trogocytosis is a transfer of fragments or patches (Tarnok 2009) and not individual molecules. As a result, all molecules adjacent to a certain membrane region are transferred between cells during trogocytosis. This can include molecules that have nothing to do with effector cells or participate in cell-to-cell communication (LeMaoult, *et al* 2007b).

It has been proposed that trogocytosis has potentially therapeutic and significant consequences. The mechanism by which trogocytosis occurs is poorly studied. As this process may influence the immune response, it is of great importance to understand the process (Aucher, *et al* 2008).

1.3.1 Physiological and functional significance of trogocytosis in the immune response

Trogocytosis has now become a recognised feature of T and B cell biology. The hypothesis is that this process may be involved in the spread of pathogen and in the control of the immune response. The exact mechanism and the overall physiological and functional significance trogocytosis plays in various cell types along with the molecules involved are yet to be discovered. There are however three theories being considered.

The first is the "immune effector clearance" hypothesis whereby acquisition of MHC class I peptides by CTLs influences these cells to fratricide (antigen specific cytolysis), thereby aiding in the elimination of CD8 effector cells. The second is the "amplification of immune responses" hypothesis whereby the transfer of the presentasome to CD4 T cells may amplify the immune response and contribute to stimulation by generating APC-like T cells. The third is the "sustainment of immune response in the absence of APCs" hypothesis where in the absence of APCs, acquisition of CD80 or CD86 and MHC class II regulates T cell proliferation and prolongs their activation (Aucher, *et al* 2008, Wiendl 2007).

Co-stimulatory molecules and proteins on the cell membrane have significant impact on cellular function. Thus the capture of various molecules not translated by lymphocytes may directly, or indirectly, alter the function and phenotype of the cells that have acquired membrane proteins (Ahmed*, et al* 2008). Lymphocytes acquire various molecules from target cells during trogocytosis, and inherited regulatory molecules like co-stimulatory molecules, or transcription factors, may alter the function and phenotype of lymphocytes (Joly and Hudrisier 2003).

Trogocytosis does not involve the transfer of individual molecules but rather the transfer of membrane patches including intra and trans-membrane proteins. Molecules that may be transferred include MHC molecules (Undale*, et al* 2004), co-stimulatory molecules (CD80 and CD86) (Tatari-Calderone*, et al* 2002) as well as adhesion molecules (CD31, CD54 and CD61). Therefore, through trogocytosis, a cell's surface molecules are altered so that the acquirer cell displays molecules which it did not previously express. Having acquired the specific molecules, the acquirer cell may also acquire some of the functions of the donor cell from which the acquired molecules originated (Caumartin*, et al* 2006, LeMaoult*, et al* 2007a).

Cells that have acquired membrane molecules through the process of trogocytosis can utilise them. For example, $CD8^+$ T cells that have acquired MHC class I ligands undergo fratricide, a process of antigen specific cytolysis, thereby contributing to effector clearance. It has been shown that $CD80^+$ T cells can stimulate resting T cells, thus behaving as APCs after trogocytosis. In the absence of APCs, T cells that have acquired CD80 can regulate proliferation signals (Cox*, et al* 2007, LeMaoult*, et al* 2007a, Tatari-Calderone*, et al* 2002).

Interestingly, the function of NK cells has been shown to be reduced when they acquire MHC Class I molecules from tumour cells. $CD8^+$ T cells are capable of inducing tumour-specific immunity when they acquire MHC class I and II and co-stimulatory molecules from APCs *in vivo* thus indicating that phenotypic expression can determine function. Activated cells were found to acquire membrane patches to a greater extent than their resting cellular counterparts (LeMaoult*, et al* 2007a).

It has been demonstrated in mouse models that transplanted T cells, originating from a different genetic MHC background to that of the host, acquire host MHC antigens, suggesting that trogocytosis can occur between host B cells and donor T cells. T cell clones that are restricted to MHC-II are able to acquire MHC-I molecules as well, indicating that specific and non-specific ligands can be acquired. Trogocytosis can occur in the presence of co-stimulatory molecules and can occur in the absence of antigen stimulation (Hwang*, et al* 2000, Rechavi*, et al* 2009).

It has been demonstrated that not all biotinylated surface proteins from target cells transfer by trogocytosis and a number of different mechanisms for specific transfer of intercellular proteins have been described. These include exosome shedding, sharing of membrane patches or proteolytic cleavage of proteins. There is, however, a correlation between the acquisition of specific membrane fragments, as determined by the acquisition of lipophilic fluorophores, and the presence of surface proteins that were localised to the membrane patch. Thus trogocytosis involves more than simply the transfer of receptors to their ligands (Rechavi*, et al* 2009, Tabiasco*, et al* 2002, Vanherberghen*, et al* 2004).

1.3.2 Involvement of co-stimulatory molecules

The adaptive immune response coordinates the balance between stimulatory and inhibitory signals which are controlled by the B7 family. B7-1 (CD80) and B7-2 (CD86) are the most widely studied co-stimulatory molecules. Other members of the B7 family include B7-H1 (CD274), B7-DC (CD273), B7-H2 (CD275), B7-H3 (CD276) and B7-H4 (Figure 1-6). Although they each have different functions, they are all important in controlling the proliferation and maturation of T cells and can be classified into three groups. Group one molecules include CD80, CD86 and CD275, group two molecules include CD274 and CD273 and group three molecules include CD276 and B7-H4 (Table 1-4) (Collins*, et al* 2005, Cox*, et al* 2007, Zang and Allison 2007).

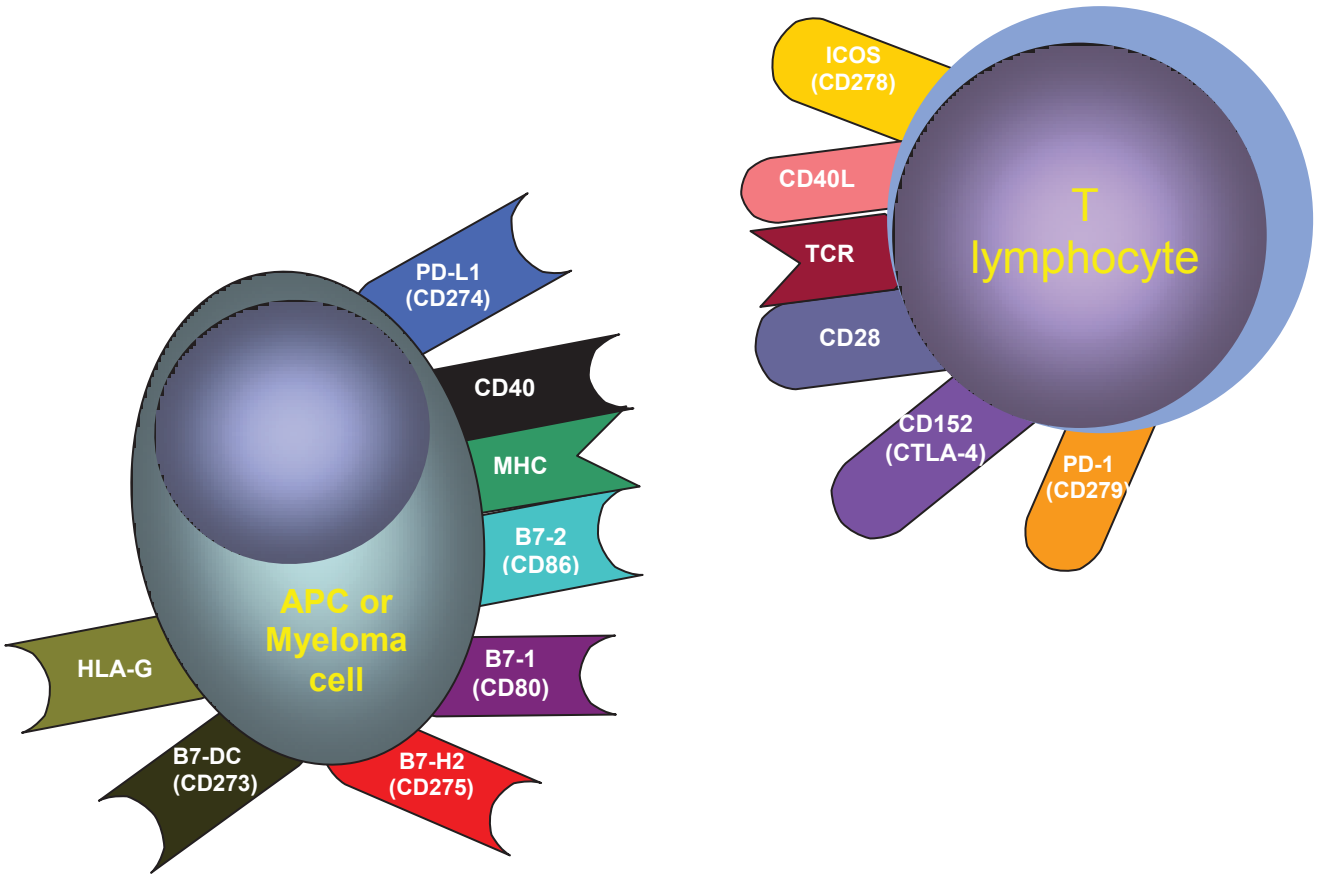


Figure 1-6: Co-stimulatory molecules and receptors found on APCs and T cells. The most common molecules found on antigen presenting cells include HLA-G, CD273, CD274, CD275, CD80 and CD86. Ligands for the molecules include CD278, CD279, CD152 and CD28 which are widely expressed on T cells.

Table 1-4: The B7 family molecules and co-receptors

Group one molecules		**Co-receptors**	
B7-1 (CD80)	[+]BB1	CD28	[+]T44, Tp44
B7-2 (CD86)	[+]B70	CD28	[+]T44, Tp44
B7-H2 (CD275)	[+]B7h, B7RP-1, GL50, ICOSL, LICOS	ICOS (CD278)	[+]AILIM, CRP-1, H4
Group two molecules		**Co-receptors**	
B7-H1 (CD274)	[+]PD-L1	PD-1 (CD279)	
PD-L2 (CD273)	[+]B7-DC	PD-1 (CD279)	
Group three molecules		**Co-receptors**	
B7-H3 (CD276)		Unknown	
B7-H4			

[+]Alternate names

1.3.2.1 The B7 family of molecules: Group one molecules

The function of CD80 and CD86 are to deliver co-stimulatory signals to naïve T cells via CD28 and co-inhibitory signals via CTLA-4 on activated T cells. These molecules are not only expressed on professional APCs but are present on activated T cells (Collins, *et al* 2005, Robillard, *et al* 1998, Yamashita, *et al* 2009).

CD80 and CD86 are members of the Ig gene superfamily and are predominately expressed on professional APCs. CD28, on the other hand, is expressed on thymocytes and on most activated and resting T cells. CD80 has also been shown to react with cytolytic T lymphocyte-associated antigen 4 (CTLA-4) on activated T cells. Interaction of CD80 and CD28 prevents induction of T cell apoptosis and clonal anergy. It has been reported that CD80 is also expressed on human T cell clones and T cells activated *in vitro*. Murine studies have demonstrated that the amount of CD80 on T cells, varies with activation status. Resting T cells display little to no CD80, however CD80 levels increase upon activation (Sabzevari, *et al* 2001).

Sabzevari and colleagues analysed CD80 levels on murine T cells upon stimulation with assorted APCs expressing varied levels of CD80. While it was previously thought that CD80 is endogenously up-regulated, their study showed that CD80/CD86 is physically acquired by T-lymphocytes through APCs soon after T cell activation. The amount of CD80 expressed on T cells varies and depends on the state of activation. The level of CD80 acquired relates to the strength of signal 1 and the level of CD80 expressed by APCs. The factors that affect ligand acquisition and ligand-receptor interactions remain unclear (Hwang, *et al* 2000, Sabzevari, *et al* 2001).

CD28 is predominately found on T cells and may also be present on normal plasma cells and myeloma cell lines. The ligands for CD28 are CD80 and CD86 which are predominately expressed by APCs and DCs. In conjunction with TCR signaling, these two molecules deliver co-stimulatory signals that result in T cell proliferation and enhanced survival by up-regulation of anti-apoptotic gene expression. The function of CD28 on T cells has been widely studied but its function in myeloma cells is not yet known (Bahlis, *et al* 2007).

CD28 expression on myeloma cells is seen in newly diagnosed myelomas, medullary recurrences, extramedullary relapses and secondary plasma cell leukemias as well as on MM cell lines. It has been suggested that CD28 expression aids in enhancing survival of myeloma cells. Unlike T cells, CD28 activation does not induce IL-6 secretion by myeloma cells. It does, however, induce the up-regulation of IL-8 (a proangiogenic chemokine) (Bahlis*, et al* 2007, Pope*, et al* 2000, Robillard*, et al* 1998).

If myeloma cell survival is due to CD28, it is possible that *in vivo* activation is likely caused by contact with other $CD86^+$ cells like other CD86 expressing myeloma cells, B cells and the interaction with normal APCs and DCs. It has been reported that patients with $CD28^+$ myelomas also co-express $CD86^+$ (Robillard*, et al* 1998), and that myeloma patients with $CD86^+$ expressing myeloma cells have a significantly poorer prognosis (Pope*, et al* 2000). In addition, it has been reported that $CD28^+$ myeloma cell lines also co-express CD86 (Bahlis*, et al* 2007).

Pope *et al.* (2000) reported that CD80 is rarely expressed and that there is variable expression of CD86 on malignant plasma cells. They reported that it is possible to up-regulate B7 family molecules with human CD40 ligand (huCD40LT) thereby allowing the transformation of malignant plasma cells to APCs. An interesting finding was that huCD40LT up-regulates the expression of CD80 but it does not induce $CD86^+$ myeloma.

The study conducted by Yamashita *et al.* (2009) showed that CD86 expression on $CD38^{++}$ plasma cells was increased in patients with MM compared to MGUS patients and haematologically normal individuals. Of the patients studied, three patients with MM expressed CD275. It was concluded that myeloma cells that expressed CD86 and CD275 had a greater potential for cell proliferation and increased cell cycling. Futhermore, they were able to demonstrate that $CD86^+$ and $CD275^+$ plasma cells enhance $CD4^+$ T cell proliferation, stimulating them to produce IL-10 and hence stimulating myeloma cell proliferation.

CD275, which is expressed on macrophages, B cells and non-lymphoid tissues, is induced by TNF-α and interacts with its receptor, inducible co-stimulator (ICOS

[CD278]), to induce T cell proliferation and secrete cytokines such as IFN-γ and IL-4 but not IL-2. This interaction further induces the production of IL-10. B7 family molecules are expressed mainly on APCs but can also be expressed on tumour cells and this may be responsible in modulating anti-tumour immunity in the host (Cox, *et al* 2007, Yamashita, *et al* 2009).

1.3.2.2 The B7 family of molecules: Group two molecules

Group two molecules include CD274 (programmed cell death-1 [PD-L1]) and CD273 (PD-L2). Expression of CD274 has been investigated in haematopoietic malignancies such as acute myeloid leukemia (AML) and multiple myeloma, as well as in solid cancers such as breast cancer (Iwasaki, *et al* 2011), renal cell carcinoma (Vanherberghen, *et al* 2004), pancreatic carcinoma (Yamashita, *et al* 2009), glioblastoma (Rechavi, *et al* 2009), squamous cell carcinoma (Yi, *et al* 1997) and esophageal cancer (Tabiasco, *et al* 2002). Expression of CD274 and CD273 has also been reported in non-small cell lung cancer (Cox, *et al* 2007, Rosenits, *et al* 2010).

CD274 is expressed on T cells, APCs, NK cells, mesenchymal stem cells, endothelial cells and pancreatic islet cells. Expression of CD274 can be up-regulated in response to inflammatory cytokines. CD273 is expressed on DCs and macrophages. CD279 (PD-1), the receptor for CD274 and CD273, is expressed widely on activated monocytes, T cells, B cells and DCs. TCR and BCR signalling is modulated by the interaction between these three molecules. The interaction between the co-stimulatory molecules CD274 and CD273 on APCs, and CD279 on T cells, inhibits T cell responses (Collins, *et al* 2005, Cox, *et al* 2007).

1.3.2.3 The B7 family of molecules: Group three molecules

Group three molecules include B7-H3 (CD276) and B7-H4. CD276 is found on lymphoid and non-lymphoid cells and peripheral organs whilst B7-H4 is expressed by peripheral tissues and on most haematopoietic cells. A receptor has not yet been discovered for these molecules and therefore the functional significance of these molecules is not yet understood. There is however evidence which suggests that CD276 stimulates T cell proliferation and the production and release of cytokines.

There is also evidence that CD276 inhibits NK cell mediated cytolysis and impairment of a Th1 response (Cox, *et al* 2007).

CD275 and CD276 are found to be co-expressed with other B7 family molecules on solid tumours. Expression of CD275 on solid tumours inhibits immune cells and expression on all cells in haematological diseases promotes expansion of tumour cells. B7-H3 is expressed in renal cell carcinoma and prostate cancer, however its function is not well understood (Cox, *et al* 2007).

Like CD274, B7-H4 is expressed on the surface of many cancers including prostate cancer, breast cancer, ovarian cancer, uterine endometrial carcinoma, renal cancer and non-dividing brain tumour cells (Cox, *et al* 2007).

1.3.3 Trogocytosis and T cell activation

The interaction between APCs and T cells involves presentation of antigens to the TCR through the MHC and a secondary signal that is produced by the linkage of the co-stimulatory molecules on the APC with their receptors on T cells. Thus to generate an effective T cell stimulation, there needs to be a second signal between CD28 and/or CD152 on T cells with the co-stimulatory molecules CD80 and CD86 on APCs. TCR engagement in the absence of co-stimulation results in T cell anergy. Apoptosis occurs as a result of signaling via CTLA-4 in the absence of CD28. A study by Pope *et al.* (2000) has suggested that the scarcity in B7/CD28 co-stimulation between malignant plasma cells and T cells is partly accountable for the failure to mount an anti-tumour immune response (Pope, *et al* 2000).

The interaction between co-stimulatory molecules and their receptors promotes stability of the TCR/MHC complex. Co-stimulatory molecules can also intensify signal 1 at the early stages of T cell activation, enhancing TCR cross-linking. This contributes to the overall avidity of the complex and therefore leads to the transfer of CD80 (Sabzevari, *et al* 2001).

Initial antigen encounter stimulates naive T cells to proliferate and differentiate into activated effector/memory T cells. It has been postulated that most of the effector

cells have a short life span and undergo apoptosis. Two observations presented by Sabzevari *et al.* (2001) suggest that CD80 acquisition may play a role in activating or regulating the immune response. The first is that effector T cells are able to acquire CD80 when signal 1 is reduced and the second is that apoptosis of these cells occurs with increased levels of signal 1 and CD80 acquisition. Further investigation is required to determine the consequences in the regulation of an anti-tumour response (Sabzevari*, et al* 2001).

1.3.4 T cells as non-professional APCs

Naive $CD4^+$ T cells acquire MHC molecules and CD80 when activated and are therefore capable of acting as inefficient APCs, that is, they are able to deliver both signal 1 and 2 to other bystander T cells therefore amplifying the immune response. Activated T cells are also capable of providing signal 2 to T cells that have received signal 1 from non-professional APCs. Acquisition can be reduced by TCR, CD28 or CD80 blocking. Mostbock *et al.* (2007) have reported that acquired p-MHC/co-stimulatory complexes are able to sustain activation and proliferation of naive T cells. It has been suggested that T:T cell stimulation can assist with the regulation of the immune response (Hwang*, et al* 2000, Mostböck*, et al* 2007, Sabzevari*, et al* 2001, Tatari-Calderone*, et al* 2002).

1.4 The Non-Classical HLA-G Molecule

Human leukocyte antigen G (HLA-G) has been termed a “non-classical” HLA class I molecule as it differs from other HLA class I molecules by its expression, genetic diversity, function, structure and its low polymorphism (8 known protein variants compared to 462 for HLA-A and 789 for HLA-B). HLA-G is largely expressed in foetal trophoblast cells, however in adults its presence is restricted to pancreatic islets, thymic medulla, endothelial and erythroid-cell precursors, it is also found in the cornea (Carosella*, et al* 2008, Menier*, et al* 2004, van der Ven*, et al* 2000).

1.4.1 HLA-G isoforms

Soluble isoforms of HLA-G are secreted by DCs and myelomonocytic cells under normal physiological conditions. It has recently been shown that HLA-G expression is up-regulated in pathological conditions including transplantation (Lila, *et al* 2007), cancers (Paul, *et al* 1998, Rouas-Freiss, *et al* 2005), viral infections (Lozano, *et al* 2002), and in autoimmune and inflammatory conditions (Wiendl, *et al* 2005).

Alternative splicing of the primary HLA-G transcript generates four membrane-bound (G1-G4) and three soluble (G5-G7) isoforms (Carosella, *et al* 2003), with HLA-G1 and HLA-G5 being the most widely studied. Expression of the different isoforms is believed to be dependent on the cell type (Lila, *et al* 2000). HLA-G1 is composed of a heavy chain (three identical α chains) non-covalently bound to β_2M. HLA-G5 has an identical structure to HLA-G1 but lacks the transmembrane domain. HLA-G2 and HLA-G4 contain only two α chains and are not bound to β_2M (Figure 1-7). Soluble isoforms are generated by the shedding or proteolytic cleavage of membrane-bound HLA-G (Apps, *et al* 2007).

HLA-G is considered to be an immunomodulatory molecule as it interacts and binds to the inhibitory receptors immunoglobulin-like transcript receptor (ILT)-2 (CD85j/LILRB1) and ILT-4 (CD85d/LILRB2) which are expressed on all monocytes, DCs, B cells and some T and NK cells. The inhibitory receptor KIR2DL4 (CD158d) is expressed on monocytes and DCs as well as on $CD56^{bright}$ NK cells present in uterine tissues. ILT-2 recognises isoforms that are bound to β_2M, namely HLA-G1 and HLA-G5, whereas ILT-4 recognises isoforms containing only two α chains (Colonna, *et al* 1998, Goodridge, *et al* 2003).

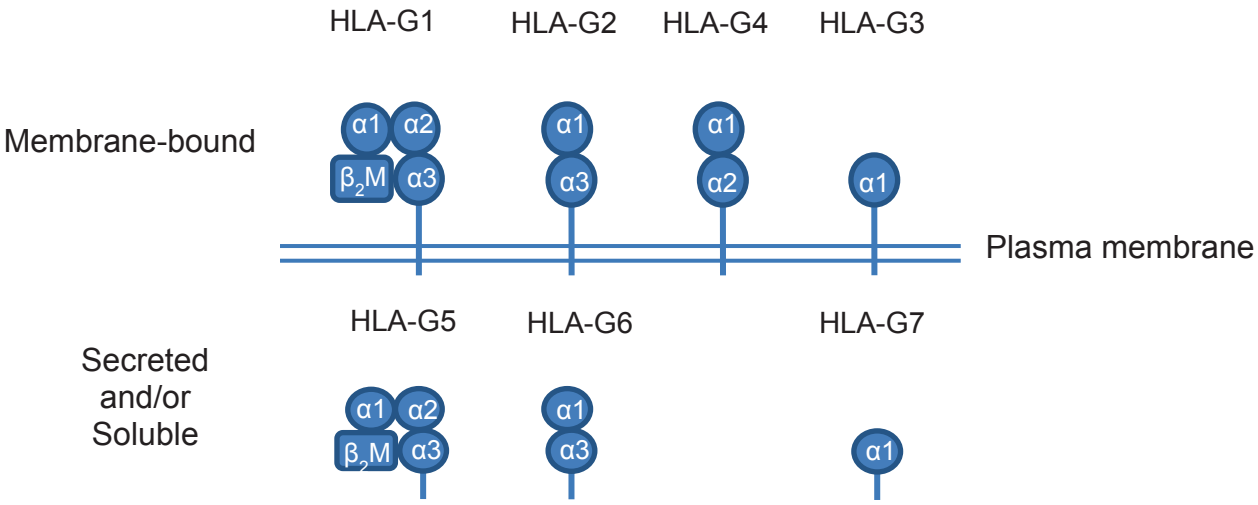


Figure 1-7: Alternative splicing of the primary HLA-G transcript results in seven HLA-G isoforms.
Alternative splicing of the primary HLA-G transcript generates four membrane-bound and three soluble isoforms. HLA-G1 is composed of a heavy chain (three identical α chains) non-covalently bound to β_2M. HLA-G5 has an identical structure to HLA-G1 but lacks the transmembrane domain. HLA-G2, HLA-G4 and HLA-G6 contain only two α chains and are not bound to β_2M. HLA-G6 lacks the transmembrane domain (adapted from Carosella, *et al* 2008).

1.4.2 Functional significance of HLA-G

Over the past decade HLA-G function has been the focus of much interest. Much of our understanding of its function has come from the expression of HLA-G on cytotrophoblasts at the foeto-maternal interface where HLA-G is known to inhibit the cytolytic function of maternal NK cells allowing immune tolerance of the semi-allogeneic foetus (Rouas-Freiss, *et al* 1997). Membrane-bound and soluble forms of HLA-G have been shown to prevent cytolytic T cells from carrying out antigen-specific cytolysis, inhibit the function of circulating NK cells, prevent allo-proliferation of $CD4^+$ T cells (Riteau, *et al* 1999) and inhibit maturation of dendritic cells (Gros, *et al* 2008). In addition, Fournel *et al.* (2000) have demonstrated induction of apoptosis of $CD8^+$ T cells by soluble HLA-G (sHLA-G) (Figure 1-8) (Fournel, *et al* 2000).

HLA-G expression by both effector and target cells controls the immunomodulatory and inhibitory functions of these cells. For instance, APCs expressing HLA-G may interact with inhibitory receptors on effector cells. This reverses the function of these cells, such that they become suppressive cells. HLA-G may also be acquired by T or NK cells through the process of trogocytosis giving rise to $HLA\text{-}G1^{acq}$ T and NK cells. $HLA\text{-}G1^{acq}$ T/NK and $HLA\text{-}G^+$ regulatory cells are hypo-responsive and hypo-proliferative to stimulation (Naji, *et al* 2007).

HLA-G expression has been found in an increasing number of pathological conditions including haematological and non-haematological malignancies, HIV and autoimmune and inflammatory diseases. These observations have led to the hypothesis that expression of 'tolerogenic' HLA-G may aid in the escape of tumours from immune surveillance (Naji, *et al* 2007).

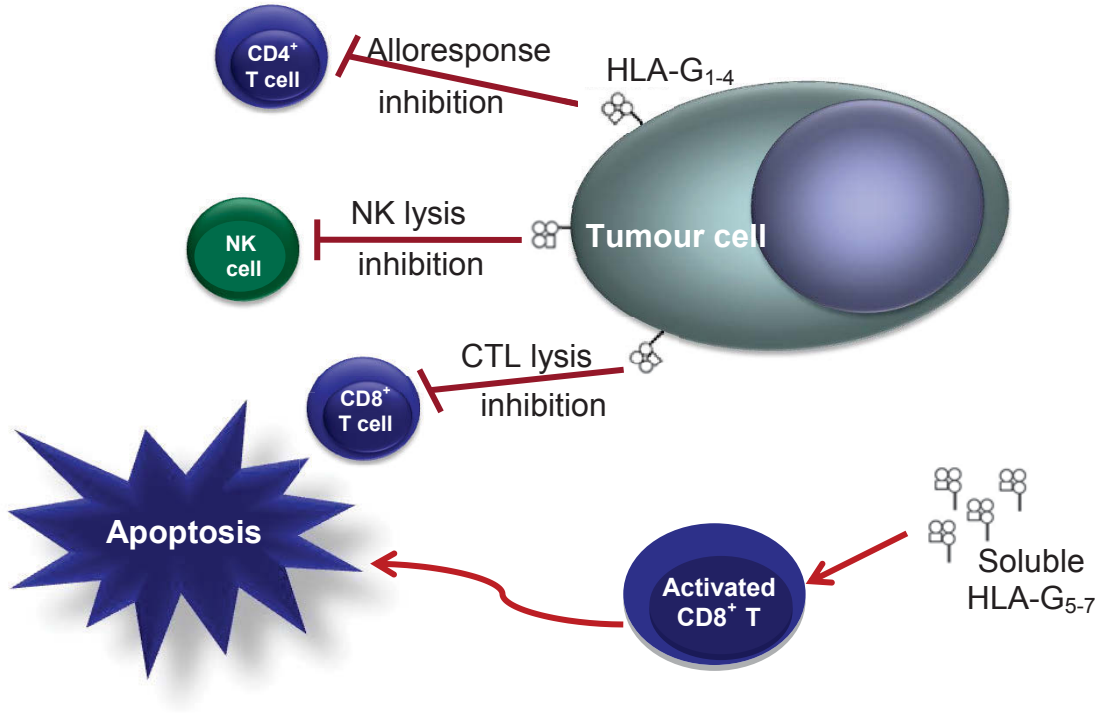


Figure 1-8: Schematic representation of the inhibitory effects of membrane-bound and soluble HLA-G on various effector cells.
The interaction between HLA-G on tumour cells and ILT receptors on CD4 cells results in the inhibition of T cell proliferation. Interaction with receptors on NK cells and CTLs results in inhibition of the cytolytic function of these cells (adapted from Rouas-Freiss, *et al* 2005).

1.4.3 Physiological role of HLA-G

HLA-G is predominately expressed on extravillous cytotrophoblast cells at the foeto-maternal interface and is believed to protect the semi-allogeneic foetus from maternal rejection by NK or cytotoxic T cell mediated cytolysis. The mechanism by which there is inhibition of cytolysis is thought to be through the interaction of HLA-G with its inhibitory receptors KIR2DL4, ILT-2 and ILT-4 (Apps, *et al* 2007).

Expression of HLA-G on trophoblasts is associated with a reduced proliferation of allogeneic lymphocytes. There is further evidence supporting the role of HLA-G, and the interaction with its receptor in inducing changes in the cytokine profile of maternal uterine leukocytes. An increase in immunosuppressive function was observed with the production of IL-6 and IL-10 (Apps, *et al* 2007, Shiroishi, *et al* 2006).

Because of this extensive inhibitory function, which is capable of targeting multiple immune cell subsets, attempts have been made at determining whether HLA-G is pathologically relevant and whether it can be used as a therapeutic or diagnostic tool and/or target in cancer or autoimmunity (Carosella, *et al* 2008).

1.4.3.1 Allogeneic transplantation and HLA-G expression

In the context of transplantation, HLA-G expression is valuable and aids in the promotion of tolerance to grafts. HLA-G has been widely studied over the past decade and, more recently, has been demonstrated to play a role in graft acceptance in patients undergoing heart (Lila, *et al* 2007, Lila, *et al* 2000), liver (Naji, *et al* 2007), kidney (Qiu, *et al* 2006) and liver-kidney transplantation (Creput, *et al* 2003). A considerably better outcome has been observed in patients that express higher levels of HLA-G.

Titers of HLA-G, in transplanted patients, may be used as a monitoring tool to establish, as well as follow, tolerance status which could then be used to regulate immunosuppressive therapies. For example, those patients with high HLA-G titers would have a reduction in immunosuppressive treatment, whereas patients who are

HLA-G negative would have a higher risk of rejection. Furthermore, HLA-G itself might be used as a therapeutic tolerogenic agent, provided exogenously to HLA-G negative patients as an alternative and/or complementary therapy (Carosella*, et al* 2008).

In a study involving patients undergoing cardiac transplantation, HLA-G was present in serum and myocardial tissue in a minority of patients. In this HLA-G positive population, acute post transplantation rejection was significantly reduced compared to HLA-G negative patients. This suggests that HLA-G may promote graft acceptance possibly by inhibiting NK and CTL mediated cytolysis. HLA-G up-regulation may occur due to cytokine (eg IL-10) release. Other mechanisms of HLA-G upregulation may involve environmental factors as well as immunosuppressive therapy (Lila*, et al* 2002, Lila*, et al* 2000).

As in the patients undergoing cardiac transplants, no acute or chronic rejection of kidney or liver grafts was observed in those patients with high levels of HLA-G undergoing liver-kidney transplant. It was also noted that suppressor $CD3^{+}$ $CD4^{low}$ and $CD3^{+}$ $CD8^{low}$ T cells were observed in peripheral blood of patients with high levels of HLA-G. These results suggest that HLA-G expression by the graft, or in serum, may contribute to graft acceptance. HLA-G may potentially be useful in predicting which transplant patients are likely to accept their graft and may also be used as a novel immunosuppressive agent (Carosella*, et al* 2008, Naji*, et al* 2007).

1.4.4 HLA-G expression and malignancies

In the context of oncology, Carosella *et al.* (2008) reported that HLA-G transcription and protein expression are turned on in tumour lesions and protects them from cytolysis. It was later demonstrated that protection against NK cytolysis by HLA-G expression correlated with malignancy in breast and ovarian carcinomas as well as in melanocytic lesions. An unfavorable outcome was observed in gastric and colorectal cancers as well as in chronic lymphocytic leukemia (CLL) expressing HLA-G. HLA-G expression has been observed in various haematopoietic malignancies, predominantly in B chronic lymphocytic leukemia (B-CLL), acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) (Carosella*, et al* 2008).

1.4.4.1 Involvement of HLA-G in haematological malignancies

Studies involving HLA-G expression in malignant haematopoietic diseases are limited and the functional significance of HLA-G expression is not well understood. It was originally shown that expression of HLA-G in AML was dependent on IFN-γ expression. In a study performed by Carosella *et al.* (2008), it was found that none of the AML patients tested expressed HLA-G. However, they found that prior incubation of the malignant cells with IFN-γ induced HLA-G expression in approximately 20% of patients (Carosella*, et al* 2008).

Investigations on HLA-G protein expression in B-CLL, proposed that HLA-G plays a role in tumour escape from the immune system. Flow cytometry was used to assess HLA-G expression on the surface of circulating B-CLL cells. It was found that expression of HLA-G varied from 1% to 54%, as determined by both RT-PCR and flow cytometry. Patients with more than 23% HLA-G positive cells appeared to have a significantly shorter progression-free survival time compared to those patients who had less than 23% positive cells. HLA-G transcription and expression in CLL patients demonstrated tumour cell protection against autologous NK lysis, thus permitting tumour development by impairing "anti-tumour immunity" (Carosella*, et al* 2008, Maki*, et al* 2008, Nuckel*, et al* 2005).

NK cell cytotoxicity assays involving HLA-G expressing CLL cells have demonstrated tumour cell resistance to NK mediated cytolysis. Blocking HLA-G causes a significant increase in cytotoxicity. This finding suggests an important role for HLA-G in protecting CLL cells from NK mediated cytolysis (Maki*, et al* 2008).

CLL patients with less than 23% HLA-G expressing cells demonstrate an improved progression free survival (PFS) compared to patients with greater HLA-G expression (median PFS: 120 months versus 23 months; p=0.0001). Furthermore, higher HLA-G expression correlated with increased immunosuppression within the patient cohort as measured by IgG levels, $CD4^+$ T cells and total T cell numbers (Nuckel*, et al* 2005, Sebti*, et al* 2007).

HLA-G expression was also studied in patients with Non-Hodgkin lymphoma (NHL). One study showed no HLA-G expression (Poláková, *et al* 2003) while other studies showed 65-100% HLA-G expression in B-NHL (Amiot, *et al* 1996) and 58% expression in T-NHL (Urosevic, *et al* 2004). Correspondingly, a study investigating HLA-G expression in AML showed that there was no expression (Poláková, *et al* 2003). This observation was in contrast to another study demonstrating HLA-G expression in 20% of patients. HLA-G expression is variable in these malignancies (Yan, *et al* 2008).

1.4.4.2 Involvement of HLA-G in non-haematological malignancies

Cytotoxic T cells recognise antigens presented by MHC class I molecules. Many tumours are able to down-regulate the expression of these molecules which results in tumour escape from CTL immune-surveillance. HLA-G expression by tumours may also favour tumour escape by the interaction of HLA-G with inhibitory receptors on T, B and NK cells (Carosella, *et al* 2003, Ibrahim, *et al* 2001, Paul, *et al* 1998, Rouas-Freiss, *et al* 2005, Wiendl, *et al* 2002).

The mechanism by which HLA-G is up-regulated by tumours is not understood, however, transcriptional activation by the tumour micro-environment may be involved in the up-regulation of HLA-G expression. This may be caused by stress, IL-10, GM-GSF, IFN-γ or IFN-β expression (Carosella, *et al* 2003). Studies indicate that expression of HLA-G by 10% of tumour cells will be enough to inhibit T cell-mediated cytotoxicity thereby protecting HLA-G negative tumour cells (Wiendl, *et al* 2002).

To date over 1000 tumours have been investigated for HLA-G expression. Results indicate that HLA-G is expressed at varying levels in a wide variety of tumours. Analysis of tissue specimens indicate that HLA-G expression is related to malignant transformation as HLA-G expression has not been detected in adjacent normal tissues (Rouas-Freiss, *et al* 2005).

Of the seven HLA-G isoforms, four are found membrane-bound and three are found circulating as soluble isoforms. Soluble HLA-G may be secreted by cells or may occur through the release of membrane-bound forms (Pistoia, *et al* 2007). Soluble HLA-G may contribute to the anti-tumour effect at the tumour site and/or systemically and is frequently found to be expressed in pathological conditions (Rebmann, *et al* 1999). A substantial increase in soluble HLA-G has been observed in patients with gliomas, melanoma, ovarian and breast carcinomas (Rebmann, *et al* 2003) as well as lymphoproliferative disorders (Nuckel, *et al* 2005).

1.4.5 HLA-G and inflammatory diseases

HLA-G expression has been observed in muscle fibre biopsies from patients with inflammatory myopathies such as inclusion body myositis and polymyositis, but has not been observed in muscle biopsies from normal control individuals (Wiendl, *et al* 2000). HLA-G has also been shown to be strongly expressed on macrophages, microglial cells, T cells and endothelial cells in brain specimens from patients with multiple sclerosis (MS) and other inflammatory and non-inflammatory brain diseases (Feger, *et al* 2007, Wiendl, *et al* 2005).

1.4.6 Expression of HLA-G by antigen presenting cells

The role of HLA-G in down-regulating the immune response has come from studies investigating the tolerance of the semi-allogeneic foetus at the foeto-maternal interface where foetal trophoblasts express HLA-G. There is recent evidence that HLA-G may be expressed in a number of pathological conditions, including cancers, resulting in protection of the antigen-presenting tumour cells from NK and T cell cytolysis (Amiot, *et al* 2010).

There are currently two proposed mechanisms by which HLA-G is able to down-regulate the immune response:

i. HLA-G inhibits NK cell-mediated cytotoxicity

Due to their production of various cytokines, NK cells play a pivotal role as effectors of the innate, and regulators of, the adaptive immune response. NK cells kill target cells without prior sensitisation. NK cells recognise MHC class I molecules via killer cell immunoglobulin-like receptors (KIRs) and immunoglobulin-like transcript receptors (ILTs), thus inhibiting their cytolytic function (Karre 2002, Sjöström, *et al* 2001).

KIR2DL4 is a specific receptor for HLA-G and is predominantly present on maternal uterine $CD3^-$, $CD56^+$ NK cells. The interaction of KIR2DL4 and HLA-G induces inhibition of NK cell lysis (Rajagopalan and Long 1999). The inhibitory receptor ILT-2 is a transmembrane receptor found on a subset of NK cells. The interaction between HLA-G and ILT-2 also inhibits lysis of HLA-G expressing target cells (Navarro, *et al* 1999, Saverino, *et al* 2000, Shiroishi, *et al* 2003).

In a study described by Rouas-Freiss *et al*. (1997), NK cell-mediated cytotoxicity was examined using polyclonal NK cells. Cytotoxicity was inhibited by HLA-G transfected cell lines and restored by the addition of HLA-G blocking antibodies suggesting that there is down-regulation of the inhibition produced by HLA-G expression (Rouas-Freiss, *et al* 1997).

ii. HLA-G on APCs regulates $CD4^+$ and $CD8^+$ T cell responses

ILT-2 is also expressed by a large proportion of $CD4^+$ and $CD8^+$ T cells (Saverino, *et al* 2000). $CD4^+$ and $CD8^+$ T cell proliferation was inhibited following incubation with a HLA-G5 transfected cell line. Anti-HLA-G blocking antibodies restored proliferation, thereby providing evidence for the integral role of HLA-G in delivering inhibitory signals. Impaired T cell proliferation was due to failure of progression from G1 to S phase of the cell cycle rather than induction of apoptosis (Bahri, *et al* 2006).

Re-stimulation of the hypo-proliferative $CD4^+$ T cells with allogeneic peripheral blood mononuclear cells (PBMCs) was unable to restore proliferation, suggesting that HLA-G^+ APC exposure induces long-term antigen-specific unresponsiveness. In another study, HLA-G1 expressing APCs induced T cell differentiation into suppressor cells. These suppressor T cells expressed reduced levels of CD4 and CD8 and exhibited significantly impaired allo-proliferation (Naji, *et al* 2007).

Cytokine profiles also differed in T cells incubated with HLA-G5 cell lines. Lower levels of IL-2, IFN-γ, and granulocyte macrophage – colony stimulating factor (GM-CSF) were produced which resulted in impaired stimulation of immune cells involved in the acute phase of an allogeneic reaction (Bahri, *et al* 2006). It has also been shown that HLA-G^+ APCs inhibit antigen-specific cytotoxic T lymphocytes (Gal, *et al* 1999).

1.4.7 HLA-G and trogocytosis

Transfer of membrane fragments, containing HLA-G, onto T and NK cells has been documented to occur primarily in activated cells. It has been hypothesised that trogocytosis of HLA-G from HLA-G expressing tumours to T and NK cells may contribute to tumour evasion of immune surveillance (Caumartin, *et al* 2007, LeMaoult, *et al* 2007b, Sjöström, *et al* 2001). Acquisition of HLA-G from APCs, by polyclonal CD4 and CD8 T cells, occurs by membrane exchange and causes a switch in their function from effector cells to regulatory cells (Wiendl 2007).

1.4.8 HLA-G and a new subset of regulatory T cells

LeMaoult *et al.* (2007) have shown that IL-2 activated $CD4^+$ and $CD8^+$ T cells can rapidly acquire HLA-G1 from HLA-G1-expressing APCs by trogocytosis *in vitro*. From their transwell studies, they concluded that direct cell-to-cell contact was essential for HLA-G transfer (LeMaoult, *et al* 2007b). In another study, intracellular and surface labelling of $CD4^+$ T cells was used to demonstrate that HLA-G is not endogenously expressed. However, after incubation with HLA-G expressing APCs, HLA-G positive areas could be detected on the surface of T cells with no intracellular HLA-G detected. These results demonstrate that HLA-G was transferred via

trogocytosis. Blocking the receptors of the immune synapse had no effect on trogocytosis suggesting that neither formation of the immunological synapse, nor direct interaction of HLA-G with its inhibitory receptor, was necessary for the transfer of HLA-G (LeMaoult*, et al* 2007b).

LeMaoult's group performed T cell activation studies using anti-CD3, phytohemagglutinin (PHA), and PHA + IL-2 which indicated that activated T cells, especially those in the late stages of activation, can efficiently capture APC surface molecules regardless of their antigen specificity. The study then assessed the proliferative properties of purified $CD4^+$ $HLA\text{-}G^{acq}$ T cells and found that proliferation of the HLA-G acquiring cells was reduced. Moreover, IL-2 re-stimulation of $CD4^+$ $HLA\text{-}G^{acq}$ T cells failed to overcome the acquired HLA-G induced inhibition (LeMaoult*, et al* 2007b).

HLA-G blocking studies indicate that blocking HLA-G does not prevent trogocytosis but does block the function of HLA-G on the recipient T cells. In these studies $CD4^+$ $HLA\text{-}G^{acq}$ T cells proliferated normally indicating that it is the acquired HLA-G that inhibits T cell proliferation. Functional assays have shown that $CD4^+$ $HLA\text{-}G^{acq}$ T cells inhibit the proliferation of resting T cells when mixed with allogeneic APCs. This inhibition was overcome by the addition of anti-HLA-G antibodies used to block HLA-G function. It was shown that HLA-G1 is present on the surface of T cells for less than 24h and has a half-life of 14h (Park*, et al* 2001).

The acquisition of HLA-G on the surface of T cells switches their function from effector to immune suppressor cells and therefore effectively generating a novel subset of regulatory T cells. The $HLA\text{-}G^{acq}$ T cells may provide an increase in the number of $HLA\text{-}G^+$ regulatory T cells, which may exert their regulatory effect on neighbouring T cells and block the function of effector T cells that are recruited to the area. This induces immune tolerance and hence the down-regulation of anti-tumour responses (LeMaoult*, et al* 2007b).

Over the last decade, a number of regulatory T cell subsets have been identified including the naturally occurring $CD4^+$ $CD25^+$ regulatory T cells (Tregs) which are present in healthy individuals under normal conditions (Dieckmann*, et al* 2001, von

Boehmer 2005). More recently, a subset of HLA-G expressing $CD4^+$ and $CD8^+$ T cells have been discovered under normal physiological conditions in peripheral blood from healthy donors (Feger*, et al* 2007).

Using monoclonal antibodies against HLA-G, MEM/G9 and 87G to assess surface expression of HLA-G, flow cytometry experiments found an average of 1.6% (range 0.1-8.3%) of $CD4^+$ and 3.3% (range 0.6-5.2%) of $CD8^+$ T cells express HLA-G. Furthermore, the proliferative capacity of HLA-G^+ T cells was found to be reduced in both T cell subsets following allogeneic stimulation. Secondary stimulation by anti-CD3/CD28 beads or IL-2 failed to overcome the hypo-proliferation of these cell subsets. Following stimulation, cytokine profiles showed reduced IFN-gamma and IL-10 mRNA levels. These results distinguish HLA-G^+ T cells from other Treg subsets that produce increased levels of IL-10 and TGF-β. It has been suggested that reduced production of IFN-γ may contribute to the non-pro-inflammatory signals (Feger*, et al* 2007).

Feger *et al.* (2007) performed suppression assays in which HLA-G^+ T cells inhibited the proliferation of autologous CFSE-labelled HLA-G^- T cells when cultured with allogeneic APCs. Inhibition was shown to be partially antagonised by the addition of anti-HLA-G antibodies. This suggests that HLA-G is not only a phenotypic marker but is also responsible for the impaired proliferation. Transwell experiments demonstrated that cell contact was not required for suppression of proliferation (Feger*, et al* 2007).

Phenotypic analysis showed that suppressive HLA-G^+ T cells are also CD25 and FoxP3 (forkhead transcription factor 3) negative, thereby differentiating HLA-G^+ regulatory T cells from $CD4^+$ $CD25^+$ Tregs. The exact mechanism of immune suppression by HLA-G^+ T cells is still unknown. However, it has been postulated that it may occur through the interaction with its receptor, ILT-2, which may play a role in maintenance of peripheral tolerance (Feger*, et al* 2007).

1.5 Project Rationale

Multiple myeloma is a cancer of plasma cells in the bone marrow. Although it is considered incurable, recent developments have shown that novel therapies offer patients an improved prognosis. A variety of host-tumour cellular interactions have been described in MM patients and it is important that we gain a better understanding of these interactions as they may offer the potential for novel therapeutic strategies. Trogocytosis is a recently described cell-to-cell interaction which involves the transfer of cell membrane material between cells during antigen presentation. The Royal Prince Alfred Hospital (RPAH) laboratory has previously reported that T cells from patients with multiple myeloma often express the co-stimulatory molecules CD80 and CD86 (Pope*, et al* 2000) and this has led to the suggestion that these molecules are acquired from the presentasome by T cells during cell interactions at the time of antigen presentation.

These findings suggest that the tumour has been recognised and T cells have interacted with malignant plasma cells but there has developed a mechanism to escape cytotoxic and NK cell cytotoxicity. Recent studies in other cancers suggest that tumour escape occurs when T cells acquire HLA-G from tumour cells and become suppressive or regulatory cells. The incidence and significance of trogocytosis in multiple myeloma has not yet been explored. If trogocytosis suppresses the normal anti-tumour cytotoxic T cell response, novel forms of therapy could be developed which block this suppression and re-establish T cell cytotoxicity.

1.6 Project Objectives

The hypothesis is that trogocytosis is common in patients with multiple myeloma and has an important role in the suppression of T cell cytotoxicity and tumour escape from host immune responses. The specific aims of this project were:

- To determine the incidence of trogocytosis in multiple myeloma compared to normal controls and other haematological malignancies by demonstrating trogocytosis *in vitro* through the culture of myeloma cell lines and primary

myeloma cells with HLA-A2 compatible and incompatible T cells as well as a T cell line that does not have a T cell receptor.

- To determine the expression of a wide range of co-stimulatory molecules related to antigen presentation on both T cells and malignant plasma cells as candidate molecules involved in trogocytosis at the immune synapse and to determine the function of these molecules once acquired.

- To determine if HLA-G expression may be a cause of tumour escape in myeloma patients by determining the incidence and significance of HLA-G expression on myeloma patient plasma cells and examining the incidence and function of HLA-G^{pos} T cells.

Chapter Two: Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Study samples

After informed consent, peripheral blood (PB) and bone marrow (BM) samples were collected from healthy donors and patients with MM who attended the clinic at Royal Prince Alfred Hospital for routine assessment. The cells used in these studies were the residual of samples collected for routine diagnostic testing.

The study was approved by the Sydney South West Area Health Service Human Ethics Review Committee (RPAH Zone), protocol X07-0201. All samples were de-identified according to the ethics protocol.

2.1.2 Bone marrow collection

Bone marrow was collected by haematology registrars as part of routine collections. Samples were collected in 20mL tubes (Sarstedt, Nümbrecht, Germany) containing 1mL of Hanks Balanced Salt Solution (HBSS) (Thermo Scientific, Massachusetts, USA), supplemented with foetal calf serum (FCS) (SAFC® Biosciences, business division of Sigma-Aldrich Missouri, USA) and heparin (Pfizer, New South Wales, Australia).

2.1.3 Peripheral blood collection

Peripheral blood was collected by haematology nursing staff as part of routine collections into 9mL Vacuette® blood tubes (Greiner Bio-One, Stuttgart, Germany) containing either lithium heparin or ethylenediamine tetra-acetic acid (EDTA) as an anticoagulant.

2.1.4 Complete culture medium

Complete culture medium was made with Roswell Park Memorial Institute-1640 (RPMI-1640) containing 25mM HEPES (MP Biomedicals, Ohio, USA) and supplemented with 20 µg/mL gentamicin/100 IU/mL penicillin/100 µg/mL streptomycin (PSG) (Invitrogen, California, USA) and 10% FCS (SAFC® Biosciences) (Refer to Table 2-1).

2.1.5 Human myeloma and T cell lines

Human myeloma cell lines, RPMI-8226, U266, OPM2 and H929 and the T cell line, Jurkat variant; JRT-T3.1 were acquired from pre-existing laboratory stocks (originally purchased from the American Type Culture Collection, Cryosite, New South Wales, Australia) and cultured in complete culture media (Table 2-1) to a density of 1 x 10^6 cells/mL. Cell lines were maintained in 25 cm^2 flasks (Corning Incorporated, New York, USA) and passaged twice weekly at a split of 1:10.

Early passage cells (that is, in continuous culture for less than 2 months) were used for all experiments, and all cell lines were more than 75% viable, determined by DAPI (Invitrogen) (Table 2-1) exclusion, at the beginning of all experiments. Primary myeloma cells were obtained from bone marrow aspirates from patients with recurring myeloma.

2.1.6 Reagents

Table 2.1 lists the preparation for reagents and solutions, unless otherwise stated. Table 2.2 lists the monoclonal antibodies (mAb) and their clones used for flow cytometry. It also outlines the volume of mAb used and associated incubation times for labelling.

Table 2-1: Reagents and solutions used in multiple experiments

Reagents	Preparation	Storage
Phosphate buffered saline (PBS)	10x oxoid tablets (Oxoid Ltd., Hampshire, UK) dissolved in 1L of Milli-Q™ water (Millipore, Madison, USA)	4ºC
Ammonium chloride lysing solution	4.15 g ammonium chloride (Merck, Darmstadt, Germany), 0.45 g sodium hydrogen carbonate (VWR, New Jersey, USA) and 0.018 g Sodium ethylenediamine tetra-acetic acid (Na_2 EDTA.$2H_20$) (Searle, Nebraska, USA) in 500mL of Milli-Q™ water (Millipore)	25 ºC
Complete culture medium	RPMI-1640 containing 25mM HEPES (MP Biomedicals) and supplemented with 20 µg/mL gentamicin/100 IU/mL penicillin/100 µg/mL streptomycin (PSG) (Invitrogen) and 10% FCS (SAFC® Biosciences)	4ºC
DAPI (Invitrogen) stock solution (5 mg/mL)	10mg reconstituted in 2mL of Milli-Q™ water (Millipore)	4ºC
T cell activation/expansion kit (Beads 1 x 10^8) (Miltenyi Biotec)	500µL anti-biotin MACSiBead particles (Miltenyi Biotec), 100µL CD2-Biotin (Miltenyi Biotec), 100µL CD3-biotin (Miltenyi Biotec) and 100µL CD28-Biotin (Miltenyi Biotec), 200µL PBS	4ºC
CellTrace™ CFSE cell proliferation kit (5mM) (Invtirogen)	50µg of lyophilised powder reconstituted with 18µL DMSO (Sigma-Aldrich)	-80ºC

Table 2-2: Antibodies used for flow cytometry and their associated incubation time for labelling

Antibody	Clone	Company	Volume	Incubation
IgG_1 PE	X40	BD	20µL	15min in the dark at RT (unless otherwise stated)
IgG_1 FITC	X40	BD	20µL	
Mouse anti-human CD38 PE	HB7	BD	20µL	
Mouse anti-human CD38 PerCP-Cy5.5	HIT2	BD	5µL	
Mouse anti-human CD38 FITC	AT13/5	DAKO	10µL	
Mouse anti-human CD3 PE	SK7	BD	2.5µL	
Mouse anti-human CD3 PE-Cy7	SK7	BD	20µL	
Mouse anti-human CD3 AlexaFluor® 488	UCHT1	BD	10µL	
Mouse anti-human CD80 FITC	L307.4	BD	20µL	
Mouse anti-human CD86 FITC	2331 (FUN-1)	BD	20µL	
Mouse anti-human CD138 FITC	B-A38	IQ Products	10µL	
Mouse anti-human HLA-A2 FITC	N/A		20µL	
Streptavidin PE	N/A	BD	10µL	
Streptavidin APC	N/A	BD	10µL	
Streptavidin AlexaFluor® 594	N/A	BD	10µL	
Mouse anti-human CD56 APC	B159	BD Pharmingen	5µL	
Mouse anti-human CD19 FITC	4G7	BD	15µL	
Mouse anti-human CD16 FITC	NKP15	BD	5µL	
HLA-G FITC	MEM G/9	Abcam	10µL	
Mouse anti-human CD273 PE	MIH18	BD	20µL	
Mouse anti-human CD274 PE	MIH1	BD	20µL	
Mouse anti-human CD275 PE	2D3/B7-H2	BD	20µL	
Mouse anti-human CD278 PE	DX29	BD	20µL	
Mouse anti-human CD279 PE	MIH4	BD	20µL	

2.2 Methods

2.2.1 Cell preparation

2.2.1.1 Isolation of peripheral blood mononuclear cells by ficoll-paque plus density gradient

Following collection of peripheral blood from patients, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden) which separates blood into layers of erythrocytes, plasma, granulocytes and mononuclear cells. Whole blood was diluted 1:2 in phosphate buffered saline (PBS) (Invitrogen California, USA) and gently layered over the Ficoll-Paque™ Plus (GE Healthcare) in a 50mL polypropylene tube (Becton Dickinson Bioscience, New Jersey, USA) in the ratio of 1:1; 15mL Ficoll-Paque™ Plus (GE Healthcare) to 15mL of diluted peripheral blood. Tubes were then centrifuged at 360g for 20 min at room temperature (RT) without braking (Spintron GT-10S, Victoria, Australia). Cells in the mononuclear layer were harvested into 8mL polystyrene round bottom tubes (Sarstedt) using a plastic transfer pipette (Sarstedt). Cells were washed once with PBS (Table 2-1) and centrifuged (Hettich EBA 21 Centrifuge, GMI Inc. Minnesota, USA) at 360g for 5min at RT. The resulting cell pellet was resuspended in 1mL of PBS and counted as described in section 2.2.1.3.

2.2.1.2 Bone marrow aspirates

Bone marrow aspirates were diluted with PBS (15mL) and centrifuged at 360g for 10min (Spintron GT-10S). The supernatant was removed and the cells were resuspended in 3mL PBS. Cells were then layered over 3mL Ficoll-Paque™ Plus (GE Healthcare) and centrifuged at RT for 15min at 360g. The mononuclear layer was transferred to an 8mL polypropylene tube (Sarstedt), washed once with PBS, the supernatant discarded and the remaining cell pellet was resuspended with 1mL RPMI-10. Cell counts were carried out as described in section 2.2.1.3.

2.2.1.3 Cell counting

Following isolation of cells from peripheral blood, cells were counted on a Neubauer Improved haemocytometer (American Optical Corporation, New York, USA) using a Carl Zeiss Microscope. The number of cells in each of the five large squares was counted. The number of cells per millilitre of suspension was then determined according to the following equation:
Total number of cells/mL = number of cells in large squares x 1 x 10^4 cells x volume cells were resuspended in.

2.2.2 Cell culture techniques

2.2.2.1 Maintenance of cell lines

Tissue culture was performed in an Email Class II Biological Safety Cabinet (BSC) (Email Westinghouse Pty. Ltd., Australia) using aseptic technique. All cell lines were maintained in a humidified environment at 37^{o}C in the presence of 5% CO_2 (NuAire CO_2 incubator, MN, USA). Complete culture medium (Table 2.1) was pre-warmed to 37^{o}C prior to use. Cells were cultured at a concentration of 1x10^6 cells/mL.

2.2.2.2 Cyropreservation and thawing of cryopreserved cells

Before freezing, cells were cryopreserved in complete culture medium containing 10% of the cryoprotectant, dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The cell mixture was transferred into a cryovial and placed in a Nalgene® Mr Frosty freezing container (Sigma-Aldrich). The container was then placed in a –80^{o}C freezer for at least 3h before being transferred to liquid nitrogen for long term storage.

Following removal from liquid nitrogen storage, cryovials were rapidly thawed in a 37^{o}C water bath and cells were transferred to an 8mL polypropylene tube (Sarstedt). 5mL of pre-warmed complete culture medium was added and cells were pelleted by centrifugation at 360g for 5min (Spintron GT-10S) and the supernatant discarded. The cells were subsequently washed twice in media to remove residual DMSO (Sigma-

Aldrich) before being seeded into a 25cm^2 tissue culture flask (Corning) with 10mL of culture medium. Cells were incubated at 37^{o}C as described in section 2.2.2.1.

2.2.3 Cell surface staining and flow cytometry

PBMCs were either isolated (as described in 2.2.1.1) or 100µL of red cells were lysed with ammonium chloride for 10min (Table 2-1) and 100µL cell suspensions of each bone marrow specimen were immunostained (in the dark) with primary antibodies targeting antigens of interest (Table 2.2) at RT for 15 min. Control aliquots were stained with an isotype-matched antibody (IgG_1 FITC or IgG_1 PE) to evaluate non-specific binding to target cells. Following incubation cells were washed once with PBS and resuspended in 200µL PBS for flow cytometric analysis.

Flow cytometry was performed using an Epics-XL-MCL analyser (Beckman Coulter, USA) or BD FACS Aria II (Becton Dickinson) analyser. Additional data analysis was performed using Expo32 (Analis, Suarlée, Belgium) or FlowJo (Tree Star, Inc. Oregon, USA) software. A minimum of 10,000 cells in the lymphocyte gate or 10,000 cells in the plasma cell gate were collected. Cell populations were identified on the basis of their forward scatter (FSC) and side scatter (SSC) properties, which are indicative of cell size and granularity, respectively. Control aliquots were stained with an isotype-matched antibody to evaluate non-specific binding to target cells.

2.2.4 *In vitro* model of trogocytosis

2.2.4.1 Biotinylation of plasma cells and co-culture with PBMCs

Plasma cells (5 x 10^4/mL) were labelled with 500uL of 1mg/mL EZ link Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Illinois, USA) by incubating for 10min at 25°C. An equivalent volume (500uL) of foetal calf serum was added to the cells and incubation was carried out for a further 10min at 4°C. Cells were washed three times in complete culture medium. Biotinylated plasma cells were co-cultured with isolated PBMCs (at a ratio of 5:1) in round bottomed, BD Falcon 96 well micro-titre plates (BD Biosciences) and placed in a 5% CO_2 incubator (NuAire) at 37°C for 2h. Following co-incubation, cells were transferred to 5mL, 12mm x 75mm, fluorescent-activated

cell sorting (FACS) round-bottom tubes (BD Bioscience) and centrifuged at 360g for 5 min and the supernatant discarded.

2.2.4.2 Confocal microscopy examination of trogocytosis

For confocal immuno-fluorescence analysis of trogocytosis 100µL of cell suspensions (plasma cells, lymphocytes, or mixed cultures of plasma cells + lymphocytes) were cytospun (Shandon Cytospin 2, Block Scientific, Inc. New York, USA) for 5 min at 500rpm. Slides were fixed in methanol for 10 min then washed in H_2O. Slides were stained with 10µL anti-CD3 AlexaFluor® 488 and 10µL Streptavidin AlexaFluor® 594 (Invitrogen). Images were acquired on a Carl Zeiss LSM 510 Meta Confocal microscope (Carl Zeiss Pty Ltd, New South Wales, Australia) with Argon 488nm and 561nm DPSS lasers and a Plan-Apochromat 63x/1.40 Oil objective, with assistance from Dr Louise Cole at the Bosch Institute (University of Sydney).

2.2.4.3 Flow cytometry examination of trogocytosis

Following co-culture (section 2.2.4.1), cells were stained with anti-human CD38 PerCP-Cy5.5, anti-human CD3 PE-Cy7, anti-human CD16 APC, anti-human CD19 FITC and Streptavidin PE (Table 2.2) for 15min the dark. Cells were then washed once with PBS, the supernatant was discarded and cells were resuspended with 200 µL PBS. Flow cytometry was performed using a BD FACS Aria II (BD Bioscience) analyser. A minimum of 50,000 cells were acquired. Cell populations were identified on the basis of their FSC and SSC properties, which are indicative of cell size and granularity, respectively. Using the plasma cell gate and lymphocyte gate, expression of biotin was determined on $CD38^{++}$ plasma cells and the transfer of biotin onto $CD3^{+}$ T cells, $CD19^{+}$ B cells and $CD16^{+}$ NK cells was analysed.

2.2.5 Flow sorting

2.2.5.1 Sorting of CD86^{pos} or HLA-G^{pos} plasma cells

Flow sorting was performed on the Becton Dickinson FACS ARIA II. The procedure described was performed in the class II BSC (Email Westinghouse Pty. Ltd.). Ficoll-separated bone marrow cells were fluorescently labelled with anti-CD38 PerCP-Cy5.5 and either anti-CD86 FITC or anti-HLA-G FITC (Abcam, Sapphire Biosciences Pty Ltd, New South Wales, Australia) for 30min in the dark at 4^{o}C. Stained cells were washed once with PBS and resuspended in 500µL PBS. The cell suspension was then transferred to a sterile 12x75 mm FACS Tube with a cell strainer cap (BD Bioscience) to remove any cell clumps. Cells were then placed on ice and remained on ice until required for sorting. CD86^{pos} or HLA-G^{pos} and CD86^{neg} or HLA-G^{neg} plasma cells were sorted into FACS tubes (BD Bioscience) containing complete culture medium. All tubes were kept on ice before and after sorting. During the sorting process, the temperature on the flow cytometer was maintained at 4^{o}C.

2.2.5.2 Sorting and collection of CD86^{pos} or HLA-G^{pos} T cells

The procedure described was performed in the class II BSC (Email Westinghouse Pty. Ltd.). Isolated PBMCs were fluorescently labelled with anti-CD3 PE and either anti-CD86 FITC or anti-HLA-G FITC (Abcam) for 30min in the dark at 4^{o}C. Stained cells were washed once with PBS and resuspended in 500µL PBS. The cell suspension was then transferred to a FACS tube with a cell strainer cap (BD Bioscience) to remove any cell clumps. Cells were then placed on ice and remained on ice until sorting was conducted. CD86^{pos} or HLA-G^{pos} and CD86^{neg} or HLA-G^{neg} T cells were sorted into FACS tubes containing complete culture medium. All tubes were kept on ice before and after sorting. During the sort, the temperature on the flow cytometer was maintained at 4^{o}C.

2.2.6 Culture and stimulation of PBMCs

The procedure described was performed in the class II BSC (Email Westinghouse Pty. Ltd.). Peripheral blood mononuclear cells isolated from whole blood (as described in

section 2.2.1.1), were resuspended in complete culture medium. Cell suspensions (1 x 10^4 cells) were transferred into 96-well round bottomed plates (BD Biosciences) and cultured under stimulating and non-stimulating conditions for the analysis of trogocytosis by flow cytometry. Cells were stimulated by culturing in the presence of anti-CD3/anti-CD28 beads (Miltenyi Biotec, New South Wales, Australia) at a ratio of 1 bead to 1 T cell for 2h at 37^{o}C in a CO_2 incubator (NuAire). Plasma cells (5 x 10^4) were then added to the lymphocyte populations and incubation was continued for a further 2h. Following culture, co-cultures were removed, washed with PBS and stained with anti-human CD38 PerCP-Cy5.5, anti-human CD3 PE-Cy7 and streptavidin PE (Table 2.2).

2.2.7 CFSE labelling of HLA-G^{neg} T cells and suppression assay

CD3^{pos} HLA-G^{neg} sorted T cells (described in section 2.2.5.2) were washed once with PBS, the supernatant discarded and cells resuspended in 1mL complete culture medium. In a class II BSC (Email Westinghouse Pty. Ltd.) pre-warmed complete culture medium (150µL) was added to 6µL of 5mM CFSE (Invitrogen). Diluted CFSE (Invitrogen) (25µL) was then added to the cell suspension. Cells were placed in a 37^{o}C water bath for 10min. Cold culture medium (3mL) was then added to the cell suspension which was then placed on ice for a further 10min. Cells were centrifuged for 5min at 300g, the supernatant was discarded and the remaining cell pellet was resuspended in 100 µL of complete culture medium.

To assess the suppressive nature of HLA-G^{pos} T cells, HLA-G^{neg} CD3^{pos} CFSE^{pos} T cells (0.5-2 x 10^5) were stimulated with anti-CD3/anti-CD28 beads (Miltenyi Biotec) at a ratio of one bead per T cell in the presence and absence of HLA-G^{pos} CD3^{pos} T cells or HLA-G^{pos} CD38^{pos} plasma cells. Proliferation was measured on day 4 by flow cytometry.

2.2.8 Statistical analysis

All statistical anaylsis was performed using GraphPad Prism Version 5.01 (GraphPad Software Inc. San Diego, USA). A p value of <0.05 was considered statistically significant. When different patient samples were tested a mean ± SEM was reported.

CHAPTER THREE: TROGOCYTOSIS IN MULTIPLE MYELOMA

3. Evidence of Trogocytosis

3.1 Introduction

Trogocytosis, a term coined by Joly and Hudrisier (2003), describes the rapid transfer of membrane proteins during direct cell-to-cell contact (Joly and Hudrisier 2003, Puaux*, et al* 2006). Following the formation of an immunological synapse, T cells acquire membrane fragments, as well as associated molecules, from donor cells that contain MHC molecules, co-stimulatory molecules, membrane receptors and adhesion molecules. The exact mechanism of trogocytosis between T cells and APCs is not understood.

There is, however, evidence which suggests that the recipient cells acquire at least a portion of both the antigenic phenotype and functional characteristics of the donor cells (Caumartin*, et al* 2006, LeMaoult*, et al* 2007a). Once T cells acquire MHC class II and co-stimulatory molecules from APCs, they gain the ability to promote proliferation of bystander T cells and prime an immune response in the absence of APCs (Joly and Hudrisier 2003, Puaux*, et al* 2006, Waschbisch*, et al* 2009).

The objective of this chapter is to report the results of experiments that investigated the incidence of trogocytosis in MM compared to age-matched haematologically normal controls and other haematological malignancies. To demonstrate trogocytosis *in vitro*, myeloma cell lines, or primary bone marrow plasma cells, were biotinylated and co-cultured with lymphocytes. The degree of biotinylation of plasma cells and evidence of trogocytosis was observed visually by confocal microscopy and quantified by flow cytometry. The requirement of a T cell receptor and HLA-A2 compatibility for trogocytosis was also investigated.

3.2 Results

3.2.1 Biotinylation of plasma cells

The plasma cell population was identified by flow cytometry according to forward and side scatter characteristics in the IgG FITC/PE negative control. Compensation was performed using the CD38 FITC/IgG PE and IgG FITC/CD38 PE stained control cells and non-specific binding was not detected. Analysis of non-biotinylated plasma cells showed no detectable biotin (Figure 3-1C). Biotin was detected on 100% of biotinylated plasma cells (Figure 3-1D). This indicated that the surface proteins of all plasma cells were labelled with biotin.

3.2.2 Optimisation of the biotinylation assay

To optimise the biotinylation assay, biotin detection was carried out at various time points (Figure 3-2). Based on their forward and side scatter characteristics, lymphocytes were gated and from these, T cells were identified based on CD3 expression (column two). Biotin expression on $CD3^+$ T cells was then measured.

Figure 3-3A and B show the mean expression and mean fluorescence intensity (MFI) (n=3) of biotin at time 0min (mean=0%), 10min (mean=4.8%), 30min (mean=6.4%), 1h (mean=6.9%), 2h (mean=8.7%), 4h (mean=8.8%) and 24h (mean=11.5%). Analysis of T cells before culture indicated an absence of biotin and the optimal time for biotin transfer was determined to be at 2h.

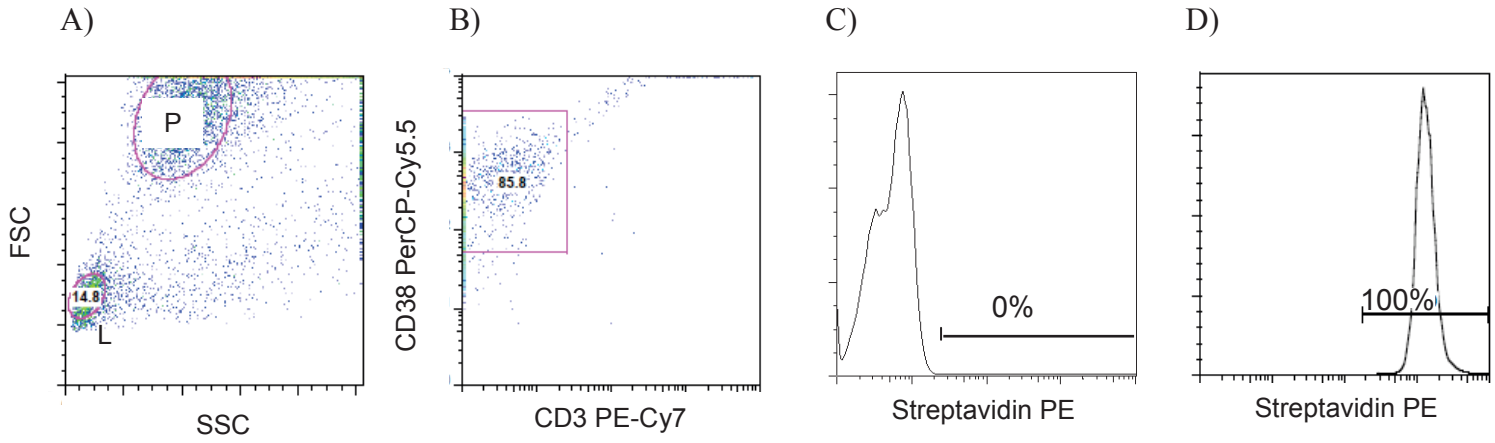


Figure 3-1: Biotinylation of a plasma cell line.
Representative flow cytometric scatter plots and histogram of plasma cells labelled with biotin. Plasma cells (P) were gated according to their forward and side scatter characteristics (A). Gating of $CD38^{++}$ and $CD3^{-}$ plasma cells (B). Example of a histogram showing background staining of non-biotinylated plasma cells (C). Example of a histogram showing streptavidin staining of biotinylated plasma cells (D).

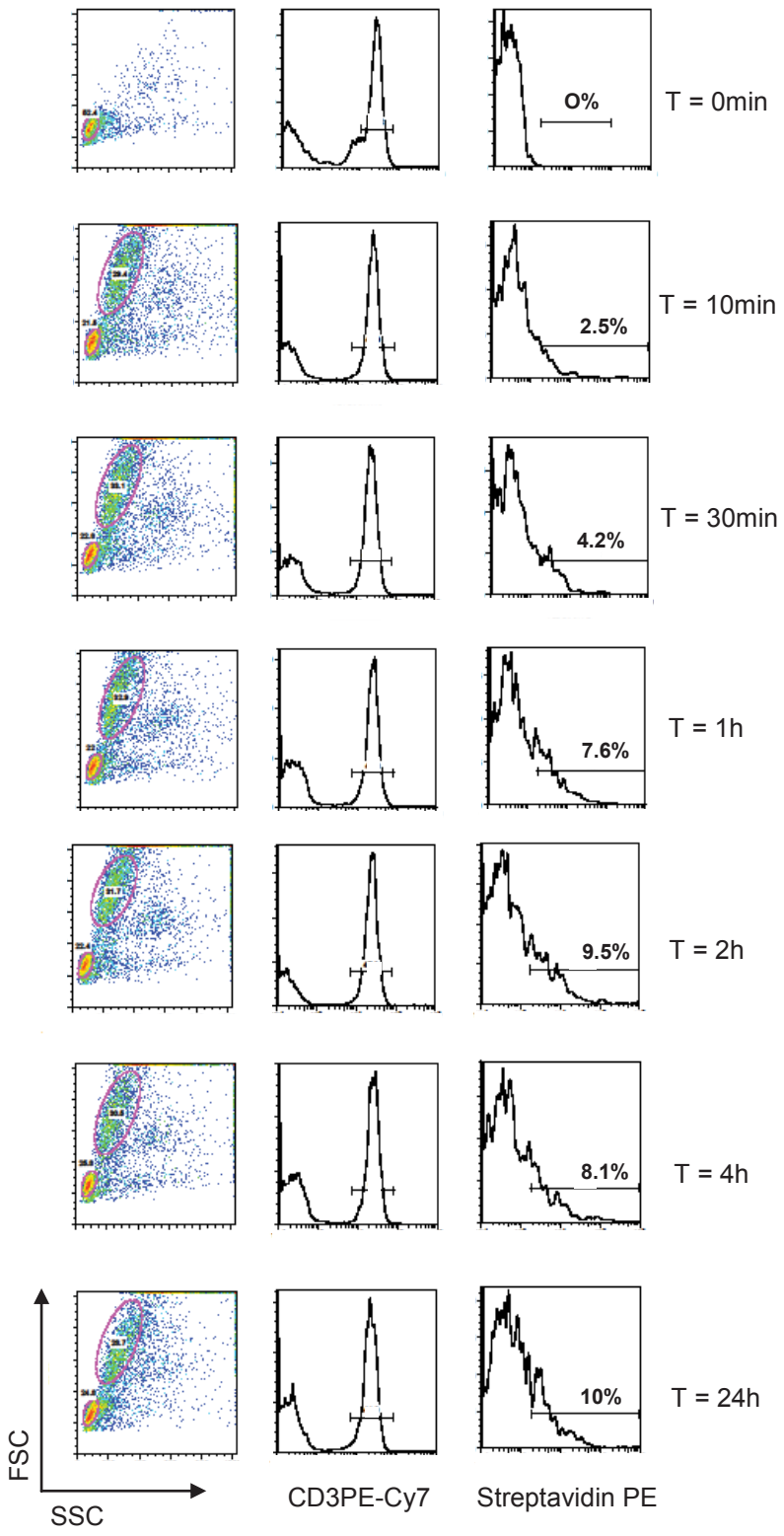
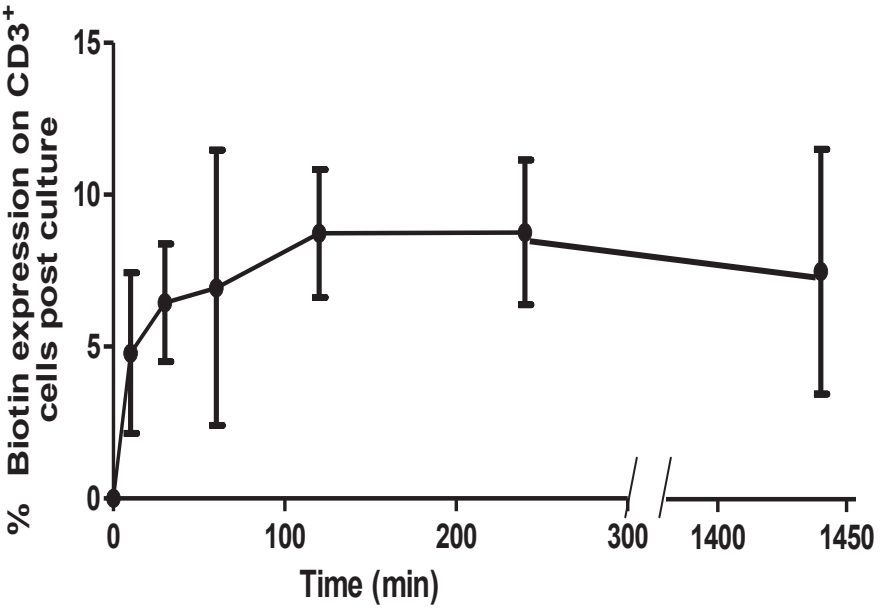


Figure 3-2: Transfer of biotin from plasma cells to mononuclear cells.
Plasma cells were gated based on their forward and side scatter characteristics (left column of each panel). Histogram of $CD3^+$ T cells (middle column) and expression of biotin following transfer to T cells (right column).

A)



B)

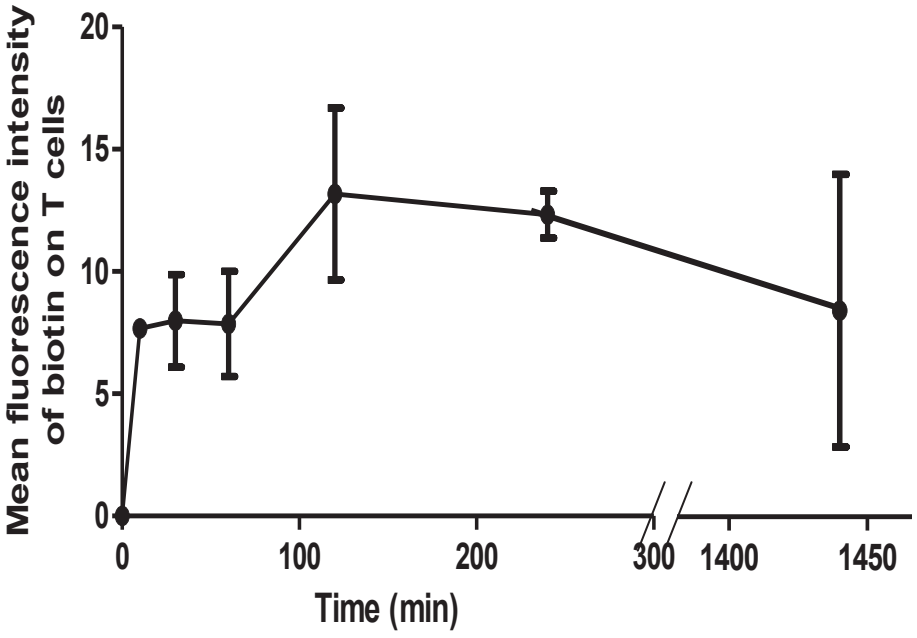


Figure 3-3: Transfer of biotin from a plasma cell line, OPM2 to $CD3^+$ T cells.
The plasma cell line OPM2, was biotinylated and placed in culture with, and without, T cells. The extent of transfer of biotin on $CD3^+$ T cells was determined by flow cytometry (A). Mean fIuorescence intensity (MFI) was also recorded (B). Each dot represents the mean (± SD) expression of biotin (n=3) and the optimal time for biotin transfer was recorded at 2h.

3.2.3 Evidence of trogocytosis detected by confocal microscopy

After biotinylation, suspensions of biotinylated plasma cells, T cells and co-cultures of cells were visualised by confocal microscopy. Images were acquired on a Zeiss LSM 510 Meta Confocal microscope and biotinylated membrane fragments were visible on a plasma cell line, U266 (Figure 3-4A). There was an absence of biotin on T cells (Figure 3-4B). Overall there was varied expression of biotin on T cells following culture (Figures 3-4C and D). These results indicated that T cells undergo trogocytosis when in the presence of biotinylated plasma cells.

3.2.4 Evidence for trogocytosis by flow cytometry

Representative flow cytometric analysis of biotin transfer is shown in Figure 3-5. The plasma cell and lymphocyte populations were identified according to their forward and side scatter characteristics in the IgG FITC/PE negative control. Biotin was detected on 100% of biotinylated plasma cells (Figure 3-5B). Following 2h culture, 75% of T cells (Figure 3-5C), 9.5% of B cells (Figure 3-5D) and 30% of NK cells (Figure 3-5E) had acquired biotinylated membrane fragments from plasma cells.

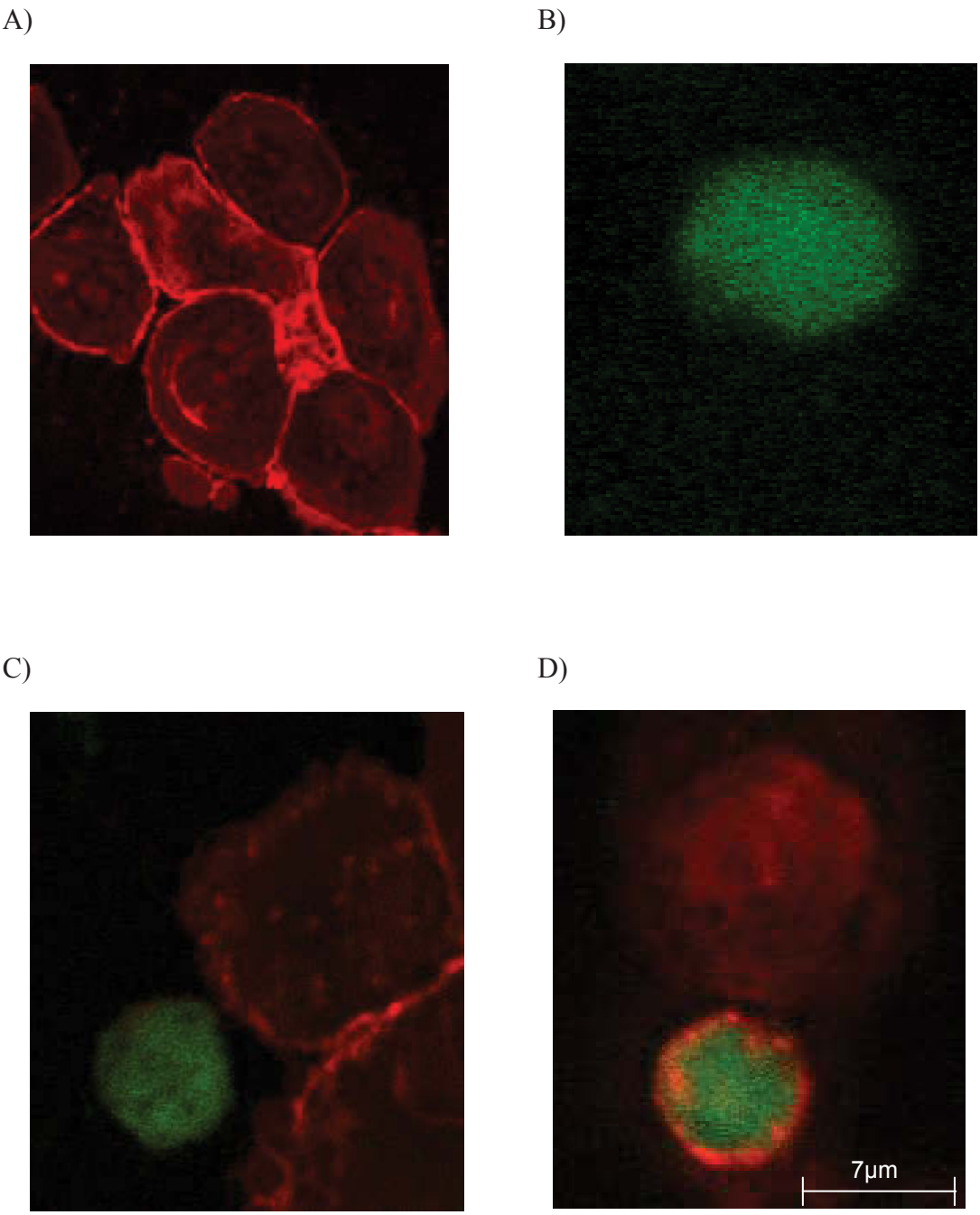


Figure 3-4: Visualisation of trogocytosis by confocal microscopy.
Biotinylated membrane fragments on the plasma cell line U266 (red, streptavidin Alexa Fluor 594) (A); $CD3^+$ T cells showing an absence of surface expression of biotinylated proteins (green, anti-CD3 Alexa Fluor 488) (B); U266 plasma cell line co-incubated with T cells for 2h (C); T cell wrapped in biotinylated membrane proteins acquired from target cells (D). Red fluorescence is Alexa Fluor 594 and green fluorescence is Alexa Fluor 488.

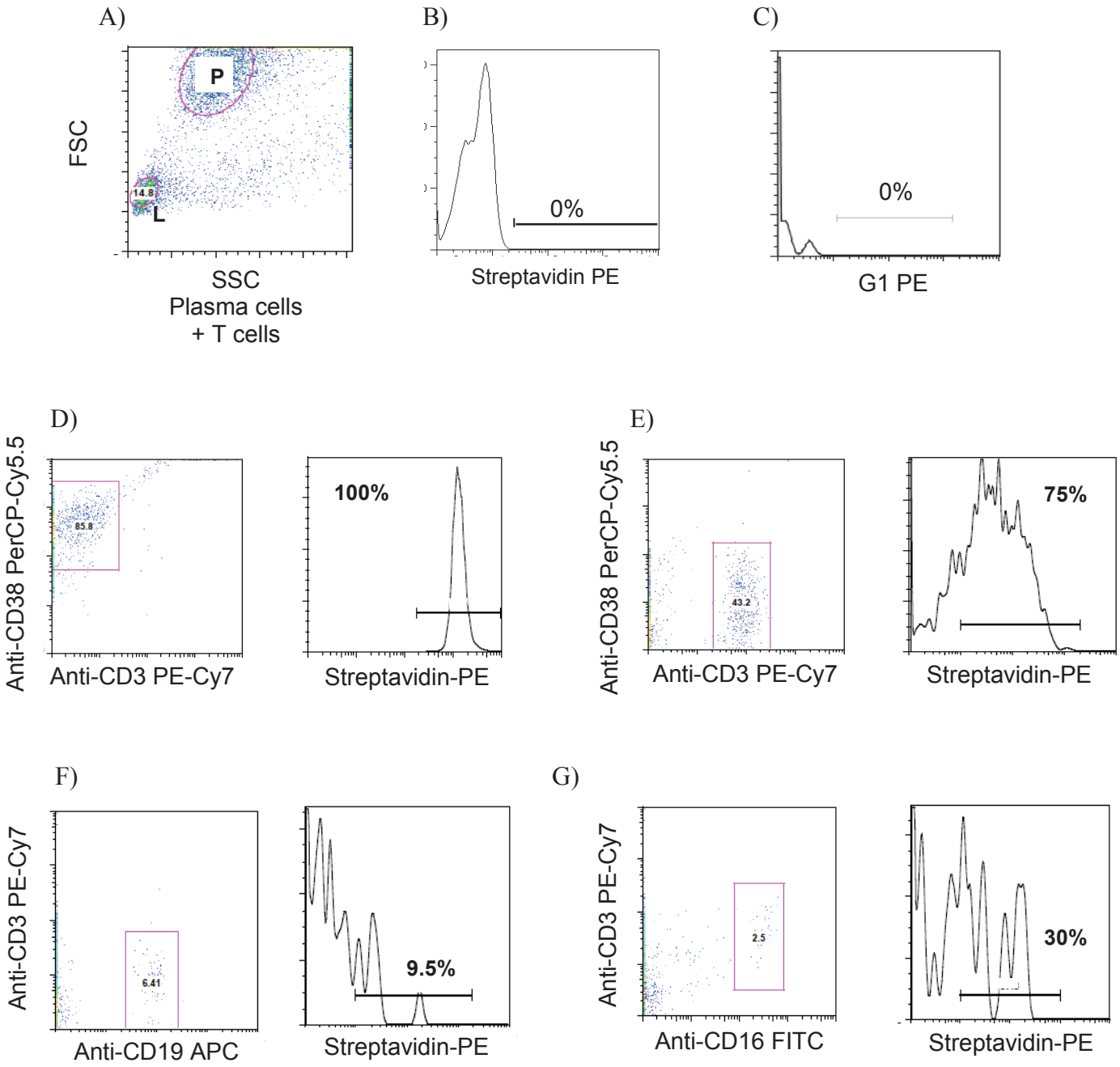


Figure 3-5: Flow cytometry detection of biotinylated membrane fragments.
Representative flow plots and histograms for the detection of trogocytosis are shown. The plasma cell (P) and lymphocyte (L) populations were gated using the forward scatter (FSC) and side scatter (SSC) dot plot (A). The regions for biotin/streptavidin positive cells were set on isotype control histograms (B and C). Using P, $CD38^{++}/CD3^{-}$ cells were then gated (D) and analysed for biotin expression. Using L, $CD38^{-}$ $CD3^{+}$ T cells (E), $CD3^{-}$ $CD19^{+}$ B cells (F), $CD3^{-}$ $CD16^{+}$ NK cells (G) were gated and anaylsed for biotin expression.

3.2.5 Trogocytosis is independent of T cell engagement and HLA matching

Representative flow cytometric analysis of Jurkat variant cells which do not express a TCR (J.RT3-T3.5), before culture and following culture are shown in Figure 3-6. Jurkat J.RT3-T3.5 cells are CD3 negative, CD7 positive and biotin negative. Following culture with biotinylated plasma cells, 63% of the Jurkat variant cells acquired biotin. The fact that these cells acquired biotin indicates that a TCR is not required for trogocytosis.

Furthermore, biotin acquisition by isolated autologous and allogeneic T cells, respectively, when cultured with biotinylated primary myeloma plasma cells was also observed (Figure 3-7 and 3-8). T cells from patients with myeloma (n=7) acquired 11.4% biotinylated membrane fragments from autologous plasma cells, whilst allogeneic T cells (n=4), from other myeloma patients, acquired 9.7% biotinylated membrane fragments ($F= 0.083$; $p=0.78$) (Figure 3-9A).

Furthermore, when the plasma cell line, U266, which is HLA-$A2^{pos}$ was co-cultured with isolated lymphocytes from HLA-A2 compatible, age-matched, haematogically normal individuals (n=4), 71.9% of T cells acquired biotin. This was higher than the amount of biotin acquired by B cells and NK cells (25.2% and 49.4% respectively). When incubated with incompatible lymphocytes, 82.1% of T cells, 27.0% of B cells and 48.2% of NK cells expressed biotin (Figure 3-9B). Statistical significance was not achieved between transfer to HLA compatible and incompatible T cells, B cells and NK cells ($F=1.21$; $p=0.93$, $F=1.62$; $p=0.70$, $F=1.67$; $p=0.68$ respectively). This was an indication that HLA matching is not required for trogocytosis to occur as there are similar amounts of biotin transfer in both cases.

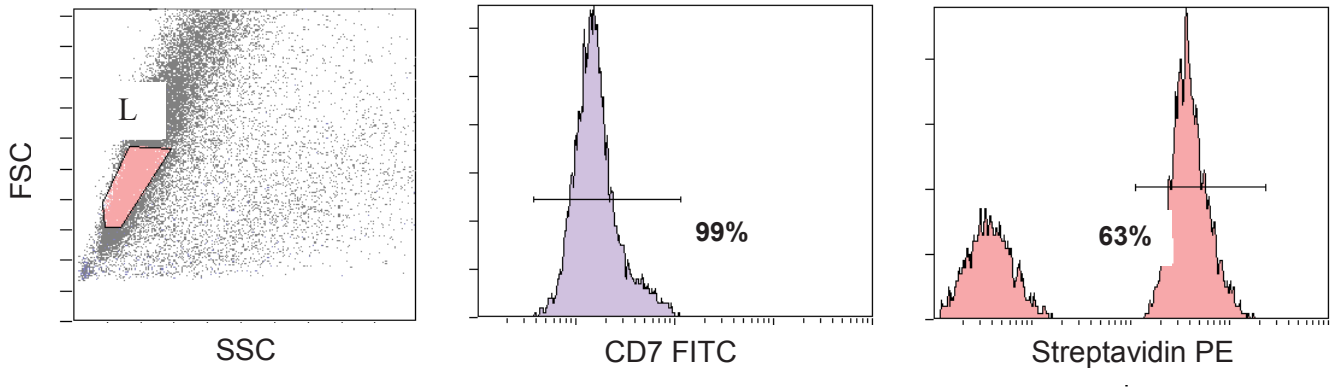


Figure 3-6: Biotin transfer from biotinylated plasma cells to the TCR negative Jurkat cell line.
The lymphocyte (L) population was gated using the FSC and SSC dot plot (left dot plot). Using L, Jurkat cells (J.RT3-T3.5) were gated based on their expression of CD7 (middle histogram). These cells were then analysed for the expression of biotin (right histogram). The regions for biotin/streptavidin positive cells were set on isotype control histograms.

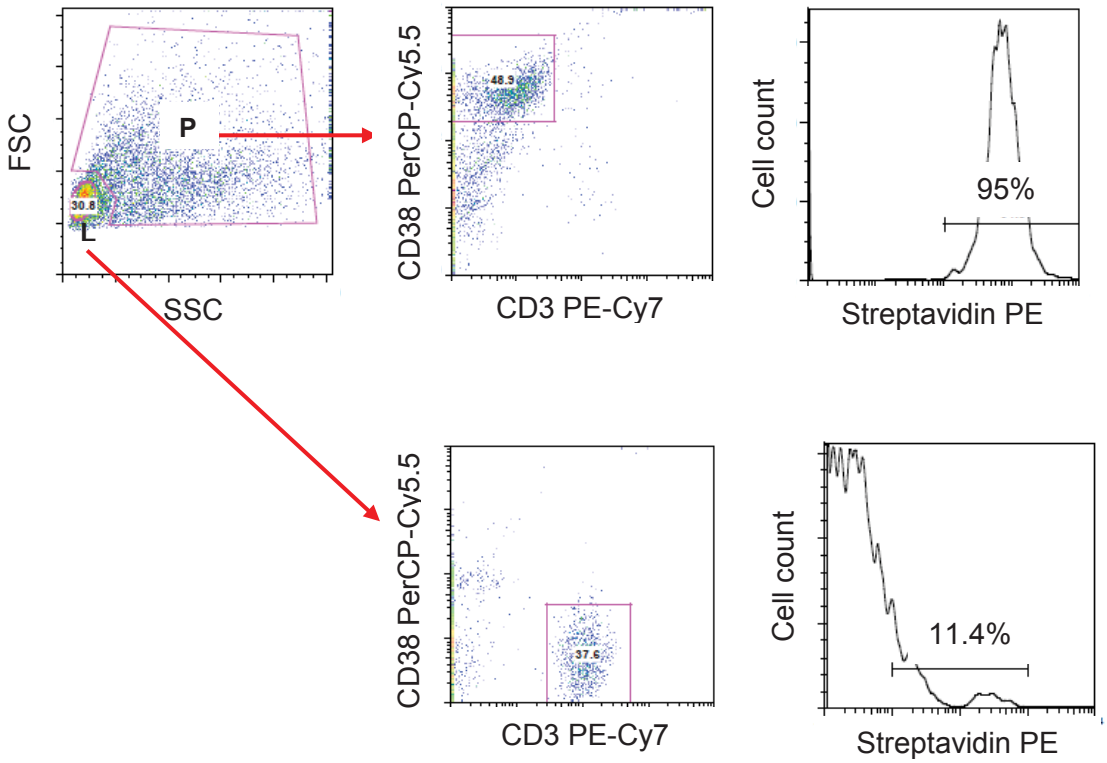


Figure 3-7: Biotin transfer from flow sorted bone marrow plasma cells to autologous T cells.
To measure biotinylation and transfer of biotin to autologous T cells, the plasma cell (P) and lymphocyte (L) populations were gated using a FSC and SSC dot plot (left dot plot of top panel). Using P, $CD38^{++}$ $CD3^{-}$ plasma cells were gated (middle dot plot of top panel) and analysed for biotin expression (histogram, top panel on right). Using L, $CD38^{-}$ $CD3^{+}$ T cells were gated (left dot plot, bottom panel) and analysed for biotin (histogram, bottom panel right).

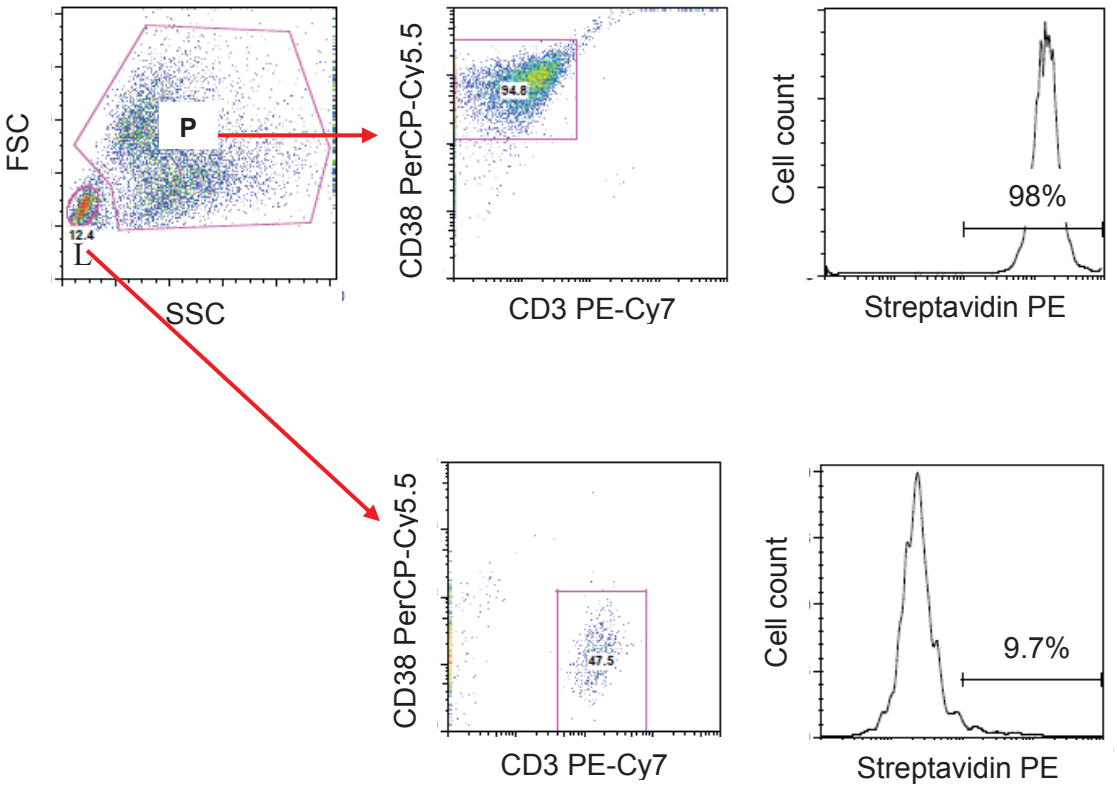
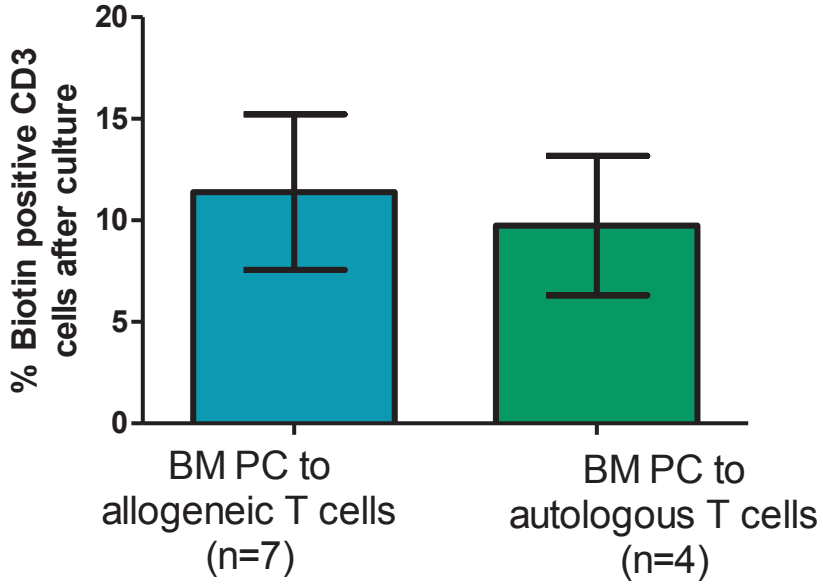


Figure 3-8: Biotin transfer from flow sorted bone marrow plasma cells to allogeneic T cells.
To measure biotinylation and transfer of biotin to allogeneic T cells, the plasma cell (P) and lymphocyte (L) populations were gated using a FSC and SSC dot plot (left dot plot of top panel). Using P, $CD38^{++}$ $CD3^{-}$ plasma cells were gated (middle dot plot of top panel) and analysed for biotin expression (histogram, top panel). Using L, $CD38^{-}$ $CD3^{+}$ T cells were gated (left dot plot, bottom panel) and analysed for biotin (histogram, bottom panel right).

A)



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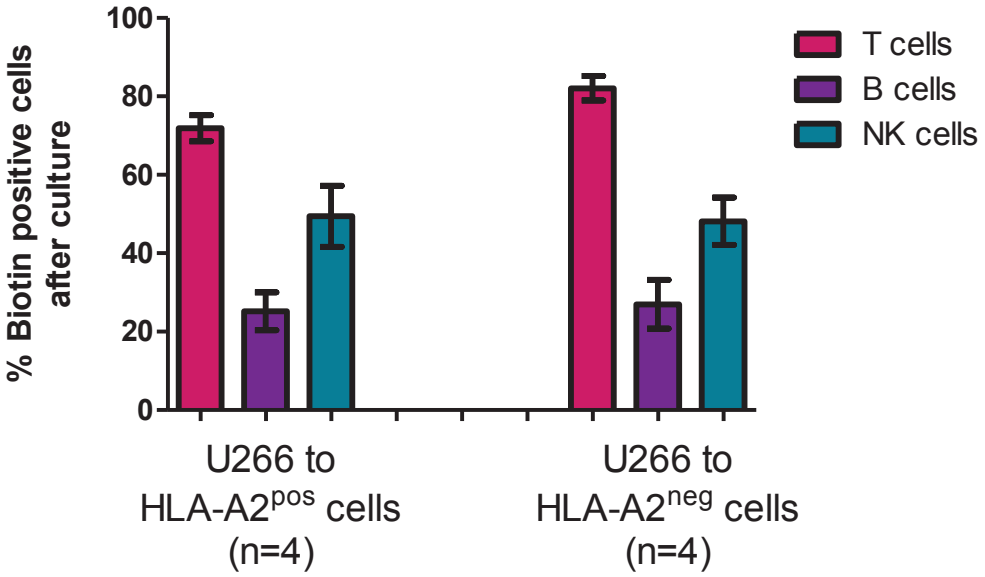


Figure 3-9: HLA compatibility is not required for trogocytosis.
Mean expression of biotin on allogeneic T cells (blue column) and autologous T cells (green column) following culture with biotinylated plasma cells (A). Data are represented as mean ± SEM. HLA-A2 compatible and incompatible lymphocytes were co-cultured with biotinylated plasma cells. Average expression (n=4) of biotin was determined by flow cytometry. T cells were observed to acquire greater amounts of biotin compared to B cells and NK cells from both cohorts (B). Data are represented as mean ± SEM.

3.2.6 Trogocytosis is predominately uni-directional

Plasma cells from myeloma patients (n=7) acquired an average of 2.5% biotinylated membrane patches from T cells whilst T cells from age-matched normal controls (n=4) acquired significantly ($p<0.05$) greater amounts of biotinylated membrane fragments (mean=13.55%) from plasma cells. This observation indicates that trogocytosis is predominately a uni-directional process with the transfer to T cells being more favoured over the transfer of membrane fragments to plasma cells (Figure 3-10).

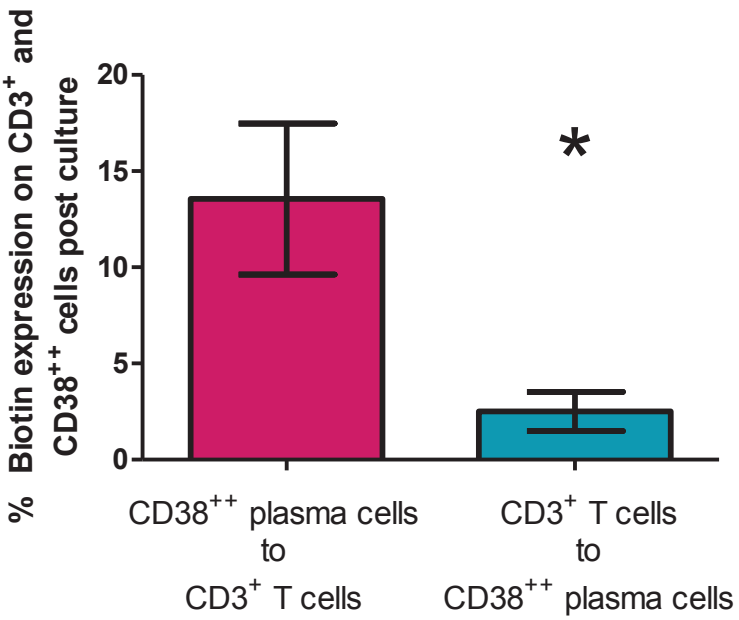


Figure 3-10: The directionality of biotinylated membrane fragment transfer. This graph shows percent average biotin expression on $CD3^+$ T cells following culture with biotinylated plasma cells and average biotin expression on $CD38^{++}$ plasma cells following co-culture with biotinylated T cells as determined by flow cytometry. T cells (n=7) were observed to acquire significantly (* indicates $p<0.05$) greater amounts of biotin (mean=13.55%) compared to plasma cells (n=4; mean=2.5%). Data are represented as mean ± SEM.

3.2.7 Trogocytosis in multiple myeloma

Peripheral blood mononuclear cells from myeloma patients (n=6) were co-cultured with a plasma cell line, U266, and following culture, cells were analysed for biotin expression. Representative flow cytometric analysis of biotin expression following culture with biotinylated plasma cells is shown in Figure 3-11. Surface expression of biotin was detected on 15.1% of T cells, 0.1% of B cells and 3.2% of NK cells following co-culture. Figure 3-12 shows expression of biotin on myeloma patient peripheral blood mononuclear cells and age matched control individual PBMCs. Statistical significance was not achieved between the two groups ($F=3.68$; $p=0.19$, $F=2.05$; $p=0.59$, $F=2.79$; $p=0.43$).

3.2.8 Trogocytosis in other B cell malignancies

Malignant B cells from a WM patient and malignant B cells from CLL patients (n=4) were biotinylated and incubated with T cells. Biotin expression was determined by flow cytometry. Figure 3-13 shows the expression of biotin on T cells following 2h culture. Less than 1% of T cells had acquired biotinylated membrane fragments from B cells from a WM patient. Flow sorted peripheral blood, $CD19^+$ $CD5^+$ B cells from CLL patients were biotinylated. $CD3^+$ T cells from normal controls were flow sorted and labelled with CFSE. Biotinylated B cells and CFSE labelled T cells were co-cultured for 2h. Less than 1% of T cells acquired biotinylated membrane fragments (Figure 3-14). Expression of biotin on T cells was significantly lower ($p<0.05$) when cultured with biotinylated B cells from WM and CLL patients (Figure 3-15). Biotin transfer was significantly increased on T cells from myeloma patients and age matched normal control individuals.

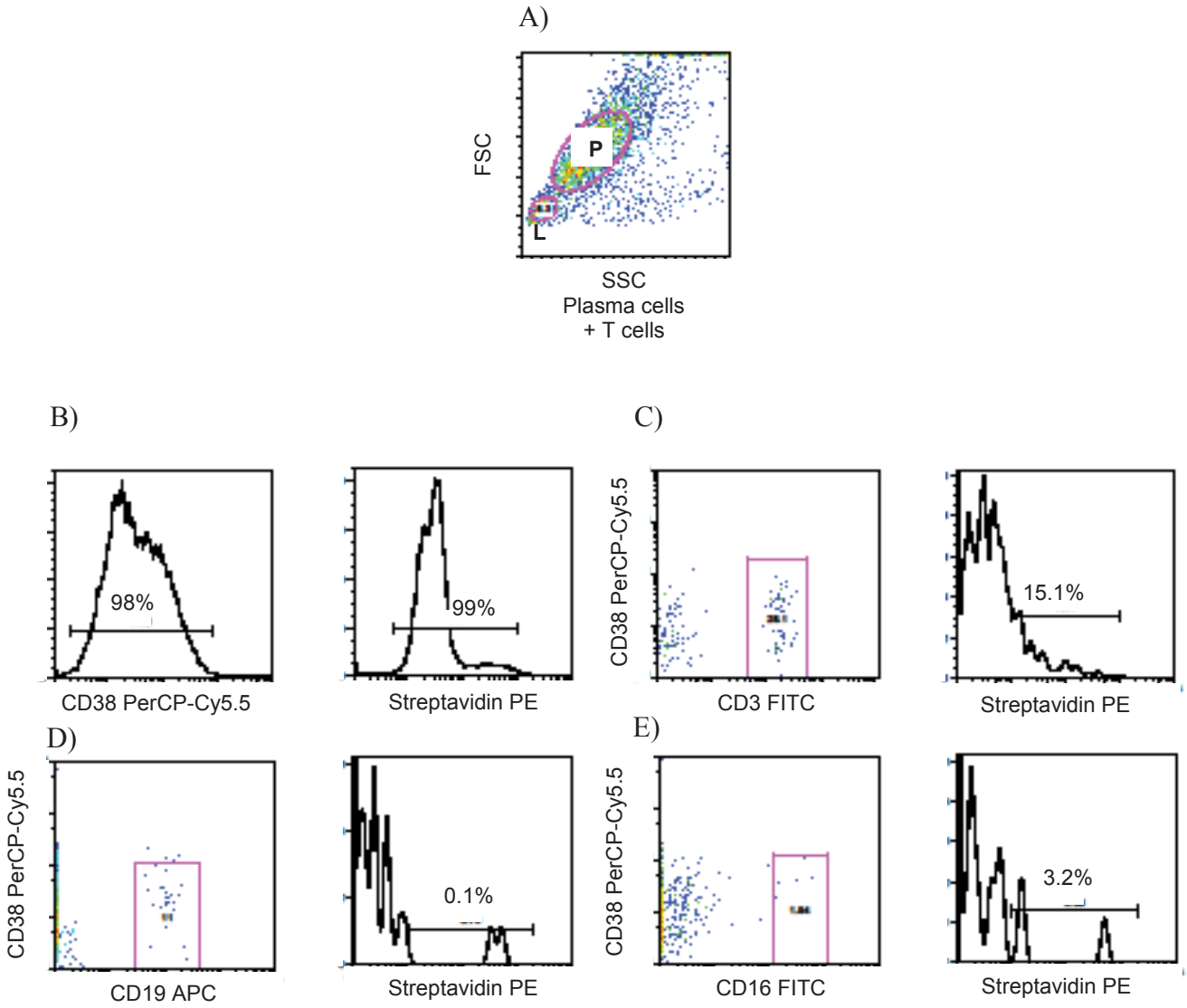


Figure 3-11: Biotin expression on a plasma cell line, U266 and peripheral blood mononuclear cells following co-culture.
The plasma cell (P) and lymphocyte (L) populations were gated using the FSC and SSC dot plot (A). Using P, $CD38^+$ plasma cells were then gated (B) and analysed for biotin expression. Using L, $CD38^-$ $CD3^+$ T cells (C), $CD38^-$ $CD19^+$ B cells (D), $CD38^-$ $CD16^+$ NK cells were gated and anaylsed for biotin expression. The regions for biotin/streptavidin positive cells were set on isotype control histograms.

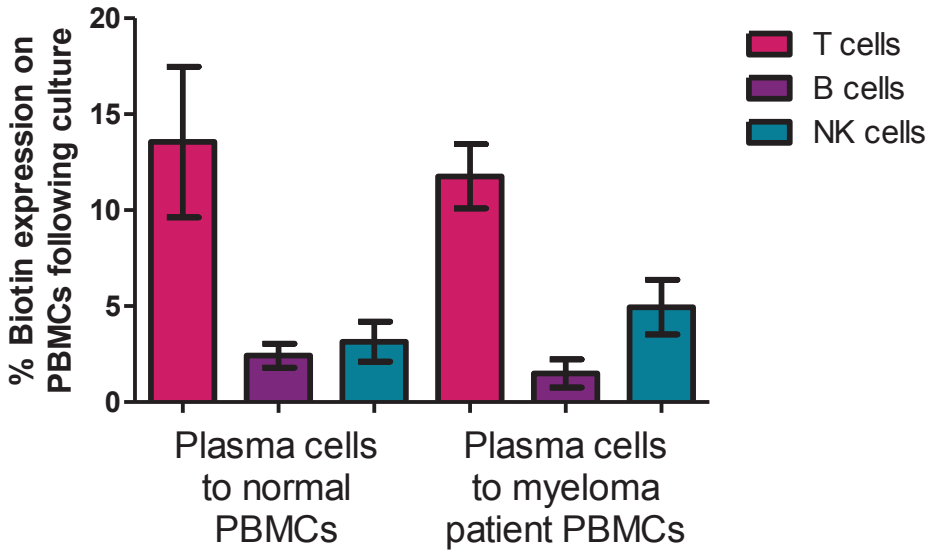


Figure 3-12: Biotin transfer to peripheral blood mononuclear cells from normal controls compared to myeloma patients.
Biotinylated plasma cells were co-cultured with PBMCs from myeloma patients (n=6) and haematologically normal control individuals (n=4). The graph shows percent biotin expression on normal patient and myeloma patient T cells (p=NS), B cells (p=NS) and NK cells (p=NS) following culture. T cells from both cohorts acquired more biotin following culture compared to B cells and NK cells. Data are presented as mean ± SEM.

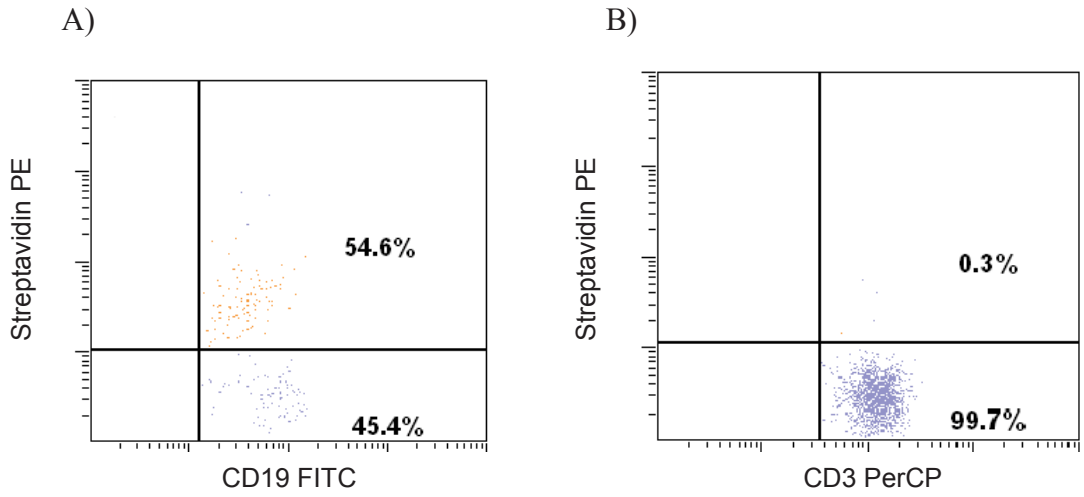


Figure 3-13: Expression of biotin on T cells following culture with bone marrow B cells from a WM patient.
Following identification of the B cell population, $CD19^+$ cells were analysed for biotin (A) expression before culture. Expression of biotin on $CD3^+$ T cells (B) following culture was also determined.

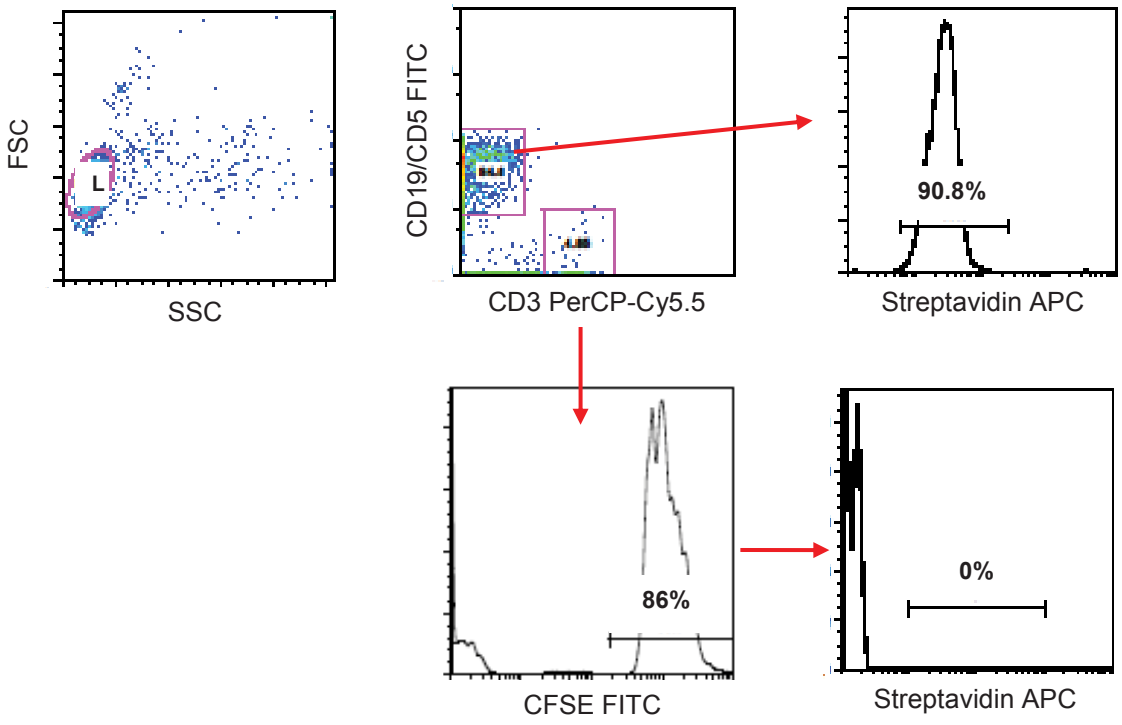


Figure 3-14: Biotin expression on T cells following culture with B cells from a CLL patient.

The lymphocyte (L) population was gated using the FSC and SSC dot plot (left dot plot, top panel). Using L, $CD3^-$ $CD19^+$ $CD5^+$ B cells were gated (middle dot plot, top panel) and analysed for biotin expression (right dot plot, top panel). Also using L, $CD3^+$ $CD19^-$ $CD5^-$ T cells were gated (middle dot plot, top panel) and CFSE positive cells (left dot plot, bottom panel) were then analysed for biotin expression (right dot plot, bottom panel).

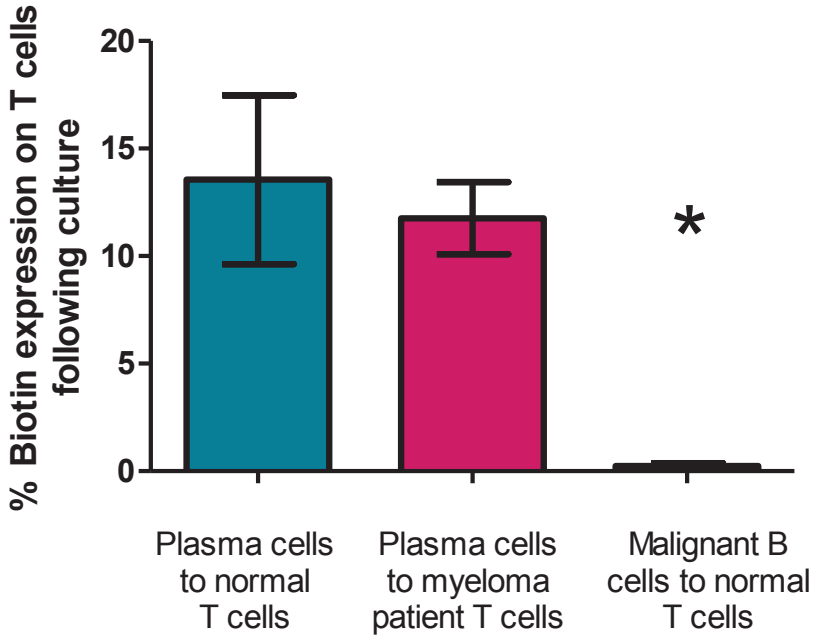


Figure 3-15: Trogocytosis is common in myeloma compared to other B cell malignancies.
This graph shows percent biotin expression on normal (n=4) and myeloma patient (n=6) T cells following culture with either biotinylated plasma cells or B cells from WM and CLL patients (n=5). Biotin transfer to T cells was significantly lower (* indicates $p<0.05$) when cultured with B cells from other B cell malignancies. Data are presented as mean ± SEM.

CHAPTER FOUR: INVOLVEMENT OF B7 MOLECULES IN TROGOCYTOSIS

4. B7 family molecules at the immunological synapse

4.1 Introduction

Of all the possible sites of cell-cell contact, the interactions between ligands, receptors and counter-receptors at the immunological synapse is the most well defined. The B7 family of co-stimulatory molecules, and in particular CD80 and CD86, has been widely studied. The interaction of these molecules with their counter-receptors CD28 and CTLA-4 may result in T cell activation, apoptosis or anergy (Key 2004, Medzhitov 2007, Weil and Israel 2006). Less is known about the expression and function of the other B7 family members but it is believed that they may also lead to T cell activation, death or anergy. T cells are known to acquire CD80 and CD86 molecules from antigen presenting cells and this is likely to be by a process of trogocytosis. An important question which needs to be addressed is whether malignant plasma cells that express co-stimulatory molecules can act as inefficient antigen presenting cells to donate these molecules to T cells and whether the T cells that acquire novel B7 surface molecules then have altered function associated with the acquisition of these molecules.

The objective of this chapter is to determine the expression of the B7 and CD28 family members on T cells in the peripheral blood of myeloma patients and haematologically normal age-matched individuals. The expression of these molecules on bone marrow plasma cells is also determined using flow cytometry. This chapter also investigates whether T cells acquire B7 molecules from malignant plasma cells. This will be done by determining the expression of B7 molecules on T cells in bone marrow samples when B7 is highly expressed on plasma cells. These investigations raise the possibility of increased B7 expression on tumour-infiltrating lymphocytes. Furthermore, *in vitro* mixing of cultures will be studied to demonstrate the acquisition of co-stimulatory molecules. The function of T cells with high levels of B7 will also be determined and T cell function will be analysed by flow cytometry.

4.2 Results

4.2.1 Immunophenotype of myeloma cell lines

The surface expression of CD80, CD86, CD138 and HLA-A2 was determined for the plasma cell lines RPMI-8226, KMS-11, NCI-H929, OPM2 and U266 (as described in section 2.1.6). After staining with the appropriate antibodies, the cell lines were analysed by flow cytometry to determine the surface expression of the molecules of interest (Figure 4-1). From the analysis of the flow plots obtained, it could be determined that CD86 was present on 97% of RPMI-8226 cells and on 8% of H929 cells. A small proportion of RPMI-8226 cells also expressed CD80 compared to the other cell lines. CD138 was observed to be expressed on all cell lines except KMS11 and only U266 was observed to express HLA-A2 (Table 4-1).

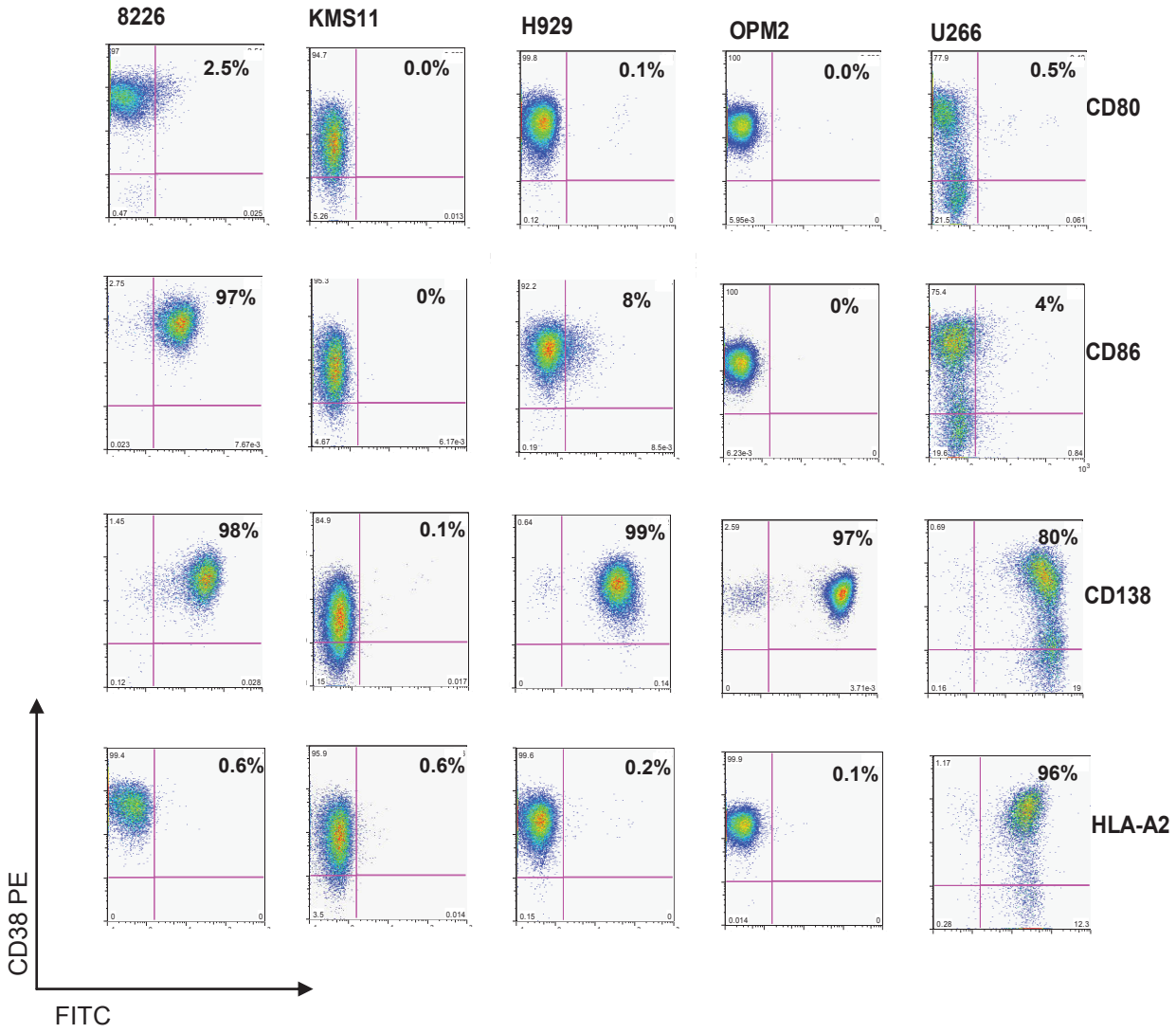


Figure 4-1: Immunophenotype of plasma cells.
Surface expression of CD80, CD86, CD138 and HLA-A2 was measured on 5 different myeloma cell lines (RPMI-8226, KMS-11, H929, OPM2 and U266). All cell lines were shown to express CD38 as seen in the upper quadrants of all scatter plots. Expression of surface molecules on $CD38^{++}$ cells, if present, was evident in the upper right quadrant of the scatter plots.

Table 4-1: Expression of surface markers on plasma cell lines

	RPMI-8226	KMS-11	H929	OPM2	U266
CD80	+	-	-	-	-
CD86	+++	-	+	-	+
CD138	+++	-	+++	+++	+++
HLA-A2	-	-	-	-	+++

- Indicates no expression

+ Indicates low expression

+++ Indicates high expression

4.2.2 Immunological synapse molecules on plasma cells of patients

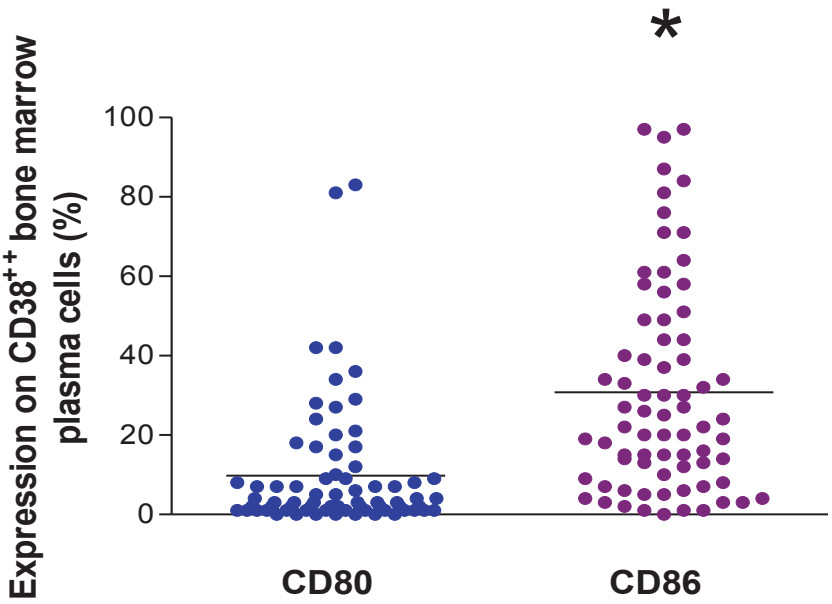
Flow cytometric analysis indicated that there was variable expression of CD80 (n=73; range 0–83%; mean=9.8%) and CD86 (n=73; range 0 – 97%; mean=30.8%) on primary bone marrow plasma cells. Expression of CD86 was significantly higher than the expression of CD80 ($p<0.0001$) as seen in Figure 4-2A. Although there was minimal expression of the other B7 family molecules on $CD38^{++}$ plasma cells, statistical significance was not achieved. CD274 was found to be present on the cells of all patients tested (n=13, range 2–42%, mean=11.8%). Expression of CD273 (n=13, mean=2.4%) and CD275 (n=13, mean=1.8%) ranged from 0 – 7% and 0 – 5% respectively (Figure 4-2B).

4.2.3 Immunological synapse molecules on T cells of patients

CD80 and CD86 expression on T cells of age-matched normal controls (n=20) ranged from 0 – 3.9% (mean=1.6%) and 0 –2.7% (mean=1.7%) respectively. The expression of CD80 on T cells from MM patients (n=143) ranged from 0 – 39% (mean=3.2%), whilst CD86 expression ranged from 0 – 54% (mean=4.5%). Myeloma patient T cells were shown to express significantly greater levels of CD80 ($\chi 2=4.7$; $p<0.02$) and CD86 ($\chi 2=15.30$; $p<0.001$) compared to T cells from age matched normal individuals (Figure 4-4A).

Surface expression of CD273 (mean=0.25%), CD274 (mean=5.09%), CD275 (mean=3.30%), CD278 (mean=1.25%) and CD279 (mean=2.25%) was measured on $CD3^{+}$ T cells from normal individuals (n=5) by flow cytometry (Figure 4-3 and Figure 4-4B). Expression of CD273 (mean=0.13%), CD274 (mean=3.34%), CD275 (mean=3.15%), CD278 (mean=0.46%) and CD279 (mean=1.39%) was also measured on T cells from myeloma patients (n=11). Furthermore, expression of co-stimulatory molecules and counter-receptors on T helper cells (Figure 4-5A) and cytotoxic T cells (Figure 4-5B) was quantified by flow cytometry. As statistical significance was not achieved between the two groups tested it was concluded that the major difference in expression of the B7 molecules between normal and myeloma related to CD80 and CD86.

A)



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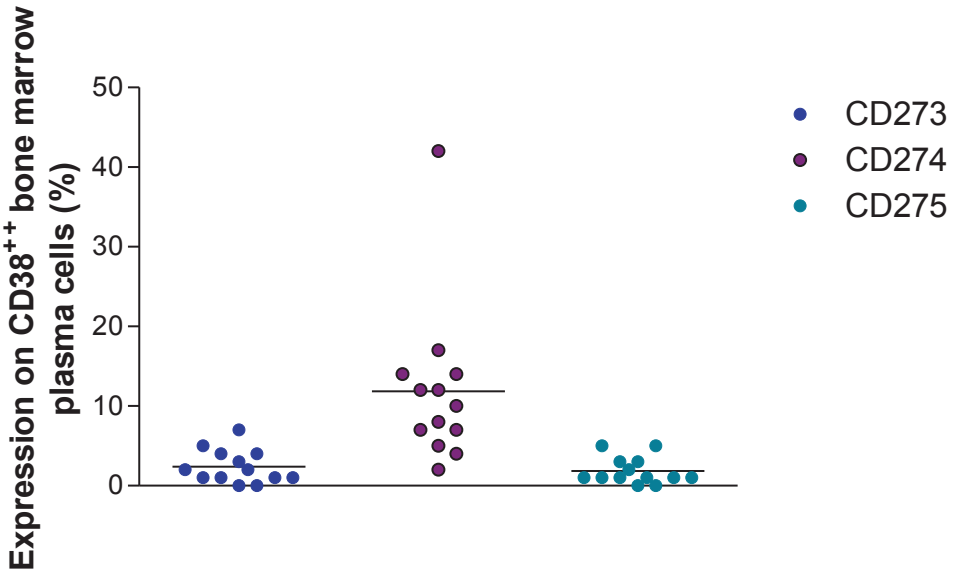


Figure 4-2: Expression of co-stimulatory molecules and counter-receptors on bone marrow plasma cells from myeloma patients.
Bone marrow plasma cells were ficoll separated and expression of the B7 family molecules was determined by flow cytometry. Percent expression of the co-stimulatory molecules CD80 and CD86 on $CD38^{++}$ plasma cells from patients with MM (A) (* indicates $p<0.05$). Percent expression of CD273, CD274 and CD275 on $CD38^{++}$ plasma cells from patients with MM (B). Each dot represents a patient and the horizontal bars represent the mean in each case.

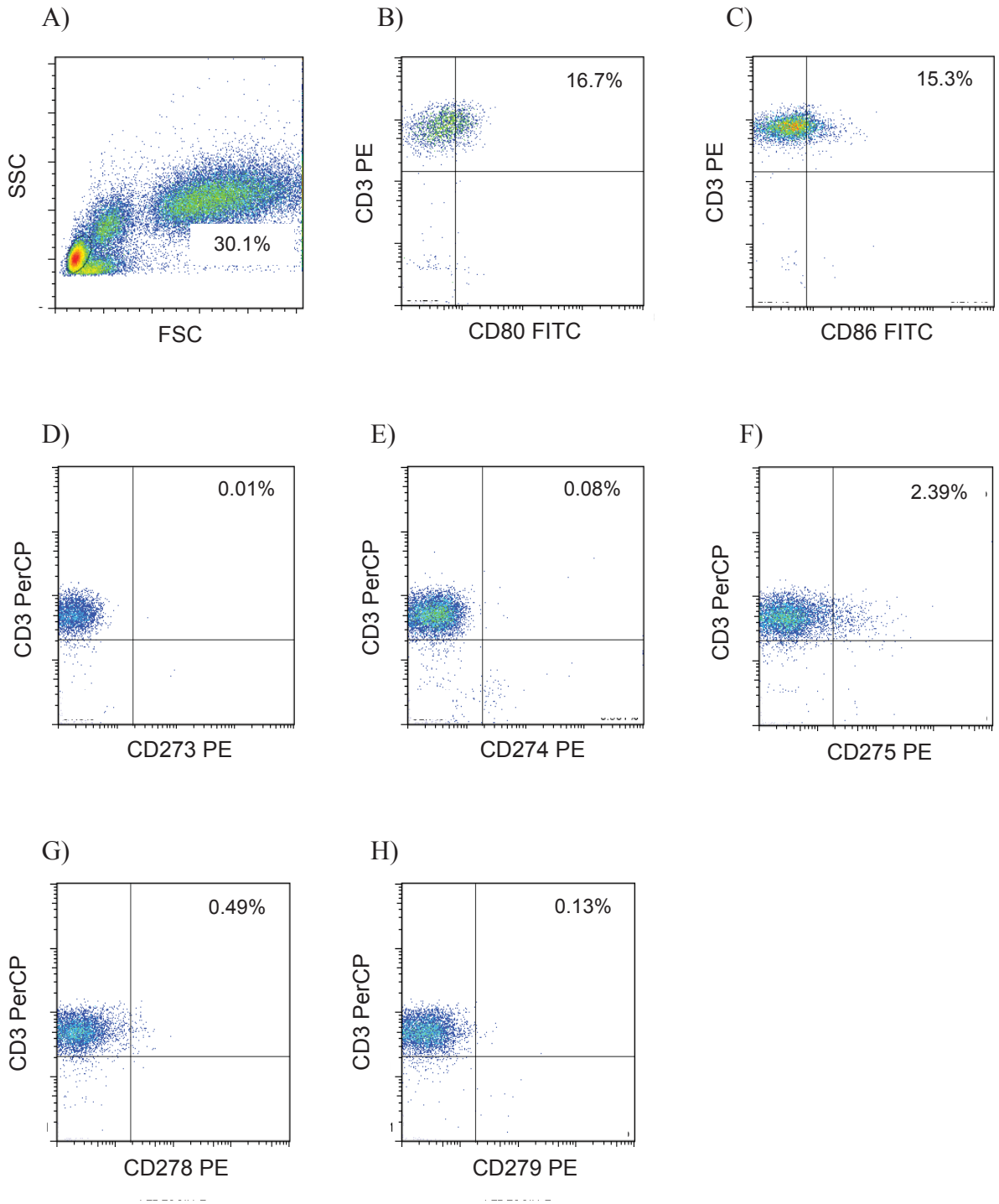
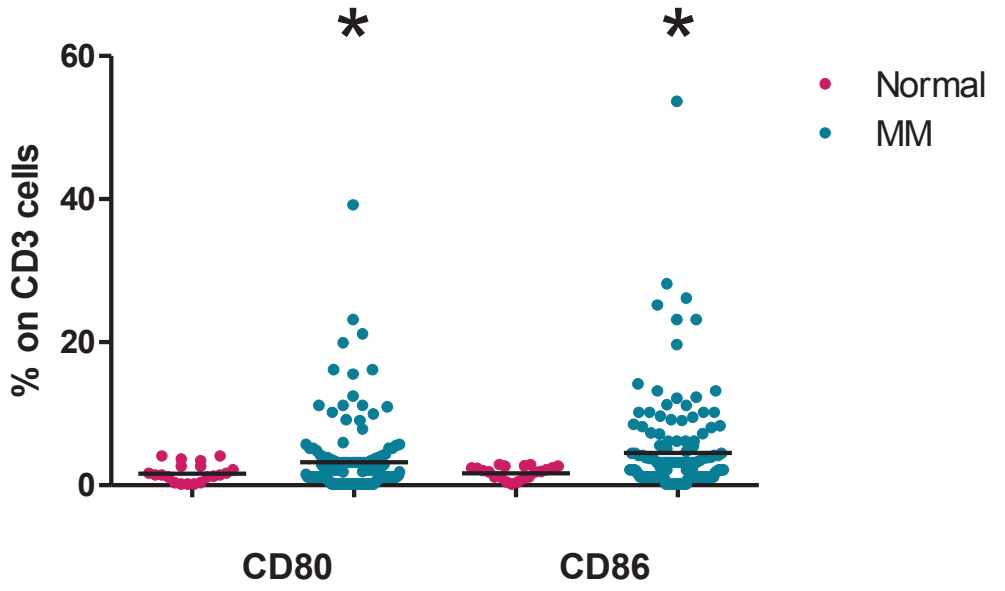


Figure 4-3: Flow cytometric analysis of B7 family marker expression on CD3 positive cells.
Lymphocytes were gated according to their forward and side scatter properties (A). Expression of CD80 (B), CD86 (C), CD273 (D), CD274 (E), CD275 (F), CD278 (G) and CD279 (H) was measured on T cells as seen in the upper right quadrant of the scatter plots.

A)



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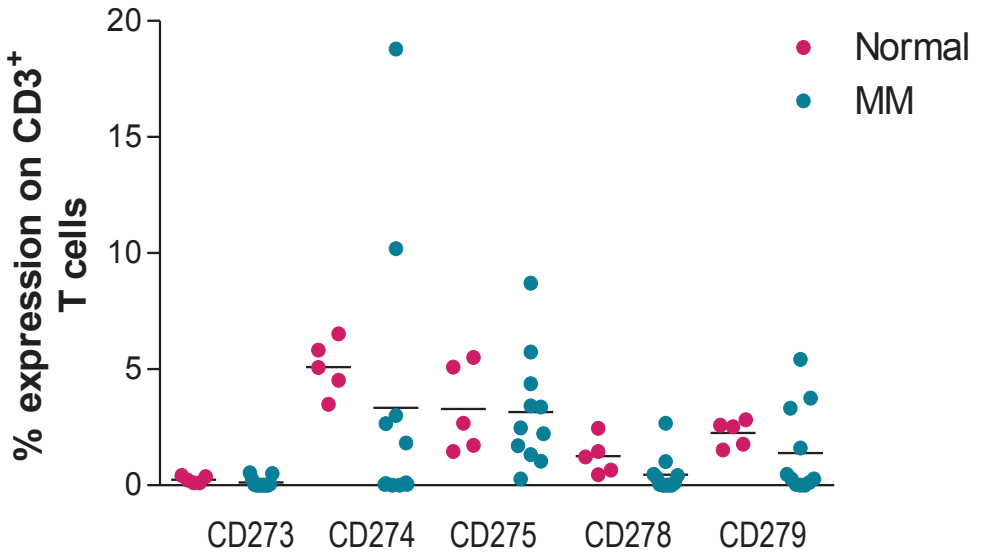
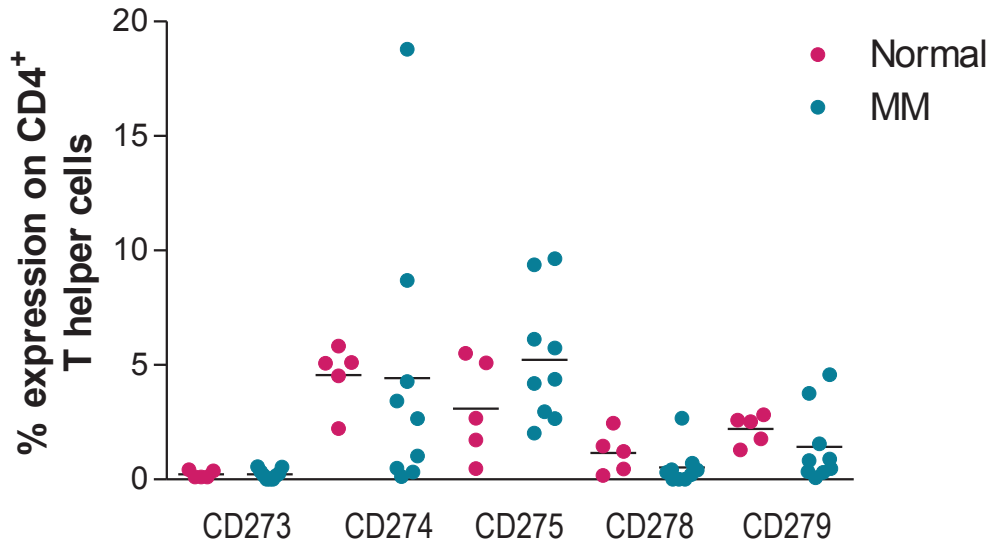


Figure 4-4: Expression of co-stimulatory molecules and counter-receptors on peripheral blood T cells from myeloma patients and normal control individuals. Percent expression of CD80 and CD86 on T cells of MM patients and haematologically normal individuals (A) (* indicates $p<0.05$). Percent expression of other B7 family members and counter-receptors on T cells from MM patients and haematologically normal individuals (B). Each dot represents a patient and the horizontal bars represent the mean expression.

A)



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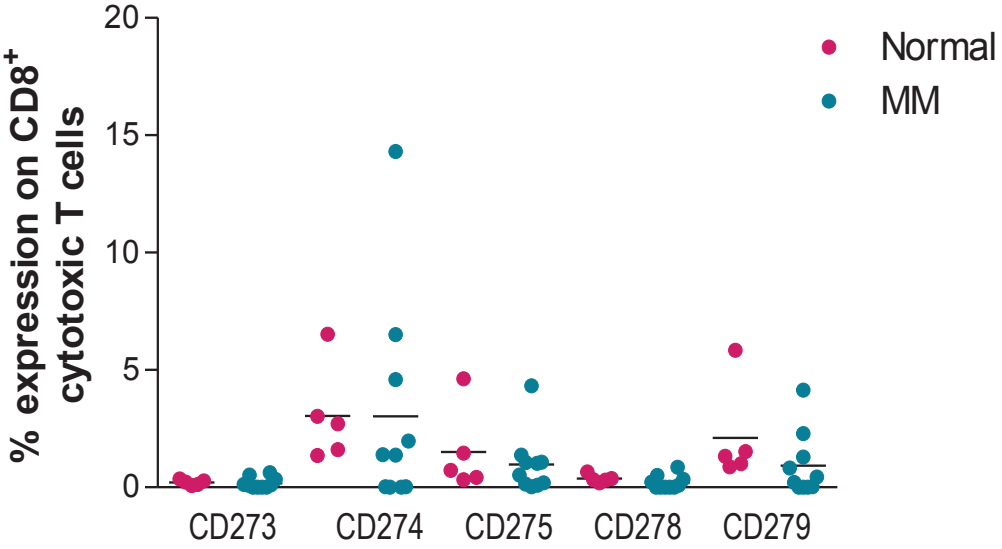

Figure 4-5: Expression of co-stimulatory molecules and counter-receptors on T cell subsets.
Expression of B7 family molecules and counter-receptors was determined by flow cytometry. Percent expression on $CD4^+$ T helper cells (A) and $CD8^+$ cytotoxic T cells (B) of multiple myeloma patients and normal control individuals. Each dot represents a patient and the horizontal bars represent mean expression.

4.2.4 CD86 expression on T cells and malignant plasma cells in the same bone marrow sample

If malignant plasma cells donate B7 molecules to T cells, it may be more important to study T cells in bone marrow samples rather than peripheral blood. Figure 4-6 shows a representative flow plot of CD86 expression on a patient's bone marrow plasma cells and bone marrow T cells. The expression of CD86 on plasma cells (Figure 4-6B) and T cells (Figure 4-6D) is seen in the histograms (Figure 4-6C and Figure 4-6E respectively). Figure 4-7 illustrates CD86 expression on bone marrow plasma cells and T cells in the same sample (n=11). There was an excellent correlation between CD86 expression on T cells and malignant plasma cells ($r=0.895$; $p<0.002$) (Figure 4-6 and Figure 4-7). Thus all patients with high CD86 expression on their bone marrow T cells had a high level of CD86 on their plasma cells.

4.2.5 CD86 expression on peripheral blood T cells and malignant plasma cells from myeloma patients

Figure 4-8 illustrates CD86 expression on bone marrow plasma cells and peripheral blood T cells from the same patient (n=15). There was no correlation between CD86 expression on T cells and malignant plasma cells ($r=0.35$; p=NS).

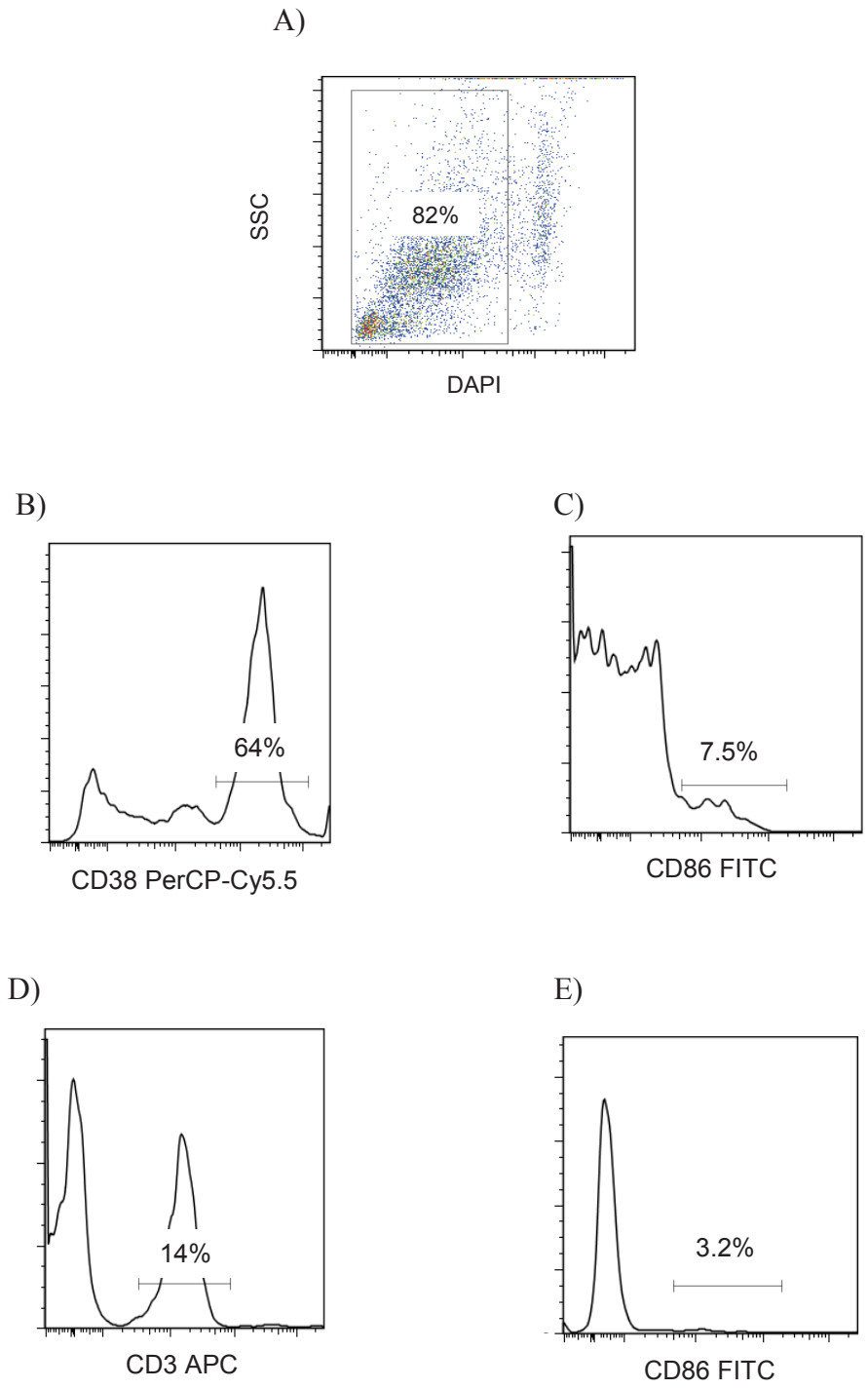

Figure 4-6: Representative flow plots of CD86 expression on bone marrow plasma cells and bone marrow T cells.
Scatter plot of viable cells (A). Histograms of CD86 expression on bone marrow plasma cells (B and C) and bone marrow T cells (D and E) from a myeloma patient.

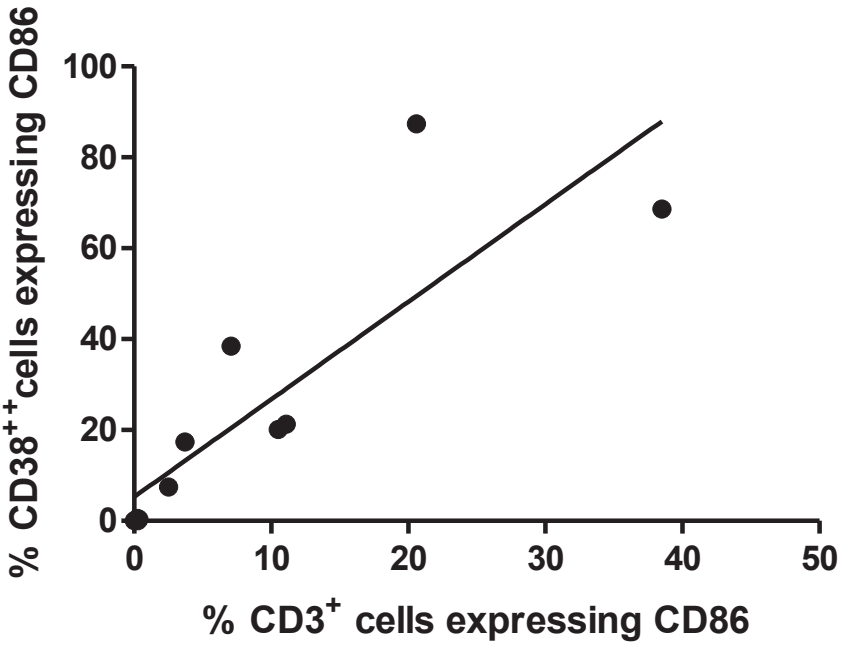

Figure 4-7: Correlation between CD86 expression on T cells and malignant plasma cells in bone marrow samples from patients with myeloma.
Expression of CD86 was measured on $CD38^{++}$ plasma cells and $CD3^{+}$ T cells in the bone marrow of myeloma patients ($r=0.895$; $p<0.002$). Each of the points in the graph represents a single patient.

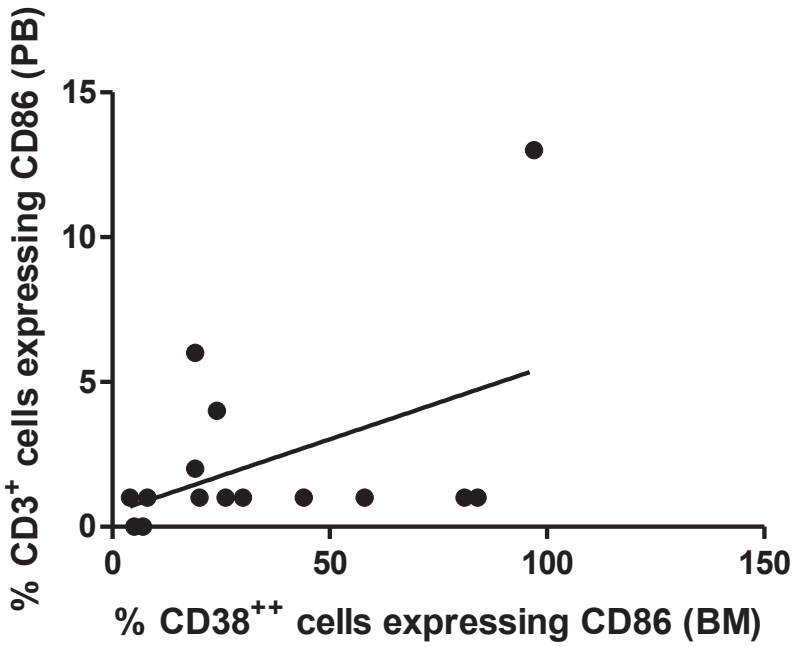

Figure 4-8: Expression of CD86 on bone marrow plasma cells and peripheral blood T cells.
Expression of CD86 was measured on $CD38^{++}$ bone marrow plasma cells and $CD3^{+}$ peripheral blood T cells from the same myeloma patient (p=NS). Each point in the graph represents a patient.

4.2.6 Acquisition of CD86 by T cells

Experiments related to active trogocytosis of CD86 *in vitro* are shown in Figure 4-9. T cells from myeloma patients (n=7), when co-cultured with a CD86 expressing plasma cell line, RPMI-8226 for 2h, were found to express significantly ($t=7.22$; $p<0.0001$) higher levels of CD86. Significantly higher ($t=6.45$; $p=0.003$) expression of CD86 was also observed when activated T cells from myeloma patients (n=3) were co-cultured with the CD86 expressing plasma cell line, RPMI-8226. Thus it was clearly demonstrated that trogocytosis of CD86 occurred *in vitro*.

4.2.7 Inhibition of T cell proliferation

The effect of CD86 expressing T cells on the proliferation of CD86 negative T cells is seen in Figure 4-10. T cells were shown to proliferate (46.8%) when stimulated with anti-CD3/CD2/CD28 beads. Proliferation was increased by approximately 5% when co-cultured with CD86 expressing T cells. The effect of CD86 expressing plasma cells on the proliferation of CD86 negative T cells was also determined (Figure 4-11). Approximately 5% inhibition was observed when stimulated T cells were co-cultured with CD86 expressing plasma cells. Proliferation of CD86 depleted T cells was inhibited by CD86 expressing plasma cells (n=3, mean=3.4%, range 1.3 – 6.1%). Similarly, proliferation of CD86 depleted T cells was inhibited by autologous CD86 expressing T cells (n=5, mean=12.6%, range -1.7 – 14.5%) (Figure 4-12).

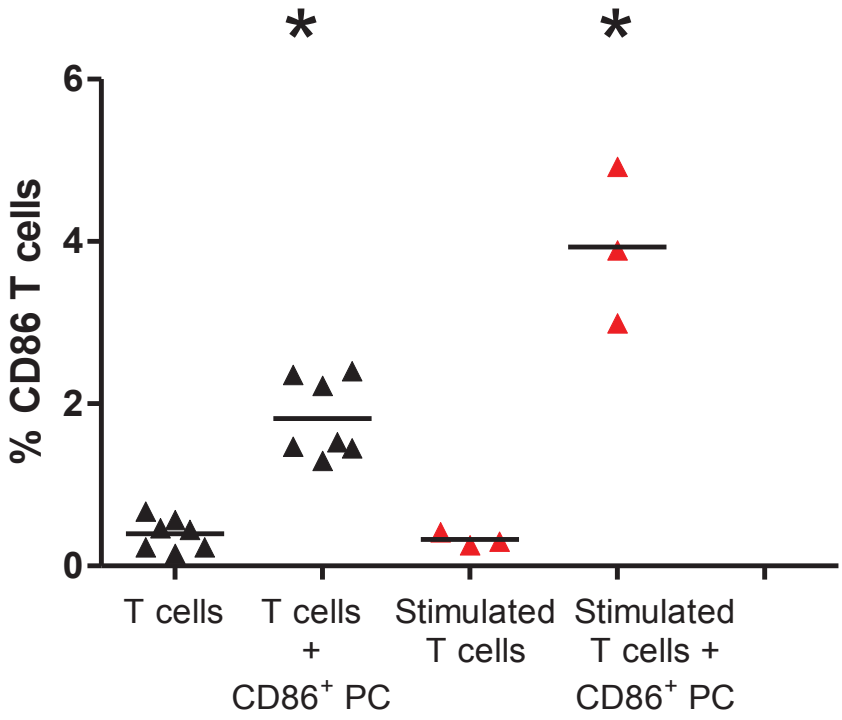

Figure 4-9: Correlation between CD86 expression on T cells and malignant plasma cells in bone marrow samples from patients with myeloma.
Expression of CD86 on T cells pre-culture (column one) and following culture (column two) with CD86 expressing plasma cells (* indicates $p<0.005$, each dot represents an experiment). Stimulation of T cells (column three, red triangles) and culture with CD86 expressing plasma cells (column four, * indicates $p<0.005$). Horizontal bars represent mean expression of CD86.

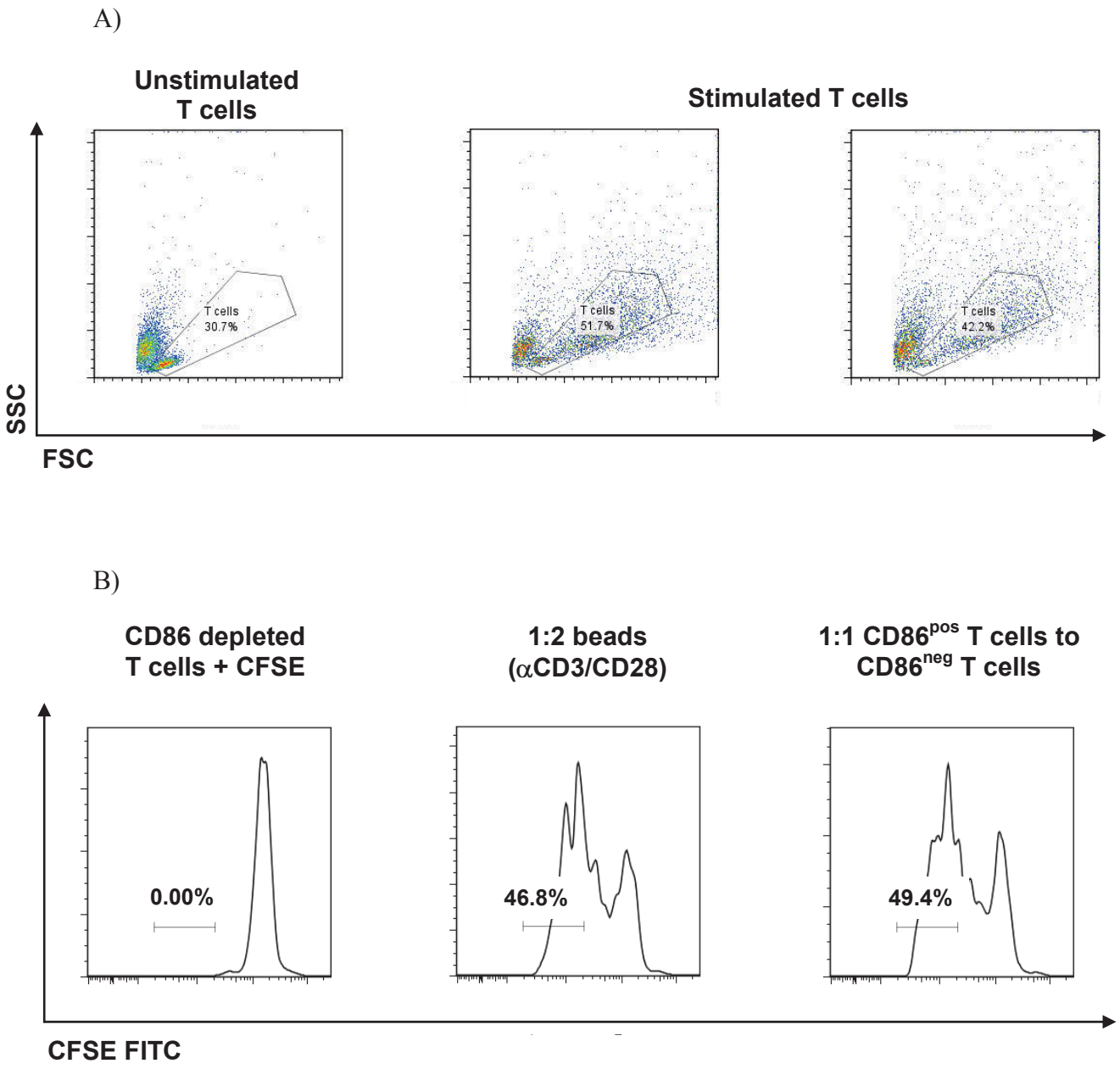

Figure 4-10: Representative flow plots showing T cell proliferation by CD86 expressing T cells.
Peripheral blood CD86 negative T cells were positively sorted and labelled with a tracking dye (CFSE). Cells were then stimulated with anti-CD3/anti-CD28 beads (A) as well as co-cultured with flow sorted CD86 positive T cells. Percent proliferation was recorded (B). Unstimulated cells were used as a control in this experiment.

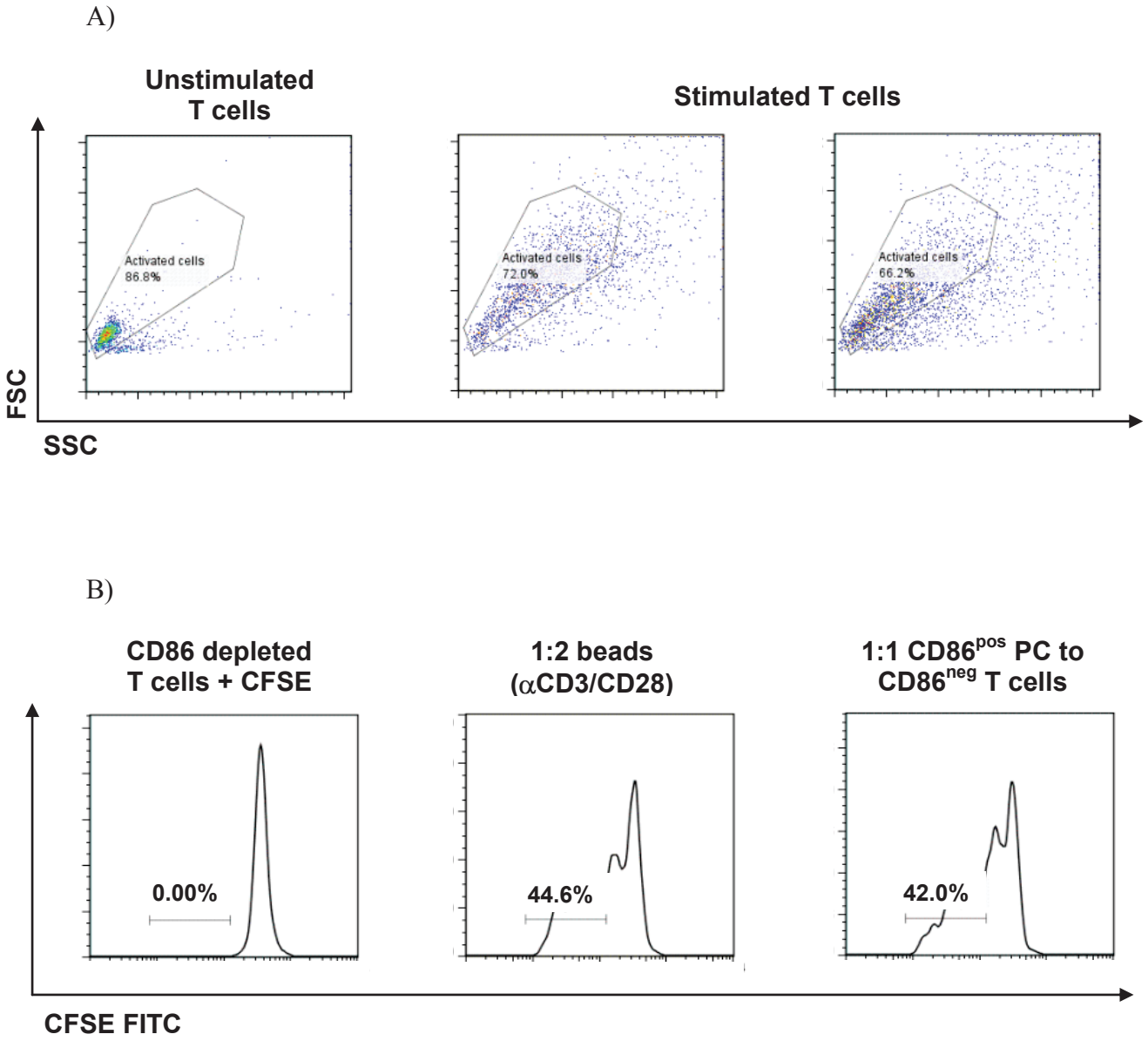

Figure 4-11: Representative flow plots showing T cell proliferation by CD86 expressing plasma cells.
Peripheral blood CD86 negative T cells were positively sorted and labelled with a tracking dye (CFSE). Cells were then stimulated with anti-CD3/anti-CD28 beads (A) as well as co-cultured with CD86 expressing plasma cells. Percent proliferation was recorded (B). The unstimulated cells were used as a control.

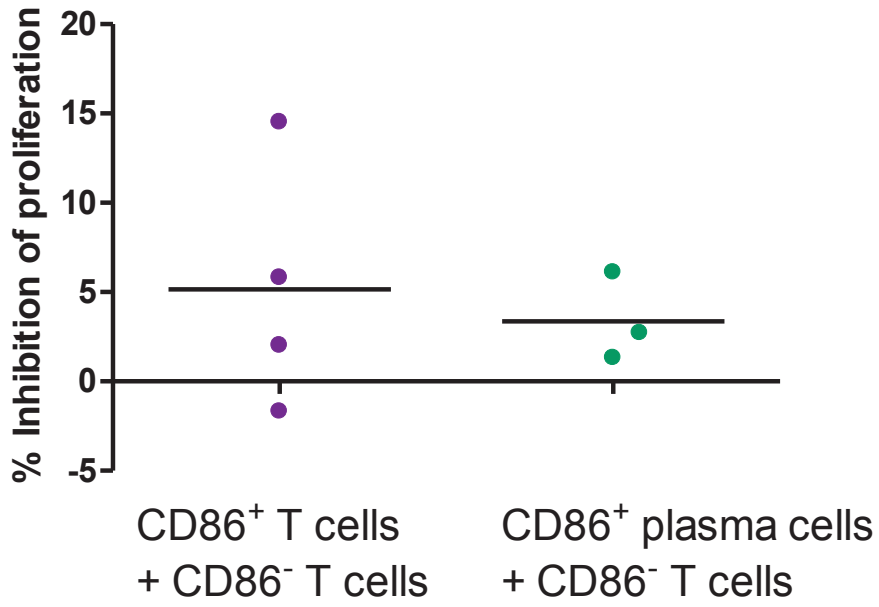

Figure 4-12: Inhibition of proliferation by CD86 expressing cells.
CD86 depleted T cells were co-cultured with CD86 expressing T cells and plasma cells. T cells were tracked with CFSE. Inhibition of proliferation was observed when CD86 negative T cells were stimulated with anti-CD3/anti-CD28 beads and cultured with CD86 expressing plasma cells.

CHAPTER FIVE: HLA-G^{POS} T CELLS: A NEW SUBSET OF REGULATORY T CELLS

5. HLA-G expression

5.1 Introduction

The relative expression of HLA-G on the cell membrane controls the immuno-modulatory and inhibitory functions of a variety of cells. For instance, APCs expressing HLA-G may be able to interact with receptors on effector cells, thereby reversing the function of these cells, such that they become suppressor cells which can induce tolerance (Naji*, et al* 2007). Due to acquisition of HLA-G, these cells behave differently to the way they are supposed to.

In patients with malignancies, it is proposed that the acquisition of HLA-G on the surface of T cells generates a novel subset of regulatory T cells whose function is switched from effector to immune suppressor. The HLA-G^{acq} T cells provide an increase in the number of HLA-G^{+} regulatory T cells, which may exert an inhibitory effect on neighbouring T cells and thus block the function of effector T cells that are recruited to the microenvironment. This induces immune tolerance and hence the down-regulation of anti-tumour responses.

The objective of this chapter is to determine the role of HLA-G as a potential immune-modulator in myeloma as this has not yet been clearly established. The expression of HLA-G on bone marrow plasma cells and peripheral blood T cells of myeloma patients will be determined and compared to HLA-G expression on T cells of haematologically normal age-matched individuals. This chapter will further investigate whether HLA-G can be acquired by T cells from HLA-G expressing malignant plasma cells.

5.2 Results

5.2.1 HLA-G expression on plasma cells of multiple myeloma patients

Surface expression of HLA-G was determined on plasma cells of myeloma patients (n=51). Representative flow plots of HLA-G expression on bone marrow plasma cells from two myeloma patients are seen in Figure 5-1A-D. Expression of HLA-G in patient 1 was less than 1% (Figures 5-1A and B) whilst patient 2 had greater than 90% expression of HLA-G on their bone marrow plasma cells (Figures 5-1C and D). Expression of HLA-G on bone marrow plasma cells of MM patients as measured by flow cytometry, ranged between 0.2 to 96% with a mean expression of 21.3% (Figure 5-2). This data confirmed the initial variability detected in HLA-G expression.

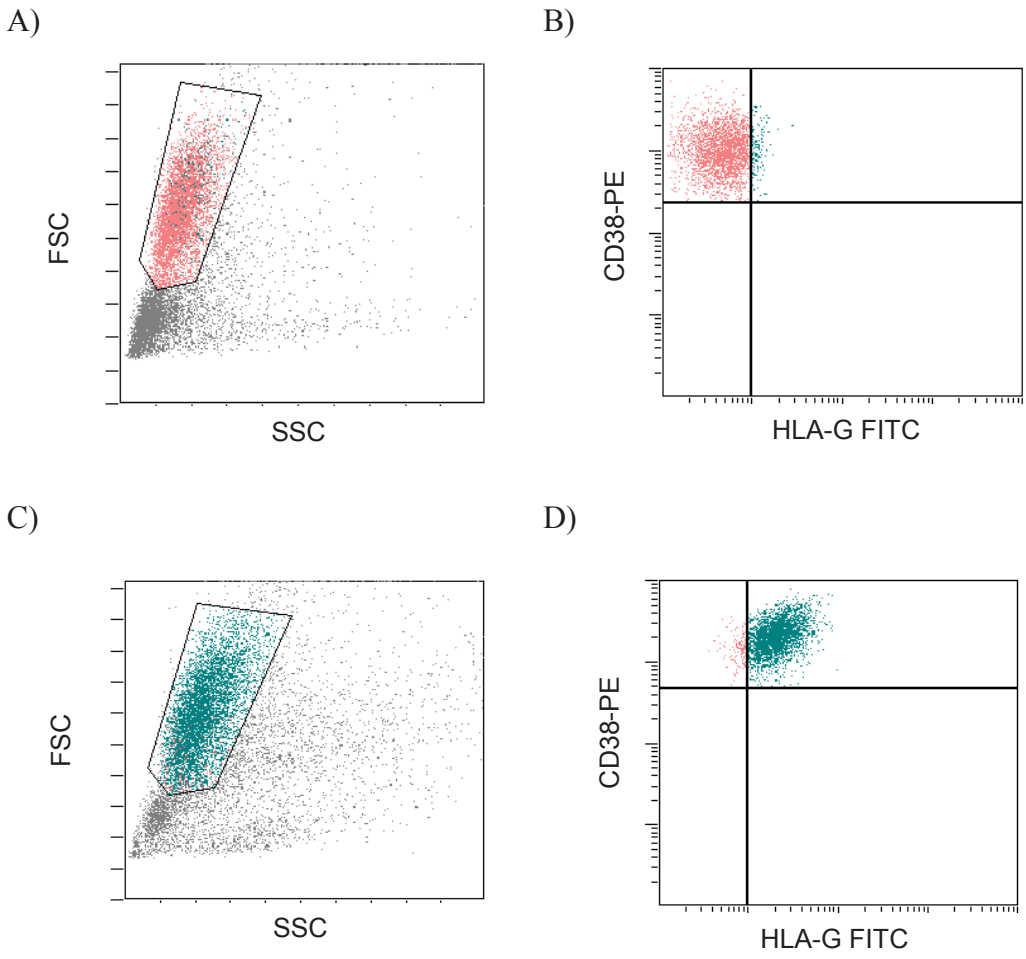

Figure 5-1: Representative flow plots of HLA-G expression on bone marrow plasma cells of multiple myeloma patients.
Forward scatter and side scatter plots indicating the position of plasma cells (A and C). Expression of HLA-G on plasma cells from patient 1 (B) and patient 2 (D) are also shown in the upper right quadrant of the scatter plots.

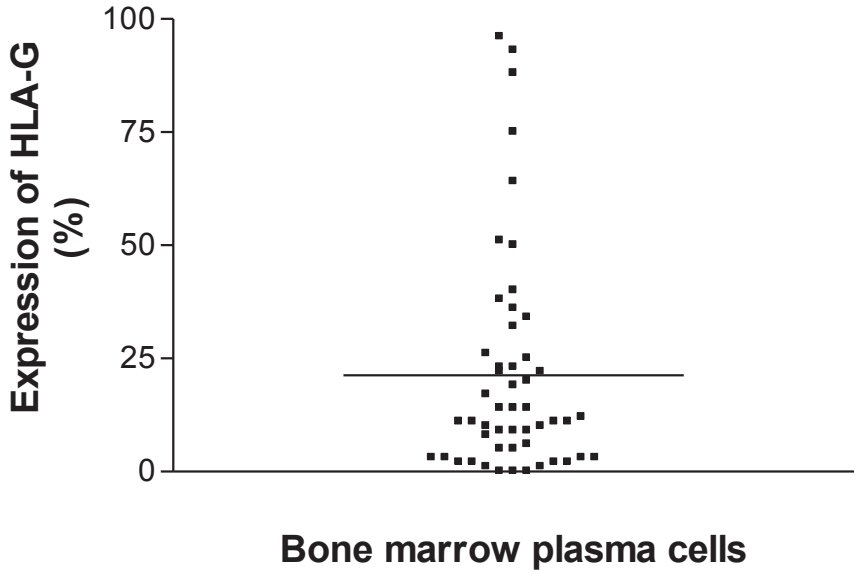

Figure 5-2: HLA-G expression on bone marrow plasma cells varies between patients.
Flow cytometric analysis of $CD38^{++}$ bone marrow plasma cells from MM patients showed varied expression of HLA-G ranging from 0.2% to 96%. The horizontal bar represents mean HLA-G expression. Each dot on the graph represents a single patient (n=51).

5.2.2 HLA-G expression is not related to stage of myeloma

Analysis of HLA-G expression based on stage of myeloma (stage I vs stage II/III) was carried out to determine if HLA-G expression was related to stage. HLA-G expression on plasma cells of patients with ISS stage I myeloma ranged from 0.4 – 96% (n=25; mean 28.6%) whilst expression on plasma cells of patients with ISS stage II/III disease ranged from 0.2 – 75% (n=20; mean 14.4%). Unpaired t-test analysis showed that significant differences were not observed ($t=1.9$; $p=0.77$) between patients in the two groups (Figure 5-3). Patients with progressive disease did not express significantly higher levels of HLA-G on their plasma cells.

5.2.3 Correlation of HLA-G expression and overall survival in multiple myeloma

A study of HLA-G expression and overall survival in myeloma patients indicated that the overall survival was significantly worse for the 11/46 patients with HLA-G^{+} plasma cells (>5% $CD38^{++}$ cells and expressing >12% HLA-G; $\chi 2=12.4$; $p<0.0004$) (Figure 5-4). Thus expression of HLA-G appears to be a potential marker of disease survival in multiple myeloma.

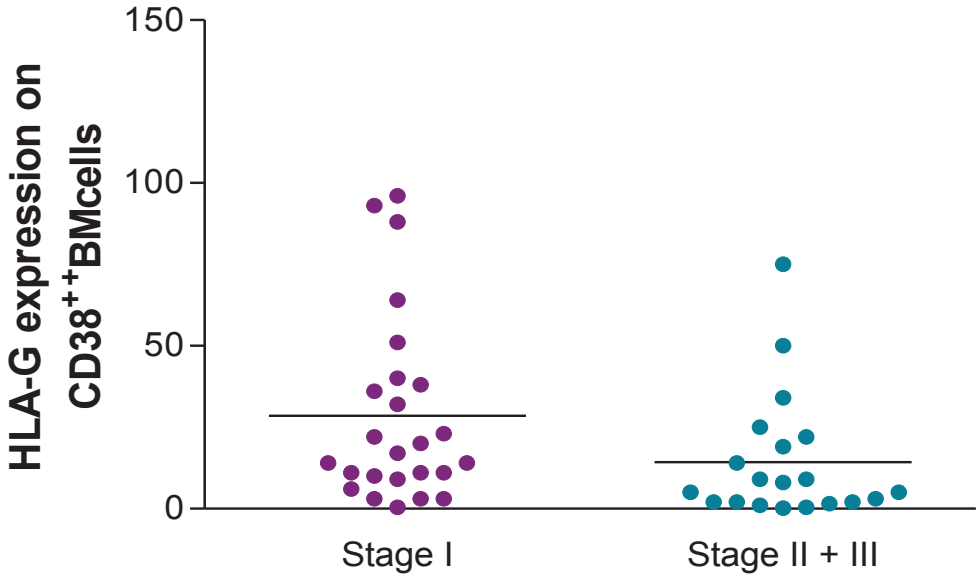

Figure 5-3: Correlation of HLA-G expression and stage of multiple myeloma.
Based on the international staging system criteria (β_2M and albumin level) stage I categorised one grouping. The second grouping consisted of patients with stages II and III. HLA-G expression was determined by flow cytometry. Each dot on the graph indicates a patient sample (p=NS).

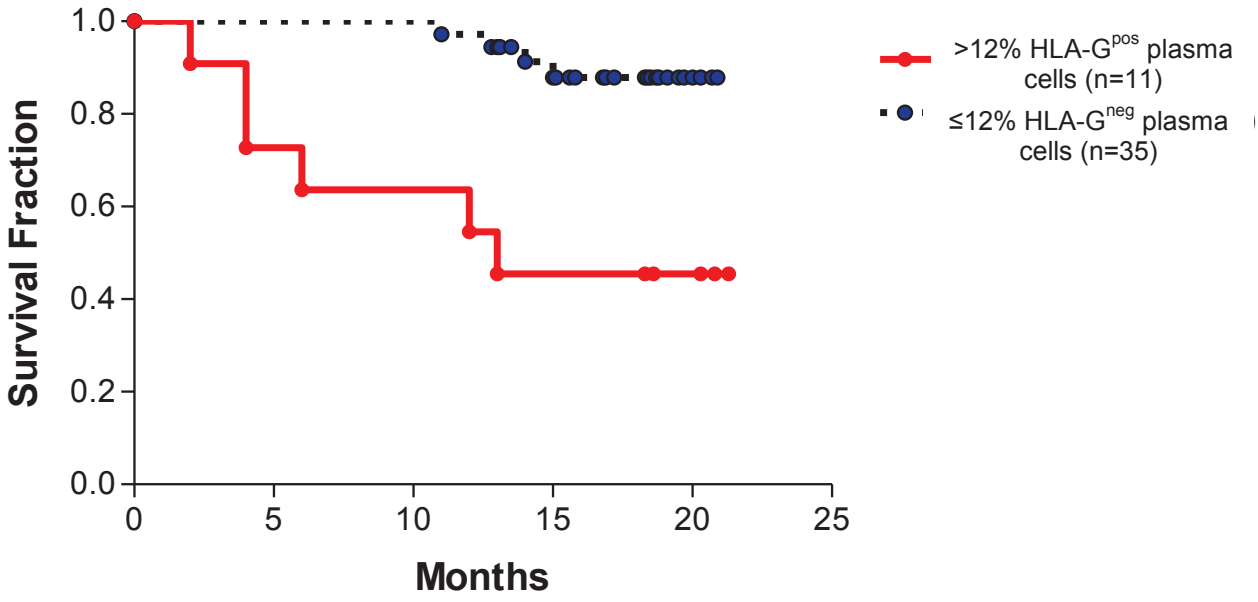

Figure 5-4: Correlation of HLA-G expression and overall survival in myeloma. Survival curves showing the relation of HLA-G expression with overall survival. Patients with HLA-G expressing plasma cells are shown in red whilst patients with non-HLA-G expressing plasma cells are indicated in blue (Log-rank analysis on Graph-Pad PRISM v5.01, $\chi 2=12.4$; $p<0.0004$).

5.2.4 Expression of HLA-G on T cells of myeloma patients and normal individuals

HLA-G expression on peripheral blood T cells was determined for multiple myeloma patients and control individuals. Flow cytometry results indicated that HLA-G was present on 0.02 – 0.56% (mean 0.24%) of $CD3^+$ cells in the peripheral blood of age-matched controls (n=15). Significantly ($F=3.7$; $p<0.01$) higher levels of HLA-G were present on $CD3^+$ cells from patients with MM (n=56; range 0.03 – 1.12%; mean 0.31%). Although expression is low, 20% of MM patients (11 of 56) demonstrated a level of $HLA\text{-}G^+$ $CD3^+$ T cells above the normal range, determined by mean + 2SD (Figure 5-5).

5.2.5 HLA-G expression on peripheral blood T cells and bone marrow plasma cells from the same patient

Figure 5-6 illustrates HLA-G expression on bone marrow plasma cells and peripheral blood T cells from the same patient (n=6). Correlation between HLA-G expression on T cells and malignant plasma cells ($r= -0.09$; p=NS) was not observed.

5.2.6 HLA-G expression on bone marrow T cells and bone marrow plasma cells from the same sample

Analysis of HLA-G expression on a patient's bone marrow plasma cells and corresponding bone marrow T cells showed that approximately 5.5% of plasma cells expressed HLA-G (Figures 5-7B and C) and 1.4% of T cells were determined to express HLA-G (Figures 5-7D and E). There is a significant correlation between HLA-G expression on bone marrow plasma cells and bone marrow T cells in the same sample (n=11; $r=0.895$; $p<0.002$) (Figure 5-8).

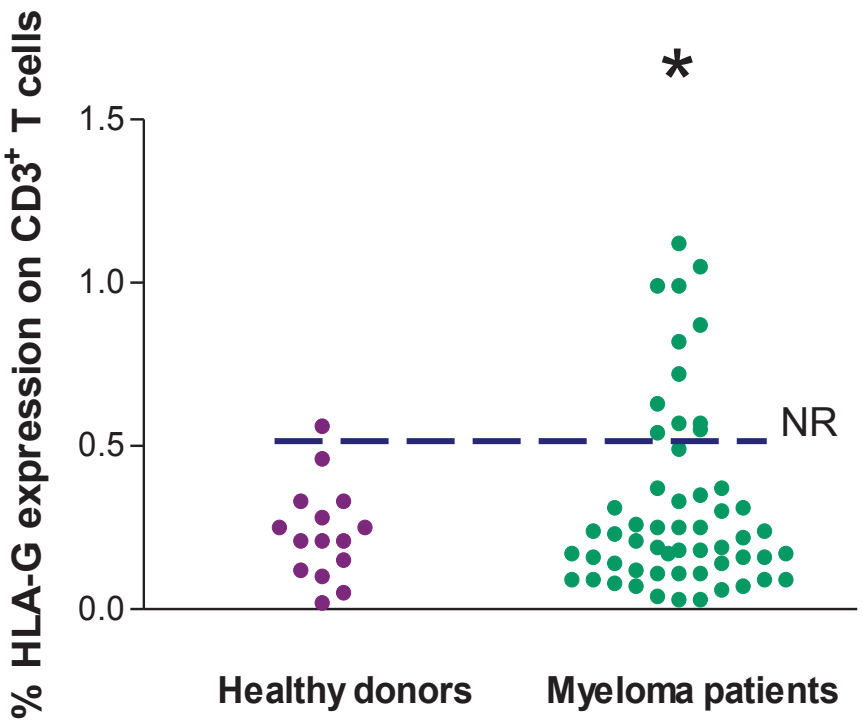

Figure 5-5: HLA-G expression on T cells of myeloma patients and age-matched control individuals.
Flow cytometry analysis of circulating HLA-G^{pos} T cells in the peripheral blood of myeloma patients (* indicates $p<0.05$) compared to haematologically normal individuals. The dotted line in the graph indicates the cut off for HLA-G expression for the normal range (mean + 2SD).

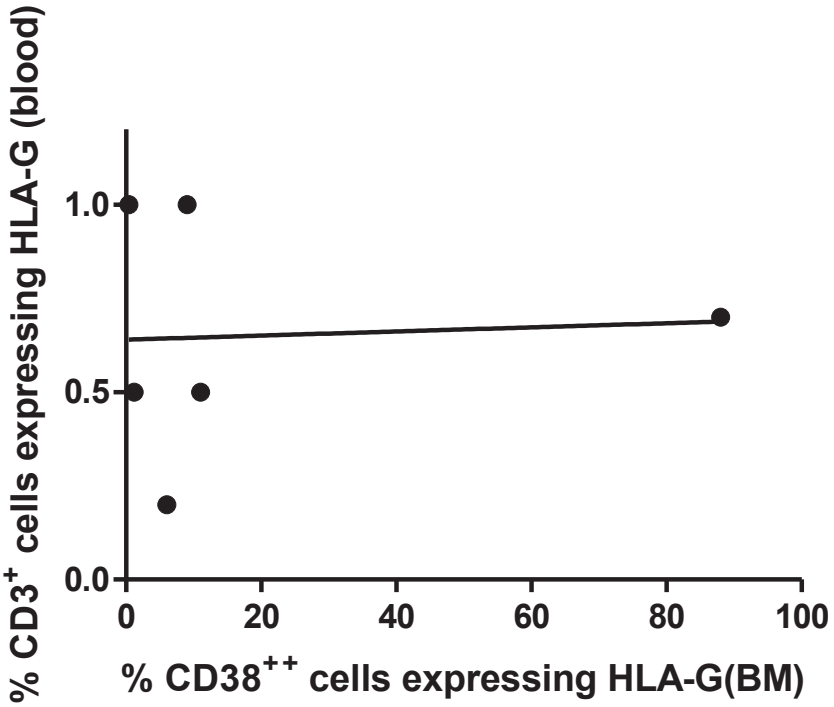

Figure 5-6: HLA-G expression on bone marrow plasma cells and peripheral blood T cells within individual patients.
There was no correlation (r= -0.09; p=NS) between expression of HLA-G on peripheral blood T cells and bone marrow plasma cells from the same patient as determined by flow cytometry. Each dot in the graph represents the analysis for a single patient.

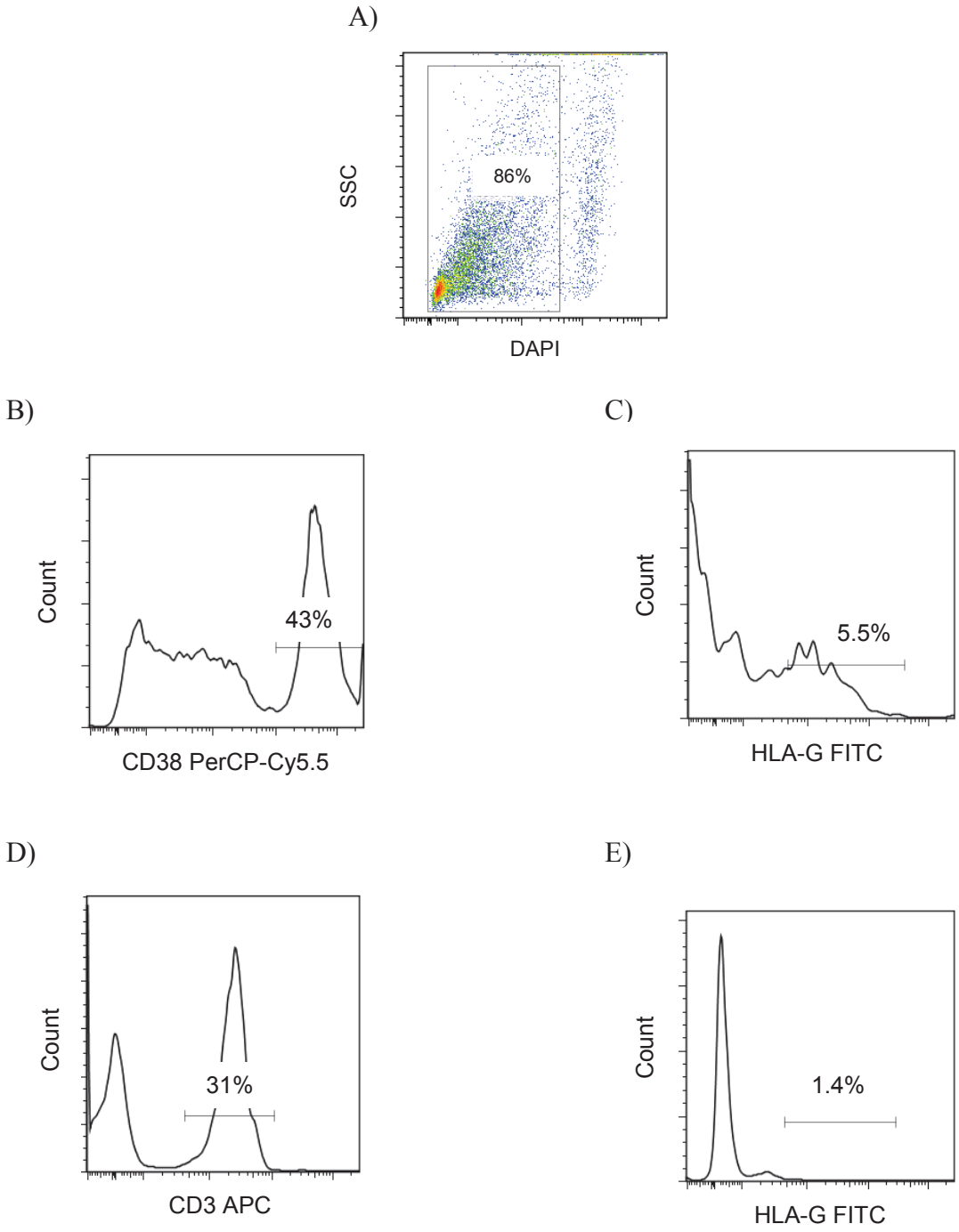

Figure 5-7: Representative flow plots of HLA-G expression on bone marrow plasma cells and T cells from the same patient.
Viable bone marrow cells were gated based on their side scatter properties (A). $CD38^{++}$ plasma cells (B) and $CD3^{+}$ T cells (D) were gated and HLA-G expression was determined on these cells (C and E respectively).

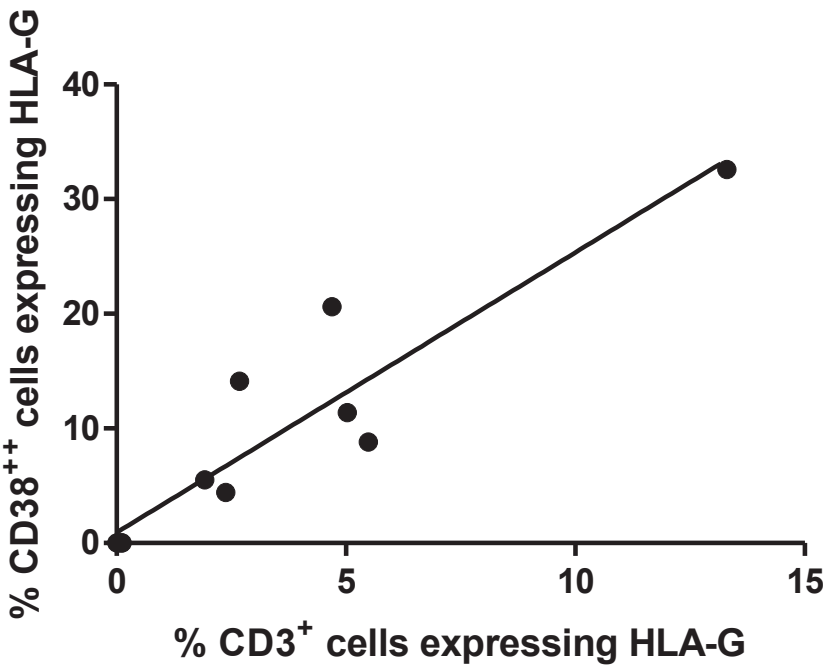

Figure 5-8: HLA-G expression on bone marrow plasma cells and T cells within individual patients.
HLA-G expression on bone marrow plasma cells and T cells was determined by flow cytometry (n=11; r=0.895; p<0.002). Each dot in the graph represents a single patient.

5.2.7 HLA-G acquisition by trogocytosis

Results from the *in vitro* HLA-G trogocytosis assays are shown in Figure 5-9. T cells from myeloma patients (n=8), when co-cultured with HLA-G expressing plasma cells for 2h, were found to express higher levels of HLA-G. However, statistical significance was not achieved (t=1.60; p=0.13). When stimulated with anti-CD3/CD28 beads, T cells did not necessarily acquire higher levels of HLA-G. This suggests that resting T cells and not activated T cells, acquire HLA-G.

5.2.8 HLA-G^{pos} T cells: a new subset of regulatory T cells

Figure 5-10 is a representative flow plot demonstrating the function of HLA-G expressing T cells. When stimulated with anti-CD3/anti-CD28 beads, HLA-G depleted T cells were found to proliferate and proliferation could be inhibited by the addition of HLA-G positive T cells.

Proliferation of HLA-G depleted T cells was also inhibited by HLA-G expressing plasma cells (n=7, mean=24.2%, range 12 – 40%). Proliferation of HLA-G depleted T cells was inhibited by HLA-G expressing T cells (n=9, mean=25.0%, range 3 – 59%), therefore indicating that both HLA-G positive T cells and plasma cells have suppressive properties (Figure 5-11).

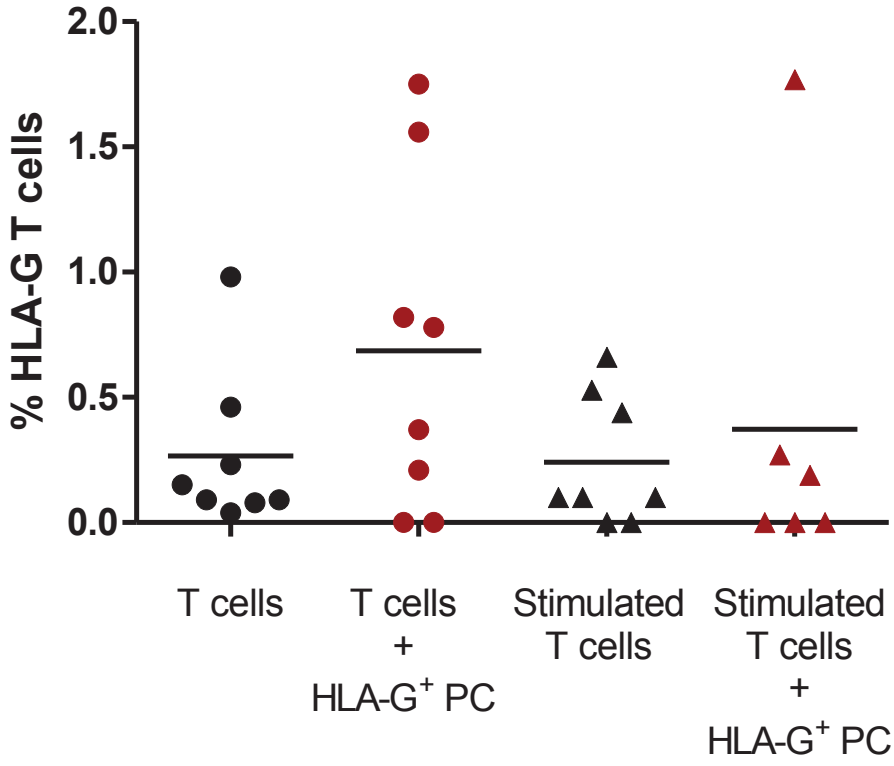

Figure 5-9: HLA-G expression on T cells following trogocytosis.
HLA-G^{neg} T cells were cultured with HLA-G expressing plasma cells (red circles) and/or stimulated with anti-CD3/anti-CD28 beads and co-cultured with HLA-G expressing plasma cells (red triangles). T cells were found to acquire HLA-G. When stimulated, HLA-G^{neg} T cells did not acquire greater levels of HLA-G. Each dot indicates a single experiment and the triangles represent a single experiment involving stimulation of T cells.

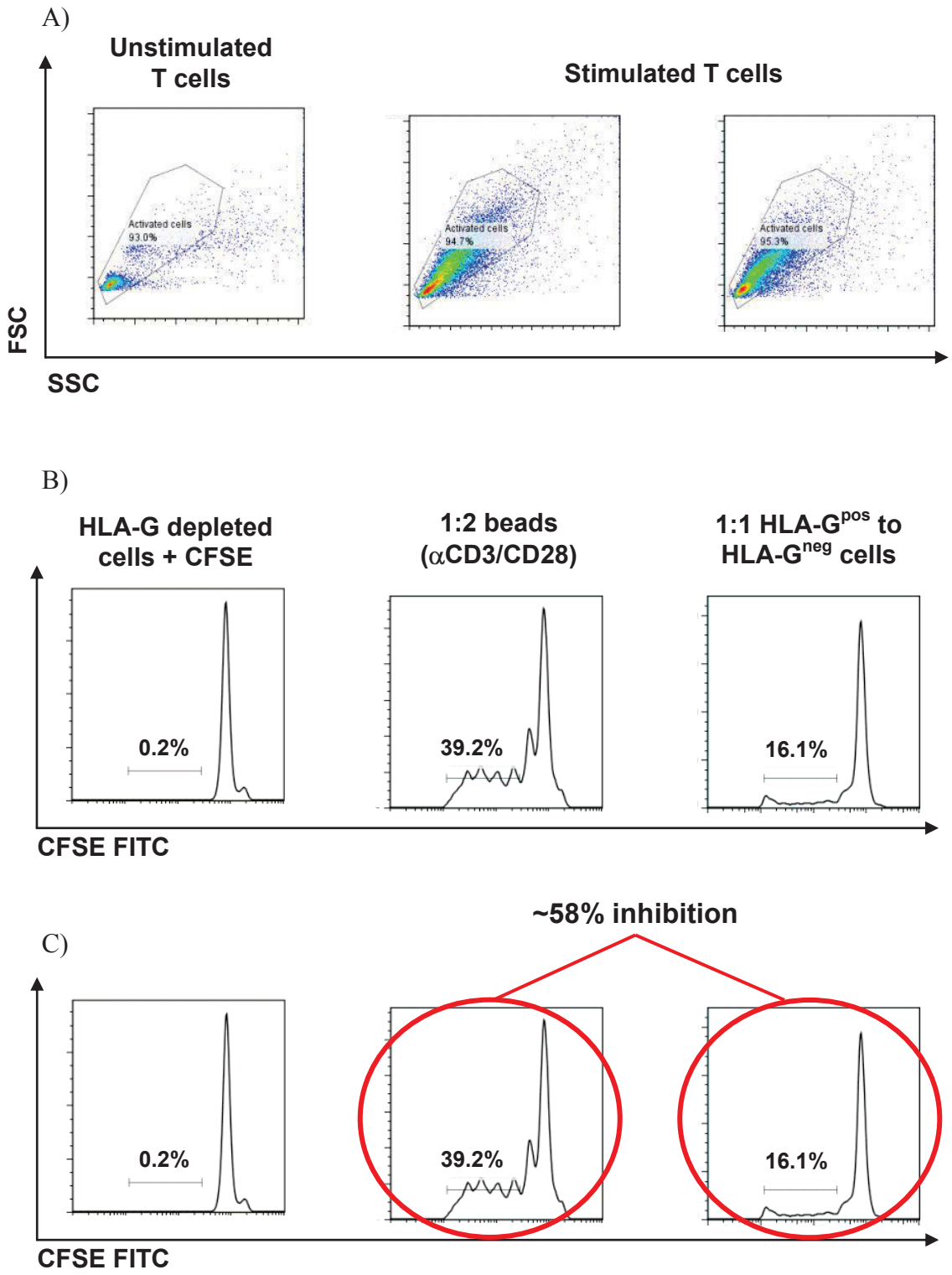

Figure 5-10: Representative flow plots of T cell proliferation and inhibition of proliferation.
HLA-G depleted T cells were labelled with CFSE and stimulated with an equivalent concentration of anti-CD3/anti-CD28 beads. The scatter plot and histograms on the left of each panel represent the unstimulated cells. HLA-G^{pos} flow sorted T cells were cultured with HLA-G^{neg} T cells. Percent proliferation (B) and percent inhibition of proliferation was determined (C). The unstimulated cells were used as a control.

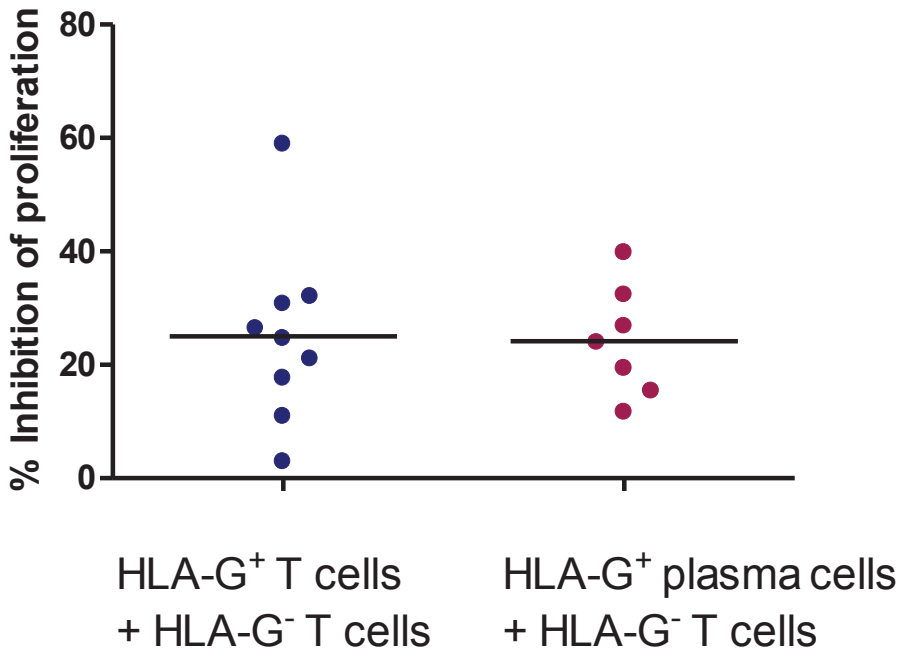

Figure 5-11: Inhibition of proliferation of HLA-G^{neg} T cells by HLA-G expressing cells.
HLA-G^{neg} T cells were labelled with CFSE and co-incubated with either HLA-G expressing plasma cells or T cells. Inhibition of proliferation was achieved by both HLA-G expressing plasma cells and T cells. Inhibition was not achieved when HLA-G^{neg} plasma cells were co-cultured with HLA-G^{neg} T cells.

CHAPTER SIX:

DISCUSSION AND

FINAL SUMMATION

6. Discussion

The acquisition of novel cell membrane proteins during cell-to-cell contact, now known as trogocytosis, has not been widely studied in the context of human cancer. However the studies in this project provide evidence for the clinical importance of this phenomenon as recipient cells may have altered function and may bring about changes in the normal homeostasis between cells. This project has provided, for the first time, information on the incidence and significance of trogocytosis in patients with multiple myeloma and has identified several molecules, derived from malignant plasma cells, which can change the function of recipient T cells. The study has shown that cytotoxic effector cells can become regulatory cells. This offers one explanation for the lack of immunological control by cytotoxic effector cells in malignancies.

6.1 Trogocytosis is a Common Finding in MM

The results from this project have demonstrated that trogocytosis is a common finding in patients with MM compared to other chronic B cell malignancies like CLL and WM. This has not previously been recognised. The transfer of cell membrane proteins from a malignant plasma cell to a T cell was visualised by confocal microscopy and quantified by flow cytometry. Trogocytosis was shown to be predominately uni-directional and independent of both HLA matching and engagement of the T cell receptor. These are novel findings which will add to the current understanding of trogocytosis and its impact in malignancy.

The advantage of using a biotinylation assay, compared to other methods of trogocytosis detection, such as lipophylic dyes, lipids and cytosolic markers (Daubeuf*, et al* 2008, Daubeuf*, et al* 2006, Puaux*, et al* 2006), is that it is easily detected on T cells, B cells and NK cells. Biotin acquisition by lymphocyte populations has not been previously investigated in patients with multiple myeloma.

In Chapter 3, it was clearly demonstrated that trogocytosis occurred between biotinylated plasma cells and either T cells, B cells or NK cells from both normal controls and myeloma patients. However, although all lymphocyte populations

acquired biotin, T cells acquired more biotinylated membrane fragments compared to either B cells or NK cells. It is interesting to note that another study investigating biotin transfer concluded that all lymphocyte populations acquired similar amounts of membrane fragments (Puaux*, et al* 2006). However this study was in the context of post-vaccination mice and not in patients with myeloma or human cancer.

The direction of membrane transfer during trogocytosis has also been investigated by Joly and Hudrisier in which they concluded that trogocytosis is a uni-directional process of plasma membrane fragment transfer from APCs to lymphocytes (Joly and Hudrisier 2003). In this project, the transfer of membrane fragments and proteins was investigated between biotinylated plasma cell lines and T cells as well as biotin transfer from biotinylated T cells to plasma cell lines. Although there was limited bi-directional transfer of biotin, trogocytosis was observed to be predominately uni-directional (Figure 3-9).

The results in Chapter 3 also investigated the interaction of specific cells during trogocytosis. In this study both $CD4^+$ and $CD8^+$ peripheral blood T cells acquired membrane fragments from malignant plasma cells. Rosenitis *et al.* (2010) investigated trogocytosis between biotinylated splenocytes (APCs) and memory T cells, concluding that P14 memory T cells acquired biotinylated membrane fragments. In a murine study by Puaux *et al.* (2006), surface biotinylated EL4 target cells were exposed to CTLs specific for OVA peptides from OT-I mice and they report that $CD8^+$ CTLs, $CD4^+$ Helper T cells, B cells and also activated T cells had acquired biotinylated membrane fragments from EL4 cells.

In another study, 3T3-Db cells were biotinylated and pulsed with gp33, then these cells were co-cultured with P14 CTLs. It was observed that biotin was transferred to P14 CTLs when cultured with gp33-pulsed biotinylated cells compared to culture with un-pulsed cells. It was suggested that CTL recognition of the pMHC complex of donor cells promoted the preferential transfer of selected membrane patches to CTLs (Hudrisier*, et al* 2001). These results suggest that trogocytosis is a process of specific reactivity rather than non-specific interaction.

Transfer of membrane fragments from biotinylated B cells from patients with CLL and WM to normal T cells was also investigated in Chapter 3. Although the transfer or loss of CD20 by B cells in CLL patients has been detected (Daubeuf*, et al* 2010, Iwasaki*, et al* 2011), biotinylation of malignant B cells and the detection of the subsequent transfer to T cells has not been previously examined.

6.2 Co-stimulatory Molecules Transferred by Trogocytosis

Of the plasma cell lines studied, RPMI-8226 was shown to express CD86, with weak expression observed on NCI-H929. These results are in contrast to the results obtained by Robillard *et al.* (1998). In their investigation of CD86 on human myeloma cell lines, they report that CD86 is not expressed on RPMI-8226 cells. On the other hand, an analysis of CD86 expression on myeloma cell lines by Pellat-Deceunynck *et al.* (1996) indicated that CD86 was weakly expressed on all cell lines they tested which included RPMI-8226, NCI-H929, OPM2 and U266. Another study also reported that U266 expresses high levels of CD86 (Yamashita*, et al* 2009). However, CD80 expression was not observed on any of the cell lines tested in this project. Similar results were also observed by other studies (Pellat-Deceunynck*, et al* 1996, Robillard*, et al* 1998). Since CD86 was only expressed on RPMI-8226, this cell line was used in co-cultures with CD86 negative T cells to determine CD86 transfer by trogocytosis.

The expression of B7 and CD28 family markers on plasma cells and T cells was investigated in MM patients and age-matched haematologically normal controls. Of the molecules studied, all but CD80 and CD86 had relatively normal expression. The inference is that the expression of CD80 and CD86 on T cells occurs after acquisition by trogocytosis (Figure 6-1).

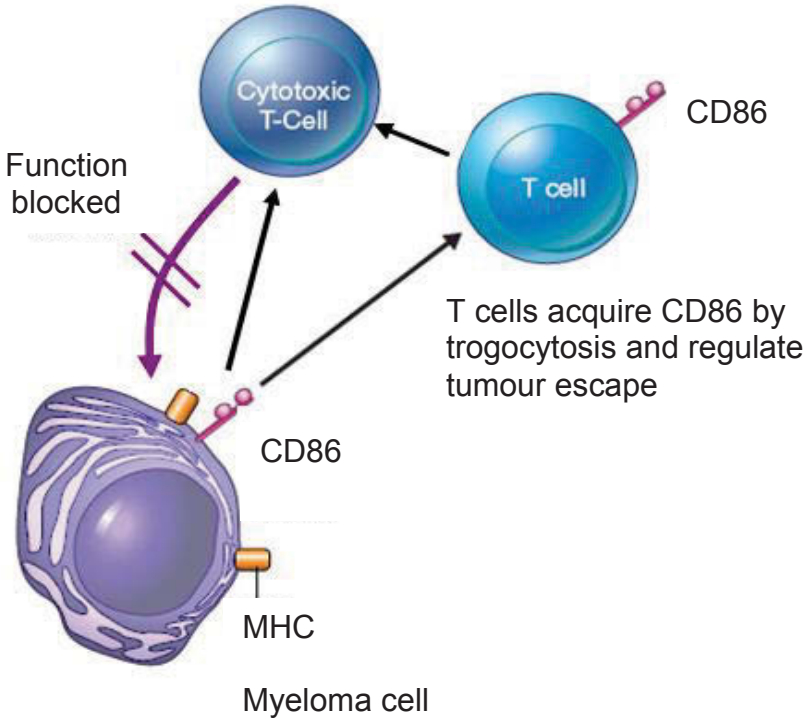

Figure 6-1: Transfer of CD86 to T cells can regulate tumour escape.
T cells acquire CD86 from myeloma cells by trogocytosis. The $CD86^{acq}$ T cells can then act as an inefficient antigen presenting cell or a regulatory T cell. That is, it can either stimulate T cell proliferation or inhibit proliferation of bystander T cells. (Adapted from (Joshua*, et al* 2008).

The expression of CD86 on myeloma cell lines and in myeloma patients has been reported by several groups (Bahlis, *et al* 2007, Brown, *et al* 1998, Pellat-Deceunynck, *et al* 1996, Pope, *et al* 2000, Robillard, *et al* 1998, Yamashita, *et al* 2009). Results from the study in Chapter 4 indicate that bone marrow plasma cells from myeloma patients had a wide range of CD86 expression. These results are consistent with those of Pope *et al.* (2000) and Yamashita *et al.* (2009). The study conducted by Yamashita *et al.* (2009) showed that CD86 expression on $CD38^{++}$ plasma cells was increased in patients with MM compared to MGUS patients and haematologically normal individuals. The normal range of CD86 expression on $CD38^{++}$ cells was established by Pope *et al.* (2000) to range from 27% to 62%. In this study 14% of myeloma patients expressed CD86 on their plasma cells.

Pope *et al.* (2000) have demonstrated the association between CD86 expressing plasma cells and prognosis in multiple myeloma. Overall survival was significantly worse in patients who expressed CD86 compared to patients that had $CD86^{-}$ myeloma. Similar results were observed in several other studies involving haematological malignancies. For example, *in vitro* studies involving the analysis of blasts from patients with AML that expressed CD86 and CD275 showed that they inhibited immunity and patients with CD86 and/or CD275 had a poor prognosis (Tamura, *et al* 2005). Similarly, it was observed that a poor prognosis was associated with expression of these molecules on AML cells and lymphoma cells (Maeda, *et al* 1998, Stremmel, *et al* 1999). The study conducted by Yamashita *et al*. (2009) showed that of the patients studied, 3 patients with MM expressed CD275. It was concluded that myeloma cells that expressed CD86 and CD275 had a greater potential for cell proliferation.

Surface expression of CD80 and CD86 was found to be greater in T cells from peripheral blood of patients with MM compared to age-matched normal controls. The mean plus 3SD was calculated to determine the upper limit of the normal ranges for CD80 (5%) and CD86 (4%). Similar results were also obtained by Brown *et al*. (2004), who reported the upper limit of the normal range of expression of CD80 to be 6% and 4% for CD86 expression.

6.3 The Immunomodulatory Molecule: HLA-G

Results from Chapter 5 indicate that there is a wide range of HLA-G expression on bone marrow plasma cells from patients with MM. Increased HLA-G expression was observed on 20% of T cells from myeloma patients and is not related to stage but is associated with overall survival. HLA-G expressing T cells and plasma cells inhibit proliferation of bystander T cells.

In this chapter, HLA-G was found to be expressed on malignant plasma cells from patients with MM. The expression of HLA-G in other haematological malignancies was rather interesting. HLA-G expressing cells in patients with Non-Hodgkin lymphoma (NHL) were reported to be absent in one study (Poláková, *et al* 2003), whilst another study reported 65-100% HLA-G expression in B-NHL (Amiot, *et al* 1996) and 58% expression in T-NHL (Urosevic, *et al* 2004). Interestingly, a study investigating HLA-G expression in AML showed that there was no expression (Poláková, *et al* 2003) with another study demonstrating HLA-G expression in 20% of patients (Yan, *et al* 2008).

The acquisition of HLA-G on the surface of T cells generates a novel subset of regulatory T cells whose function is switched from effector to immune suppressor. The HLA-G^{acq} T cells may provide an increase in the number of HLA-G^{+} regulatory T cells, which may exert their regulatory effect on neighbouring T cells thereby blocking their function. As a consequence, there would be an induction of immune tolerance and anti-tumour responses would be down-regulated.

It was observed that acquisition of HLA-G by resting T cells was greater than HLA-G acquisition by activated T cells. In a similar study conducted by LeMaoult and colleagues (2007), they demonstrate HLA-G positive areas on the surface of T cells after incubation with HLA-G expressing APCs. These results demonstrate that HLA-G was transferred by trogocytosis. Other studies conducted by several other groups (Carosella, *et al* 2008, Caumartin, *et al* 2006, LeMaoult, *et al* 2007b, Rouas-Freiss, *et al* 2005) have reported that activated $CD4^{+}$ and $CD8^{+}$ T cells, and not resting T cells acquire HLA-G from HLA-G expressing APCs. It is important to note that HLA-G acquisition was investigated between APCs and T cells in all these latter studies.

Phenotypic analysis showed that suppressive HLA-G^+ T cells are CD25 negative, thereby differentiating HLA-G^+ regulatory T cells from $CD4^+$ $CD25^+$ Tregs. The exact mechanism of immune suppression by HLA-G^+ T cells is still unknown. Although it has been hypothesised that it may occur through the interaction with its receptor ILT-2 (Feger*, et al* 2007). In this study, surface expression of ILT-2 was absent on T cells.

The results in Chapter 5 indicate that HLA-G^{acq} T cells behave as regulatory T cells, inhibiting the proliferation of autologous HLA-G^{neg} T cells *in vitro*. HLA-G expressing plasma cells were also found to inhibit T cell proliferation. Similar results on functional assays have been reported by several groups (Feger*, et al* 2007, LeMaoult*, et al* 2007a, LeMaoult*, et al* 2007b, Park*, et al* 2001) and all have concluded that HLA-G expressing T cells inhibit proliferation of HLA-G^{neg} T cells. However, the suppressive property of HLA-G expressing plasma cells reported in this project, is a novel observation and has not been previously reported.

Although studies have shown the association between soluble HLA-G and survival (Amiot*, et al* 1996), the association between membrane bound HLA-G and survival has not been previously demonstrated in patients with MM. A novel and important observation reported here is that patients who have HLA-G expressing plasma cells have a significantly poorer prognosis compared to patients with HLA-G negative plasma cells. In a study of another haematological malignancy, it was found that CLL patients with less than 23% HLA-G expressing cells demonstrated an improved progression free survival (PFS) compared to those patients with greater expression of HLA-G (median PFS: 120 months versus 23 months; p=0.0001) (Nuckel*, et al* 2005). As a result of the studies in this project, HLA-G expression can now be considered to be a prognostic factor for patients with MM.

HLA-G expression in CLL patients has demonstrated tumour cell protection against autologous NK lysis thus permitting tumour development by impairing "anti-tumour immunity" (Maki*, et al* 2008, Nuckel*, et al* 2005, Sebti*, et al* 2007). In the context of oncology, it was reported that HLA-G expression by tumour lesions protected against NK cytolysis correlated with malignancy in breast and ovarian carcinomas as well as

in melanocytic lesions. This suggests that expression of HLA-G may aid in the escape of tumours from immune surveillance.

6.4 Conclusion

The results presented in this thesis provide clear evidence of a significant degree of trogocytosis in patients with multiple myeloma compared to other chronic B cell malignancies. Trogocytosis was shown to be predominately uni-directional and independent of HLA matching. Of all the B7 family of molecules, CD86 is commonly associated with trogocytosis. There is a wide range of HLA-G and CD86 expression on bone marrow plasma cells and peripheral blood T cells of patients with MM which can inhibit proliferation of bystander T cells. HLA-G expression is not related to stage but its association with overall survival is a novel observation.

These studies add further support to the concept that malignant cells evade the immune system by altering the immune response. This study provides good evidence that the uni-directional transfer of a range of cell surface molecules can be considered to be a part of the suppression of the immune response by malignant cells.

6.5 Future Directions

This study investigated the expression and transfer of co-stimulatory and immunomodulatory molecules to T cells from primary myeloma cells. Functional studies were performed in order to understand the functional significance of these molecules. It was observed that T cells expressing HLA-G were able to suppress proliferation of bystander T cells. Further investigation into the mechanisms responsible for this suppression is required which may provide potential therapeutic targets to prevent tumour escape. Any changes during the evolution of the disease should be determined.

T cells that acquire co-stimulatory molecules are capable of behaving as APCs themselves. To investigate this further, studies are required where HLA-G^{acq} or $CD86^{acq}$ T cells are sorted following co-culture with HLA-G or CD86 expressing plasma cells and co-cultured with HLA-G or CD86 negative T cells. Furthermore,

following trogocytosis assays HLA-G or CD86 acquired T cells are to be sorted, stimulated and cultured with HLA-G or CD86 negative T cells. This will confirm that trogocytosis plays a key role in the switch from effector to suppressor T cells. A better understanding of the basic biology of cell interactions involved with trogocytosis opens up new opportunities for novel therapies.

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