In vitro generation of cytotoxic T cells with potential for adoptive tumour immunotherapy

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Abstract

In vitro generation of cytotoxic T cells with potential for adoptive tumour immunotherapy of Multiple Myeloma.

By Wafaa Seifalnaser Sedik Khalaf

Multiple myeloma (MM) is a life-threatening haematological malignancy, which is rarely curable by conventional therapies. Immunotherapy, using autologous antigen specific cytotoxic T-lymphocytes (ASCTL), may represent a useful adjunct therapy for MM. In this study, I assessed the ability of previously described hybrid cell lines, generated by chemical fusion of myeloma tumour cells and the EBV B-lymphoblastoid cell line (EBV B-LCL) HMy2, to induce ASCTL *in vitro* from peripheral blood lymphocytes from patients with MM (and from healthy individuals). The tumour associated antigens (TAAs) hTERT, MUC1, MAGE-C1 and CS1 were selected as potential inducers of ASCTL, based on their prevalence of expression in MM patients. Expression of these TAAs was assessed in four B-LCL/myeloma hybrid cell lines, using real time PCR and flow cytometry, and two of the hybrid cell lines were selected as *in vitro* stimulator cell lines in long-term activated T-cell cultures, using PBMCs from HLA-$A2^+$ healthy donors and multiple myeloma patients. Induction of ASCTLs was assessed by HLA-A2-peptide pentamer staining and flow cytometry, Europium release cytotoxicity assays, and interferon-gamma and perforin ELIspot assays, using known HLA-A2 restricted peptide epitopes of the TAAs. The hybrid cell lines induced ASCTLs to the 4 selected TAAs, after 4 rounds of *in vitro* stimulation with the hybrid cell lines, in PBMCs from both (HLA-A2+) healthy donors and MM patients. In contrast, the (HLA-$A2^+$) myeloma cell line, U266, failed to activate ASCTL *in vitro*. Hybrid cell lines, generated by fusion of EBV B-LCL and myeloma tumour cells, can induce ASCTL in PBMCs from healthy individuals and multiple myeloma patients *in vitro*, and may represent a novel strategy for use in immunotherapy of MM.

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With Love

Wafaa

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Abbreviation

ACT	Adoptive cellular transfer
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloblastic leukaemia
APC	Antigen presenting cell
ASCT	Autologous stem cell transplantation
BCG	Bacillus Calmette-Guerin
B-LCL	B-lymphoblastoid cell line
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDR	complementarity - determining regions
CLIP	Class II-associated invariant polypeptide
CR	Complete remission
CT	Cancer testis
CTLA-4	Cytotoxic T lymphocyte antigen-4
CTLs	Cytotoxic T lymphocytes
CS1	SLAMF 7
Das	Differentiation antigens
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EBV	Epstein-Barr virus
ELISpot	Enzyme Linked ImmunoSpot
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FB	Fixing buffer
FcR	Fc receptor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GBM	Glioblastoma multiform
GalCer	Galactosylceramide
GM-CSF	Granulocyte macrophage -colony stimulating factor
GVHD	Graft versus host disease
GVL	Graft versus leukaemia
HMY2 x tumour cells	HMy2 hybrid of this tumour cells
HAT	Hypoxanthine aminopterin thymidine
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HLA	Human leukocyte antigen
HSCT	Haematopoietic stem cell transplantation
IF	Immunofluorescence
IFNγ	Interferon gamma
IL	Interleukin

ITAM	Immunoreceptor tyrosine-based activation motif
KIR	killer cell Ig-like receptor
mAbs	Monoclonal antibodies
MAGE	Melanoma antigen
MDSC	Myeloid derived suppressor cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
ml	Millilitre
MM	Multiple myeloma
MUC1	Mucin-1
MØ	Macrophage
NK	Natural killer
NPC	Nasopharyngeal carcinoma
PAMPs	Pathogen associated molecular patterns
PASD1	Circadian clock protein PASD1
PBMC	Peripheral blood mononuclear cells
PE	Phycoerytherin
PEG	Polyethylene glycol
PRAME	Preferentially expressed antigen of melanoma
qRT-PCR	Quantitative reverse transcriptase PCR
RCC	Renal cell carcinoma
RNA	Ribonucleic acid
Rpm	Round per minute
RPMI	Roswell park memorial institute
RT	Room temperature
SCT	Stem cell transplantation
SGM	Supplemented growth medium
SR	Spontaneous remission
TAA	Tumour associated antigen
TAP	Transporter associated with antigen processing
TBH	Tumour B-cell hybrid
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
TIL	Tumour infiltrating lymphocytes
TLR	Toll like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cells
T:E	Target: Effector
WB	Washing buffer
WT-1	Wilm’s tumour-1
µl	micro litre

Chapter 1 Introduction

1.1 The role of the immune system in development and progression of tumour

Over the last two decades, improved understanding of constituents of tumour microenvironments and immunosurveillance, have paved the way for manipulation of the immune system in the treatment of various types of tumours. Many clinical studies have demonstrated the existence of tumour specific immune responses in numerous types of cancer, proving its role against tumour progression. However, this response is usually ineffective to protect the host against cancer progression. This is because, in the tumor microenvironment, tumor cells can produce extracellular signals, to promote tumor angiogenesis and enhance peripheral immune tolerance, leading to unregulated cell growth through which tumor cells can spread to all parts of the body through the lymphatic system and blood. Initially, the immune cells present in the tumor microenvironment are trying to dampen the growth and evolution of the tumor cells. This phenomenon is known as immunosurveillance (Burnet, 1957). Tumor immunosurveillance might eradicate various transformed cells before their growth, through patrolling of the body by the effector cells of innate and adaptive immunity, looking for any microscopic tumors before they become evident (Restifo et al., 2012). However, this does not always happen, as in established tumours, the immune cells may promote rather than inhibit tumour development (Whiteside, 2008). This is the reason why many researchers have tried to develop effective immunotherapy, by using different immune system compartments to stimulate antitumor activities of the immune system for treatment of various types of tumour, thereby breaching the peripheral immune tolerance, which has become established around the tumour.

By extending the research about tumor immunology and escape mechanisms, the immunosurveillance concept has been extended to a wider concept known as tumor immunoediting. This process is responsible for the tumour sculpting and host-protective immunity actions in cancer (Dunn et al., 2002). During this process, the immune system can shape the tumour fate into three phases; elimination, equilibrium and escape, and can be abbreviated into the three Es of cancer immunoediting. Each of these phases has different features. The first event is Elimination, which relates to immunosurveillance, meaning identifying and removal of the cancerous cells by the immune mechanisms. The second phase is Equilibrium, that refers to a state of unbiased interaction between tumour cells and the immune effectors (tumour remains at a subclinical level without development or elimination). During this phase, the tumour cells are being edited into a

new variant, which increases resistance of the tumour to the immune system, leading to tumour growth. The third step is Escape, which indicates tumour progression after evading the tumour immunosurveillance (Mittal et al., 2014). Numerous immune compartments are involved during immunosurveillance activity, and are discussed in the following sections.

1.2 Different components of tumour immunosurveillance:

The immune system, responsible for biological defences against infection or to avoid any unwanted biological invasion, is characteristically categorized into innate immunity and adaptive immunity. In this section, different components of innate and adaptive immunity, which are of crucial importance in detection and removal of the tumour cells, are detailed. The following chart (Figure 1.1.) illustrates important compartments of the immune system.

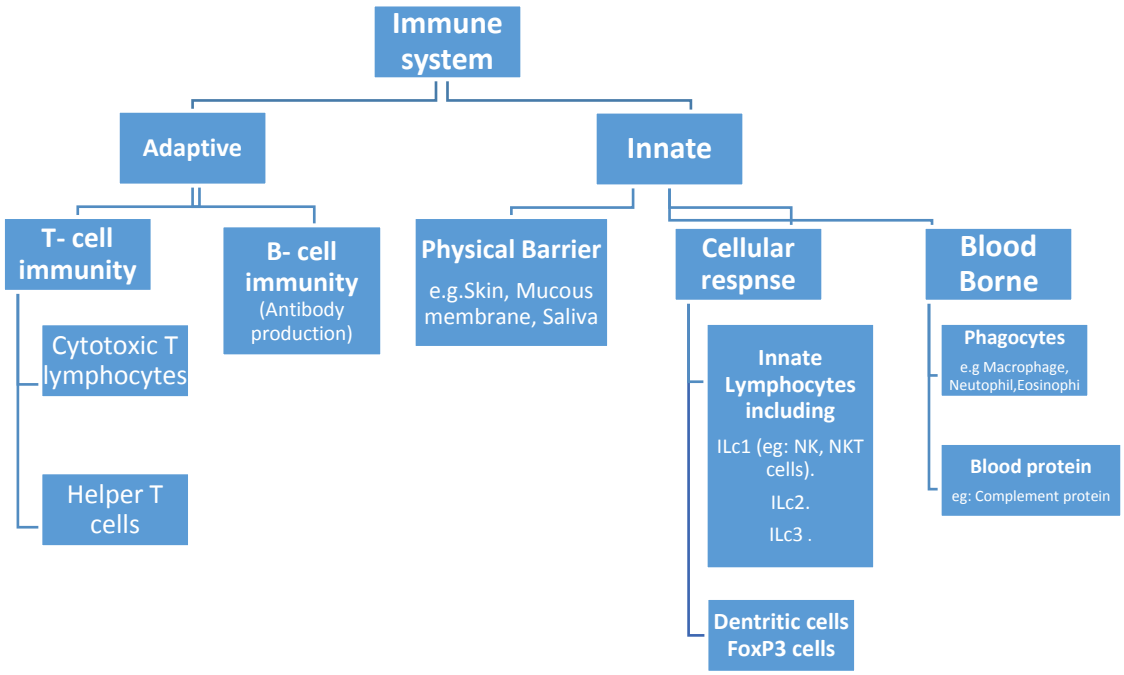

Figure 1.1: Different components of the immune system. *While typically the depicted distinction is made in relation to the antimicrobial response, all factors have a role in chronic inflammation. There is communication between both arms of the immune system, whereby the innate modulates and prepares the adaptive immune system.*

1.2.1 Innate tumour immunosurveillance

The innate immune response is normally characterised by its non-antigen-specific nature; however, it can discriminate self and nonself, and generate fast acting immune responses. Many components are involved in innate immunity, for instance physicochemical barriers (e.g. tears, mucosa, skin, ciliary movement of the cilia of mucosa, mucous secretions, etc.), cytotoxic cells (including natural killer (NK) and NKT cells), phagocytic cells (such as neutrophils, macrophages, monocytes), and blood proteins, for example the complement system, and a set of chemokines and cytokines, which work cooperatively to raise the antitumor immune response. Natural antibodies are low affinity immunoglobulins, which are part of the innate system and provide initial reactivity before the specific antibody response is mounted. The distinct features of innate cellular immunity usually refer to diversity of myeloid and lymphoid cells which can exert an effector function through a range of germline-encoded receptors (Vivier et al., 2011).

1.2.1.1 Natural Killer Cells

Natural killer (NK) cells originate in the bone marrow from the common lymphoid progenitor cell. They can be discriminated from the cells of the adaptive immune response, as they lack CD3 and CD19 surface marker expression, and antigen-specific cell surface receptors, so they are classified as a component of the innate immune system, whose action is completed in the absence of specific antigenic stimulation and can induce direct lysis of tumour cells and virus-infected cells, without prior sensitisation with specific antigen. So, they are characterised as a direct cytolytic effector cells (Annunziato et al., 2007; Vivier et al., 2011). Natural killer cells normally express an array of stimulatory receptors such as NKG2D (their ligands are MHC-class-I-related molecules expressed on the target cells), in addition to inhibitory receptors such as killer cell Ig-like receptors (KIR). The ligands of these receptors are members of HLA class I (Iannello et al., 2008; Cooper et al., 2001). Consequently, modification or down regulation of MHC class I molecules expression on the surface of the tumour cells, causes reduction of the inhibitory signals of NK cells and dominance of their stimulatory molecules (Cooper et al., 2001). Also, NK cells can express many lymphoid markers, such as NK1.1 or NK1.2, and express CD56 and CD16 (expressed also in neutrophil). The role of CD56 is to mediate the adhesion of NK cells to their target cells. NK cells mediate their cytotoxic effect either through antibody dependent cellular cytotoxicity or though cytolytic protein secretion. The antibody dependant cellular cytotoxicity function of NK cells is fulfilled

by using CD16, which can interact with the Fc fragment of the antibody (Watzl and Long, 2010; Leibson, 1997). The second mechanism of cytotoxicity is mediated through secretion of cytolytic granules including perforin and granzymes, leading to either osmotic cell lysis or apoptosis of the target cells (Topham and Hewitt, 2009).

Walzer *et al* (2005) described NK cells as major producers of many cytokines, including interferon-γ (IFNγ), some immunosuppressive and proinflammatory cytokines, for instance interleukin (IL)–10 and tumour necrosis factor–α respectively. In addition to that, NK cells can secrete some growth factors such as granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and IL-3 (Walzer et al., 2005). Furthermore, they require some priming factors, such as IL-15, IL-12 or IL-18, in order to perform their function. Regarding the function of NK cells, in addition of their direct cytotoxicity effect, they help to shape the response of T cells, either through IFNγ production or through indirect effects on DCs, which cause stimulation of T lymphocytes. On the other hand, they can produce some inhibitory cytokines as IL-10, which has an inhibitory effect on T cells and macrophages (Vivier et al., 2011). The following image (Figure 1.2) shows how NK cells contribute to immunity.

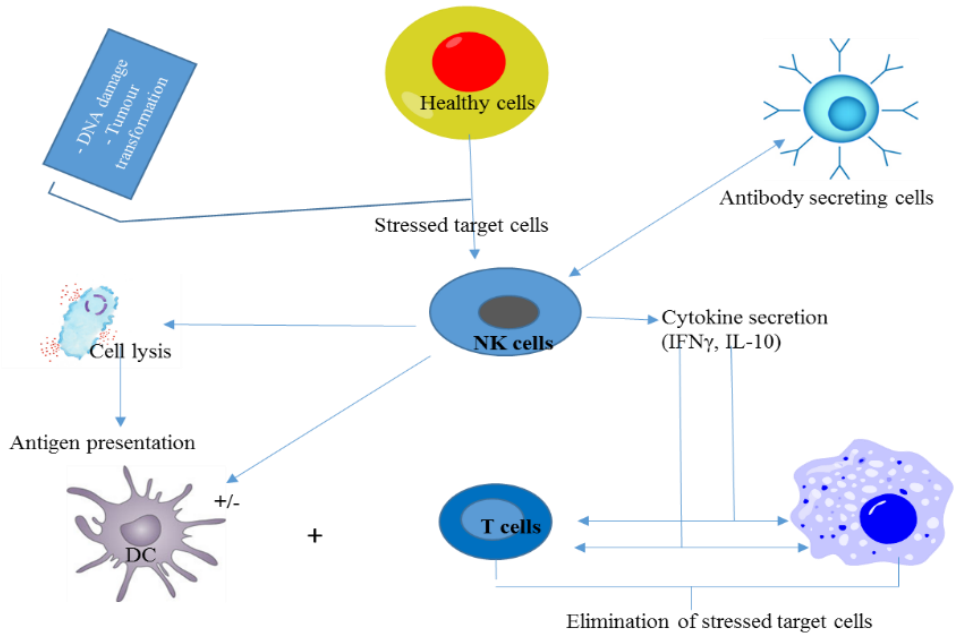

Figure 1.2: The role of NK cells in tumour immunosurveillance. *NK cells can stimulate or supress T and B cells of the host. In addition to that, NK cells mediate cytokine production and cytotoxicity. So, NK cells participate in many different ways in immunity to fulfil these functions. Image adapted from (Vivier et al., 2011).*

1.2.1.2 Natural Killer T cells (NK T cells)

Natural Killer T (NK T) cells are innate lymphocytes, as they function as a part of innate immune system, and express NK cell markers including NK1.1 and CD94 (Wu et al., 2009). They also exhibit features of T lymphocytes, as they can express CD3 cell surface marker, and somatically rearranged antigen receptors, however of lower diversity than those of T lymphocytes (Brennan et al., 2013). Generally, NK T cells recognise the lipid or glycolipid antigens (particularly alpha-galactosylceramide (α-GalCer)) presented in the context of CD1d (MHC class I like molecules) (Arrenberg et al., 2009). There are two major subsets of NKT cells. NKT cells type 1, play an immunostimulatory role through production of Th1- pro-inflammatory cytokines (e.g., IFNγ, TNF-α). NKT cells also can play an immunosuppressive role, which is the main function of type II NKT cells, through production of Th2- anti-inflammatory cytokines such as IL-10. Hence, type I and type II NKT cells counter-regulate each other (Terabe and Berzofsky, 2008; Robertson et al., 2014). A hallmark of NKT cells upon antigenic stimulation is their production of various types of cytokines, such as (IFNγ), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukins (IL)-2, -4, -10, -13, -17, -21, and 22, and TNF-α. Thereby they determine the milieu of subsequent immunity. They are mostly CD4 and CD8 double negative cells, however a considerable significant portion are $CD8\alpha^+$ positive cells (Wu et al., 2009). NKT cells are of importance in protection against tumour growth by enhancing tumour surveillance. They can supress tumour through production of IFNγ, which activates NK and $CD8^+$ T cells. They also, contribute to tumour surveillance by activating dendritic cells to produce IL-12 (Terabe and Berzofsky, 2008). Some reports described reduced target cell apoptotic effect of NKT cells after activation with α-GalCer and blocking the production of perforin release, suggesting that perforin release was the chief mechanism through which NKT cells mediate their cytotoxic effect (Kawano, 1998).

1.2.1.3 Antigen presenting cells

Antigen presenting cells (APCs), including dendritic cells (DC), B cells and macrophages (Mφ), are of crucial importance for the innate immune system, and also they are vital for effective adaptive immune responses. It is well accepted that MHC class II molecules are constitutively expressed on the surface of APCs. In contrast, MHC class I molecules are expressed on the surface of all nucleated cells. Moreover, the level of expression of MHCs expression on the surface of the APCs is always controlled by the efficiency of their

antigen uptake. Furthermore, professional APC constitutively express important costimulatory molecules such as CD80 and CD86. The expression of all these molecules results in potent ability of APC to present the processed endogenous and exogenous antigens (including tumour antigens), using their MHC molecules, in addition to delivering of costimulatory signals, leading to activation of antigen specific T lymphocytes. However, non-professional APCs express MHC class I, but do not constitutively express MHC class II, and lack the ability to express the co stimulatory molecules on their surface, so they can only be recognised by previously activated T lymphocytes (Jensen, 2007).

Immature DCs capture antigens, and undergo maturation when inflammatory stimulus and danger signals, including PAMPs (Toll like receptor TLR agonists), pro-inflammatory cytokines, or heat shock proteins are sensed. This maturation leads to upregulation of the expression of MHC class II molecules, CD80 and CD86 co-stimulatory molecules. After that, APCs migrate to the lymph nodes, where they present processed antigen to naïve CD8+ and $CD4^+$ T cells specific for the same antigen, which elicits their T cell receptors' signalling and activation. In addition to that, professional antigen presenting cells such as mature DC produce many cytokines, including IL-12. The produced IL-12 participates in the differentiation of the activated T cells into T helper1, which in turn act as effector cells and produce interferon-γ (IFNγ). The produced IL-12 also enhances the proliferation of NK and NKT cells. Therefore, APCs lead to activation of both innate and adaptive immune system (Medzhitov, 2001; Wilson et al., 2004). It is possible that activated DCs migrate to a lymphoid structure similar to a lymph node that can be found in the vicinity of the tumour (tertiary lymphoid organ). It is thought that cancers with this histopathological feature have a better prognosis, because antigen presentation is active and elicits tumour antigen specific T cells.

Dendritic cells (DC) are the most potent antigen presenting cells of the immune system, and are the main primers of T cells by presenting antigens processed from other cells, such as virus-infected cells or tumour cells. So, they are in focus when developing different vaccines to be used in immunotherapy against tumours (Melief, 2003; Ho et al., 2006; Rosenblatt et al., 2013). Also, DC and macrophages can act as phagocytic cells, because their surfaces have many receptors for complement and the Fc region of antibody. Therefore, they can undergo a process of opsonisation of tumour cells by engulfment of the antibody attached tumour cells (Kelly et al., 2005). Regarding B cells, direct binding

of membrane Ig to antigen enhances antigen presentation and dependent T cell activation. Helper T cells activate B cells through secretion of lymphokines, which are growth and differentiation factors, and additional signals which enhance B cells response to lymphokines, such as CD40-CD40 ligand interaction (Parker, 1993).

1.2.1.3.1 The importance of CD80 and CD86 as costimulatory molecules:

The costimulatory molecules CD80 and CD86, members of B7 family, are cell surface glycoproteins, expressed on the surface of professional APCs, such as DCs, B cells and Mϕ. They act as a ligand for CD28 and CTLA4 receptors on the T lymphocytes, and regulate their stimulation (Hathcock et al., 1994). Greene and Cosand (1996) described the presence of a single binding site of each monomer of CD80 and CD86 for CTLA4 or CD28, and that binding of both CD80 and CD86 required homodimers of CD28 or CTLA4 for T cell activation. Also, they showed that CD28 and CTLA4 binding are both characterised by rapid on and off dissociation rates, and that binding may control the signal transduction by regulating the time of receptor occupancy. In addition to that, they suggested low affinity of binding of both CD80 and CD86 to CD28, however, their binding is of higher affinity with CTLA4. The binding affinity of CTLA4 to CD80 and CD86 is 500 to 2500 times stronger than CD28, which suggests that the described competitive inhibition is the principal mechanism of inactivation of T cells (Chan et al., 2014). Also, the dissociation rate of CD86 is higher than that of CD80 (Greene and Cosand, 1996). Ellis et al. (1996) identified that the structure of the binding sites of CD80 and CD86 on CD28 and CTLA4 have some similarity, but are not identical. Additionally, the resting T cells express CD28 only, whereas, activated T cells express CD28 and CTLA4 (Chan et al., 2014). These costimulatory molecules have been described as the most extensively characterised T cell costimulation pathway.

Resting B cell express little or no costimulatory molecules. However, after activation with different stimuli, including ligation of CD40, MHC class II or contact with the antigen via antigen receptors, the expression of these two costimulatory molecules is rapidly upregulated (Palena, 2004).

1.2.2 Adaptive immunity

The adaptive immune system is distinguished by self and non-self-discrimination, antigen specificity, and ability to respond more vigorously and rapidly to the secondary exposure of the same antigens i.e.: immunological memory (Burmester, 2003). Adaptive immunity

is categorised into two types of cellular immunity, B and T lymphocytes. These lymphocytes clonally express antigen specific receptors that are produced by somatic recombination, which make T cell receptor (TCR) and B cell receptor (immunoglobulin). Functionally, naive B and T cells encounter antigens present in specialised lymphoid organs and go through a process of cell proliferation and maturation in a suitable environment before differentiation into effector cells.

1.2.2.1 T lymphocytes

T cells represent the core of the adaptive immune system, with a unique ability to recognise and respond to a specific peptide antigen. They represent 48.7–77.1% of peripheral blood mononuclear cells (PBMCs) (Autissier et al., 2010). T lymphocytes can be mainly categorised into $CD4^+$ or $CD8^+$ lineage, according to their phenotypic characterisation and their effector functions. Cytotoxic T cells ($CD8^+$ T cells) mainly recognise peptides produced from degraded endogenous (or less commonly, exogenous antigen), presented in the context of major histocompatibility complex class I (MHC class I) molecules, whilst helper T cells (CD4+ T cells) recognise the peptide presented in the context of MHC class II molecules (Kisielow, 1988; Von Boehmer and Kisielow, 1990). Usually, T lymphocytes can recognise such antigens through their αβT cell receptor. Antigen receptors on T lymphocytes are clonally distributed, meaning that each clone has a unique TCR and recognises different specific antigen/MHC complex. Large numbers of distinct T cell clones form the entire T cell repertoire (Davis, 1988). To appreciate how this vast diversity have been generated, we firstly have to discuss the structure of TCR.

1.2.2.1.1 T cell receptor (TCR)

Antigen recognition by naïve T lymphocytes is the initiating step in triggering a specific immune response. This recognition is the function of specific membrane bound, heterodimeric, T cell receptors, which are comprised of α and β chains. These chains are bound together by disulphide bonds. Each α and β chain is composed of a constant (C) and a variable (V) region. The variable domains V α and V β are folded together forming the monovalent antigen-binding site, which varies between different clones of lymphocytes. The constant domain is involved in structural integrity and transmission of effector functions; therefore, it is relatively conserved within all clones. However, in the variable region, most variability is concentrated in defined stretches, which are called complementarity - determining regions (CDRs), or hypervariable regions (Figure 1.3).

Within the V region of each chain of the TCR, there are three CDRs (CDR1, CDR2 and CDR3). Amongst different T cell clones, CDR3 is the most variable domain (Goldrath, 1999; Wucherpfennig et al., 2010; Smith et al., 2013).

The maturation of T cells is intimately linked to the generation of the numerous diversity of TCR. There are potentially as many as 10^{11} different clones of T cells which, importantly, arise before an encounter with antigenic material. In fact, there are not enough genes in the human genome to encode for all of these different clones, however, the immune system develops a mechanism which enables a limited number of genes to produce these extremely diverse lymphocyte receptors (von Boehmer & Kisielow, 1990).

The first suggested mechanism to produce such diversity is known as combinational diversity. This mechanism occurs in the germ line, where the gene segments responsible for encoding the antigen receptors (V, D and J gene segments) are separated, and undergo somatic recombination throughout their maturation, ensuring the diversity of the antigen receptors produced. This type of diversity is limited by the number of the available V, J and D gene segments. The other type of the diversity is the Junctional diversity, where the nucleotide sequence of the V, J and D gene segments undergo several changes such as deletion or insertion of some nucleotides. This type provides unlimited diversity (Goldrath, 1999; Abbas et al., 2014).

The main function of the somatically rearranged TCR is antigen recognition, however, it does not have the ability to transmit the signals to T cells. Therefore, TCR is usually associated with a complex of CD3 co-receptor and zeta (ζ) proteins, which have the ability of transmitting signals when the TCR recognises its own specific antigen. CD3 is expressed on all T cells, and can be used as a cell surface marker for identification of T cells (Wucherpfennig et al., 2010; Chlewicki et al., 2005). Moreover, transmitting the activation signals to the T lymphocytes requires the association of co-receptor molecules CD4 and CD8 that bind to non-polymorphic regions of MHC molecules, leading to triggering of antigen-induced cytokine production (Miceli and Parnes, 1991; Li et al., 2013).

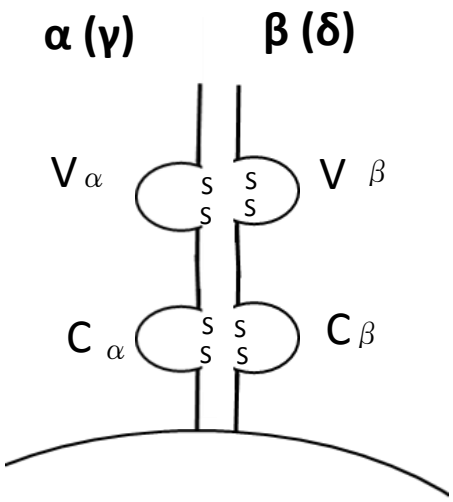

Figure 1.3: T cell receptor structure.

1.2.2.1.2 Development of T cell repertoires

Commitment of hematopoietic stem cell progenitors to T cell lineage involves three different events: proliferation, maturation and selection. Firstly, double negative T cells ($CD4^-$ and $CD8^-$ T cell progenitor) migrate from bone marrow to the thymus (Figure 1.4), where they receive numerous micro environmental signals and undergo extensive proliferation to express both CD4 and CD8 co-receptors, and become CD4+ and CD8+ T cells. Then maturation step; throughout T cell development in the thymus, sequential TCR gene rearrangements take place, mainly by varying the sequence of the nucleotide at the recombination site, in addition to insertion or deletion of nucleotides at the junctional segment between V and J in case of α; or V, J and D loci in case of β (or gamma γ and delta δ in case of γ δ T cells), which lead to more diversity and endless repertoire of specific T cell receptors as previously described. The last step is the selection, where T cells which recognise self-peptide – MHC complexes with low affinity are positively selected, and T cells which recognise self-peptide MHC complexes with high affinity are negatively selected or deleted. The positively selected cells whose TCR recognise MHC class I reserve the expression of CD8 co-receptor and lose the ability to express CD4, and are committed to $CD8^+$ lineage. Conversely, T lymphocytes which recognise MHC class II – peptide complex preserve the expression of CD4 co-receptor and lose the ability to express CD8, and are committed to the CD4+ lineage (Von Boehmer and Kisielow, 1990; Davis, 1988; Shortman, 1996; Goldrath, 1999; Starr et al., 2003).

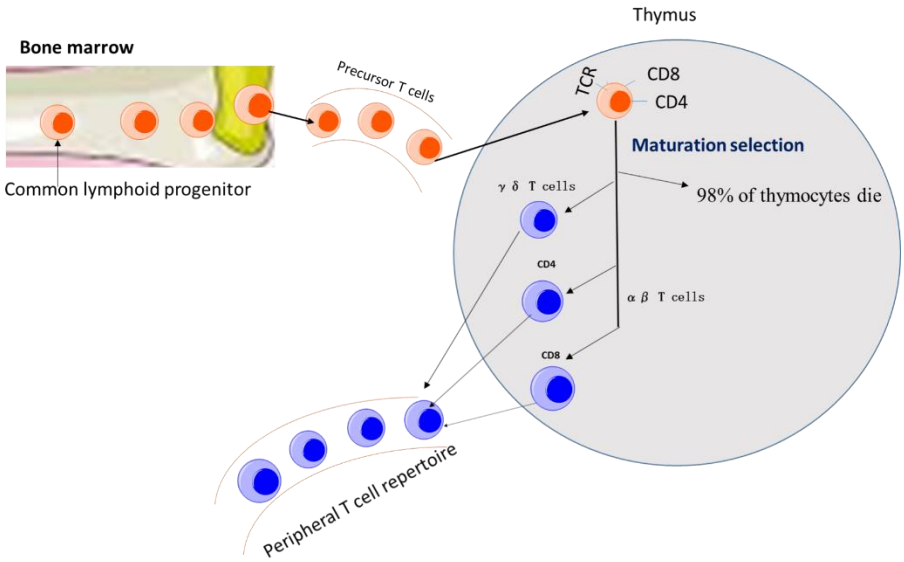

Figure 1.4: T cell development in the thymus. Figure modifies from (*Malmstrom et al., 200).*

1.2.2.1.3 Activation of T cells

Naive T cells have the ability to recognise the antigen, but they do not have the ability to perform effector functions. Recognition of the antigen by naive cells is the initiating event in the process of T cell activation. On encounter with the specific antigen presented by autologous MHC molecules, naive T cells receive additional signals mediated through the interaction of the costimulatory molecules of APCs, such as B7 (CD80 and CD86) and CD40 with their receptors (CD28) and CD40 L (CD154) respectively, resulting in their proliferation and clonal expansion. A fraction of those activated T cells is converted into effector cells, with the ability to perform cytotoxic function (effector $CD8^+$ T cells) or cytokine release (effector CD4+ T cells). $CD4^+$ T cells, also, can influence the antibody mediated immune response. The other fraction of those cells which experienced the antigens are turned into a memory cell pool, which are ready for rapid and vigorous response upon repeated exposure to the same antigen. Also, the activation of T cell responses is affected by the presence of inflammatory cytokines. Curtsinger *et al.*, (1999) studied the effect of some cytokines on naive T cells. They found that the proliferation of $CD4^+$ and $CD8^+$ T cells in response to antigen and IL-2 required IL-1 and IL-12 respectively. These results support the concept that distinct inflammatory cytokines are required as third signals on naive $CD4^+$ and $CD8^+$ T, with antigen and IL-2, to activate their differentiation and clonal expansion (Curtsinger at al, 1999). After elimination of the antigen, all the activation stimuli are downregulated through induction of the expression of the regulatory receptor CTLA4, which binds to B7 molecules with higher

binding affinity than CD28 molecules. As a result, the activated effector cells die, and the immune system is returned to its resting state (Brenchley et al., 2002; Abbas et al., 2012).

1.2.2.1.4 Major histocompatibility molecules (MHC) and their importance

Major histocompatibility (MHC) molecules are membrane bound proteins that present peptides derived from microbial or tumour antigens, for activation of antigen specific T lymphocytes (MHC restriction), as a first step in activation of adaptive immune response. There are two main subsets of MHCs, MHC class I and MHC class II. MHC class I molecules present the antigen-derived peptide for activation of $CD8^+$ T lymphocytes, while the antigen-derived peptide required for activation of CD4+ T lymphocytes are presented on the context of MHC class II molecules. MHC molecules were discovered as a genetic locus, which was a principal determinant of the graft rejection, in the case of graft exchanged between individuals with mismatched MHC molecules (Abbas et al., 2012; Coico and Sunshine, 2015). In humans, MHC class I is termed HLA class I, and encoded by three highly polymorphic genes (HLA-A, HLA-B, and HLA-C). HLA-A2 is the most common MHC class I molecule. It is noteworthy that HLA-A2 is of critical importance for restriction of antigen specific cytotoxic T lymphocytes recognition in tumour immunity (Mimura et al., 2011). The loss of expression of this allele from tumour cells may be explained by the escape mechanism from recognition by T cells, which suggests its significant role in tumour immunosurveillance (Komlos et al, 1995). Moreover, the frequency of HLA-A2 gene expression is high among different ethnic groups (Krausa & Browning, 1996). Consequently, HLA-A2-restricted CTL epitopes might be valuable for the immunotherapy of many diseases all over the world.

1.2.2.1.4.1 Structure of MHC class I and MHC class II

The two classes of MHCs are similar in overall structure, but different in their subunits, as shown in Figure (1.5). MHC class I molecules contain a peptide binding cleft, which is formed of α1and α2 domains, through which a peptide of 8-11amino acid can be accommodated. The polymorphic residues of MHC class I molecules are the amino acid located in the α1 and α2 domains, which vary among different individuals' MHC molecules. Variation of these residues either leads to variation in the floor of the peptide binding cleft that results in variation of the peptides bound, or variation in the sides or the top of the cleft, thus contributing to the selection of the T lymphocytes via their T cell receptors. In MHC class I, the α3 domain is the binding site of CD8 co-receptor of T cells,

which is important for CD8+ T cell activation. The α3 domain is associated with B2 microglobulin, which stabilize the binding groove of MHC class I. MHC class I is constitutively expressed on almost all nucleated cells. Downregulation of MHC class I expression is one of the immune escape mechanisms that can happen during malignant conversion (Bjorkman and Parham, 1990; Bubeník, 2004; Janeway Jr et al., 2012; Abbas et al., 2012). Regarding the MHC class II molecule, it consists of an α and β chain, and the peptide biding cleft is composed of the α1 and β1 domains at the distal end of the chains. The peptide binding cleft of MHC class II molecule can accommodate a peptide of 10-30 amino acid length. The β2 domain is a non-polymorphic domain, which contain the binding site of CD4 coreceptor of T cells. MHC class II molecules are expressed mainly on the surface of antigen presenting cells (Bubeník, 2004; Abbas et al., 2012; Janeway Jr et al., 2012).

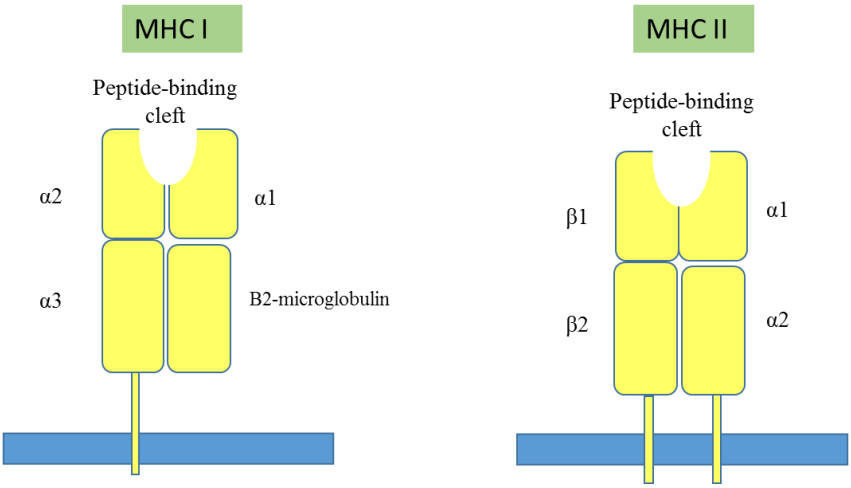

Figure 1.5: schematic diagram of MHC class I and MHC class II molecules. *Figure adapted from (Janeway et al., 2012).*

1.2.2.1.5 T Lymphocyte categories

By virtue of the abundant phenotypic and functional variability of T lymphocytes, they can be divided into different functional categories. There are two main types of T lymphocytes: CD8+ T lymphocytes and CD4+ T lymphocytes. In general, CD8+ T lymphocytes mediate the specific and direct cytotoxicity of the target cell, for instance virally infected or tumour cells, through responding to specific peptides presented in the context of MHC class I of the target cell, as described previously. Two basic approaches of CTL mediate their cellular cytotoxic effect. The first pathway is a granule dependent cytotoxicity, through release the lytic granules including perforin, which has the ability

to polymerise in the presence of calcium and form cylindrical pores in the target cell membrane. The perforin pores act as passive conductors of granzyme molecules, and allow an ionic exchange, which leads to an osmotic imbalance and cell death. Granzyme granules activate the caspase pathway, leading to DNA fragmentation and protein hydrolysis. In the second approach, the cytotoxic effect is arbitrated through induction of interaction between Fas receptor (expressed on the membrane of the target cell) and Fas-L (membrane-bound molecule expressed on T cell surface). These different pathways induce apoptosis and destroy the target cell (Groscurth, 1998). Additionally, cytotoxic CD8+ T cells produce IFNγ, which is important for T helper cell activation, in addition to inhibition of viral replication and induction of macrophages activation (Janeway et al., 2001).

In contrast, CD4+ T cells play a crucial role in achieving an effective immune response through release of cytokines. Naïve CD4+ T cells are activated throughout the interaction with peptide- MHC class II complex. CD4+ T cells are differentiated into distinct categories, according to modification of specific genes and activation of the appropriate lineage specific transcription factors, coupled with the produced cytokine signalling. Figure 1.6 shows the major subtypes of CD4+ T lymphocytes, which are T helper1 and T helper 2. Beside these categories, other categories have been classified, such as regulatory T cells, T-helper 9, T-helper 17 and follicular helper T cells. Each of these lineages mediates its effector function through secretion of a particular set of cytokines (Luckheeram et al., 2012; Mosmann and Coffman, 1989). The category, Th1 normally produces IL-2, IFNγ and TNF-α, and therefore has a principal function in activation of CTL, so it is of crucial importance in combating intracellular pathogens and tumour cells. Moreover, Th1 cells and IFNγ production can be stimulated in the presence of IL-12. The second subset are Th2 cells. These cells principally produce IL-4, IL-5 and IL-13. Th2 cells activate the antibody mediated immune response through stimulation of B cell proliferation. The Th2 cells are also important for clearance of extracellular pathogen parasitic infection, by releasing IL-4 and IL-13. Th2 cells are stimulated by cytokines such as IL4 (Min et al., 2004).

Some reports have demonstrated an effector function of Th17 in degradation of cancer cells, by activation of CTL through recruitment of the DC to the tumour site (Martin-Orozco et al., 2009). In addition, Th 17 cells have a protective function against extracellular microbes, in particular. Th17 cells are characterised by the release of effector

cytokines, such as IL-17 (or IL-17A), IL-17F, and IL-6 (Weaver et al., 2006). In respect to Th9 cells, Kaplan et al. (2015) described the antitumor effect of Th9 cells in melanoma and in lung carcinoma by increase lymphocyte infiltration in tumour and increase the antitumour activity of mast cells, which is IL-9 dependent. The development of Th9 cells requires the presence of TGF-β and IL-4 in order to reprogram T helper cells to produce IL-9. IL-9 is a mast cell growth factor, which has a potent anthelminthic effect (Bonilla and Oettgen, 2010; Kaplan et al., 2015).

Another category of $CD4^+$ T cells, whose function is to suppress the deleterious effect of $CD4^+$ T cells, is Treg cells. The suppressive activities of T reg cells is antigen-specific, just as the rest of T cell subtypes. Regarding the molecular mechanism for their development, Hori (2003) described Foxp3, an autoregulatory gene, which encodes a transcription factor, important for development of regulatory T cells (Hori & Sakaguchi, 2003). Treg, also, constitutively express CD25 at high level, whereas, conventional CD4 T cells transiently express CD25 at low level, following activation. Moreover, Treg express low or no IL-7R. So, the presence of FoxP3, and a high expression of CD25 in the absence of IL-7 R, can provide good markers to distinguish T reg from the other T cells (Zhu and Paul, 2008). Treg cells suppress autoimmunity through cell contact dependent and cell contact independent mechanisms. Cell contact dependent mechanisms include expression of CTLA4 or inadequate activation of DC. Cell contact independent mechanisms include secretion of some inhibitory cytokines such as IL-10 and TGF-β.

Treg cells have a dual roles in tumour immunology, as they are a significant contributors to tumour growth, and enhancement of the immune escape (Whiteside, 2015). The suppressor function of T reg is mediated by inhibition of the responder cell activation through deactivation of APCs by CTLA-4/B7 interaction, or by secretion of inhibitory cytokines, or through competition for cytokines that signal via γ-chain cytokine receptors (IL-2, IL-4 and IL-7). On the other hand, they are important to control inflammation and tissue damage associated with cancer (Whiteside, 2015; Zou, 2006).

Another way of characterizing T cells is as naïve or memory T cells. Memory $CD4^+$ and $CD8^+$ T cells provide a memory function for the previously experienced antigen, a unique feature of adaptive immunity. These cells can respond to the repeated exposure of specific antigen faster and much vigorously than naïve cells can do. Memory cells comprise two main categories, central memory T cells and effector memory T cells, according to expression of CCR7 cell marker. Central memory T cells ($CCR7^+$ T cells) migrate through

the lymphoid organs, and can be activated only upon re-exposure to the antigen. The effector memory CD8+ T cells ($CCR7^-$ memory T cells) migrate to the peripheral tissues, and have the capability to produce perforin granules. Effector memory CD4+ T cells can produce different cytokines, such as IFNγ and IL-4 (Sallusto et al, 1999; Mueller et al., 2013).

Furthermore, memory cells are distinguished from naïve T cells on the basis of expression of isoforms of the leukocyte tyrosine phosphatase CD45, thus the cells that express high-molecular weight isoform (CD45RA) are considered as naïve cells, which circulate in the lymphoid organs, and those which express the low molecular weight isoform (CD45RO) are considered to be memory T cells, which circulate throughout the body to encounter the repeated exposure of specific antigen (Janeway, 1992).

Within the T lymphocyte repertoire, there are 5% to 10% of T lymphocytes expressing TCR with gamma (γ) and delta (δ) chains, instead of α and β chains. These receptors are of different specificities, as they can recognise protein and non-protein antigens which are not usually presented by MHC molecules, so they can perform non-MHC restricted cytotoxicity. This category of T lymphocytes is usually abundant in epithelial tissue, and of low frequency in the thymus and peripheral lymphoid organs (Kunzmann et al., 2000; Chien et al., 2014).

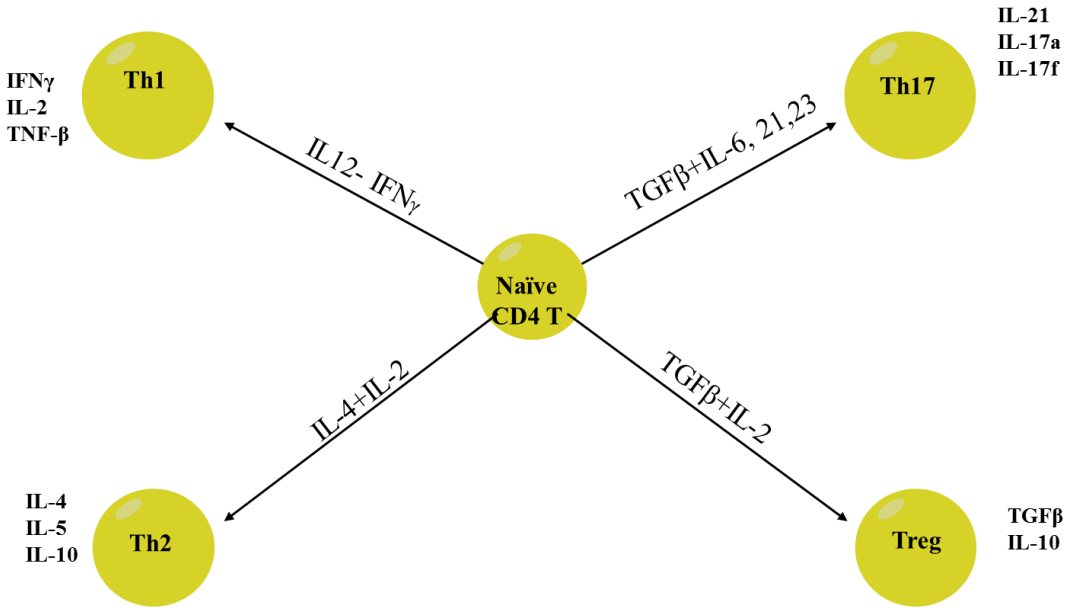

Figure 1.6: Different categories of CD4+ T cells. *Picture modified from (Zhu and Paul, 2008).*

1.2.2.1.6 Tumour infiltrating lymphocytes and its importance

There is a growing body of clinical data showing that antigen specific lymphocytes infiltrate in tumour (TIL). The majority of TIL are T lmphocytes. This infiltration occurs immediately after they encounter their specific cognate tumour antigen in the tumour environment, suggesting that T cells play an important role in controlling tumour growth (Clark, 1991).

It is well accepted that tumour infiltrating lymphocytes (TIL) mediate an indirect antitumor immune response, through secretion of cytokines, such as interferon γ (IFNγ) and tumour necrosis factor (TNF) cytokines, that stimulate the innate immune response by inducing the expression of MHC molecules on the surface of both APCs and tumour cells. In addition to that, TIL also mediate cytotoxic responses, either through release of cytolytic granules or expression of apoptosis inducing molecules (Barth et al., 1991; Finke et al., 1994). Numerous reports identified the presence of infiltrated tumour specific cytotoxic T lymphocytes in various tumours, including multiple myeloma (Noto et al., 1997; Noonan et al., 2015), breast and pancreatic carcinoma (Jerome et al., 1993), melanoma (Van Eynde et al., 1989) and renal cancer (Finke et al., 1994).

The low functional ability of tumour infiltrating T lymphocytes in rejection and clearance of tumour could be explained by chronic antigen exposure, which could lead to clonal exhaustion. In addition to the presence of T lymphocytes in low numbers, as the ratio of tumour cell: cytotoxic T lymphocytes is equal or higher than 10:1 (Mantovani et al., 2008). For these reasons, tumour infiltrating T lymphocytes have been isolated by many research groups and expanded *in vitro* to increase the proportion of T lymphocytes to tumour cells, in the presence of T cell growth factor (IL-2), in order to enhance their ability to selectively lyse autologous tumour cells and produce cytokines (Barth et al., 1991; Finke et al., 1994). The produced stimulated T lymphocytes were adoptively transferred into humans, as an immunotherapeutic treatment of cancer, with an objective rate of improved response (Rosenberg et al., 1988; Dudley et al., 2002).

On the other hand, diverse tumour antigens have been identified at a molecular level that are recognised by tumour specific T cells. The use of HLA class I restricted tumour antigenic epitopes of these well characterised antigens, expressed and presented by the tumour cell, and recognised by autologous CTL *in vitro*, has been shown to enhance T

cell response and tumour regression (Boon, 1996; Coulie et al., 2014), thereby concluding the relevance of T cell immune response and its importance in tumour immunotherapy.

1.3 Tumour immunogenicity

The theory of immunoediting suggests that tumours are immunogenic. Initially, at least, immunogenicity could be due to the ability of most tumours to express a wide range of tumour associated antigens, which are different from self-antigens (Escors, 2014). Evidence of the presence of immunogenic TAA was reported about 50-60 years ago (Haughton and Amos, 1968). Many years ago, scientists described high levels of tumour associated antigens (TAAs) expression by tumours induced by viral infection. However, other reports provided evidence that strong immune responses and regression of tumours after immunization may possibly be raised against cancers of non-viral origin, which supported the existence of TAA expressed in tumours of non-viral origin (Morton et al., 1992; Ichim, 2005; Escors, 2014). This supports the use of cancer vaccination in treatment of a variety types of cancer, tailored to their expression of TAA (Escors, 2014). Up till now, a growing body of TAAs has been discovered, which has been used as a target of immunotherapy for many tumours, due to their ability to induce antigen specific immune responses (Guinn et al., 2007; Cheever et al., 2009).

The required characteristics of the widely used antigens in tumour immunotherapy are worthy of mention here. The ideal antigens to be used in immunotherapy should (1) play a vital role in maintenance of tumours; (2) have specific expression in tumour cells, but not in normal tissue, to avoid development of autoimmune disease, by limiting the immune response possibility to direct against the normal host tissue; (3) be stably expressed in the majority of tumour cells; (4) and have the ability to stimulate tumour antigen-specific cytotoxic T lymphocytes (Simpson et al., 2005; Krishnadas et al., 2013). By taking these requirements into consideration, various antigens have been chosen for use as anticancer treatments. These antigens have been divided into different categories, as follows:

1.3.1 Categories of tumour antigens:

Tumour antigens can be classified into oncofetal antigens (expressed by cancerous somatic cells and fetal tissues); oncoviral antigens (caused by tumorigenic transforming viruses); cancer-testis antigens (expressed only by germ line tissues and neoplasia); accumulated / overexpressed antigens (encoded in both healthy and tumour tissues, with

highly elevated level of expression in tumour cells); mutated antigens (expressed as a result of genetic mutation in cancer cells); and idiotypic antigens (resulting from highly polymorphic genes that result in expression of specific clonotypes by tumour cells, i.e. B cells and T cells) (Zarour et al, 2003). Examples of these different antigen categories are included in the following table (Table 1.1).

Table 1.1: Different categories of tumour antigens.

Category	Example Antigen	Cancer Histology
Oncofetal	CEA	Colorectal carcinoma
	Immature laminin receptor	Renal cell carcinoma
	TAG-72	Prostate carcinoma
Oncoviral	HPV E6, E7	Cervical carcinoma
Cancer-Testis	BAGE family	Multi
	CAGE family	Multi
	GAGE family	Multi
	MAGE family	Multi
	SAGE family	Multi
	XAGE family	Multi
	NY-ESO-1/LAGE-1	Multi
	PRAME	Multi
Overexpressed/accumulated	BING-4	Melanoma
	Ep-CAM	Breast carcinoma
	Her2/neu	Multi
	Telomerase	Multi
	SAP-1	Colorectal carcinoma
	Survivin	Multi
Mutated	β-catenin	Melanoma, Prostate, HCC
	BRCA1/2	Breast, ovarian carcinoma
	CML66	CML
	Fibronectin	Multi
	MART-2	Melanoma
	Ras	Multi
	TGF-βRII	Colorectal carcinoma
Idiotypic	Ig, TCR	B, T leukemia, lymphoma, myeloma

HPV = human papilloma virus; Ep-CAM = epithelial cell adhesion molecule; CDK4 = cyclin-dependent kinase-4 MART-1/-2 = melanoma antigen recognized by T cells; MC1R = melanocortin-1-receptor; CML66 = chronic myelogenous leukemia (antigen) 66; SAP-1 = stomach cancer- associated protein tyrosinephosphatase-1; BRCA = breast cancer antigen; TAG-72 = tumor antigen-72; TGF-βRII = transforming growth factor-β receptor II; TRP = tyrosinase-related protein; Ig = immunoglobulin.Table adapted from (Zarour, 2003).

1.3.2 Commonly expressed antigens in multiple myeloma

1.3.2.1 Cancer Testis Antigens

Cancer Testis Antigens (CTA) are exclusively expressed in the germline tissue such as testis, ovaries and placenta, in addition to a variety of tumour types, including various solid and haematological tumours such as leukaemia and myeloma (Table 1.2). The absence of MHC class I molecule expression by the germline tissues protects them against auto immunity, due to lack of the peptide presentation in the context of MHC class I, which is necessary for CTL stimulation. In addition, the existence of a blood testis barrier guarantees the safety of these germline tissues in case of CTA application (Westbrook et al., 2004; Hudolin et al., 2013). The restricted expression and powerful immunogenic characters of CTAs offer a research option for developing new tumour immunotherapies, which has attracted lots of attention for developing a safe anticancer immunotherapy. Atanackovic et al. (2009) explained many reasons for using CTA as immunotherapeutic targets for multiple myeloma (MM) patients. Firstly, CTA have the ability to induce T-cell–mediated immunity and antibody-mediated immune responses in multiple myeloma (MM) patients (Atanackovic et al., 2009). Secondly, for these commonly expressed antigens, down-regulation is not a regular tumor escape mechanism. Thirdly, a dramatic increase of the relapse frequency has been observed after allogeneic stem cell transplantation in patients who express MAGE-C1/CT7. MAGE-C1 is a CTA, which has high level (about 70%) of expression in MM patients, and it promotes proliferation of MM cells. For these reasons, CT antigens are supposed to be promising immunotherapeutic agents for multiple myeloma patients (Atanackovic et al., 2009; Krishnadas et al., 2013; He et al., 2014).

Table 1.2: CT antigen families and their proportion of MM patients whose tumours express them.

Gene family	frequency of distribution in MM	Antigen members	References
MAGE-A	85-100%	MAGE-A(1,2,3,..,12)	(Jungbluth and Chen-Kiang, 2005)
MAGE-C	70%	MAGE-C1	(Jungbluth and Chen-Kiang, 2005)
BAGE	14%	BAGE- (1,2,3,4,5)	(van Baren et al., 1999)
GAGE-1	41%	GAGE- (1,2,...,8)	(van Baren et al., 1999)
NY-ESO	60%	NY-ESO-1	(van Rhee et al., 2005)
PRAME	48%	PRAME	(van Baren et al., 1999)

Table modified from (Meklat et al., 2007).

The earliest discovered family of CTA is MAGE, which comprises 25 functional genes, located in three regions of the X chromosome, MAGE-A, MAGE-B, and MAGE-C1 (Chomez et al., 2001; van der Bruggen and Traversari, 1991). The members of the MAGE family play an important role in embryogenesis, in addition to their role in the germ cell genesis and apoptosis. However, their role in tumour cells is unclear (Xiao and Chen, 2004). Xiao et al (2004) speculated that after embryogenesis, the gene could be deactivated by methylation, then, when a tumour is formed, the methylated genes could be reactivated again and produce proteins that can be recognised by the immune system. Therefore the presence of the MAGE antigen is not the cause of a tumour, but it is a result of tumour genesis (Xiao and Chen, 2004). PRAME, is also considered to be one of the CT antigens (Meklat et al, 2007), which functions through interfering with retinoic acid receptor signalling. Retinioc acid receptor is important in cell differentiation and haemostasis. Therefore, loss of retinoic acid signalling is of benefit to cancer cells (Epping and Bernards, 2006). There are some reports of decrease of PRAME expression level with the reduction of tumour burden (Turtle et al., 2004; Gaponova, 2009).

1.3.2.2 Overexpressed antigens

Self-proteins can be overexpressed, compared with their level of expression in healthy tissue, in a wide range of tumours, which reflects their role in tumorigenesis. An example of these antigens is mucin 1 (MUC1). The MUC1- oncoprotein is widely expressed (73%) in multiple myeloma cells (Yin et al, 2012; Cloosen et al., 2006). In normal cells, the function of MUC1 is generally attributed to hydration and lubrication of the cell surface, in addition to protection against infection. However, in malignant cells, MUC1 is overexpressed, displays an aberrant glycosylation, and inhibits extracellular matrix interaction (Brayman et al., 2004). Also, human telomerase reverse transcriptase (h TERT), a ribonucleoprotein enzyme, is one of the most commonly overexpressed antigens in cancer cells (about 90%) (Weiss et al., 2012). In around 85% of human cancers, overexpression of h TERT is associated with immortality of cancer cells (Wu et al., 2003). In addition to that, CS1 is highly expressed in primary tumour cells from MM patients (about 97%) (Tai et al., 2008). CS1 is a member of the signalling lymphocytic activation molecule (SLAM) family. Overexpression of CS1 enhances MM cell growth and survival, through promoting the adhesion of myeloma cells to bone marrow stromal cells (Tai et al., 2009). Also, the receptor for hyaluronan mediated motility (RHAMM) is

overexpressed on the surface of the malignant cells in MM patients. This receptor controls the MM cell motility, and therefore enhances the invasiveness of the malignant plasma cells in blood or in the bone marrow of the patients (Crainie et al., 1999). In addition, it has been shown that MM secrete Dickkopf-1(DKK1), which has a correlation with MM associated bone lesions (Gavriatopoulou et al., 2009), as DKK1 has an important role in inhibiting pre-osteoblast differentiation (Qiang et al., 2008).

These antigens are widely expressed throughout both solid and haematological malignancies. They are expressed at a greater level in cancerous cells than that of healthy tissue, meaning that their use as immunotherapeutic agents could lead to autoimmunity against normal cells (Ramanathan et al., 2005). However, Minev et al. (2000) showed that hTERT peptide-specific CTL have the ability to recognise similar peptides on tumour cells in the absence of killing of normal haematopoietic autologous cells expressing the same antigens. This report gives promise for using hTERT in immunotherapy. Also, the preliminary vaccination trial using MUC1 peptide in breast and ovarian cancer patients did not show any autoimmune reactions (Brossart et al., 2000). More importantly, numerous studies demonstrated the effect of MUC1-specific CTLs in treatment of MUC1 positive tumours, without any apparent infiltration or destruction of normal secretory epithelial cells that express MUC1, owing to the low level of MUC1/MHC class 1 complexes on the normal cells which express this antigen (Gong and Kuf, 2000; Ocadlikova et al., 2009). So, overexpressed tumour antigens are used widely as candidates for cancer vaccines.

Based on the previous studies, we conclude that MUC1, hTERT, CS1 and MAGE C1 are of high prevalence of expression in MM patients. Moreover, these antigens are potential targets for induction of antigen specific CTL responses (Minev et al., 2000; Schmitt et al., 2008; Lendvai, 2010; Kim et al., 2013; Brossart et al., 2015; Brossart et al., 1999). Therefore, we chose these antigens for detection of presence of antigen specific CTL in our long term stimulated cultures.

1.3.3 Processing and Presentation of intracellular and extracellular antigens

Intracellular antigens are usually displayed in the context of class I MHC molecules, and recognized by CD8+ T cells. Cytosolic antigens or intracellular antigens may be produced from virus infection, some phagocytosed microbes that are leaked from or transported out of the phagosome, or from mutated host genes that encode nuclear or cytosolic proteins such as tumour antigens. All of these types of cytosolic antigens are displayed (as short

peptides) by MHC class I molecules. For antigen processing, the intracellular protein is tagged with a peptide called ubiquitin, then threaded through the proteasome. In the proteasome, the proteins are degraded by proteolytic enzymes into peptide sizes that enable the produced peptides to bind to MHC class I. Afterward, the transport associated with antigen processing (TAP) protein binds to the processed peptide and actively pumps them into the endoplasmic reticulum (ER), where they can bind to the newly synthesized MHC class I molecules, and form stable complexes, which are transported to the cell surface (Bjorkman and Parham, 1990; Abbas et al., 2012).

In contrast, extracellular protein antigens are internalized by APCs, processed in their endocytic vesicles. In the endoplasmic reticulum, the newly synthesized MHC class II molecules are complexed with an invariant chain (li) chain, to prevent binding of MHC molecule with self-peptide, then transported to endocytic vessels. In the endocytic vessels, the cathepsin enzymes cleave the li chain and form CLIP, which remain in the MHC class II peptide biding cleft until replaced by antigenic peptide by the aid of HLA-DM. After removal of CLIP and form stable MHC class II- peptide complex, the complexes are then delivered to the cell surface, and can be recognized by TCR of CD4+ T lymphocytes (Abbas et al., 2012; Janeway Jr et al., 2012; Blum et al., 2013).

1.3.4 Pathogenesis of Multiple Myeloma

Multiple myeloma is one of the most common hematologic tumours. It represents about 10% of all hematological malignancies, and around 1% of new cases (Kocoglu and Badros, 2016). The MM is a post germinal centre neoplasm, characterised by malignant proliferation of plasma cells (Palumbo, 2011). Most commonly myeloma evolves from a monoclonal gammopathy of undetermined clinical significance (MGUS), which is an asymptomatic state, in which a monoclonal immunoglobulin (Ig), also called paraprotein, is found in the serum and urine (Kyle and Vincent Rajkumar, 2006). Genetic alterations can occur at the Ig heavy chain locus, which is abnormal in the majority of MM patients (Tricot, 2002). The myeloma tumour is formed from clonally diverse subset of myeloma cells (Figure 1.7). This diversity evolves during the disease progression, and usually shifts between dominant and subdominant clones during therapy or relapse (Röllig et al., 2015). During disease progression, myeloma cells produce many immunological inhibitory cytokines, for instance IL-10 and TGF-β. These inhibitory cytokines can alter the antitumor immune response. For example they can abrogate DC function, by inducing constitutive expression and activation of STAT3 (Nguyen-Pham et al., 2012).

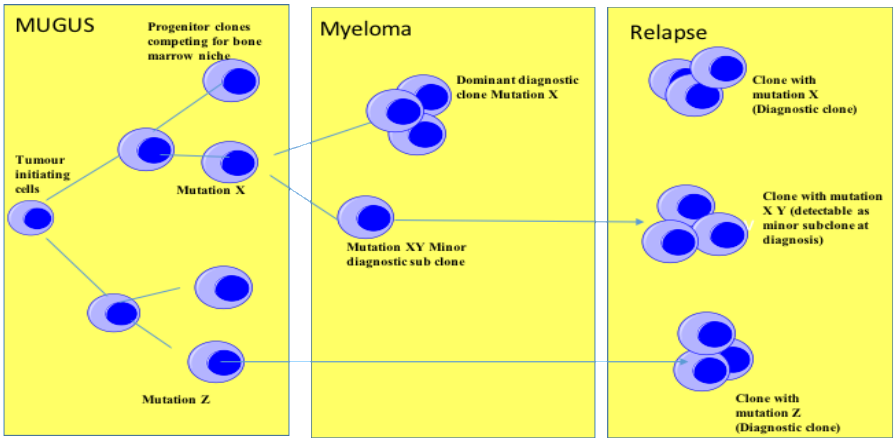

Figure 1.7: Clonally diverse subsets of myeloma cells. *Different subsets evolving during the disease progression and therapy. Figure adapted from (Röllig et al., 2015).*

The malignant plasma cells crowd out the normal healthy red blood cells and white blood cells from the bone marrow, leading to many complications such as anaemia and immune suppression. Also, myeloma cells can inhibit the reactive T lymphocytes through the interaction with the carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) expressed on the surface of myeloma cells (Witzens-Harig et al., 2013). In early stages of MM, the growth and survival of tumour cells are dependent upon their tumour microenvironment. The interaction of tumour cells with tumour micro-environment leads to bone destruction, which is caused by increased osteoclast activity and decrease of osteoblast activity, through release of DKK1 that is a major inhibitor of osteoblast formation, and also through release of certain cytokines such as tumour necrosis factor α (Galson et al., 2012).

In the advanced stage of MM, the tumour cells become independent of the tumour microenvironment through the accumulation of sufficient genetic alterations, which help them to grow at the extramedullary sites. Drug resistance is a major feature of myeloma cells in the advanced stage, that is caused by resistance to apoptosis which normally occurs during cell adhesion-induced growth arrest, and by FAS and TRAIL activation (Tricot, 2002).

1.3.5 Multiple myeloma-mediated suppression of T Cells

Tumour immune evasion is one of the principal mechanisms of tumour progression and metastasis in MM, and is a major obstacle for tumour immunotherapy. Downregulation of HLA class I expression by the tumour cells is an important mechanism for survival and progression of the disease, due to the importance of HLA class I molecules for induction

of the anti-myeloma T cell immune response (Spanoudakis et al., 2009). However, loss of HLA class I expression leads to absence of NK cells inhibitory signals, and results in their activation against the tumour cells (reviewed in Dahlberg et al., 2015).

In addition, there are some other reasons described for the T lymphocytes' unresponsiveness *in vivo* (Figure 1.8). Firstly, MM microenvironment *in vivo* inhibits DC maturation, and also causes DC dysfunction (Pratt et al., 2007). This DC dysfunction could lead to T cell inactivation due to absence of the costimulation (Pratt et al., 2007). Also, it was reported that some adhesion molecules, such as CEACAMs expressed on the cell surface of freshly isolated myeloma cells, upon the interaction with the T cells, can result in inhibition of T cell activation and cause their unresponsiveness (Witzens-Harig et al., 2013). Moreover, the existence of non-functional T cells could be attributed to the interaction of PD-1 on the surface of T cells with PDL1 on the surface of MM cells (Fichtner et al., 2015). Furthermore, suppressive factors, such as transforming growth factor b1 (TGF-b1), secreted by tumour cell lines, have the ability to suppress dendritic cell function and T-cell proliferation through inhibition of their responses to IL-2 (Cook et al., 1999). Also, Villunger et al. (1997) described that tumour evasion may arise through expression of FAS-L on the surface of malignant myeloma cells, as it leads to activation induced cell death of T cells (Villunger et al., 1997). Regarding the cell mediated suppression, T regs are one of the major contributors, as they can suppress the immune response and induce tolerance through various direct and indirect mechanisms, as described previously in section 1.2.2.1.5 (Zou, 2006; Murakami et al., 2004; Whiteside, 2015). Therefore, tumour immunotherapy using conventional T cells has to overcome all of these suppressive mechanisms to efficiently eradicate myeloma tumours.

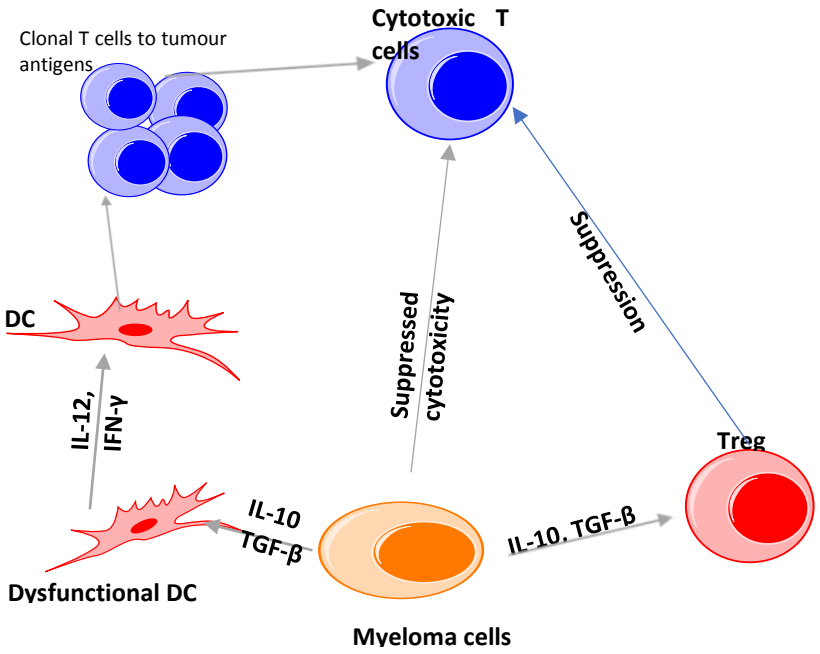

Figure 1.8: Mechanisms of tumour induced T cell suppression of multiple myeloma. *Figure modified from (Brown et al., 2013).*

1.4 Tumour Immunotherapy

Failure to stimulate the immune system to eradicate tumours, in spite of the high antigenicity of most of tumours, could be attributed to the immune escape mechanisms of the tumour cells, as discussed previously. Therefore, most of the on-going researches of cancer immunology all over the world aim to harness immune compartments to recognise and destroy the tumours through acceleration and increase of the anti-tumour immune responses, which may provide an alternative therapeutic approach, termed tumour immunotherapy. Tumour immunotherapy is based on using different anti-tumour immune effectors with tumour specificity and tolerance to normal tissues. Also, it should lead to long lasting prophylaxis, which could prevent tumour recurrence. The first observation of the antitumor effect of the immune system was in 1890, when William Coley observed regression of sarcoma after severe bacterial infection, the bacteria involved were Streptococcus pyogenes, and Serascia marcescens (reviewed in (Bremers and Parmiani, 2000). The other conventional strategies used for management of tumours, such as chemotherapy, which targets rapidly dividing cells, affect normal cells (like haematopoietic stem cells, hair follicles and gastric mucosa), leading to many side effects such as hair loss, peptic ulcer, and bone marrow suppression leading to aplastic anaemia and recurrent infections. The other option is radiotherapy. It also affects both tumour cells and normal cells, such as blood cells and their progenitors, leading to considerable side

effects. In spite of the effectiveness of these conventional therapies for management of many tumours, their significant side effects, in addition to development of resistance mechanisms in some tumour cells, lead to limitation of their use, and increase the scientists' interest in trying different immunotherapeutic approaches.

MM is a haematological malignancy, and is considered incurable by conventional therapies. Although its prognosis has been improved recently due to use of novel therapeutic agents, relapse occurs in the majority of patients, which is ultimately fatal due to immune suppression, anemia, paraproteinemia, and renal function loss. Progress in understanding the pathophysiology of MM, and improvement in the knowledge of tumour immunology, will be the way to assist these patients. Immunotherapy is thought to be a powerful intervention which maintains a long-lasting control without relapse of the disease, or even eradicates the disseminated tumour cells (Ruffini and Kwak, 2001; Zhang et al., 2012). Some immunotherapeutic approaches used in treatment of MM and other tumours are described herein.

1.4.1 Adoptive cellular transfer

Adoptive cellular transfer (ACT) is one form of passive immunization, which has been used over the past 50 years, and proven to have the potential to improve antitumor immunity (June, 2007). The anticancer effect of the immune system was definitely identified when Weiden et al. (1979) reported strong anti-leukaemia effect after bone marrow transplantation. This effect was raised as a result of graft versus host disease (GVHD) (Weiden et al., 1979). The researchers reported that T cells were the main mediators of GVHD after bone marrow transplantation (Korngold and Sprent, 1978). Adoptive cellular transfer involves various immunotherapeutic approaches such as stem cell transplantation and adoptive T cell immunotherapy.

1.4.1.1 Stem cell transplantation

Allogeneic hematopoietic stem cell transplantation (HSCT) is the earliest form of adoptive cellular transfer used with anticancer purpose. It has been used effectively in the treatment of a number of haematological cancers, rather than allogenic whole bone marrow transplantation (Fabre, 2001; Welniak et al., 2007). To extend the recovery of MM patients who are subjected to allogenic HSCT, donor lymphocyte infusion may be used potentially as a saving therapy due to its graft-versus-myeloma effect (Nguyen-Pham et al., 2012). However, due to lack of specificity of the allogenic transplant for tumour

cells, it is difficult to discriminate its effect on tumour and on host, leading to GVHD (Kolb et al., 1995). Also, autologous stem cell transplantation (ASCT) is one of the most common methods for treatment of MM (Attal et al., 2007). Nevertheless, ASCT is not considered as a perfect treatment, because the majority of patients relapse within 3 years, due to the remaining malignant spots (Danylesko et al., 2012). However, Condomines *et al*. (2010) described that certain chemotherapeutic agents such as myeloablative therapy can be used before ASCT, showing immunomodulatory activities and reducing risk of relapse in treatment of myeloma (Attal et al., 2007; Condomines et al., 2010).

1.4.1.2 Adoptive T cell immunotherapy

The use of T cells in cancer immunotherapy has long been of interest, due to their numerous beneficial properties such as (1) Their specificity, so they can separate their effect on cancerous and normal tissues; (2) After activation of T cells, they expand clonally up to 1000 fold; (3) Immunotherapy using T cells may have memory properties, therefore their effect can remain for many years after initial therapy; and (4) T cells have trafficking properties, so they can travel to the site of antigens, for targeting tumour cells *in situ* (Perica et al., 2015). Therefore, adoptive T cell transfer is a promising approach for treatment of various tumours. In ACT, autologous or allogenic T cells are cultured *ex vivo* in the presence of certain cytokines, and infusion of activated T cells into cancer patients is undertaken with the goal of targeting and destroying tumour cells (Watanabe et al., 1992; Manzo et al., 2015).

Adoptive leukocyte transfer was first used in the 1960s, when Southam and his colleagues established that using leukocyte autografts in cancer patients inhibited tumour growth in approximately 50% of patients. They proposed that leukocytes have a definite inhibitory effect on growth and implantation of cancer cells, so they can be used as powerful candidates of adoptive immunotherapy (Southam et al., 1966).

For autologous adoptive immunotherapy, T cells are separated from the patient's PBMCs, TILs, or draining lymph nodes. The first trials of isolating T lymphocytes from TILs of cancer samples was in 1988, following a study that demonstrated the cytotoxic activity of *in vitro* cultured TILs in presence of IL-2. Responses were seen in 11 of 20 patients with metastatic melanoma (Muul et al., 1987; Rosenberg et al., 1988). In another report in 1994, only 5 out of 29 patients with metastatic melanoma had a complete response, with a median duration of response of only four months (Rosenberg et al., 1994). A major

breakthrough occurred when researchers suggested that the occurrence of lymphopenia after various chemotherapy or immunosuppressive treatments may enhance the efficacy of ACT of T cells (Baxevanis et al., 2009). There were various explanations for the synergistic effect created by lymphodepletion before adoptive transfer of T cells. Firstly, proliferation and activation of CTL improved, by depletion of Treg cells from the tumour environment (Lizée et al., 2006). Secondly, increase the production of the endogenous cytokines such as IL-2, IL-7, and IL-15 following lymphodepletion generates a more favourable condition for lymphocyte activation (Lizée et al., 2006; Baxevanis et al., 2009). Thirdly, lymphodepletion eliminates tolerogenic APCs, leading to more activation of the infused T cells (Noonan et al., 2015).

Clinical applications of adoptive transfer of antigen specific T cells have various potential benefits for treatment of many malignancies, owing to their competence to selectively target tumour antigens, through their cognate receptors, and their ability to persist and proliferate, to mediate the long-term antitumour protection (Gerdemann et al., 2011; Manzo et al., 2015; Redeker and Arens, 2016). Allogeneic and autologous adoptive T cell therapies have been used effectively in the management of various solid and haematological tumours. Hunder et al. (2008) described long-term complete remission of metastatic melanoma, following adoptive transfer of autologous NY-ESO-1–specific CD4+ T cell clones (Hunder et al., 2008). Various studies demonstrated the feasibility and efficacy of adoptive T cell transfer of antigen specific CD8+ T cells in melanoma patients, without adverse effect (Mackensen et al., 2006; Rosenberg and Restifo, 2015). Another investigation demonstrated that adoptive transfer of vaccine primed and co-stimulated autologous T cells for patients undergoing stem cell transplantation (SCT) resulted in activation of cellular and humoral immune responses in patients with MM (Rapoport et al., 2005). Also, marrow-infiltrating lymphocytes (MILs) were used as an ACT in myeloma patients, following *ex vivo* activation and expansion of MILs, and myeloablation of the patients. Using this approach imparts a great reduction in myeloma burden, and enhanced the progression-free survival (Noonan et al., 2015). Comoli et al. (2004) induced long-term stimulated EBV specific CTLs for clinical trials, using allogeneic HLA-matched PBMC, and EBV- LCL as a stimulator for several weeks, then infused into the patients with EBV-related nasopharyngeal carcinoma. The infused EBV-specific CTLs were well tolerated, and caused temporary disease stabilization. Also, a marked increase in the TILs were observed (Comoli et al., 2004). In addition, Weber et

al. (2013a) showed their ability to generate potent anti-leukaemia antigen-specific CTLs, which successfully expanded *in vitro* (Weber et al., 2013b). The major concern of adoptive antigen specific T cell therapy is the evolution of antigen escape variants, however this problem can be circumvented by using multi-epitopic CTL clones (Marincola et al., 1999).

1.4.1.3 Genetically engineered T lymphocytes

The potential for desired specificity and limitless source of T lymphocytes can be obtained using genetically engineered autologous or allogeneic lymphocytes, to modify them to express the desired TCR (Kershaw et al., 2013). Tumour reactive TCR must initially be identified in isolated T lymphocytes, and then they can be engineered to increase the affinity by changing of the complementarity determining regions (Varela-Rohena et al., 2008). The use of genetically engineered T cells vastly expanded the range of cancers that could be treated by using ACT, such as sarcoma, neuroblastoma, and colorectal cancers (Pule et al., 2008; Morgan et al., 2013; Parkhurst et al., 2011). However, the concerns of this strategy are that the engineered T cells contain both endogenous and engineered TCR (dual specificity), which may lead to cross reactivity after activation. Also, the native and engineered T cell receptors could be paired together, and create a TCR of a new specificity. Moreover, use of engineered T cells may result in decrease in the contribution of central tolerance (bypassing normal selection) leading to increase the risk of autoimmune toxicity. Additionally, a harmful effect of the engineered cells may be due to targeting tumour antigens expressed on healthy tissues (that potentially goes for any T cell therapy, but possibly more with TCR engineered to have higher affinity for the antigen). In melanoma, use of engineered T lymphocytes did not induce as successful a response as the response of TIL-derived ACT (reviewed in Perica et al., 2015).

1.4.2 Vaccination

1.4.2.1 Dendritic cell vaccines

Active immunization using dendritic cells (DCs), the most potent (so called professional) APCs known, can induce a strong adaptive immune response, due to activation of $CD8^+$ and $CD4^+$ T cells, through presentation of the phagocytosed antigens in the context of their HLA class I and class II molecules, in addition to provision of the essential co-stimulatory molecules required for activation of the naive CTL and T helper cells through its ligands (Melief, 2003).

Several mechanisms have been proposed to load the antigen on DCs *in vitro*, such as pulsing DCs with peptides (Brossart et al., 2001), DCs pulsed with protein such as myeloma-derived heat shock protein (Qian et al., 2005), transfecting DCs with RNA or DNA (Milazzo et al., 2003; Vergati et al., 2010), fusion DCs with tumour cells (Hao et al., 2004; Vasir et al., 2005), and DC pulsed with cell lysate (Lee et al., 2007). The advantage of hybrid formation or pulsing with tumour cell lysate is the presentation of multiple antigenic epitopes in the context of MHC on the surface of DCs, so it can induce a polyclonal T-cell immune response, and reduce the probability of immune escape from a single TAA (Nguyen-Pham et al., 2012).

On the other hand, if vaccination using DCs results in tumour lysis, this would result in release of various tumour antigens, which can be processed and presented by the professional APCs, to prime the immune response to these antigens (epitope spreading phenomenon). This happened previously in a trial of dendritic cells pulsed with MUC1 peptide, in treatment of ovarian and breast cancer patients. This resulted in production of MUC1 specific T cells, in addition to emergence of CEA and MAGEA-3 specific T cells (Brossart et al., 2000).

Various studies have demonstrated the use of DCs pulsed with idiotype protein or peptide for treatment of patients with MM (Houet and Veelken, 2006; Titzer et al., 2000). Enhanced immune responses were seen in some patient, but with rare clinical response, which was attributed to the weak immunogenicity of the idiotype protein. The idiotype, the antigenic determinant of paraproteins secreted by MM cells, is regarded as individual specific or tumour specific antigens (Zhang et al., 2012)**.**

However, a major concern of self-peptide(s) pulsed DCs is the presence of tolerance against the self-peptide(s), as they have already been presented to the immune system during disease progression. Therefore, using analogue peptides is one of the effective methods to circumvent this problem. Hardwick et al (2013), showed that DCs loaded with PASD1 analogue peptides enhanced IFNγ production, using T cells from normal individuals and AML patients. They, also, showed expansion of peptide specific T cells using PASD1 analogue peptide (Hardwick et al., 2013).

1.4.2.2 Hybrid cell vaccines

Use of hybrid cells (autologous or allogeneic APC-tumour cell hybrids) in tumour immunotherapy is based on enhancing the immunogenicity of the tumour cell by several

means: (1) to provide the co-stimulatory molecules CD40, CD80 and CD86, and HLA class II expressed in the APC partner, (2) to provide the antigenic properties of identified and unidentified antigens, expressed by the tumour cell partners, and (3) to express allogeneic MHC molecules (in case of using allogeneic APC), which may act as potent activators of T cell (Trefzer and Walden, 2003; Yasuda et al., 2007). The use of the hybrid cells in treatment of various haematological and solid tumours has been examined successfully in both mouse models and clinical trials (Homma et al., 2005; Rosenblatt et al., 2013; Trefzer and Walden, 2003). Also, fusion cells prepared by fusion of autologous myeloma cells and DC induced MHC restricted myeloma specific cytolysis which could not be obtained by using the patients' tumour cells alone or DC alone (Raje et al., 2004).

Rosenblatt (2013) showed the feasibility of production of autologous hybrid vaccines of DC and myeloma cells. The produced vaccine was well tolerated in a phase II trial. In this study the patients received vaccination after autologous stem cell transplantation (ASCT), aimed to target residual disease. 47% of patients achieved complete response (CR), or a near CR, after vaccination, 24% of patients with a partial response following ASCT were changed to CR. It was concluded that vaccination with dendritic cell/myeloma fusion, when applied at the posttransplant period, caused an expansion of myeloma-specific CD8+/ CD4+ T cells, and reduction of the residual disease (Rosenblatt et al., 2013). Some clinical trials using DC-tumour fusion cells are summarised in Table 1.3. Vaccination using genetically engineered MM cell secreting IL-4, which enhance maturation and differentiation of DCs, fused with DC showed enhanced tumour specific CTL response *in vitro* and more efficient protective immunity against tumour challenge *in vivo*, compared with wild type myeloma cells fusion vaccine (Xia et al., 2005). Also, Cao et al (1999) transfected DCs with a vector expressing GM-CSF, then fused the transfected DC with tumour cells. This transfection enhanced the specific CTL response and caused protection of the immunized mice from tumour challenge, more efficacious than the untransfected vaccine.

However, some limitations had been shown for using DC hybrids, such as their low ability for replication, short lived nature, and the low number of DCs in PBMC. So, Ahmadi et al. (2008) investigated the use of activated B cells to replace DCs as APCs in tumour vaccines.

Activated B cells have some advantages over using DCs in the hybrid formation. Activated B cells (such as B cells stimulated with CD40L) can be grown for long periods

of time (Ahmadi et al., 2008). Also, activated B cells have the ability to process and present antigens using their MHC class I and MHC class II, which are required for activation of CD8+ CTL and CD4+ T cells, plus they express co-stimulatory CD80, CD86 and CD40 molecules, as do DC, which are required for stimulation of cellular immune responses (Trefzer and Walden, 2003). Also, there is a higher percentage (5-10%) of B cells in PBMC than that of DC (1-2%).

Kugler et al. (1998) demonstrated the safety and therapeutic activity of autologous and allogeneic renal cell carcinoma x B cell fusion vaccines, in a phase I clinical trial in patients with metastatic renal cell carcinoma. Moreover, on the basis of the previous findings, B lymphocytes have been used successfully for preparation of tumour hybrid vaccines, such as tumor B-cell hybridoma (TBH) vaccine for treatment of glioblastoma multiforme (GBM). The authors showed that using such vaccines had potent and rapid therapeutic response against tumour stem cells' persistence, as four out of twelve patients showed complete remission after vaccination and the remaining patients response ranged from partial response to unresponsiveness (Moviglia et al., 2008).

In contrast to the promising results achieved by using B-cells and DCs as APC fusion partner in hybrid cell formation, Souberbielle et al., (1998) did not find any improved immune response in mice after immunization with macrophage and melanoma cell hybrids. They attributed the absence of the immune stimulatory response of these hybrids to the lack of expression of CD80, CD86, MHC class I and MHC class II. This reflects the importance of careful phenotypic characterization of the hybrid cells before their use in clinical trials.

Table 1.3: Summary of some clinical trials using DC-tumour fusion vaccines

Tumour	DCs	Tumour cells	Co-administered agents	Patient number	Clinical response	Refrences
Melanoma	Allo	Auto		16	1 (CR) 1 (PR) 5 (SD) 9 (PD)	(Trefzer et al., 2000; Trefzer et al., 2005)
	Auto	Auto		17	1 (PR) 1 (SD) 15(PD)	(Krause et al., 2002)
	Allo	Auto		13	8 (SD) 3 (SD) 2 (N)	(Barbuto et al., 2004)
Renal cell carcinoma	Allo	Auto		22	14 (SD) 2 (PD) 3 (OR) 3 (N)	(Barbuto et al., 2004)(Barbuto *et al.*, 2004)
	Auto	Auto		13	5 (SD) 8 (PD)	(Avigan et al., 2004)
Breast Cancer	Auto	Auto		10	2 (PR) 1 (SD) 7 (PD)	(Avigan et al., 2004)
	Auto	Auto	rh IL-12	2	1 (SD) 1 (PD)	(Kikuchi et al., 2004)
Hepatocellular carcinoma	Auto	Auto		1	1 (PD)	(Koido et al., 2008)
Ovarian Cancer	Auto	Auto	rh IL-12	3	2 (SD) 1 (PD)	(Kikuchi et al., 2004)

CR: complete response, MR: mixed response, PR: partial response, PD: progressive disease, SD: stable disease, N: not evaluated. (Table adapted from (Koido et al., 2010).

1.4.2.3 EBV B-lymphoblastoid cells -based fusion vaccines

Epstein-Barr virus B-lymphoblastoid cell lines (EBV B-LCL), such as HMY2, have the same features of professional APCs (HLA-class I and class II, and co-stimulatory molecules expression), as activated B cells and in addition, their growth in tissue culture is immortalised. Moreover, expression of EBV gene products, may have an adjuvant anti-tumour effect through stimulation of EBV specific T cells (Kerr et al., 1992). Dunnion et al (1999) selected HMY2 as the parent antigen presenting cell for their fused hybrids for several reasons. Firstly, this cell line is sensitive to hypoxanthine, aminopterin and thymidine (HAT) and resistant to ouabain (Edwards et al., 1982), allowing hybrid cell chemical selection. Secondly, EBV B-LCL are easy to culture and their growth is

continuous, as viral transformation can be carried out using small number of B cells. In contrast, dendritic cells proliferate very poorly, and more patient samples are required to produce enough DCs to make vaccine, so they were unable to generate adequate numbers of the hybrids using DCs as parent APC (Dunnion et al., 1999). A number of solid and haematological tumour cell/ cell lines were fused with HMY2, forming hybrids which have been used in several studies done previously in my supervisor's lab, and which formed the basis of this project.

The project started in 1999, when Dunnion et al. made fusions between different melanoma and lymphoid tumour cell lines with HMY2, using PEG fusion followed by double chemical selection (HAT to exclude unfused HMY2, and ouabain to exclude homogenous fused and unfused tumour cells) (Edwards et al., 1982). The produced hybrid cell lines grew constantly in tissue culture, had high expression levels of the surface markers of their parent-APC, and excellent expression of several immunogenic TAAs. In addition, they were able to prime and activate T cells proliferation in mixed lymphocyte reaction *in vitro*, more significantly than their parent tumour cells. The produced hybrids activated T cells were able to recognise the cognate parent tumour cells and specifically kill them in 51Cr release cytotoxicity assays (Dunnion et al., 1999).

Following that, Cywinski et al. (2006) showed that T cell responses against hybrid cell lines formed by fusion of haematological tumour cells with HMy2 were significantly higher than their parents, which was attributed to their expression level of the costimulatory molecules, which was significantly lower in hybrids made by fusion of HMY2 with non-haematological tumour cells. The response of the hybrids made of non-haematological tumour cells were higher than their parents, but lower than the hybrids of the haematological tumour cells, (Cywinski et al., 2006).

Afterward, Walewska *et al*. (2007) generated a group of five stable hybrid cell lines by fusion of HMy2 with CD138+ bead-separated myeloma plasma cells. The hybrid cell lines produced in these studies were very stable, and maintained their phenotypic and functional characteristics, producing self-renewing cell lines with potential for therapeutic vaccination. In addition, they showed that these hybrid cell lines consistently expressed both MHC class I and class II molecules, as well as expression of a range of T cell co-stimulatory ligand molecules, including CD80 and CD86, and directly enhanced T cell proliferative responses *in vitro*, by comparison with the parent tumour cell lines (Walewska et al., 2007).

A number of tumour-associated antigens have been identified in tumour cells from multiple myeloma patients. These antigens have the ability to induce a specific immune response, as described in section 1.3.2. So, they could be used as targets for immunotherapy (Zhang et al., 2012). This is supported by the finding of Atanackovic who showed that immune response directed against CT antigens can be induced by the allo-SCT, which is partly mediated by donor derived T cells (Atanackovic et al., 2009).

This was confirmed by the work done in my supervisor's lab by Mohamed et al. (2012a), who described that PBLs isolated from healthy, HLA-A2+ individuals can be stimulated by the hybrid cell lines, producing tumour antigen-specific CTLs to TAAs, including survivin, MAGE-A1, NY-ESO-1 and WT-1. Hybrid cell lines such as those described may be a new immunotherapeutic approach for use in the treatment of haematological malignancies (Mohamed et al., 2012b). Also, by activation of T cells, we can generate from a small number of naive lymphocytes which have predetermined receptors for any antigen, a great number of functional effector cells which have the ability to eliminate cells expressing that antigen (Abbas et al., 2012).

On that basis, further immunological studies of these hybrid cell lines, generated by the fusion of HMy2 cells and myeloma tumour cells, to produce antigen specific T cells isolated from MM patients, formed the basis of my PhD project.

Hypothesis

We hypothesised that hybrid cell lines produced by fusion of HMy2 and myeloma cells or cell lines are capable of inducing HLA-A2-restricted tumour antigen specific cytotoxic T cells (CTL) *in vitro*, in long-term stimulated peripheral blood lymphocytes isolated from both HLA-A2-positive healthy donors and multiple myeloma patients.

Aims and Objectives

The current research project aimed to

1- Demonstrate that the previously described hybrid cell lines can induce HLA-A2-restricted tumour antigen specific (CTL) *in vitro*. This aim was done through, firstly, identifying the phenotypic characterization of the used hybrid cell lines. Then, selection of some TAAs of high prevalence of expression in MM, and detecting their expression level in the used cell lines, at mRNA and protein expression level. Afterward, the ability of these hybrids to induce antigen specific CTL in long-term stimulated cultures was determined using HLA-A2 peptide

pentamer staining assay, IFNγ and perforin release ELISpot assays. Furthermore, the response of long-term activated T cells in patients' PBMCs cultures were confirmed by CTL-mediated cytotoxicity assays, to ensure their antigen specific cytotoxic activity. This objective demonstrates the "proof of concept" that stable, allogeneic hybrid cell lines produced by fusion of HMy2 cells and myeloma cells can induce antigen-specific CTL *in vitro* that could be used for adoptive immunotherapy of myeloma, if produced to appropriate standards.

2- The second aim of my project was to optimize the process of antigen specific CTL generation and expansion *in vitro*. To do this, I investigated protocols, through the addition of exogenous cytokines, for the optimal *in vitro* generation of tumour antigen specific CTL that could potentially be used for adoptive immunotherapy of myeloma, if produced to appropriate standards.

Chapter 2 Materials and Methods

2.1 Cell lines and primary cells

2.1.1 The cell lines

HMy2 (an EBV- transformed B-LCL, used as APC for fusion with other cells (Edwards et al., 1982)), HU266, HIC, HRC, and HCM (hybrid cell lines previously made by fusion of HMy2 and different myeloma cells) and U266 (myeloma cell line, used to show the phenotypic and functional features of myeloma tumour cells) were grown in tissue culture as detailed below. The hybrid cell lines (HIC, HRC, and HCM) were made previously using *ex vivo* myeloma cells, while HU266 was made using MM established cell line (U266)(Walewska et al., 2007). T2 is a TAP deficient cell line, characterized by its ability to present exogenously loaded peptides in the context of its HLA-A2 molecules (Bossi et al., 2013). For this reason, T2 cell line was used, after loading with an appropriate peptide, as target for determination of antigen specific IFNγ and perforin release assays, and cytotoxicity assay. K562 (chronic myeloid leukaemia cell line) was used as a target, for assessment of NK cell activity in the cultures, as it lacks MHC class I expression (Nishimura et al., 1994).

2.1.2 Primary cells

After informed consent, samples of peripheral blood were obtained from patients with multiple myeloma and from healthy HLA-$A2^+$ individuals, after receiving Local Research Ethics Committee approval (study number 05/Q2502/26), and University Research Ethics Committee approval respectively (study number wssk1-012f). Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples. The details of the myeloma patients are summarised in Table 2.1. Fifteen healthy adult donors were recruited from staff and graduate students in the Department of Infection, Immunity and Inflammation, University of Leicester.

2.1.2.1 Staining with HLA-A2 antibody:

The isolated PBMCs were washed in cold PBS, and counted. Then, $1x10^5$ cells were resuspended in the least amount of PBS, and stained with 10µl of anti-HLA A2 antibody [BB7.2] (Abcam, UK and BD Pharmingen, UK), and incubated in dark on ice for 20 minutes. The cells were washed twice using 2ml of sterile pre-cooled washing buffer WB (1% BSA in PBS) and centrifuged. The pellets were dispensed in 1ml PBS, and flow cytometry were carried out using a FACS Canto flow cytometer (Becton Dickinson, UK). Fluorescently labelled isotype control antibodies were used as negative controls.

Table 2.1: Details of MM patients used during this study.

Pt no	Sex	Age	Paraprotein isotype	Paraprotein Level (g/L)	Current status	Current medication (myeloma)	Lymphocyte count (x10*9/L)	HLA-A2 status
1	M	67	IgGK	NBD	Complete remission	No current medication	2.48	negative
2	M	67	IgGK	27.7	Stable myeloma	No current medication	1.35	positive
3	M	75	IgAK	NBD	2nd relapse 2015	Lenalidomide / Dexamethasone	0.90	negative
4	F	71	IgGL	8.6	Stable myeloma	No current medication	8.95	positive
5	F	86	IgGK	26.1	3rd relapse 2016	Stopped Lenalinomide March 2016 (no response)	1.08	positive
6	M	61	IgAK	NBD	2nd relapse 2015	Lenalinomide / Dexamethasone	2.29	negative
7	F	61	IgAL	NBD	Plasmacytoma "watch and wait"	No current medication	1.29	positive
8	M	76	IgGK	14.7	1st relapse 2016	No current medication	1.14	negative
9	M	54	IgGK	6.4	Stable remission	No current medication	2.46	positive

NBD = no paraprotein band detected in serum.

2.1.3 Cell culturing

The growth of the cell lines was maintained in tissue culture in supplemented growth medium (SGM)_RPMI-1640 (with L-glutamine, streptomycin, penicillin and 10% foetal calf serum (FCS) (Sigma, UK) in 75 or 25cm^3 tissue culture flasks (Nunc, UK) (Fig. 2.1). These flasks were incubated at 37°C and 5% CO_2 in a humid atmosphere. Every 2-3 days, the cells were split 1:4 with fresh SGM to preserve the cells' growth.

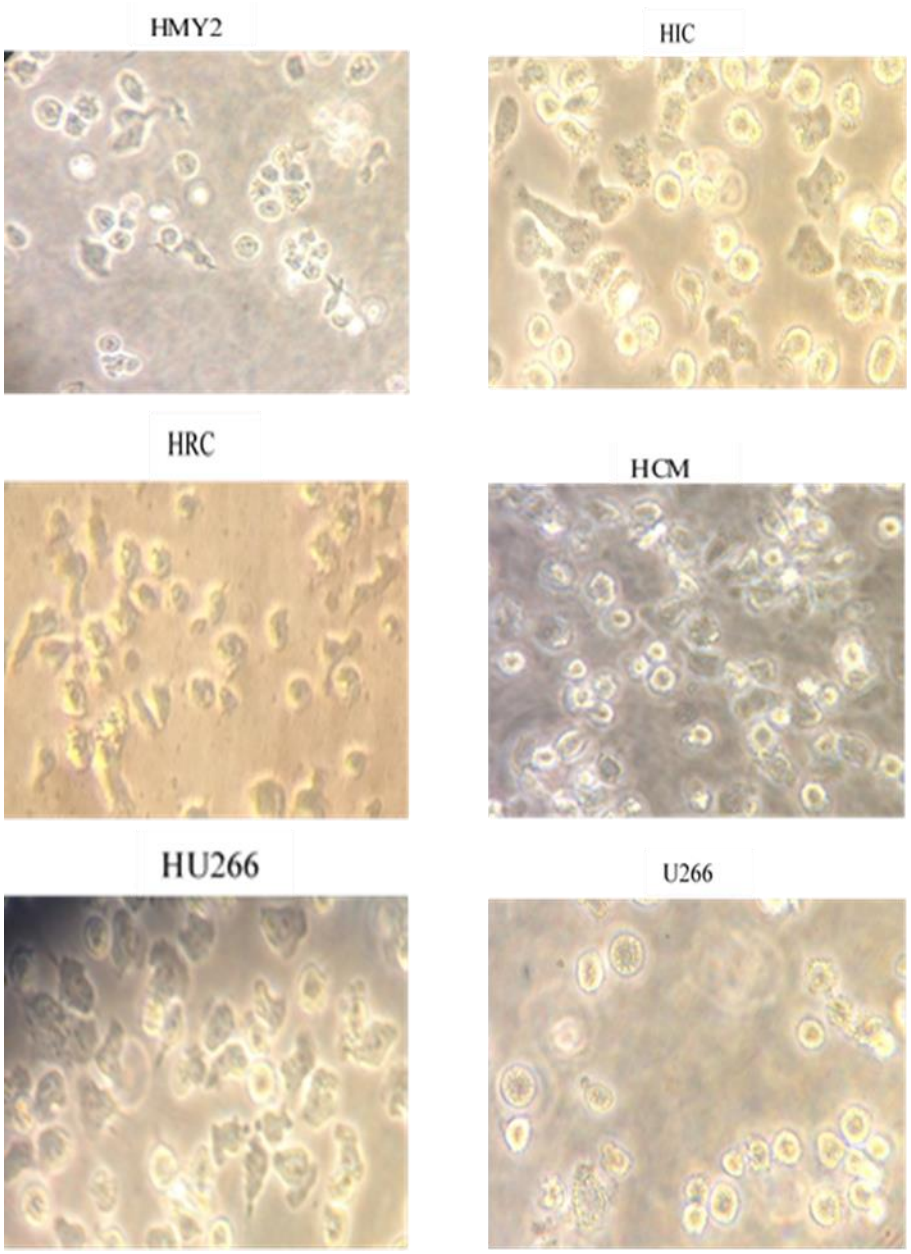

Figure 2.1: The growth and proliferation of HMY2, the hybrid cell lines (HRC, HIC, HCM, and HU266), and U266 in the cultures, using 40X magnification.

2.1.4 Cell freezing

Prior to cell freezing, the cells were subjected to trypan blue staining, to evaluate cell viability using stained cell exclusion method. Normally, around 5 – 10 $X10^6$ viable cells were obtained from each flask. Cell cultures were centrifuged at 300 xg for 10 minutes, then 1ml of cold freezing medium (10% dimethyl sulfoxide (Sigma, UK) in RPMI containing 10% FCS) was added to the cells gradually and mixed gently. After that, the cells were transferred to 1.5ml cryovials, and placed in precooled Mr Frosty box (Thermo Scientific) in a -80°C freezer. After 24 hours, the cryovials were moved into a cryobox, and placed in liquid nitrogen vessel (-196°C).

2.1.5 Cell thawing

Cryovials were removed from the liquid nitrogen storage vessel, placed on dry ice, and the cells were quickly thawed. Immediately afterward, the cells were washed using 10 ml of SGM and centrifuged. After centrifugation, the cell pellets were suspended in 10ml of SGM and transferred to tissue culture flasks, and then they were incubated in a humid incubator at 37°C, and 5% CO_2, in 25 cm^3 tissue culture flasks.

2.2 Phenotypic characterization of hybrid cell lines, HMy2, and U266

2.2.1 Cell surface markers, HLAs, CS1 and RHAMM immunofluorescent staining

For investigation of phenotypic characteristics of HMy2, U266 and the hybrid cell lines, direct surface immunofluorescence (IF) staining was performed by staining the viable cells with specific fluorescently labelled mouse anti-human monoclonal antibodies (mAbs), and flow cytometric analysis was carried out to investigate the human leukocyte antigen expression and different cell surface markers.

Firstly, cells were harvested from the culture, washed in cold PBS, then counted and distributed as $0.5x10^6$ cells in a least amount of PBS per tube. For staining, 10µl of the monoclonal antibody (mAb) was added to each tube, mixed by vortexing, and incubated in dark on ice for 20 minutes. After that, the cells were washed twice using 2ml of sterile pre-cooled washing buffer WB (1% BSA in PBS) and centrifuged. The pellets were dispensed by flicking the tube gently, and pre-cooled 0.5 ml of fixing buffer FB (1% paraformaldehyde (Sigma, UK) in PBS) was added. Finally, the tubes were stored at 4°C in the dark till flow cytometric analysis.

2.2.1.1 Flow cytometric analysis

Flow cytometry was carried out for each of the five cell lines, after staining with the antibodies listed in (Table 2.2), using a FACS Canto flow cytometer (Becton Dickinson, UK). Then, the data were analysed using FACS Diva software (Becton Dickinson, UK). Fluorescently labelled isotope control antibodies were used as negative controls. During the analysis, $1x10^4$ events were acquired, and gates were drawn to identify the living populations using forward and side scatter dot plots.

Table 2.2: Immunoglobulin antibodies used for flow cytometric analysis.

mAb	Label	Isotype	Source	Catalogue Number	Clone
Isotype control	FITC	IgG_1	BD Pharmingen, UK	551954	MOPC-21
Isotype control	PE	IgG_1	Beckman Coulter, UK	A07796	679.1Mc7
HLA-ABC	FITC	IgG_1	Beckman Coulter, UK	IM1838U	B9.12.1
HLA-A2	FITC	IgG_{2a}	BD Pharmingen, UK	551285	BB7.2
HLA-DP,DQ, DR	PE	IgG_1	Dako Cytomation, UK	F0817	CR3/43
CD19	FITC	IgG_1	BD Pharmingen, UK	555412	HIB19
CD80	FITC	IgG_1	Beckman Coulter, UK	IM1853U	MAB104
CD86	PE	IgG_1	BD Pharmingen, UK	555658	2331(FUN-1)
CD138	PE	IgG_1	Beckman Coulter, UK	IM2757	BB4
Mouse monoclonal isotype control	PE	IgG2b	Abcam UK	ab91532	PLPV219
Mouse monoclonal Anti-CS1antibody	PE	IgG2b	Abcam UK	Ab95827	162
Rabbit IgG monoclonal Isotype control	FITC	IgG	Antibodies online	ABIN1586119	polyclonal
Rabbit monoclonal Anti human RHAMM	FITC	IgG	US Biological life science	036650	polyclonal

2.2.2 Detection of the expression of tumour associated antigens using RT-PCR

2.2.2.1 RNA extraction

Extraction of total RNA from about 1×10^7 cells was done by RNeasyMinikit (Qiagen, UK), following the instructions of the manufacturer. RNAase free Falcon tubes were used to harvest the cells, centrifuged for 10 min at 300 xg. Then the pellets were loosened and washed in PBS. After washing, 600 µl RLT buffer was added and vortexed for complete dissolution of the cells. Next, the cells were homogenised using a 23 gauge needle, and mixed with an equivalent volume of 70% ethanol. After that, 700 µl of the cell lysate was placed in a RNA minicolumn, which was centrifuged for 15 seconds at 8000 xg.

2.2.2.2 Genomic DNA degradation

After RNA extraction, 350 µl RW1 buffer was added to the RNeasy column and centrifuged for 15 seconds at 8000xg. Then, 10 µl of DNase 1 stock solution was diluted with 70 µl RDD buffer. After that, this mixture (80 µl) was added to the RNeasy mini column and incubated for 15 minutes on bench top for the digestion of genomic DNA. Then, the columns were washed using 350 µl RW1 and centrifuged. Afterward, 500 µl RPE buffer was added, and centrifuged at 8000xg for 2 minutes. The column was placed in a 1.5ml collection tube, and RNA eluted with 30 µl of RNase free water, then centrifuged at 8000xg for 1minute.

2.2.2.3 First strand Complementary DNA (cDNA) synthesis

A cDNA Superscript™ First-Strand Synthesis kit (Invitrogen, UK) was used for reverse transcription of total RNA into cDNA, following the instructions of the manufacturer. Up to 2.5 µg RNA was mixed with 4 µl 5X reaction mixture and 2 µl of 10X Superscript Enzyme, and the volume was completed to 20 µl with DEPC- treated water. Afterwards, the tubes were mixed gently, and incubated for 10 min at 25°C. Then the tubes were incubated at 42°C for 60 minutes, and finally the reactions were terminated at 85°C for 5 minutes.

2.2.2.4 Reverse transcription- polymerase chain reaction (RT-PCR)

Reverse transcription-PCR was performed using cDNA template, to examine the expression of the TAAs of interest. 2 µl cDNA templates were mixed with 2.5 µl 10X buffer, 4 µl 1.25 mM dNTP, 25 mM $MgCl_2$, 2 µl 5 pmol/ µl of forward and reverse primers, 0.2 µl Taq-polymerase 5U/µl, and PCR grade water to a final volume of 25µl (all

from Sigma, UK). All these ingredients were mixed in a 0.2 ml PCR tube, placed on ice. The tubes were mixed and transferred to the Thermo-cycler (T100™ Thermal Cycler BIO RAD). After PCR, the products were run by electrophoresis on agarose gel (1.5%) with 0.5 µg/ml EtBr (ethidium bromide, Sigma, UK), which intercalates with DNA, and then visualized under ultraviolet (U.V) light. All the primers used, the amplicon size, and the annealing temp are summarized in Table 2.3.

Table 2.3: primer sequences, annealing temperatures and amplicon product sizes for RT-PCR and q- PCR for detection of the examined TAA expression.

Antigen	Primers sequences	Prod. size	Anneal-ing temp	Reference
RHAMM	5'CAG GTC ACC CAA AGG AGT CTC G-3' 5'CAA GCT CAT CCA GTG TTT GC-3'	571 bp	60°C	Greiner, et al ,2002
MAGE-A1	5'CGGCCGAAGGAACCTGACCCAG-3' 5'GCTGGAACCCTCACTGGGTTGCC-3	421 bp	68°C	Van Baren et al., 1999
PRAME	5'CTGTACTCATTTCCAGAGCCAGA-3' 5'TATTGAGAGGGTTTCCAAGGGGTT3'	561 bp	67°C	Van Baren et al., 1999
MUC1	5'GCTGCTCCTCACAGTGCTTA-3' 5'GCTGGGCACTGAACTTCTCT-3'	608 bp	59°C	NCBI: FJ226029
h TERT	5'GCTGTTTGCGGGGATTCGGC-3' 5'CCACGCAGCCATACTCAGGGAC-3'	137 bp	61°C	Mohamed, 2012
Survivin	5'CACCGCATCTCTACATTCAA-3' 5'CACTTTCTTCGCAGTTTCCT-3'	275 bp	59°C	Schmidt et al., 2003
DKK1	5'AGACCATTGACAACTACCAGCCGT-3' 5'TCTGGAATACCCATCCAAGGTGCT-3'	287bp	59°C	NCBI: NM_012242.2
CS1	5'GGACCTTCAACACAACCCCT-3' 5'AGGTGCTCGTAGACATGCAG-3'	226 bp	55°C	NCBI: NM_001282592.1
MAGE-C1	5'AGCGGAGGGAGGAGACTTAT-3' 5'TCGGCATCCCAGCAGTAGG-3'	278 bp	55°C	NCBI: NM_005462.4
β-Actin	5'GCTCGTCGTCGACAACGGCTC-3' 5'CAAACATGATCTGGGTCATCTTCTC-3'	353 bp	60°C	Invitrogen

2.2.3 Semi-quantitative detection of TAAs expression levels

Relative detection of the expression level of TAA genes in the examined cell lines (different hybrids, HMy2 and U266) was determined by real time PCR, using 7500 Fast Real time PCR system (AB Applied Biosystem), following the manufacturer's instructions. Power SYBR green PCR master mix (AB Applied Biosystem) was used for amplification of cDNA (Power SYBR green PCR master mix contains fluorescent dye (SYBR green), hot start Taq polymerase, dNTP mix and polymerase buffer).

2.2.3.1 Principle and procedure of q PCR

The power SYBR green is a fluorescent dye, which can bind to the minor grooves of the DNA double helix molecules. The fluorescence of the dye is enhanced when bound to the double-stranded DNA, allowing the Light Cycler to estimate the quantity of the double stranded DNA formed in each cycle.

The reaction mixture preparation contained; SYBR green mix 12.5µl, forward primer 0.5 µl, reverse primer 0.5µl and PCR-grade water 10.5µl. All the reagents were premixed and added to 1µl cDNA template in 96 well plates. The standard curve was constructed by four tenfold serial dilutions of the positive template, which was made by mixing all the samples together.

The reaction was started by activation of the Taq polymerase at 95˚C for 30 seconds, then 40 amplification cycles were performed (denaturation at 95˚C for 15 second, annealing for 30 seconds at the required temperature, elongation at 60°C for 45 seconds). At the end of each cycle, a data acquisition step was performed to estimate the amount of DNA amplified. At the end of the run, the temperature was gradually increased, with continuous data acquisition, from 60˚C to 95˚C to construct the melting curve, for determination of the purity and specificity of the end product. Pure end product should produce a melting curve of a single peak (Figure 2.2).

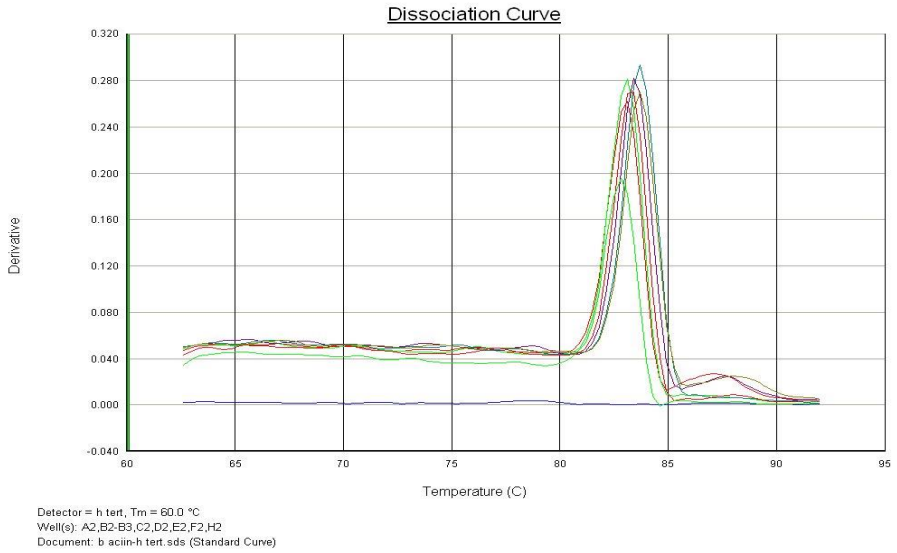

Figure 2.2: Dissociation curve with a single peak of HMY2, hybrid cell lines and U266 and base line of negative control.

2.2.3.2 Antigen expression quantification

The levels of TAA expression were calculated automatically by the software of the machine, using the constructed standard curve (Figure 2.3). The standard curve is the plotting of log concentration of tenfold dilutions (1, 0.1, 0.01, and 0.001) of the positive template versus CT (which is the threshold cycle); it is the cycle number which has a detectable increase in the fluorescence of the SYBR green. The standard curve was constructed automatically by the software by the completion of the run. Relative mRNA expression was estimated using the calculated mRNA concentration, after normalization to the beta actin expression value in the same cDNA preparation.

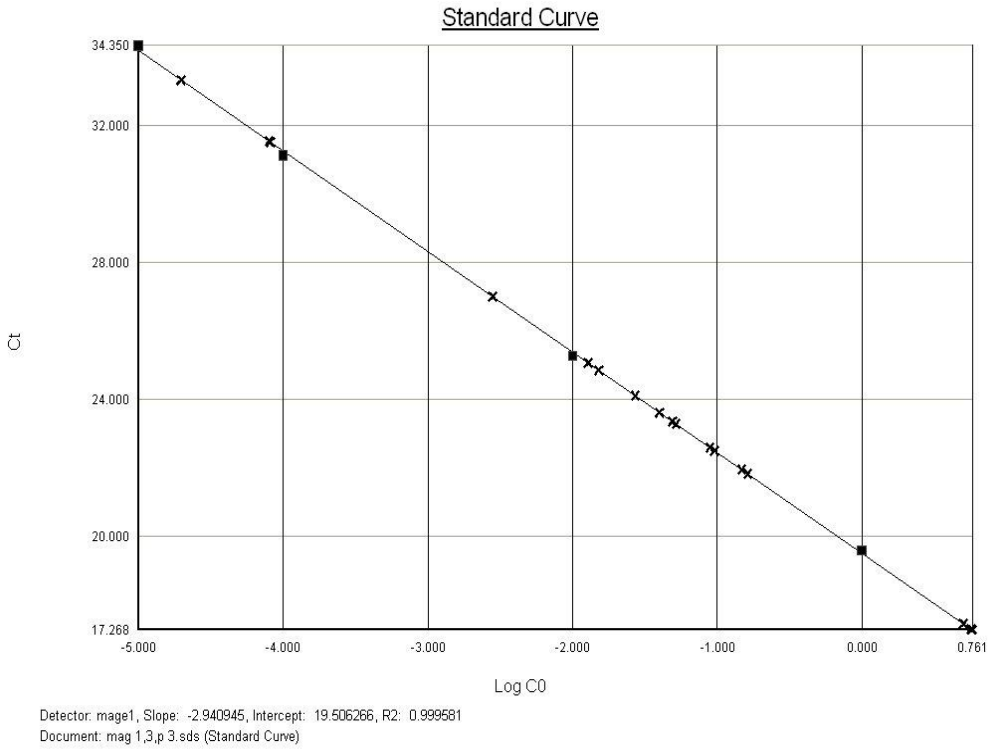

Figure 2.3: Representative standard curve of q PCR, straight line demonstrating linear relationship between the cycle numbers and log concentrations of the examined samples.

2.2.4 Protein translation level of tumour associated antigens

2.2.4.1 Detection of RHAMM and CS1 protein

Detection of RHAMM and CS1 protein level by direct Immunofluorescence Single-color Staining for Flow Cytometric analysis was mentioned previously in section 2.2.1.

2.2.4.2 Detection of MUC1 protein translation level by indirect staining

Detection of MUC1 protein translation level was done by indirect staining, as there was no directly conjugated antibody available. For indirect staining, the cells were harvested, washed, counted and adjusted to a concentration of 1 x 10^6 cells/ ml with 1% BSA/PBS buffer and centrifuged. After centrifugation, 10 µl of anti MUC1 mouse monoclonal antibody (anti-CD227; Insight Biotechnology Ltd) was added, mixed by pipetting and incubated at room temperature for 30 minutes. Then, the cells were washed with 5 ml of PBS/BSA buffer, centrifuged and the resulting supernatant was discarded. Next, 10 µl of the PE anti mouse Ig G secondary antibody (Sigma Aldrich) at the recommended dilution (1:500) was added. Afterwards, the cells were mixed well and incubated at 4°C for 30

minutes. Then the cells were washed, fixed and subjected to flow cytometric analysis as described previously.

2.2.4.3 Detection of Intracellular h TERT antigen by Flow Cytometry

The cells were analysed for expression of the intracellular human telomerase reverse transcriptase antigen (h TERT) using Fix and Perm kit (Life Technology.uk), according to the manufacturer's instructions for methanol modification method. Firstly, 100 µl of adjusted cell volume (equivalent to 1 x 10^6 cells) was added to 100 µl of Reagent A (Fixation Medium) and incubated at room temperature for 2–3 minutes. Then, 4 ml of cooled absolute methanol was added and mixed. After that, the cells were incubated at 4°C for 10 minutes, centrifuged at 300–350 x g for 5 minutes and washed with the washing buffer (PBS + 5% FBS). Afterward, 100 µl of Reagent B (Permeabilization Medium) and 10 µl of intracellular FITC anti h TERT antibody; Bioss bs-1411R or its corresponding FITC Ig G isotype control; Bioss bs-0295P, was added. Then, the cells were mixed and incubated for 30 minutes at room temperature. After incubation, the cells were washed three times with the previous washing buffer. Finally, the cell pellet was dispersed in 0.5 ml of fixation buffer (1% paraformaldehyde) and stored in the dark at 4°C. Flow cytometric analysis was carried out within 18 hours, as described in section 2.1.1.2.

2.2.4.4 Western Blotting for detection of MAGE-C1

Total cell proteins were extracted by lysis of 1×10^6 cells in 200 µl 2X lysis buffer (containing 50 mM Tris-Cl pH 6.8, 10% glycerol, 2% SDS and 0.1% bromophenol blue), then incubated for 5 min at 95°C and centrifuged at 300xg for 10 min. After centrifugation, protein lysates were separated on 8% polyacrylamide gels (SDS-PAGE) at 60 ampere for 60 min and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Sigma Aldrich, UK).

For MAGE-C1 protein detection, the membrane was blocked by incubation at room temperature in 5% non-fat dry milk in PBS for 60 min, and then incubated overnight with the primary rabbit anti MAGE-C1 polyclonal antibody (Fisher Scientific.UK) at a dilution of 1: 1,000 at 4°C. After that, the membrane was washed three times in 0.5% PBST for 15 min each, and incubated with polyclonal anti- rabbit immunoglobulin HRP (Dako, Denmark) for 1 hour at room temperature and washed three times as before. Finally, the membrane was washed and developed by using an enhanced chemiluminescence (ECL) detection system and visualised using X-ray.

After detection of MAGE-C1, the same membrane was used for detection of β actin. The membrane washed three times in 0.5% PBST for 15 min, and incubated overnight with mouse monoclonal anti- β actin antibody (Sigma – Aldrich UK) in dilution of 1: 5,000 at 4°C and washed three times in BST for 15 min each. After washing, the membranes were incubated with polyclonal goat anti mouse immunoglobulin HRP (Dako, Denmark) at a dilution of (1: 5,000) for 1.5 h at room temperature. Then ECL Western Blotting Detection Reagent was used as before for detection of antibody binding.

2.3 *In vitro* Immunostimulatory assays of PBMCs using the hybrid and parent cell lines:

The purpose of this experiment was to examine the ability of the hybrid and parent cell lines to induce lymphoproliferative T cell responses.

2.3.1 Preparation of HMy2, hybrid and parent tumour cell lines

The hybrid cell lines, HMy2 and U266 were used as stimulators for allogeneic PBMC in separate proliferation assays. The stimulator cell lines were treated with 0.5ml Mitomycin-C (50µg/ ml) (Sigma, UK) and incubated at 37°C in 5% CO2 for 30 minutes (to inhibit cell division by induction of cross linking of DNA). After that, the cells were washed at least twice in warmed RPMI-1640 medium, counted, and then re-suspended in SGM at a concentration of $1X10^6$ cells /ml.

2.3.2 Preparation of the responder cells

2.3.2.1 Separation of peripheral blood mononuclear cells

The PBMC were isolated from venous blood of nine patients suffering from MM, and from 15 normal healthy individuals, using density gradient centrifugation over lymphocyte separation medium, as follows: Firstly, venous blood was collected into heparin (10 U/ml of blood) from the donor by venepuncture, and then the blood was diluted with an equal volume of sterile PBS. Diluted blood was layered gradually over Ficoll Paque plus solution (GE healthCare). Tubes were then centrifuged at 400 xg, at 18-20°C, for 30-40 minutes. After centrifugation, PBMC were collected from the buffy interface between PBS and the Ficoll layer. Next, PBMC were washed twice with PBS medium, and centrifuged at 1200 rpm for 10 minutes. A sterile pre-warmed 0.1% BSA in PBS solution was used to re-suspend PBMC cells at concentration of $1x10^6$ cells/ ml.

2.3.2.2 PBMC labelling with Carboxyfluorescein diacetate succinimidyl ester:

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a cell labelling and tracking dye that is used for tracking and phenotyping the dividing T cells. This dye can diffuse into the cells and interact with the intracellular amines, by its ester groups, forming fluorescent dye-protein (with excitation peaks of 492 and emission peaks of 517 nm). This fluorescence is well retained by the cells. Accordingly, upon cell division, the dye will be divided equally between the new daughter cells; for this reason the intensity of the label can be used as an indicator of the cell proliferation level.

The CFSE dye was prepared according to the manufacturer's instructions by dissolving the components of one vial (50µg lyophilized CFDA-SE powder) of Cell Trace™ CFSE Cell Proliferation Kit (Invitrogen, Paisley, UK), in 18µl of DMSO (provided with the kit) to obtain 5mM stock solution. The remaining reagent was kept at -20°C in the dark.

2.3.2.2.1 CFSE labelling

1µl of 5mM CFSE stock solution was used to label each $1x10^6$ cells/ ml of PBMCs cell suspension. After labelling, the cells were incubated at 37°C for 10 minutes in 5% CO2 in a humid incubator. Five volumes of precooled RPMI-1640 media was added to quench the excess dye from the cells, and the tubes were placed for 5 minutes on ice. After incubation, the cells were centrifuged for 10 minutes at 1200 rpm then the pellet was washed twice with RPMI media. Then, the labelled cells were ready to use as responder T cells in the stimulation reaction.

2.3.2.2.2 Stimulation of the CFSE labelled-PBMC with the hybrid and parent cell lines:

In a 24 well plate, $2x10^6$ of CFSE-stained PBMCs were mixed with $1x\ 10^6$ of Mitomycin-C treated stimulator cell lines (HMy2, HU266, HCM, HIC, and U266). The plate was incubated for 5 days at 37°C. Then the cells were used for CFSE/CD markers flow cytometric analysis. The unstimulated CFSE-stained PBMC were used as negative controls.

2.3.2.2.3 Characterization of the subpopulations of the proliferating cells:

The harvested CFSE labelled cells were washed using cold PBS. The cells were suspended in the remaining PBS after decantation of the supernatant, and stained with10µl of the following mAbs for surface IF staining using CD3-PE; BD Pharmingen, UK 555333, CD4-PECY7; BD Pharmingen, UK 557852 and CD8-PerCP 5.5 mAbs BD

Pharmingen, UK 341051. Then the cells were vortexed and incubated for 20 minutes in the dark at 4°C. After that, the cells were washed in WB twice and then resuspended in 1ml PBS. To assess the proliferation of T cell subpopulations, cell acquisition was done using a FACS Canto flow cytometry system. Data were analysed by FACS Diva software (Becton-Dickinson Ltd, UK). Multiple gating was performed on CD3, CD4 or CD8 populations.

2.4 Induction of long-term activated tumour antigen specific cytotoxic T lymphocyte cell cultures:

With the intention of examining the ability of HMY2, the hybrid cell lines and U266 to induce functional antigen specific T cell responses *in vitro* (in terms of cytokine release, and cytotoxic ability), repeated stimulation of allogeneic T cells using HMy2, hybrid and parent tumour cell lines was carried out to establish long-term activated antigen specific T cell cultures.

Long-term activated T lymphocyte cultures were produced by co-culturing an allogeneic donor's PBMCs (isolated from HLA-$A2^+$ normal healthy individuals and MM patients) with stimulator cell lines for several successive rounds (each of one week). The stimulator cell lines were treated with 0.5 ml (50µg/ml) Mitomycin-C (Sigma, UK) for each $4x10^6$ cells. In the first round at day 1, PBMCs were isolated and mixed with Mitomycin-C treated stimulator cells, as $2x10^6$ responder cells in 2:1 ratio in 6 well plates, in 2 ml/ well SGM as total volume, in the presence of 1ng/ ml IL-7 (BD Bioscience, UK). Fresh SGM was added on the third and fifth day. In the second round, the cell cultures from the first round were centrifuged. After centrifugation, the cells were suspended in fresh SGM, counted and re-activated with a new lot of Mitomycin-C treated stimulators in 3:1 ratio, and in 2 ml/ well of SGM in presence of 300U/ml rhIL-2 (Prospec, Protein-Specialists) and IL-7 (1ng/ml) in 6-well plates. Fresh SGM was added on days nine, eleven, and thirteen. In the third week, on day fifteen, the cell cultures from the second round were centrifuged as before. After centrifugation, the cells were suspended in fresh SGM, counted and re-activated with a new batch of Mitomycin-C treated stimulators in 3:1 ratio, and in 2 ml/ well of SGM in presence of rhIL-2, IL-7, and 10ng/ml rhIL-15 (Prospec, Protein-Specialists) in 6-well plates, and fresh SGM was added on days seventeen, nineteen, and twenty-one (Figure 2.4). By the beginning of the fourth round, cells were harvested and counted as before, and in accordance with the number of the produced T cells, these cells were either exposed to an additional stimulation round, or used directly

in subsequent assays. The activation rounds were carried out for up to 10 rounds for the selected hybrids and their available parents in parallel under similar co-culturing conditions. The produced activated T cell cultures were used for characterization of the proliferating cell populations, and also for IFNγ releasing and perforin releasing ELISpot assays, as responder cells to investigate their activities. Moreover, pentamer staining assay was used to examine the production of tumour antigen specific T cell clones.

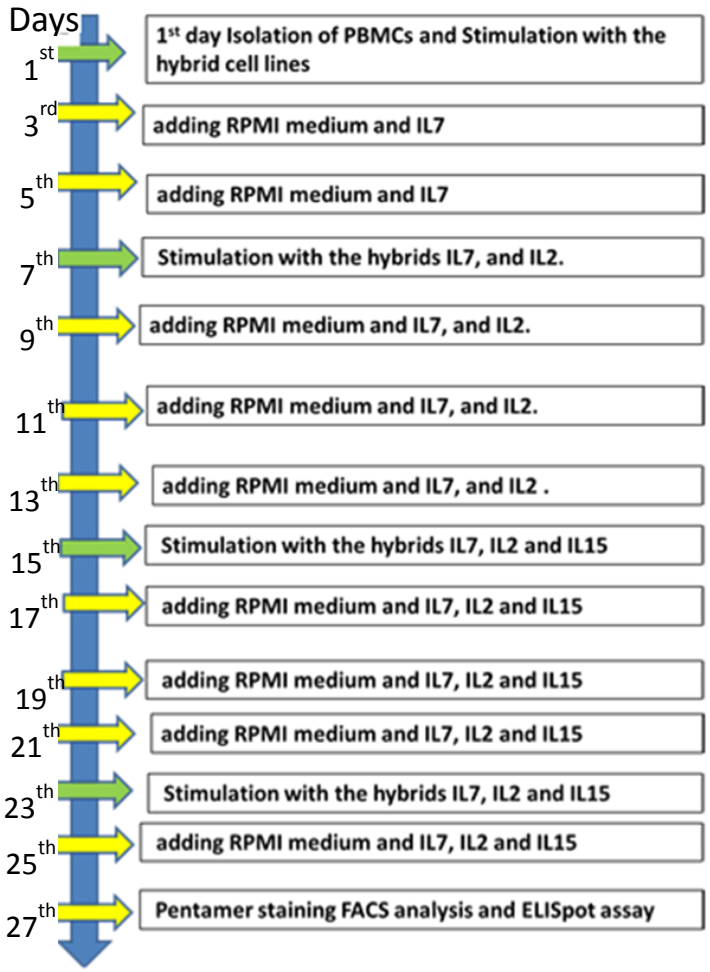

Figure 2.4: the long-term stimulation protocol. *The isolated PBMCs were stimulated weekly with the hybrid cell lines, HMY2, and U266.*

2.4.1 MHC Class I Pentamers staining

2.4.1.1 MHC Class I Pentamers staining principle:

Pro5® MHC Class I Pentamers (Proimmune, UK) include five MHC class I peptide complexes connected together through a coiled-coil domain. In addition, each Pro5® pentamer comprises five fluorescent tags for efficient and bright labelling (Figure 2.6). The Pro5® MHC Class I Pentamers bind directly to T cell receptors of a particular

specificity. This specificity is determined by the Major Histocompatibility Complex (MHC) allele and peptide combination.

Recognition and estimation of tumour antigen-specific CTLs were carried out by staining of long-term activated T cells with FITC-labelled anti-CD8 and R-PE-labelled Pro5 MHC class I pentamers incorporating different HLA-A2 binding peptides (Proimmune Limited, UK). Then, flow cytometric analysis was carried out. The four different HLA-A2-restricted peptide pentamers of the selected antigens are described in Table 2.5, as well as the control peptide.

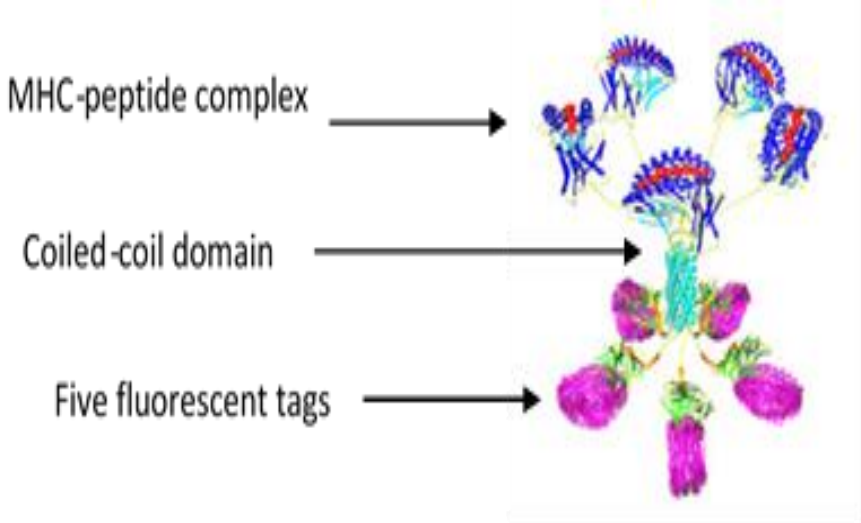

Figure 2.5:Pro5® MHC Class I Pentamers structure. *Figure from (Proimmune pentamer manual).*

Table 2.4: HLA-A2-peptide pentamers of relevant TAA, *and irrelevant WNF virus peptides, labelling, and peptide sequence.*

The antigen	HLA-A2-restricted peptide	Peptide sequence	Labelling
MUC1	MUC1 (12-20)	LLLLTVLTV	R-PE
hTERT	hTERT (540-548)	ILAKFLHWL	R-PE
MAGE-C1(CT-7)	MAGE-C1 (959-968)	ILFGISLREV	R-PE
CS1	CS1 (239-247)	SLFVLGLFL	R-PE
WNF	WNF (294-302)	LGMSNRDFL	R-PE

2.4.1.2 *Pro5® MHC Class I Pentamers staining procedure:*

Following the manufacturer's instructions, the cells were harvested, washed with WB (1% BSA in PBS), counted and dispensed as $2.5x10^6$ cells in 50µl of WB, in FACS tubes. The new vials of the pentamers were spun for 5 minutes at 14000xg at 4°C to decrease the risk of non-specific staining caused by protein aggregates. Next, 10µl of the labelled pentamers (Proimmune Limited, UK) were mixed with the cells, and incubated for 10 minutes in the dark at room temperature. Afterwards, cells were washed once using 2ml cold buffer (1% BSA in PBS), and centrifuged for 5 minutes at 500 xg. After centrifugation, the pellet was dispensed by gentle flicking and 1µl of FITC-labelled anti-CD8 mAb, (Proimmune Limited, UK) was added. Cells were incubated for 20 minutes, at 4°C, shielded from light. After incubation, cells were washed as before, and the resulting pellets were dispensed in 1ml PBS. The tubes were stored at 4°C in the dark before flow cytometric analysis, which was carried out within three hours. Unstimulated PBMCs were isolated from the same normal healthy donor or the MM patient were stained with the same anti-CD8mAb and pentamers and subjected to the same staining conditions as the stimulated cells, and used as negative controls.

Cells were acquired by a FACS Canto flow cytometer, and the analysis was done using FACS Diva software (Becton Deckinson, UK). Appropriate gating on living T cells was done, and sequential gating, 10,000 events acquired in the appropriate gate for $CD8^+$ T cell analysis.

2.4.2 Enzyme Linked ImmunoSpot (ELISpot) Assays

The enzyme-linked immunospot assay is a highly sensitive technique that allows quantification of the responding cytokine-secreting cells at the single-cell level, by detection of lymphocyte cytokine release under certain stimulation conditions. IFNγ is a very important cytokine that is released by activated helper and cytotoxic T lymphocytes, and it is a feature of their response. Furthermore, IFNγ can adjust immune responses by its effect on T cells, APCs, and B cells. Perforin is one of the essential cytolytic proteins of cytotoxic T cells. Also, it is known a key effector molecule for T-cell activation.

2.4.2.1 *Perforin and IFNγ ELISpot assays using the long-term activated T cells as responder cells.*

The assays were achieved using human IFNγ ELISpot kit and perforin ELISpot kit (Mabtech AB, Sweden), in accordance with the manufacturer's instructions. The

Multiscreen PDVF filter 96 well plates (Millipore, USA) were activated by adding 50µl/ per well of 70% ethanol, and were incubated for 1 minute at RT. After incubation, the plates were washed five times by addition of 200µl/well sterile distilled water. After washing the wells were coated by addition of 100µl/ well of the capture antibody (anti-IFNγ mAb clone 1-D1K diluted to 15µg/ml in sterile PBS or anti-perforin mAb clone Pf-80/164 diluted to 30µg/ml in sterile PBS).

Then, the plate was incubated at 4°C overnight. After incubation the excess antibody was removed and the plate was washed five times with 200µl/ well of sterile PBS. After that, 200µl/ well of SGM containing 10 % of the same serum used in cell suspension was added for blocking of any non-specific binding, and the plate was left for not less than 30 minutes at RT.

The stimulator cells were harvested, counted and treated with Mitomycin-C, to stop cell division as described in Section 2.3, and washed twice in RPMI medium. Afterward, cells were re-suspended at the desired concentration in SGM, and 100µl was added to each well. The responder cells (the long-term activated T cells) were counted, re-suspended in SGM and seeded in the desired concentration as 100µl/ well in triplicate wells, and mixed with the responder cells in 96-well plates. The ratio of the responder long-term activated T cells to stimulator cells was adjusted in a total volume of 200µl per well. Then the plates were incubated for 48 hours for detection of IFNγ and 5 days for detection of perforin at 37°C in a humid atmosphere in 5% CO_2. During the incubation time, T cells were stimulated and IFNγ and perforin were produced. The produced IFNγ and perforin were captured by the coating mAb attached to the surface of the plate. Afterward, the cells were removed and the plates were washed with 200 µl/well with sterile PBS for 5 times. Next, the secondary (Pf-344-biotin) anti-perforin mAb and the biotinylated anti IFNγ secondary antibody (7-B6-1) were diluted in sterile PBS containing 0.5% FCS to 1µg/ ml, and 100µl/ well was added and incubated for 2 hours at RT. After that, the excess antibody was eliminated, then the plate was washed as described in the last step, and Streptavidin conjugated alkaline phosphatase (Streptavidin-ALP; Mabtech, Sweden) diluted 1:500 in PBS containing 0.5% FCS, and 100µl was added in each well, followed by incubation at RT for one hour. Then, the unbound enzyme was removed by washing the plate as described before, and 100µl/ well of filtered substrate solution, TMB (tetra methylated benzidine; Mabtech, Sweden), was added, and the plate was incubated at RT for 15 minutes or until purple spots emerged in the positive wells. Then the plate was washed

extensively using nano pure water to terminate the reaction. Then the plate was left to dry and the emerged spots were counted using x40 magnification lens under a dissection microscope. Figure 2.6 shows schematic illustration of the principle of the ELISpot assay.

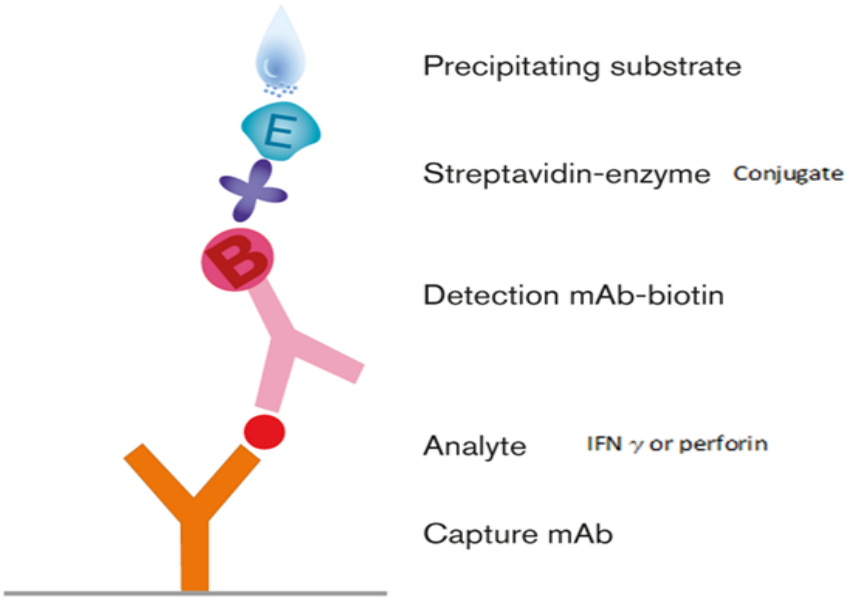

Figure 2.6: Schematic illustration of the principle of the ELISpot assay. *Figure adapted from Mabtech, UK, ELISpot assay principle.*

2.4.2.2 Tumour antigen-specific ELISpot assays:

In order to determine the activity of the produced antigen specific CTL in the long-term stimulated cultures, HLA-A2 restricted TAA peptide-pulsed T2 cell line was used as target cells in IFNγ and perforin ELISpot assay, and the cytotoxicity test, in order to investigate the presence and activity of its cognate antigen specific CTLs.

2.4.2.2.1 T2 cell line

T2 cells are a TAP deficient cell line, which is characterized by its incompetence to present the endogenous antigens in the context of their HLA class I molecules. However, the exogenous antigens of interest can be loaded on HLA-A2 cell surface molecules on T2, and used as a target in different antigen specific CTL assays.

2.4.2.2.2 T2 cell line peptide pulsing

The candidate peptides; ProImmune, Ltd, UK (Table1.5) were solubilized according to the manufacture's instruction in 10µl DMSO, and then diluted to 1ml using distilled H_2O. The dissolved peptides were aliquoted into small volumes, and stored in -80^{0}C until use.

T2 cell line was harvested, spun for 5 min at 1480 rpm, washed in SGM medium and counted. The cells were suspended to a concentration of $3X10^6$/ml in serum free SGM, and pulsed in a 12 well plate with 50µg/ml of the desired peptide, and 3µg/ml β2-microglobulin (Sigma, UK). The plates were mixed and incubated at 37°C in a humid atmosphere, and 5% CO2 for 4 hours. After incubation the cells were washed twice in serum free SGM, and used as a targets in IFNγ and perforin ELISpot assays, as previously described in section 2.5.1.1.

2.4.3 Cell sorting and expansion of the long-term activated cultures

The hybrid cell lines, HMY2 and U266 long-term stimulated cultures were carried out as mentioned in section 2.4, and stained with pentamer staining as described in section 2.4.1.1.2. The highest percentages of the antigen specific $CD8^+$ T cells were obtained from HU266 and HRC long-term stimulated cultures for MUC1, and CS1 antigen specific $CD8^+$ T cells respectively. Accordingly, HRC and HU266 stimulated cultures were FACS-sorted using FACSAriaII, in an attempt to isolate CS1 and MUC1antigen specific $CD8^+$ T cells, respectively. The data analysis was done using FACSDiva software Version 6.1.3 (BD Biocsiences, San Jose, USA). The expansion of the sorted cells was carried out using Dynabeads® Human T-Activator CD3/CD28/CD137 (Thermofisher Scientific, UK). According to the manufacturer's instructions, the sorted cells were dispensed in different wells in 96 well plate, at concentration of 100,000 cells in 100-200µl SGM/well. The Dynabeads were added to obtain beads: cell ratio of 1:10, in the presence of IL-2 (300U/ml), IL-7 (1ng/ml), and IL-15 (10ng/ml). The plate was incubated in a humidified incubator, at 37C, and 5% CO2. The culture was examined daily, and re-stimulated after 7 days.

2.4.3.1 Re-stimulation using Dynabeads® Human T-Activator CD3/CD28/CD137:

Prior to re-stimulation, the cells were removed from the plate, and transferred into suitable tubes. Then the tubes were placed in a magnet for 1-2 min, to allow the beads to move to the side of the tube. After that, the supernatants were transferred to a new tube, and the cells were dispensed back in the culture medium at a concentration of $1x10^5$ cells in 100-200 µl in presence of the same cytokines mentioned in section 2.4.4.

2.4.4 DELFIA EUTDA Cytotoxicity assay:

The antigen derived-peptide pulsed T2 cells described in section 2.4.3.1.1 were adjusted to $1x10^6$ cells/ml, and used as target cells in the cytotoxicity assays. The cells were loaded with 2.5 µl of the fluorescence enhancing ligand (BATDA), and incubated for 30 minutes

at 37^{0}C. Then, the cells were harvested and washed 3-5 times in serum free SGM. After washing, the pellet was suspended in the SGM, and adjusted to 5x10^{4} cells/ml. 100 µl of target cells were placed in a V-bottom plate, and 100µL of the effector cells (long-term stimulated T cells), suspended in the same SGM, of different concentrations (100:1, 50:1, and 25:1) was added, then incubated for 2hrs in 5% CO_2 humidified atmosphere at 37^{0}C. After incubation, the cells were centrifuged for 5 min at 500xg. 20 µL of the supernatant, and 200 µL of europium solution were transferred to flat bottom plate, and incubated for 15 min with shaking. After incubation, the fluorescence has measured in the time resolved fluorimeter, and the % specific release was calculated using the following formula:

% Specific release = Experimental release (counts) - Spontaneous release (counts) / Maximum release (counts) - Spontaneous release (counts) ×100

% Spontaneous release = Spontaneous release (counts) - background (counts) / Maximum release (counts) - background (counts) x100.

Background = media without cells, Spontaneous release = target cells without effector cells, Maximum release = lysed target cells.

2.5 Statistical analysis

The statistical significance of differences of Pentamer staining experiment data were analysed by one way ANOVA, and the ELISpot and cytotoxicity data were analysed by two way ANOVA, using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA). A statistically significant difference between parameter was considered when P value was <0.05.

Chapter 3 Phenotypic characterization of the hybrid cell lines, HMY2 and U266

3.1 Introduction:

This chapter describes the antigenic and phenotypic characterisation of the hybrid cell lines of interest, as well as HMY2 (EBV transformed B-LCL) and U266 (as an example of the parent myeloma cells). The hybrid cell lines used in my project were made previously by fusion of HMY2 (as professional antigen presenting cell) and myeloma cells or cell line (Walewska et al., 2007). The phenotypic characterisation of these hybrids included the examination of the expression of HLA class I, HLA-A2, HLA class II, and various important cell markers including CD80, CD86, CD19 and CD138. It also describes the hybrids' antigen expression profile of some important TAA, and estimation of their expression level by semi quantitative q PCR, in addition to detection of the protein expression level of TAA known to be highly expressed by myeloma cells.

3.1.1 Purpose of Hybridoma formation

Although almost all tumour cells have a significant capability to express high levels of a wide variety of TAA, their ability to induce immune responses, and especially CTL responses, is very weak (Dunnion et al., 1999). This is the reason why previous studies aimed to increase the immunogenicity of the tumour cell through various techniques, such as gene transfection of CD80 and HLA-A1 or A2, for induction of antigen specific (CTL) immune responses (Raez et al., 2004; Guckel et al., 2005). These studies showed that the efficacy of the immune response not only depends on the presentation of the respective immunogenic tumour antigens in the context of MHC molecules, but also depends on the expression of costimulatory molecules. Therefore, as an alternative to this strategy, my supervisor's lab fused a panel of tumour cells or cell lines with EBV-associated B-lymphoblastoid cells (HMY2) to form stable hybrid cell lines. These hybrid cell lines add the advantages of the co stimulatory molecules expression to the antigenic expression profile of the tumour cell, with the aim of enhancing the stimulation of antigen specific T cell responses (Dunnion et al., 1999; Cywinski et al., 2006; Walewska et al., 2007; Mohamed et al., 2012b; Mohamed et al., 2012a).

3.1.2 Importance of phenotypic characterisation of HMY2 and the hybrid cell lines:

The importance of phenotypic characterisation of Epstein-Barr virus (EBV) transformed human B lymphocytes cell line, HMY2 and its derived hybrids has been reported by (Kerr et al., 1992). They showed that HMY2 can have two distinct phenotypic forms, caused by different transcription of latent EBV, leading to different expression of cell surface

markers, adhesion molecules and some virus antigens (Kerr et al., 1992). Also, the potential of production of phenotypically stable hybrids produced by fusion of HMY2 and lymphoid or non-lymphoid tumour cell has been reported previously (Kerr et al., 1992, Dunnion et al., 1999, Cywinski et al., 2006). In a study carried out by Dunnion et al. (1999) hybrid cell lines made by fusion of HMY2 cells with a variety of haematological tumour cell lines such as promyelocytic (HL-60) and erythroleukaemia (K562) cell lines. The produced hybrids expressed MHC class I and class II molecules, high levels of adhesion molecules, and a wide range of highly immunogenic TAA, in addition to the costimulatory molecules CD40, CD80, and CD86 (Dunnion et al., 1999). Additionally, Cywinski et al (2006) produced hybrids, by fusion of HMY2 and three heamaological cell lines, that showed a consistent expression of both HLA class I and class II, and expression of range of immunologically relevant accessory molecules, including CD80 and CD86. whilst the hybrids produced by fusion of HMY2 with solid tumour cell lines cell had the same phenotypic characteristics as their parent tumour cells, lacked expression of the secondary accessory molecules including CD80 and CD86, and showed MHC class II expression only when the parent tumour cell line expressed MHC class II molecules (Cywinski et al., 2006). This study indicated that the phenotypic characteristics of the hybrids arise as a result of the difference of the type of the parent tumour cell. Extending these studies, Walewska et al. (2007) created five hybrid cell lines by hybridisation of HMY2 with *ex vivo* $CD138^+$ bead-separated malignant plasma cells isolated from patients with multiple myeloma or MM cell line. These hybrids showed stability for several rounds of freezing and thawing in liquid nitrogen, and also they grew continuously in tissue culture. In addition, these hybrids retained the phenotypic characteristics of professional antigen presenting cells and the functional ability to stimulate autologous and allogenic T cell response *in vitro* (Walewska et al., 2007).

3.1.3 Tumour-associated antigens: importance in tumour immunotherapy

The TAAs are promising targets for cancer immunotherapy, when the processed antigen is presented by an appropriate HLA class I molecule, and recognised by antigen specific CTL. These TAA can be classified into different categories, such as cancer testis antigen (CTA), which are expressed in normal testis and different tumours (but not in other somatic tissues), and self-antigens which are overexpressed in tumour tissues, as described previously (Jäger, 2001). Novellino et al. (2005) listed the epitope sequence of different TAAs and their HLA alleles, which restrict their recognition by CTL. These

TAAs have a variable role in progression of tumour and immunogenesis. Generally, the TAA is considered as a good candidate for immunotherapy only when this TAA is defined to be of crucial role in tumour progression and has reliable immunogenic epitopes presented in the context of different HLA alleles (Linley et al., 2011).

All of these studies reflect the importance of both phenotypic characterisation of the cell lines involved in the studies and the TAAs they express. The phenotypic and functional stability of the myeloma hybrid cell lines described by Walewska et al. (2007) formed the basis of my study, with the aim of using these self-renewing hybrids for induction of antigen specific CTL with potential for tumour immunotherapy. This is the reason why I examined the expression of different TAAs by the used myeloma hybrid cell lines. These TAAs are known to be expressed at high prevalence in MM tumour cells, and have ability to induce antigen specific CTL, as reviewed in the discussion section of this chapter. In addition, I characterized the surface marker profile of the hybrid cell lines, to ensure that they had retained an immunogenic phenotype.

In this chapter, I will present the phenotypic characterisation of the hybrid cell lines of interest and their available parents HMY2 (parent APC) and U266 (parent myeloma cell) in terms of

1- The expression of major histocompatibility class I and II, in addition to HLA-A2, which is of crucial importance in the presentation of the antigens to CTL.
2- The expression of CD80 and CD86, as important costimulatory molecules in the activation of naive T cells.
3- The tumour associated antigen expression profile, including h TERT, MUC1, MAGE-A1, MAGE-A3, MAGE-C1, DKK1, RHAMM and CS1 (and β- actin, as a housekeeping gene), and estimation of their levels of expression using q PCR.
4- Detection of the expression of CS1, MAGE C, h TERT and MUC1 at the protein level, using flow cytometric analysis and Western blotting. The expression of these antigens was not investigated in any of the previous studies done using these hybrid cell lines.

3.2 Results

3.2.1 Phenotypic and genotypic characterisation of HMy2, the hybrid cell lines and U266:

The hybrid cell lines used in my project were made previously by my supervisor's group using HMY2 as partner APC and different *ex vivo* multiple myeloma cells or cell lines (Walewska et al., 2007). These hybrids showed continuous growth in tissue culture, with stable phenotypic characterisation. Moreover, they also showed stability for several rounds of thawing and freezing in liquid nitrogen (Walewska et al., 2007; Mohamed et al., 2012a). Of the previously made five hybrids, I have successfully re-established four hybrids in the culture and one failed, may be due to long time storage in liquid nitrogen.

3.2.1.1 Immunofluorescence staining and flow cytometric analysis:

Immuno-phenotypic analysis of the expression of a range of important costimulatory molecules, MHCs and certain cell surface markers by HMy2, the hybrid cell lines and U266 was carried out. Selection of the assessed markers (HLA class I, HLA class II, HLA-A2, CD80, CD86) was done based on their significance as immunostimulatory agents of CTL. Additionally, CD19 as cell surface marker of B cells, from which HMY2 has been derived, and CD138 as a plasma cell surface marker.

The immuno-phenotypic analysis was carried out using direct immunofluorescence (IF) staining by specific fluorescently-labelled monoclonal antibodies (mAbs). Fluorescently labelled non-specific isotype matched control mAbs were used as negative controls. After that, flow cytometric analysis was carried out using a FACS Canto flow cytometer and the data were analysed using FACS Diva software, as described in section 2.2.1.

The flow cytometric histograms shown in Figure 3.1 demonstrate the level of expression of HLA class I, class II, HLA-A2, CD80, CD86, CD19 and CD138 by the candidate cell lines. For ease of interpretation, mean fluorescence intensities (MFI) of the expression of each of the examined cell markers by the used cell lines is summarised in Figure 3.2. The data illustrate that the hybrid cell line HU266 showed the highest level of expression of HLA class I, followed by HMY2 and the other cell lines HCM, HRC, HIC and U266 in descending order. Also, HU266 showed about twice the level of expression of HLA class II than HMY2 and HRC, and around one and half times the level of expression of this marker than HCM, and six times higher expression level of HLA class II than HIC. However, the parent myeloma cell line U266 lacked the expression of HLA class II.

Regarding HLA-A2, the highest level of its expression was shown by HMY2 and HU266. In addition, low to moderate level of HLA-A2 expression was seen by the hybrid cell lines HIC, HRC and HCM, whilst, the parent tumour cell line U266 was the lowest cell line concerning HLA-A2 expression level.

In terms of expression of the costimulatory molecules, CD80 and CD86, HU266 showed higher expression of CD80 than HMY2 and the other hybrid cell lines, as HU266 had around four times more expression level than HRC, HCM and HIC. Regarding CD86 expression, HU266 and HCM expressed CD86 at the highest level followed by HMY2. The hybrid cell line HRC had relatively low expression level of CD86, while HIC hybrid cell line expressed very low levels of CD86. In contrast with the hybrid cell lines, the parent cell line U266 lacked expression of both candidate costimulatory molecules, CD80 and CD86.

As regards the expression of CD19, HMY2 had slightly higher expression level of CD19 than the hybrids HU266, HRC, HCM, and HIC, while, U266 lacked the expression of CD19.

In contrast, U266 had the highest level of expression of CD138, compared with all the other examined cell lines. The expression of these cell markers by U266 showed consistency as a mature plasma cell, as it showed moderate expression level of HLA class I, HLA-A2, lacked the expression of the other examined cell markers (HLA class II, and the secondary accessory molecules CD80 and CD86), and had high level of expression of CD138 (plasma cell marker). Also, the hybrid cell line HU266 showed an upregulated level of expression of CD80, CD86, CD19 and MHC class I (including HLA-A2) and class II when compared with its parent cell U266, these results agreed with Walewska et al. (2007). The other parent tumour cells were not available for such comparison with their hybrids.

The expression of these cell markers by HMy2 is consistent with the phenotypic characteristics of EBV – BLCL and its role as a potential APC, and supports its use as fusion partner for the hybrids used for immunogenic stimulation of CD8+ T lymphocytes.

It is noteworthy that almost all of the hybrid cell lines expressed the co-stimulatory molecules CD80 and CD86 and HLA class II, in spite of its absence in the parent myeloma cell U266, and these expressions were consistent with the co-stimulatory molecules and

HLA class II expression by HMY2. However, the hybrids showed some variability of their expression level.

Overall, all of the hybrids expressed all the candidate cell surface markers at much higher level than the tumour cell U266 (shown in Figure 3.2), with the exception of the myeloma marker CD138. These data of phenotypic characterisation of the hybrids show agreement with the previous studies done on these hybrid cell lines (Walewska *et al.*, 2007; Mohamed *et al.*, 2012).

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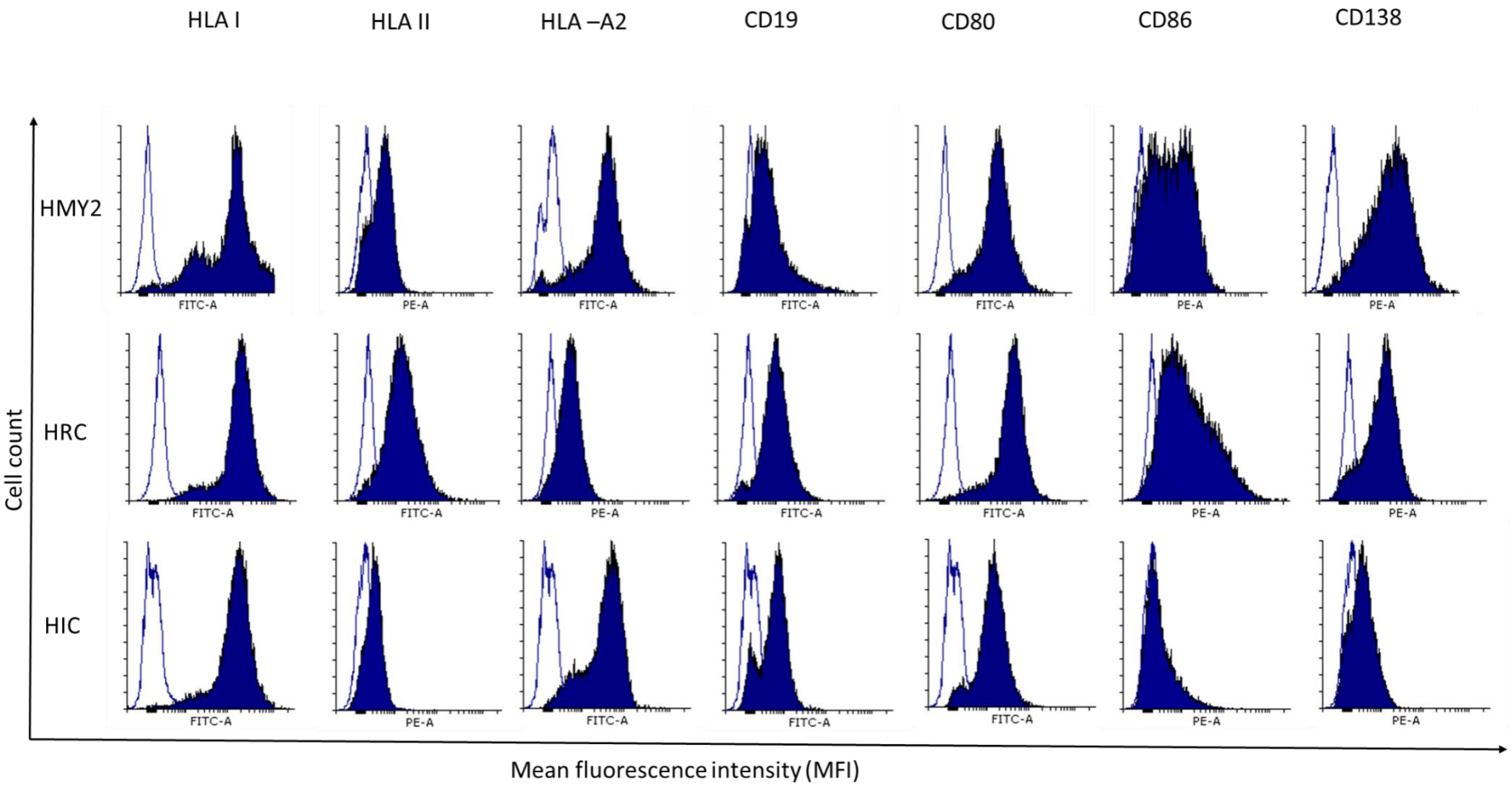

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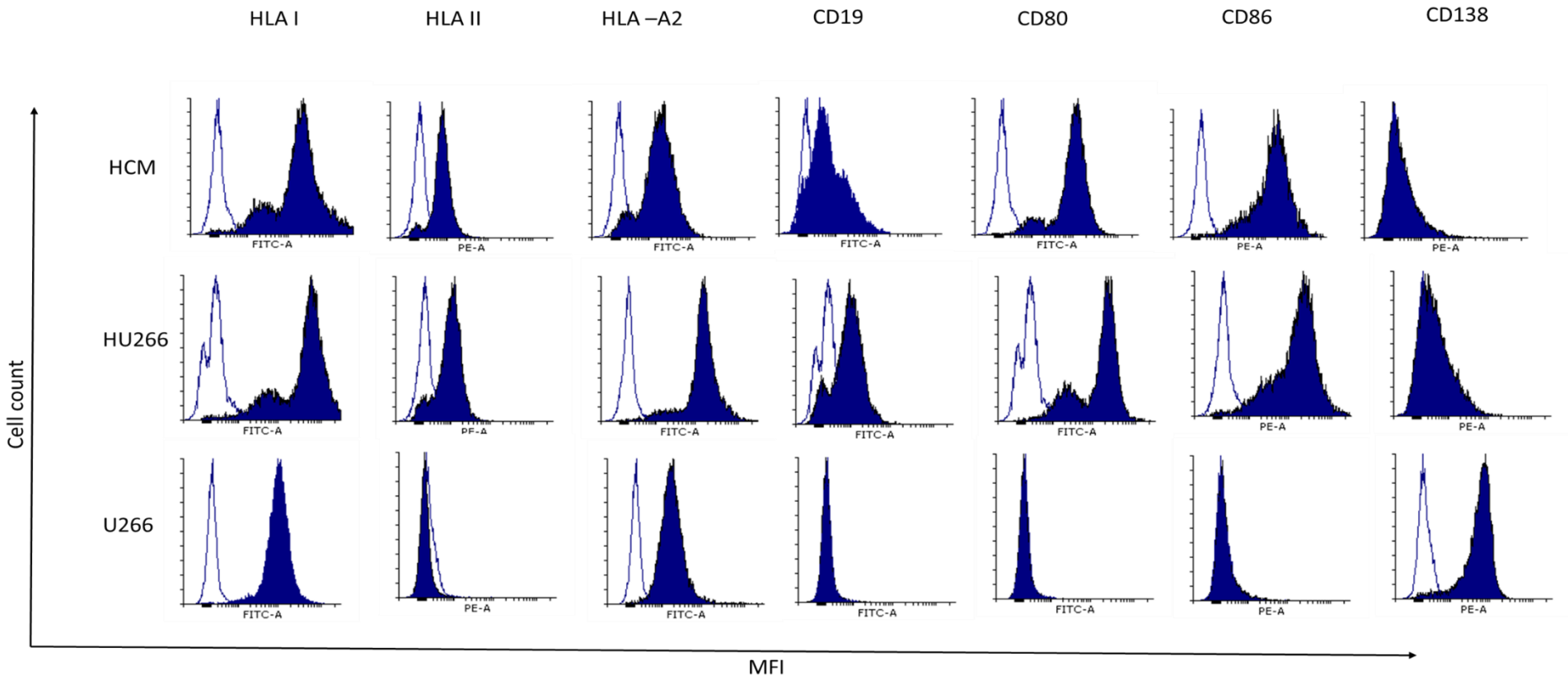


Figure 3.1: Phenotypic characterisation flow cytometric histograms of HMy2, the hybrid cell lines and U266. *The plots demonstrate the level of expression of HLA class I, class II, HLA-A2, CD80, CD86, CD19 and CD138. Grey histograms show isotype control of mAb stained cells, and blue histograms show the relevant monoclonal antibody stained cell line (HMY2, the hybrid cell lines and U266).*

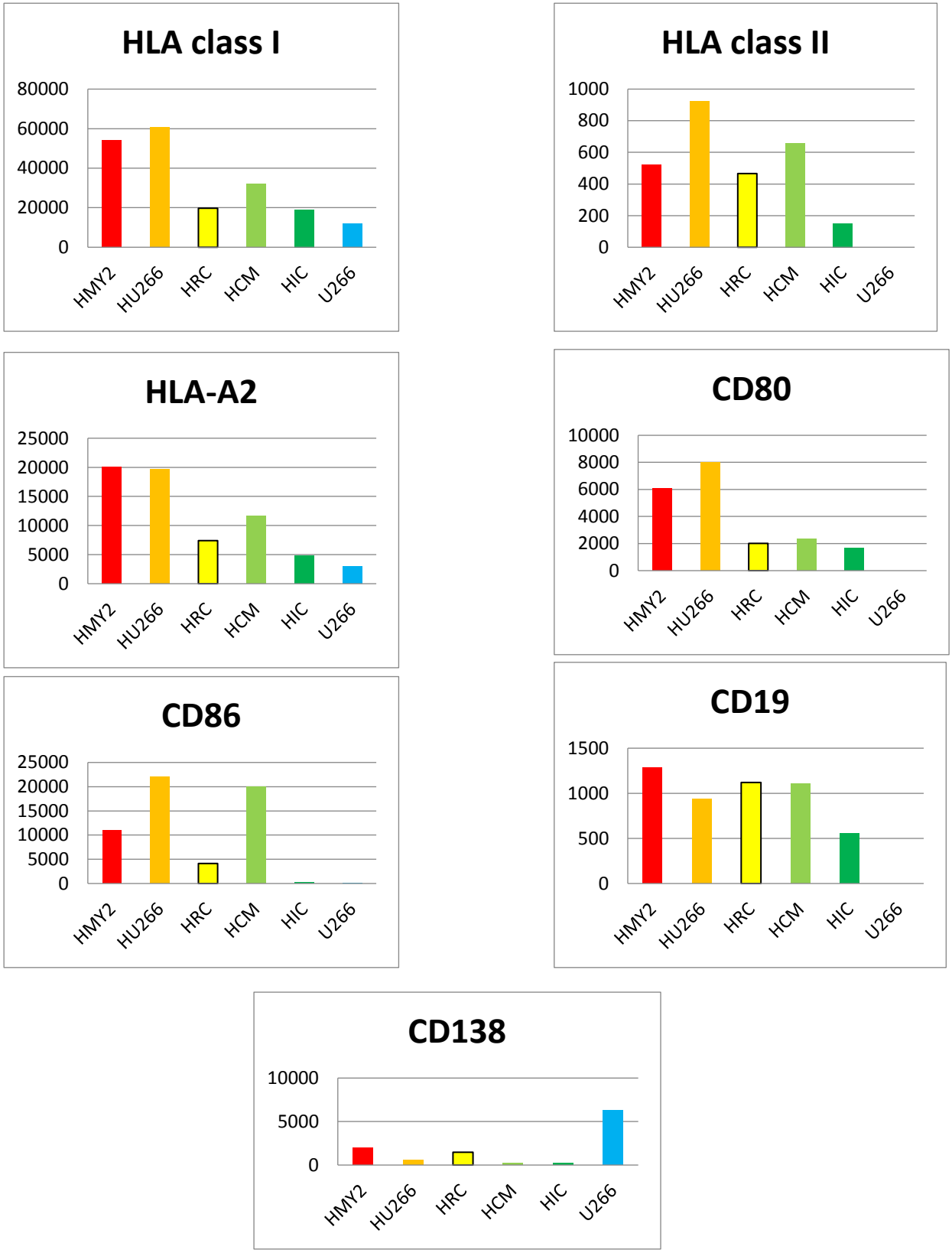


Figure 3.2: Mean fluorescence intensity of the expression of HLA class I, HLA-A2, HLA class II, CD80, CD86, CD19 and CD138 by HMY2, the hybrid cell lines and U266. *The presented MFI values are (the MFI of examined marker expression - MFI of matched isotype control). The experiment was done once only.*

3.2.2 Detection of TAAs expression by HMy2, the hybrid cell lines and U266 using reverse transcriptase-polymerase chain reaction (RT-PCR):

After detection of expression of a variety of important cell surface markers by the used cell lines, I wanted to investigate the expression of some important tumour associated antigens (TAA). The selected TAA had been identified before as potent targets for tumour immunotherapy, and especially for antigen specific $CD8^+$ T cells (Cloosen et al., 2006; Tai et al., 2008; Meklat et al., 2007; Schmitt et al., 2008; Yin, 2012). Therefore, RTPCR was carried out to examine the expression of the candidate TAA (MAGE-A1, PRAME, Survivin, MAGE-A3, MUC1, RHAMM, DKK-1, CS1, MAGE-C1 and h TERT) by HMy2, the hybrid cell lines (HU266, HRC, HCM and HIC) and U266. Firstly, total RNA was extracted from each cell line. Then single stranded complementary DNA (cDNA) was synthesised and used as a template in PCR reactions as mentioned previously in section 2.2.2. Throughout the reactions, examination of β-actin expression (housekeeping gene) was done, to confirm the DNA integrity. Two negative controls were used in these reactions, the first one was cDNA synthesised from PBMCs of normal healthy individuals, which should not express the TAAs, and the second negative control was using the reaction mixture without cDNA. U266 was used as positive control.

The results of RTPCR are shown in Figure 3.3. This figure shows that all the examined antigens were expressed by HMy2, the hybrid cell lines and U266, with the exception of DKK1, which was expressed only by the tumour cell line U266, and did not show expression by any of the hybrid cell lines, even HU266. Expression of the examined antigens by PBMCs was negative, except for RHAMM and survivin. β- actin was detected in the cDNA from all the cells / cell lines.

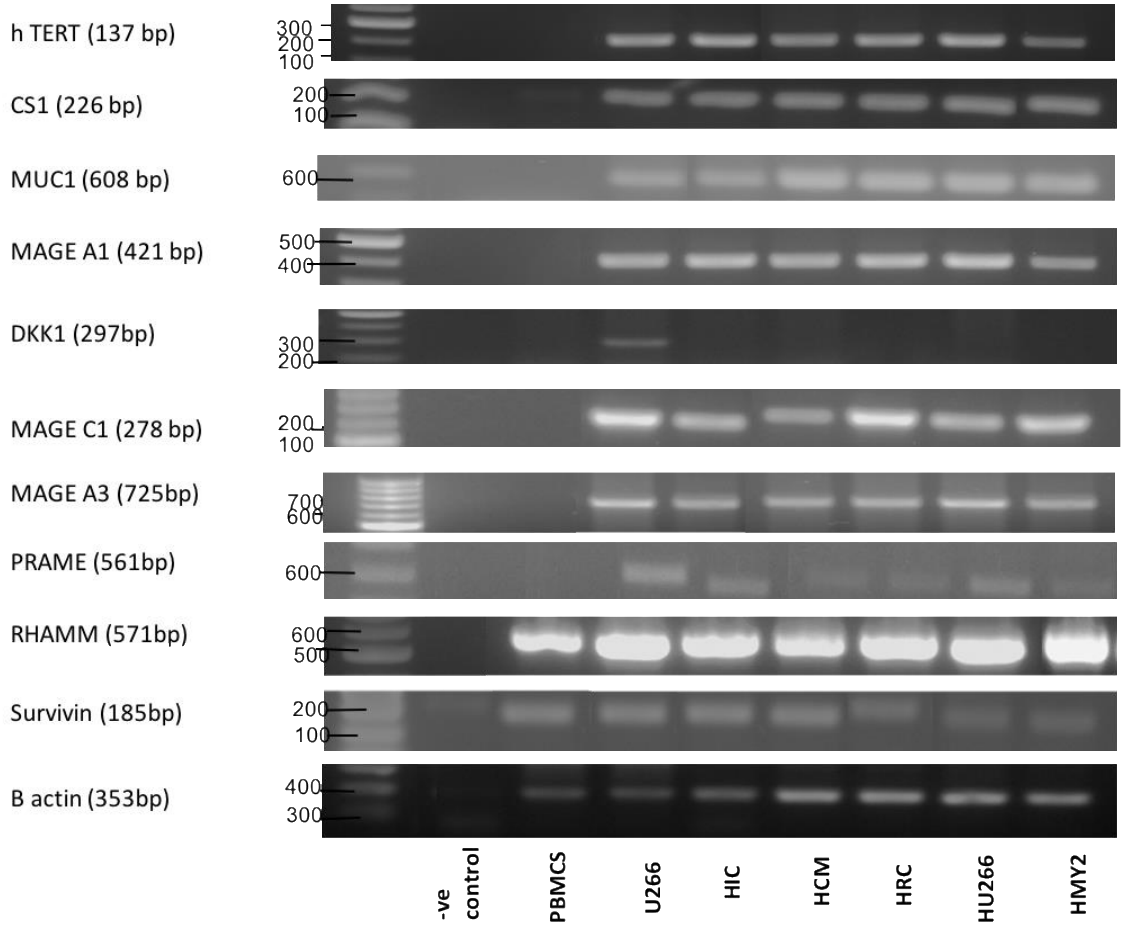


Figure 3.3: RT PCR *bands represent TAAs, and β-actin mRNA expression by the hybrid cell lines and HMy2.U266 was used as a positive control, and PBMCs as negative control. First lane shows bands for 100 bp ladder, and positive bands were of correct band size for the relevant antigen specific TAA, as indicated.*

3.2.3 Semi-quantitative evaluation of TAA expression:

Following determination of TAA expression profile by the myeloma hybrid cell lines using RT PCR, real time semi-quantitative PCR (q PCR) was used to estimate the level of mRNA expression of these tumour antigens in the examined cell lines. The significance of this experiment was to validate the PCR result, and to investigate the highest expressed antigens by most of the used cell lines. The product of these genes can be used as candidates to examine the presence of antigen specific CD8+ T lymphocytes in long-term stimulated cultures. Also, determination of mRNA expression of any of these antigens by PBMCs (as an example of somatic cell) would indicate limitation of their usage in immunotherapy, to prevent development of autoimmune disease.

Semi-quantitative PCR for relative estimation of mRNA expression levels of MAGE-A1, PRAME, MAGE-A3, Survivin, RHAMM, h TERT, MUC1, MAGE-C1 and CS1 by

HMY2, hybrid cell lines, U266 (as positive control) and normal PBMCs (as negative control) was done using 7500 fast real time PCR system. PCR master mix was used with each antigen specific primer pair. The running conditions were adjusted according to the reagents manufacturer's instructions, and to the primer pairs melting temperature, as described in section 2.2.3

Each q PCR was repeated three times under the same conditions. The mean value of mRNA concentration levels from the triplicate runs was normalized against mean values of β actin concentration. The individual column of each chart in Figure 3.4 represents the mean value ± SEM of each cell line after analysis using GraphPad Prism 5.5 (CA, USA).

Regarding h TERT, HRC showed strong expression of mRNA, about one hundred fold more than the other hybrids, HMY2 and U266. The other hybrid cell lines and HMY2 had lower levels of expression of h TERT mRNA, but elevated compared with PBMCs. The hybrid cell line HU266 had a slightly lower level of expression than its parent myeloma cell line.

All of the examined cell lines expressed CS1 mRNA. The parent myeloma cell U266 was the highest cell line for this TAA expression. However, by comparing the hybrids together, HRC showed highest expression of CS1 mRNA, followed by HU266 and HIC, whilst HMY2 and HCM expressed CS1 at a relatively low level, but higher than PBMCs.

Regarding MUC1, HU266 had about half the level of mRNA expression of MUC1 than its parent U266, and around thirty-fold higher expression than HMy2, HCM and HIC. Additionally, HU266 was followed by HRC, which showed ten times more MUC1 mRNA expression than HMy2.

Concerning the candidate members of MAGE family, MAGE-A1, MAGE-A3 and MAGE-C1, the hybrid cell line HRC showed elevation of mRNA expression of all of these respective antigens, as HRC had around one hundred fold of expression of MAGE-C1 and MAGE-A1, and about ten time more expression of MAGE-A3 than HMY2. All of the other hybrids had lower level of expression of mRNA of these antigens, but more than PBMCs. The parent myeloma cell line U266 showed the highest levels of mRNA expression for all of these antigens. Normal PBMCs expression levels for all of these examined TAA were almost zero.

The parent APC HMY2 showed low expression level of PRAME mRNA, while U266 had the highest mRNA expression of this antigen. Also, mRNA of PRAME was expressed

at similar level by HU266, HIC and HRC hybrid cell lines, whilst HCM showed very low expression level.

Myeloma cell line U266 expressed RHAMM at a high level, compared with the other examined cell lines. HIC had the highest RHAMM mRNA expression of all hybrids, while, HU266 showed a very low value of this antigen expression. The hybrid cell lines HRC and HIC, and HMY2 had slightly higher values of RHAMM mRNA expression level than PBMCs.

In terms of survivin mRNA expression level, all of the hybrids had nearly the same expression level as was seen in PBMCs, while the parent tumour cell U266 showed strong elevation of expression of this mRNA, and HMY2 was slightly higher in its expression than the hybrids.

To conclude, relative mRNA expression of most of the candidate TAAs by HMy2 was lower than in the hybrid cell lines. U266 showed the highest values of almost all of them. However, by comparisons with other hybrids, HRC gives greater values of expression of the majority of antigens such as h TERT, MAGE-C1 and CS1, MAGE-A1, MAGE-A3 and PRAME (Figure 3.4). Normal PBMCs expression level for all of the examined mRNA were almost zero, with the exception of RHAMM and survivin.

Overall, the increases in the mRNA expression of most of these antigens by the hybrid cell lines compared with HMy2 indicates that hybridisation may cause stabilisation and elevation of the expression of these antigens than their respective expression levels by the parent APC, but lower than U266. Most of these results are consistent with the previous work undertaken in my supervisors' lab (Dunnion *et al.*, 1999; Walewska *et al*., 2007; Cywinski *et al*., 2006; Mohamed *et al*., 2012). However, Mohamed et al. (2012a) showed negative expression of PRAME, MAGE-A1 and MAGE-A3 by HMY2, and I have shown weak expression of these mRNAs by HMY2. These discrepancies are discussed in the Discussion section of this chapter.

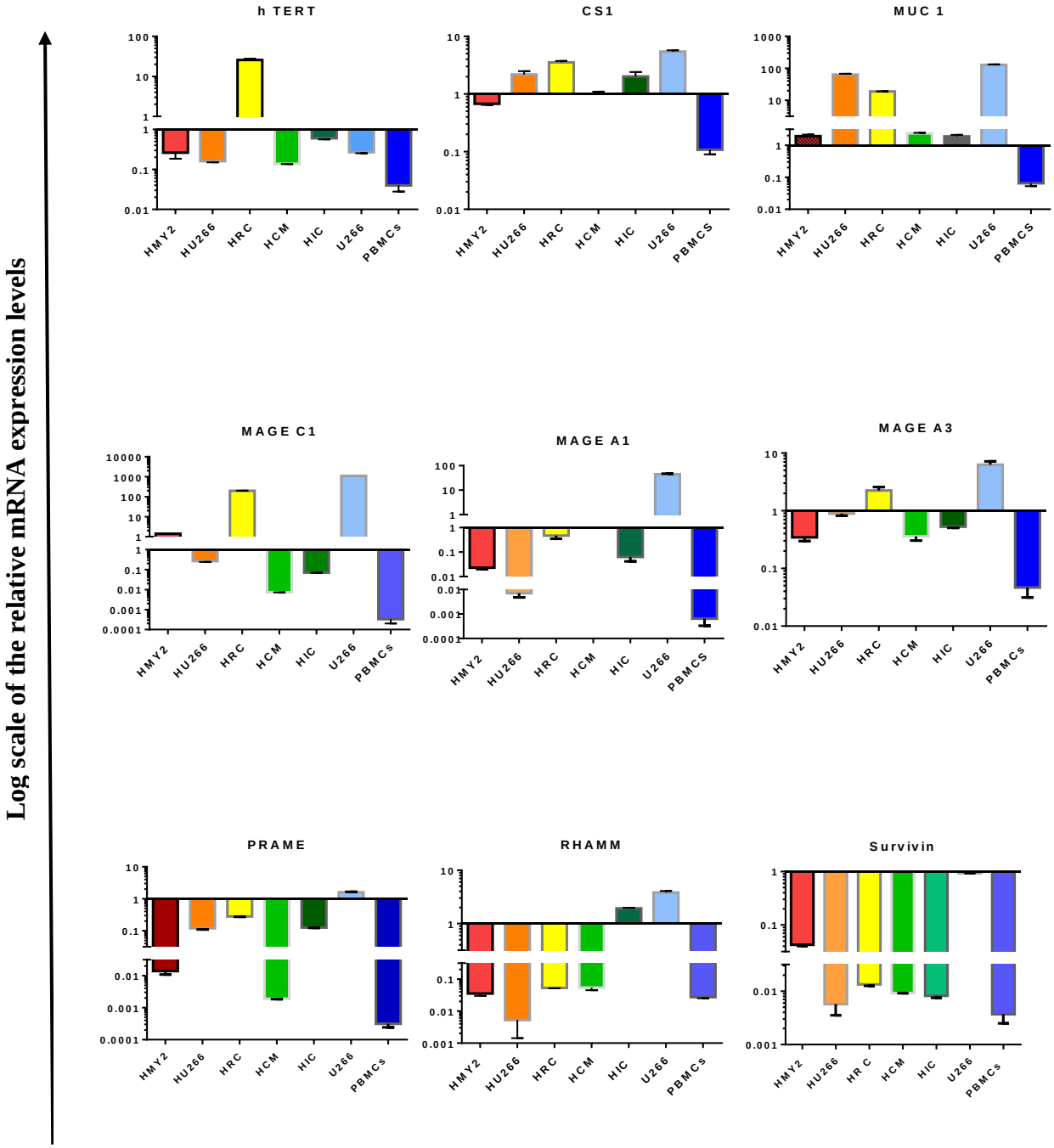


Figure 3.4: Relative tumour associated antigen mRNA expression levels of HMY2, the hybrid cell lines, U266 and normal PBMCs. *The graphs represent nine different TAAs as indicated in the individual graph heading. q PCR analysis for each antigen was repeated three times, and the individual column of each chart represents mean value ± SEM.*

3.2.4 Evaluation of protein expression for h TERT, MUC1, CS1, RHAMM and MAGE-C1 in HMY2, the hybrid cell lines and U266:

After evaluation of the level of TAA- mRNA expression, and detection of the highest relative expression for h TERT, RHAMM, CS1, MAGE-C1 and MUC1 using q RT-PCR, the next aim was to estimate the level of protein expression for these antigenic proteins in the used cell lines. This is because, although the measurement of mRNA and protein level are usually complementary to each other, the direct relationship between gene and protein expression does not always happen. This is because the regulation of the process of transcription and translation is complex, and mRNA transcripts are of lower stability compared to protein stability (Greenbaum et al., 2003; Pascal et al., 2008). So, this experiment was done to confirm the PCR and q PCR results, and to investigate the protein expression level of h TERT, RHAMM, CS1, MAGE-C1 and MUC1 (the highest expressed candidate antigens by the examined cell lines), with the view to use these antigens for detection of antigen specific cytotoxic T lymphocytes produced in the long-term stimulated T cell cultures.

3.2.4.1 Flow cytometric analysis for estimation of the level of expression of h TERT, MUC1, CS1 and RHAMM in the used cell lines:

Flow cytometric analysis was used to evaluate the protein expression level of h TERT, MUC1, CS 1 and RHAMM. These tumor associated antigens have high prevalence in multiple myeloma patients (Cloosen et al., 2006; Meklat et al., 2007; Schmitt et al., 2008; de Carvalho and Vettore, 2012; Tai et al., 2008; Yin, 2012), and q PCR data presented in section 3.2.3 showed high levels of mRNA expression of these antigens by the hybrid cell lines. Each of the examined cell lines was stained with the relevant antigen - specific antibodies, or their isotype controls as described in section 2.2.4, and the results were analyzed using flow cytometry. Additionally, normal PBMCs were included and treated under the same staining conditions as the examined cell lines, as a negative control. Detection of protein expression in this normal somatic cell may lead to limitation of use of certain TAA in immunotherapy, as described previously.

Flow cytometric histograms of protein expression for HMY2, the hybrid cell lines, and U266 are illustrated in Figure 3.5, and for easy interpretation MFI values are presented in Figure 3.6, to rank the expression level of these proteins by the used cell lines.

Regarding h TERT, the results illustrated that HRC showed highest level of h TERT protein expression. Also, HMY2 had a relatively high level, followed by U266. The hybrid cell line HU266 showed almost half expression level of its myeloma parent cell line U266. The same level of h TERT expression was shown by HU266 and HIC, however HCM and PBMCs lacked h TERT protein expression.

In contrast, HIC hybrid cell line had the highest level of MUC1 protein expression, and this value was slightly higher than that of U266 and HRC respectively. However, HU266 hybrid cell line expressed almost a quarter of the level of expression of its parent U266. The parent APC HMY2 and the hybrid cell line HCM expressed MUC1 at a very low level. The normal PBMCs showed negative expression of this protein antigen

In terms of CS1, all of the used hybrid cell lines, except HCM, expressed this antigen at a higher level than the parent myeloma cell line U266, and also higher than APC partner cell line (HMY2). HRC had the highest level of CS1 protein expression followed by HU266. HIC and HMY2 had almost similar level of CS1 expression. No expression was detected in the normal somatic PBMCs.

RHAMM was expressed only by the parent cell line, HMY2, and at low level by U266, whilst none of the examined hybrid cell lines showed expression of this protein. This could be attributed to switching off the expression of this protein during hybridisation. In addition to that, weak expression of this antigen was shown by PBMCs, which could limit the usage of this antigen in immunotherapy.

In summary, the hybrid cell lines generally expressed higher level of h TERT, MUC1, and CS1 than PBMCs, although the level of expression was variable between individual hybrid cell lines. In contrast, the hybrids did not express RHAMM. HMY2 cells expressed relatively high levels of h TERT and RHAMM, with lower levels of CS1 and MUC1. As expected, normal PBMCs either lacked expression of the antigens, or expressed them at very low levels.

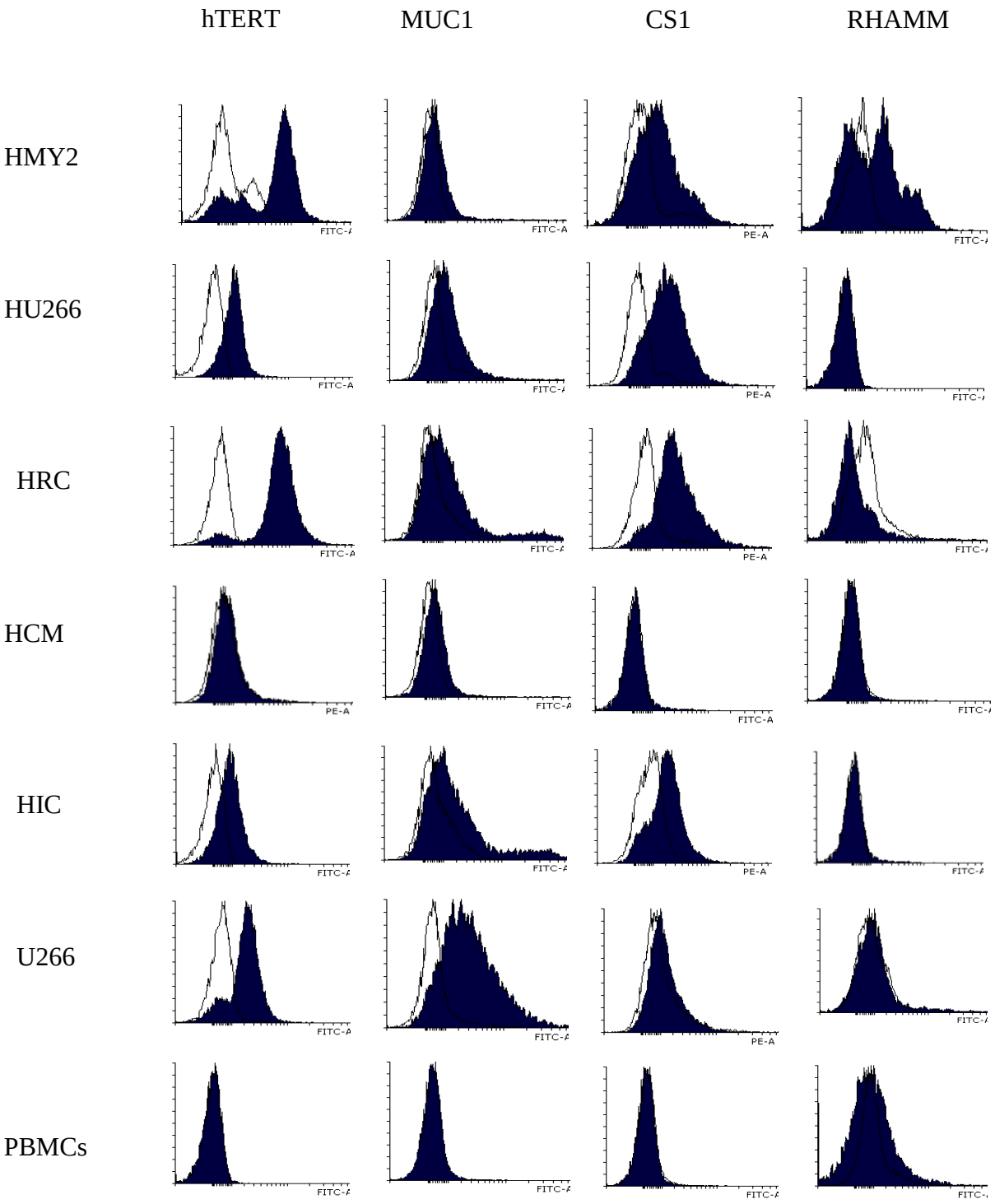


***Figure* 3.5: Flow cytometric histograms representing the level of expression of h TERT, MUC1, CS1 and RHAMM by HMy2, the hybrid cell lines and U266.** *The open histograms represent isotype mAb stained cells, the dark blue histograms represent HMy2, hybrid and U266 cells stained with the relevant mAb. The experiment was dine only once.*

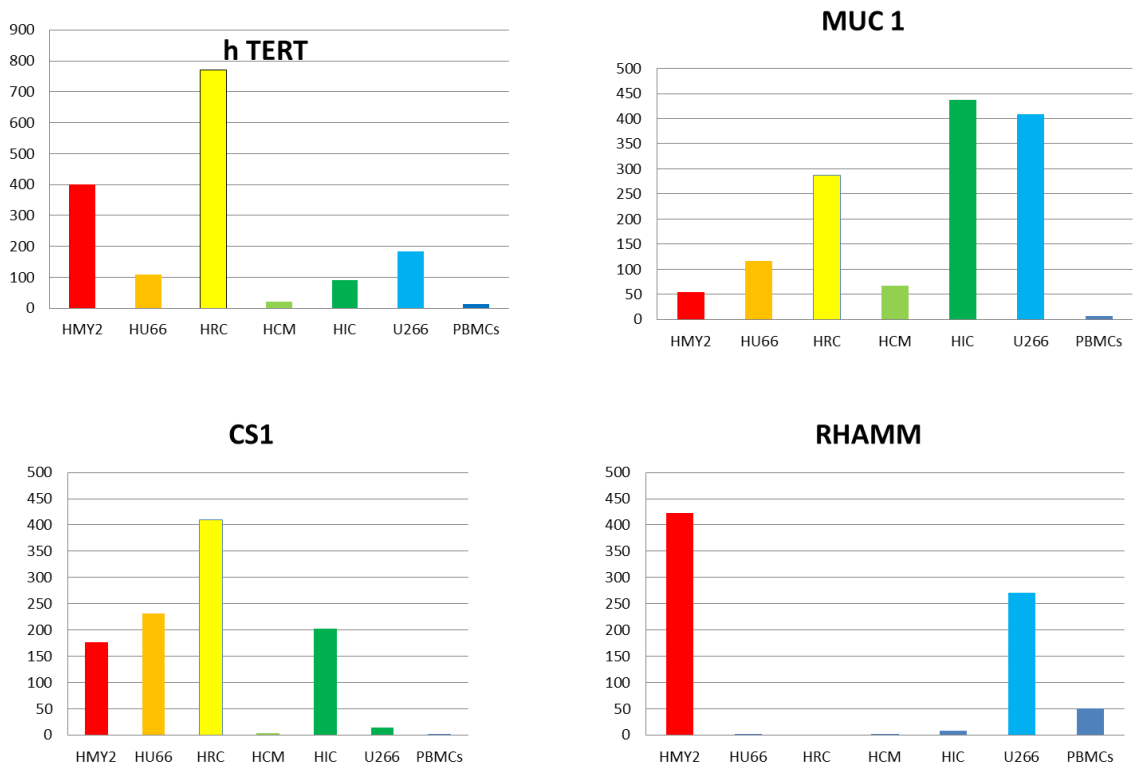


Figure 3.6: MFI of the expression of h TERT, MUC1, CS1 and RHAMM by the indicated cell lines.

The presented MFI values are the MFI of examined marker expression minus MFI of matched isotype control. The experiment was done once only.

3.2.4.2 Western blot analysis for detection of the expression MAGE-C1 by HMY2, HU266, HRC, HIC and U266

Detection of the expression of MAGE-C1 by the used cell lines was done using Western blot by whole protein extraction method as described previously (as there was no available antibody suitable for detection of this protein by flow cytometric analysis). Serial dilution of the protein was done for detection of the best protein concentration to be used in the experiment, as shown in Figure 3.7, using U266 cells. The negative control included in this reaction was PBMCs isolated from a normal healthy individual. Also, for true normalisation, β-actin was detected on the same membrane as described in section 2.2.4.5. . Housekeeping genes, such as β-actin are usually used for loading control; these proteins are generally expressed constitutively at high amounts because of their role in cell integrity (cyto-skeleton).

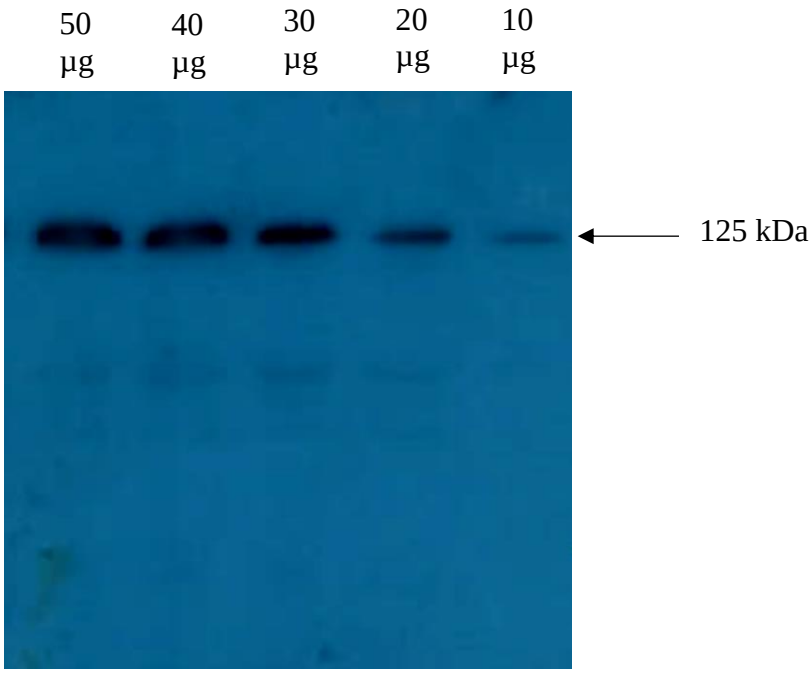


Figure 3.7: Western Blot for detection of MAGE-C1, using U266 extracted total protein. *Different protein amount were loaded as indicated.*

Based on the results shown in Figure 3.7, 20µg protein were used in the subsequent western blots for MAGE-C1. It is of note that MAGE-C1 protein has two isoforms produced by alternative splicing. The first one is high molecular weight isoform (123,643 Da) and the other is low molecular weight (23,747 Da) (uniprotkb - o60732 (magc1_human)). U266 expressed only the higher molecular weight isoform. Subsequently, western blotting for MAGE-C1 protein was carried out on HMY2, the hybrid cell lines, U266 and PBMCs, using 20g total protein from cell extracts.

The result, shown in Figure 3.8, illustrates that HMY2 and the used hybrid cell lines expressed the low molecular weight isoform, however only U266 expressed the high molecular weight isoform. This may be explained by HMY2 and the hybrid cell lines processing the MAGE-C1 protein in the same way, whilst U266 can process it in a different way. In addition to that, by comparing the band density (using BioRad imaging densitometer), then doing the data analysis using Molecular Analyst software, and normalizing the result against β-actin, it is clear that HRC had the densest band (Figure 3.9), and showed agreement with q PCR result, as HRC was the highest hybrid cell line

regarding MAGE-C1 mRNA transcription. HU266 and HIC also expressed MAGE-C1, but at lower levels, whilst HCM expressed very low level of MAGE-C1. PBMCs were negative for MAGE-C1 (and positive for β actin as expected).

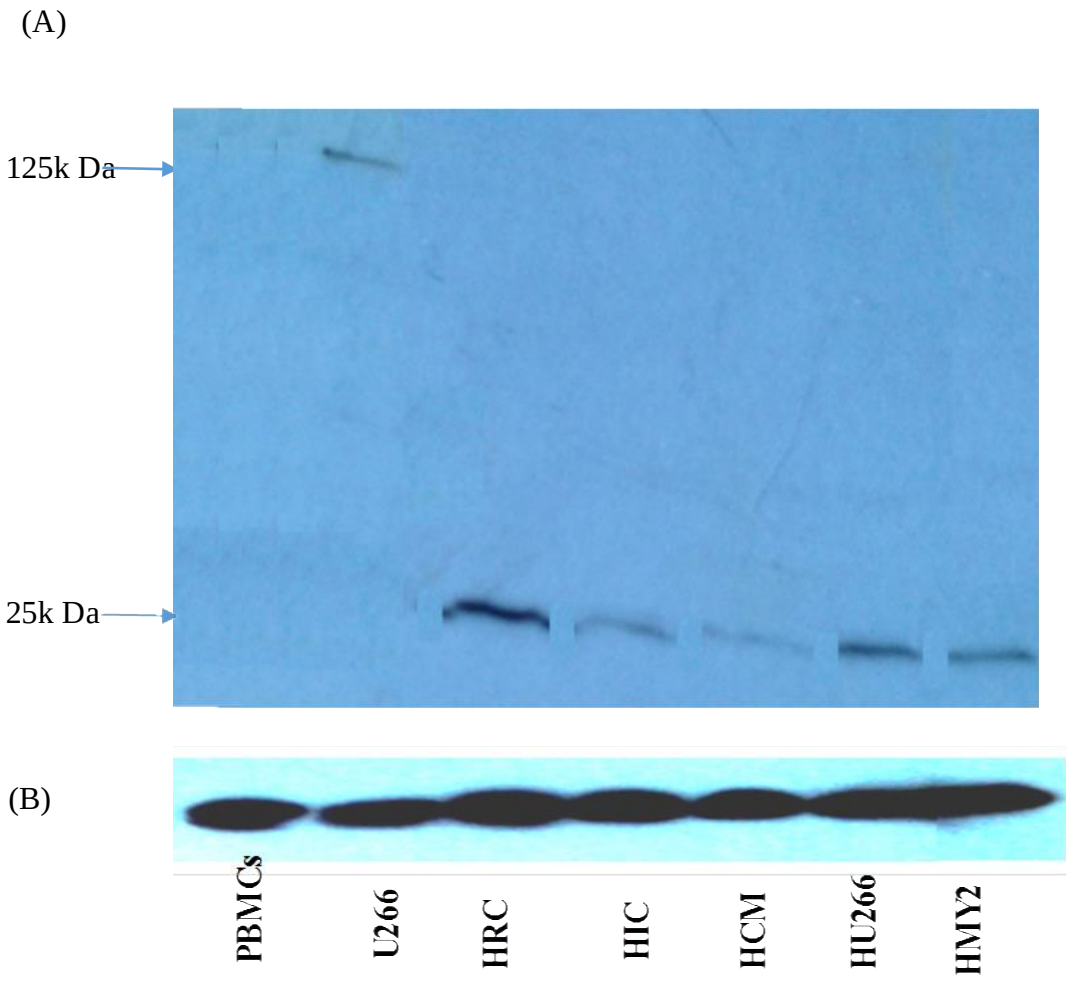


Figure 3.8:(A) Western blot analysis for MAGE-C1 expression *in HMY2, the hybrid cell lines and U266, using anti MAGE-C1 polyclonal antibody (B) β-actin expression by the same cells, using monoclonal anti-β actin antibody. The proteins were resolved on an 8% SDS-PAGE gel. 20µg total protein from cell extracts were used. PBMCs were negative for MAGE-C1. The experiment was done only once.*

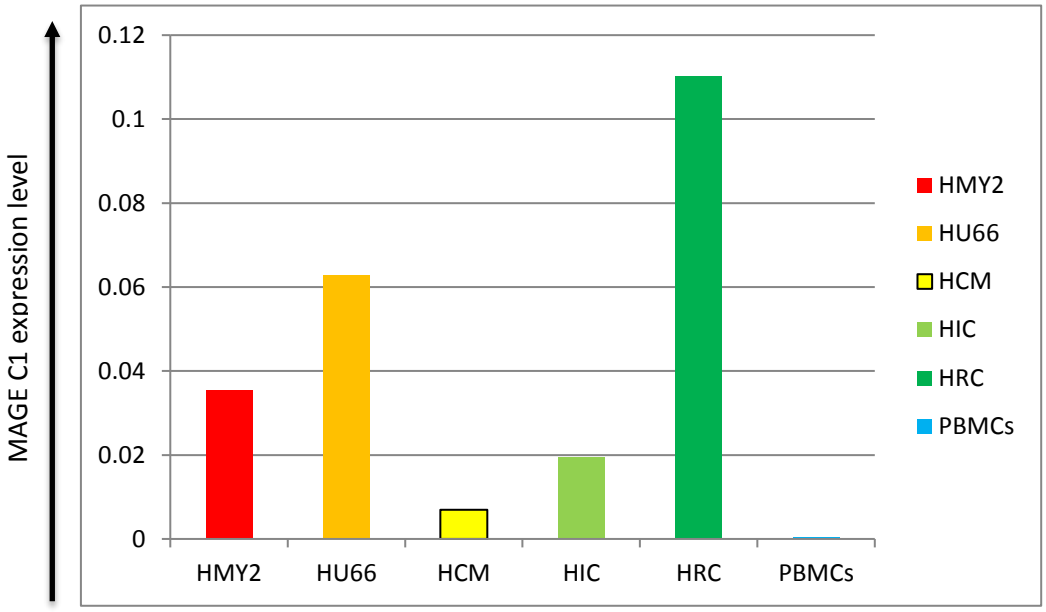


Figure 3.9: Relative MAGE-C1 protein density values for the hybrid cell lines and HMY2, *after normalisation against β-actin. The values were determined using imaging densitometer. 20µg total protein from cell extracts were used. PBMCs were negative for MAGE-C1.*

3.3 Discussion

The potential use of the hybrid cell lines (HMY2/ multiple myeloma cell) as immunogens, for induction of antigen specific immune responses in tumour immunotherapy is based mostly on the APC-derived phenotype and the effective processing and presentation of TAA to CTL. This phenotype offers several advantages in stimulation of CTL compared with using the tumour cells alone. Firstly, the hybrids effectively process and present TAAs in the context of MHC class I and class II, for recognition by $CD8^+$ and $CD4^+$ T cells respectively, which is considered as the first signal in activation of T cell immune response (Stuhler et al., 1998; Trefzer et al., 1998). Secondly, the expression of certain costimulatory molecules by APCs, such as CD80 CD86, and engagement with their cognate receptors on T cells (CD28) is another signal required for full activation of naive T cells. In contrast, the absence of the signalling pathway of CD28 may lead to induction of antigen-specific tolerance (Lenschow et al., 1996). Thirdly, the hybrids enable the presentation of a broad spectrum of the processed identified and un-identified antigens, using HLA class I and HLA class II, which leads to activation of the respective T lymphocytes, providing the opportunities for treatment of tumours of unidentified or insufficient number of identified antigens (Kim et al., 2007; Sigal, 2012). For the purposes of this study, I investigated T cell responses to a panel of known tumour antigens, and did not attempt to identify novel TAAs. Dunnion et al (1999) reported enhanced immune-stimulatory capacity produced by using LCL like HMY2 hybrid cell lines with upregulated expression of HLA I, HLA II, CD80 and CD86 compared with non LCL hybrids.

A panel of hybrid cell lines, used in my study, was made previously by Walewska et al. (2007) by fusion of B- LCL HMy2 with different *ex vivo* myeloma cells or cell lines, using the PEG fusion method. These hybrids have the ability to grow in selective growth medium containing HAT and ouabain, which maintains the growth of the heterokaryon only, and does not support the survival of homokaryon and unfused cells. Also, these hybrids survived for long time, including freezing in liquid nitrogen and subsequent thawing cycles. By re-establishment in tissue culture, their growth was maintained as suspension cells for many weeks or months, and showed retained phenotypic characterisation throughout the prior studies (Walewska et al., 2007; Mohamed et al., 2012a). Furthermore, the fused nature of these hybrids was confirmed previously using

the microsatellite analysis that showed incorporation of parental polymorphic alleles from both parents in the hybrid cell lines (Walewska et al., 2007).

The importance of careful phenotypic characterisation of the hybrids has been discussed previously in various studies, due to the possibility of chromosomal loss during hybridisation, or the potential to produce hybrids of different phenotypes from the same hybridisation, or due to the probability to alter the balance of the transcription factors during hybridisation (Dunnion et al., 1999; Tripputi et al., 1988; Kerr et al., 1992). So, based on this, I have started my study by investigation of the phenotypic and genotypic characterisation of the used myeloma hybrid cell lines.

3.3.1 Costimulatory molecules and antigen presenting molecules expression by the hybrid cell lines, HMY2 and U266

I initially examined the expression of some important markers by HMy2 (the parent APC), the hybrid cell lines, and U266 (parent myeloma cell). Some of these markers are costimulatory molecules expressed by APC such as CD80, CD86. Also HLA class I and HLA class II, which have a pivotal role in the presentation of respective cognate peptides for recognition by $CD8^+$ T cells and $CD4^+$ T cells respectively, as mentioned before, were investigated. Furthermore, the examination of the expression of HLA-A2 was done, for some reasons. Firstly, HLA-A2 is of crucial importance in presentation of multiple HLA-A2 restricted peptides derived from TAA, for specific recognition and activation of TCR of CTL. Secondly, HLA-A2 is expressed constitutively by most ethnic groups and it is of high prevalence within HLA class 1 alleles (Player et al., 1996; Sette and Sidney, 1999. As a result, using tumour associated antigenic peptides presented by HLA-A2 represent a good candidate for induction of antigen specific CTL stimulation during my study based on the assumption that HLA-A2+ patients will be suitable for such adoptive immunotherapy. Additionally the expression of CD138 (plasma cell marker) and CD19 (B cell marker from which HMy2 had been derived) were examined for all of the cell lines of the study to confirm their original cell line characteristics (Wang and and Liu, 2012; O'Connell et al., 2004).

The flow cytometric analysis showed that HMy2 and almost all of the hybrids used in my study had high to moderate levels of expression of the major T cell costimulatory molecules and antigen presenting molecules. This expression was stable over culture time and freeze-thaw cycles, as documented in previous studies (Walewska et al., 2007;

Mohamed et al., 2012b). More interestingly, by relating HU266 with its parent cell line U266, the data illustrated an elevated level of the examined markers (HLA class I, HLA-A2) in the hybrid cell line HU266. Moreover, the parent tumour cell U266 lacked the expression of HLA class II and the costimulatory molecules (CD80 and CD86), which were expressed specifically by the APC and the hybrid cell lines. So, the expression of cell markers by U266 showed consistency as a mature plasma cell, as it also expressed CD138 (plasma cell marker) at a higher level compared to the hybrid cell lines or HMY2 (Horst et al., 2002; O'Connell et al., 2004). Additionally, the phenotypic characterisation of HMy2 and the hybrid cell lines showed a high level of expression of the examined costimulatory molecules (CD80 and CD86). These results suggest that the hybrids have unequivocal phenotypes of their parent APC (HMY2), and this phenotype should provide the hybrids with the ability to stimulate $CD8^+$ and $CD4^+$ antigen specific T lymphocytes, which requires constitutive and inducible expression of CD80 and CD86 co-stimulatory molecules, and HLA expression (Edwards et al., 1982; Gool et al., 1996). Additionally, by comparing the hybrids together, I found that HU266 had the most elevated level of all the previously mentioned markers. In contrast, HIC was the lowest hybrid in terms of expression of HLA class II, HLA-A2, CD80 and almost lacked the expression of CD86. These data illustrate the significance of the careful phenotypic characterisation of the used cell lines.

To conclude, nearly all of the hybrids of interest had high to moderate levels of expression of the T cell costimulatory molecules and antigen presenting molecules. However, the pivotal importance of antigen specific CTL in the rejection of tumour cells is determined not only by the level of expression of the co-stimulatory molecules, but also by the immunogenicity of the respective TAA and effective presentation of processed peptides in the context of the relevant MHCs.

3.3.2 The importance of TAAs expressed by the hybrid cell lines (HMY2 x MM cell hybrids), and the parents

The second step in my study was the detection of TAA expression profile by the hybrid and the parent cell lines (HMy2 and U266) using RT PCR and semi-quantitative RT-PCR. Selection of some antigens as potential targets for induction of antigen specific T cells immune response was done, such as cancer testis antigens, MAGE-A1, MAGE-A3,

MAGE-C1, PRAME or overexpressed antigens such as MUC1, h TERT, RHAMM, CS1, Survivin and DKK1.

It is generally accepted that in normal tissues, CTA are exclusively highly expressed in germ line tissues, as they frequently map to chromosome X. The germ line tissues are considered as immune privileged tissues, due to presence of blood tissue barriers and the lack of expression of HLA class I, as described previously (section 1.3.2). Therefore, the use of these antigens as immunotherapeutic targets is usually associated with very low autoimmune side effects. Also, Atanackovic at al.(2009) reported the potential use of CTAs as prognostic and diagnostic markers, as well as powerful immunotherapeutic targets, for multiple myeloma. This could be attributed to many reasons. Firstly, for these commonly expressed antigens, down-regulation is not a regular tumour escape mechanism (Atanackovic et al., 2009). Secondly, CTAs have the ability to induce T-cell–mediated immunity and antibody-mediated immune responses in multiple myeloma (MM) patients (Atanackovic et al., 2009). Thirdly, (Tajima et al., 2003) found that vaccination with different CTAs increased the rate of recovery in up to 70% of the patients by using four different antigen specific vaccine. Furthermore, as a consequence of the high immunogenicity of the CTAs and their restriction to tumour, many CTA specific vaccines of different malignancies were efficiently developed (Meklat et al., 2007). For all these reasons, CTAs are supposed to be promising immunotherapeutic agents for multiple myeloma patients.

Wide expression (87.7%) of CTAs in leukaemia and lymphoma patients was reported by Nishikawa and Ito (2012) and in multiple myeloma by Atanackovic et al. (2009). In myeloma patients, MAGE-C1 and MAGE-A3 are expressed in 80 % and 70 % respectively, with increase of MAGE-C1 protein expression up to 77-100% in advanced stage of the disease (Jungbluth and Chen-Kiang, 2005; Meklat et al., 2007; de Carvalho and Vettore, 2012). Lendvai (2010) identified natural presence of MAGE-C1 specific T lymphocytes in MM patients expressing this CTA, suggesting a strong immunogenicity of this antigen (Lendvai, 2010). Also, MAGE-C1/CT7 expression is restricted to tumour cells, with a dramatic decrease of the relapse time observed after allogeneic stem cell transplantation in patients who express MAGE-C1/CT7. So, this antigen seems to be an attractive target for MM immunotherapy (de Carvalho and Vettore, 2012). Another well-known example of CTAs is PRAME, which is expressed in around 62% of multiple

myeloma patients, with decrease in its level of expression by the tumour regression (Turtle et al., 2004; Gaponova, 2009).

In certain instances, tumours can up-regulate certain cellular proteins, which support the malignant phenotype, such as expression of mucin 1 (MUC1). This antigen is expressed by adenocarcinomas and encoded by MUC1 gene. The MUC1- oncoprotein is widely expressed (73%) in multiple myeloma cells (Cloosen et al., 2006; Yin, 2012), and its tandem repeat is highly immunogenic, so it can elicit both humoral responses, and MHC unrestricted and restricted T cells (Brossart et al, 1999). Takahashi and Imai (1994) generated HLA unrestricted CTL cell lines using MM cells and different types of carcinoma cells expressing MUC1 (Takahashi and Imai, 1994). In contrast to other tumour antigens, tumour-specific T cells can recognize the intact cell surface protein of MUC1 rather than recognition of the processed antigen presented on class I or class II MHC molecules (Henderson, 1996). However, the T-cells activated by intact mucin expressed on tumour cells have a suboptimal level of proliferation (Henderson, 1996). Henderson genetically modified dendritic cells (DC) to express MUC1 at high level, to enhance the activation of T cells. More importantly, numerous studies demonstrated the effect of MUC1-specific CTLs in treatment of MUC1 positive tumours, without any apparent infiltration or destruction of normal secretory epithelial cells that express MUC1, owing to the low level of MUC1/MHC class1 complexes on the normal cells which express this antigen (Gong et al., 2000; Brossart et al., 2015; Ocadlikova et al., 2009; Brossart et al., 1999). Therefore, it is a suitable candidate for cancer immunotherapy.

Also, human telomerase reverse transcriptase (h TERT), a ribonucleoprotein enzyme hTERT, is one of the most commonly expressed antigens in cancer cells (around 90%) (Weiss et al., 2012). h TERT has the ability to participate in malignant transformation of human cells, consequently, Minev et al. (2000) showed that h TERT peptide-specific CTL have the ability to recognise and specifically lyse the tumour cells expressing this antigen. This report gives promise for using h TERT in immunotherapy as a broadly applicable human universal cancer vaccine (Minev et al., 2000; Ocadlikova et al., 2009).

In addition to that, CS1 antigen is considered as a promising target for cancer immunotherapy, because it is member of the Lymphocyte Activation Signalling Molecule family, which is expressed in multiple myeloma cells. CS1 mRNA was highly expressed in primary tumour cells from MM patients (about 97%) (Tai et al., 2009). Kim et al. (2013) identified specific HLA-$A2^{+}$ immunogenic peptides derived from the CS1 antigen,

which have the ability to stimulate peptide-specific cytotoxic T lymphocytes against HLA-A2+ multiple myeloma cells. The CTL exhibited HLA-A2-restricted proliferation, antigen-specific cytotoxicity, and degranulation versus both MM cell lines and primary MM cells (Kim et al., 2013).

In addition to the previous antigens, hyaluronic acid-mediated motility (RHAMM) antigen, is an over expressed antigen that can elicit both cellular and humoral immune responses in both solid tumour and haematological malignancy patients. It is expressed in around 80% of MM patients (Schmitt et al., 2008). Schmitt et al. (2008) described a highly immunogenic $CD8^+$ T-cell HLA-A2 restricted epitope (R3 antigen) derived from RHAMM. $CD8^+$ T cells isolated from acute myeloid leukaemia (AML) patients primed by R3 have the ability to lyse autologous AML blasts which express RHAMM. Because of these positive characteristics of RHAMM, Schmitt initiated the use of R3 peptide vaccination for HLA-A2 positive patients with MM and AML overexpressing RHAMM, to assess the feasibility and safety of this therapeutic approach, and found a considerable increase of specific CD8+ T cells against the R3 peptide in 80% of patients (Schmitt et al., 2008). Another widely overexpressed antigen in cancer cells is survivin, a member of the apoptosis inhibitors family. Numerous reports described the immunogenicity of survivin and its ability to elicit survivin-specific CD8+ T cells in multiple myeloma patients. So, immunotherapeutic strategies targeting survivin might be a good approach for treating multiple myeloma patients (Grube et al., 2007; Arber et al., 2015).

Similarly, Qian et al. (2007) examined a new tumour-associated antigen, Dickkopf-1 (DKK1) which is expressed only by MM cells. They synthesized DKK1 peptides specific for HLA-A2. Then by using peptide tetramers, they detected DKK1-specific CD8-positive T cells in myeloma patients. Afterward they generated DKK1 peptide-specific CD8 T-cell lines and clones from HLA-A2 positive blood donors and myeloma patients. These T cells had the ability to lyse peptide pulsed but not un-pulsed T2 cells, DKK1-positive HLA-A2 myeloma cell line U266 and, more importantly, HLA-A2 primary myeloma cells from patients. No killing was observed on DKK1 expressing HLA-A2 negative primary myeloma cells and myeloma cell lines. Their results indicate that these antigen specific T cells were potent cytotoxic cells which identified DKK1 peptides presented by HLA-A2 myeloma cells (Pozzi et al., 2013).

Based on these studies, the antigens described above were considered as candidates for immunotherapy in MM, and their expression was assessed in the hybrid cell lines.

The result of RT PCR showed that all of the hybrids, HMy2 and U266 expressed mRNA for all the examined antigens except DKK1, which was expressed only by U266 and was negative for all of the hybrids including HU266. This could be explained by gene shut off which may occur during hybridisation (Tripputi et al., 1988).

A surprising result of this study was the positive expression of most of the candidate antigens by HMY2, which can be attributed to the nature of HMy2 (transformed B cells infected with EBV and turned into permanently growing immortalized lymphoblastoid B cells). In this way, HMY2 may resemble myeloma cells (which are cancerous plasma cells) in its antigen expression profile. Also, for all of the examined antigens, PBMCs were negative, except for mRNA expression of survivin and RHAMM. Consistent with this work, Maxwell et al. (2004) reported expression of different isoforms of RHAMM in PBMCs (Maxwell et al., 2004). Also, Fukuda (2006) illustrated the possibility of expression of survivin in normal tissues (Fukuda, 2006). However, there are some discrepancies between the results of this study and the results of the previous work done on these hybrids by (Mohamed et al., 2012b), as he showed that HMY2 lacked the expression of PRAME and MAGE-A1, while the present RT PCR result showed positive expression of PRAME and MAGE-A1 by HMY2. This discrepancy could be explained by the presence of subdominant cells, which expressed PRAME and MAGE-A1 in heterogeneous cell population of HMY2, and these cells became dominant in the culture overtime. However, the antigen expression profile similarities between HMY2 and myeloma cells could be expected, due to the similarity of their origin from B lymphocyte, as explained previously.

Following RT-PCR, qPCR was carried out to investigate the level of mRNA expression of TAA by the hybrids, HMY2 and U266. Relative mRNA expression levels of different antigens showed that in most cases, HMy2 had a relatively lower level of the expression when compared with most of the hybrid cell lines. In contrast, the parent tumour cell, U266, had the highest expression values of most of the antigens. The hybrid cell line HU266 had lower levels of mRNA expression for all of the examined antigens than its parent U266. This can be explained by the effect of hybridisation, which has changed the balance of transcription factors within the hybrids compared with U266, leading to transcriptional reduction of the examined mRNA.

Overall, mRNA expression of the examined antigens by the used cell lines was consistent with the previous work done in my supervisor’s lab (Walewska et al., 2007; Cywinski et

al., 2006; Mohamed et al., 2012), with the exception of low to moderate level of expression of MAGE-A1 and PRAME by HMY2, and expression of survivin and RHAMM by PBMCs, as previously discussed.

3.3.3 Protein abundance of h TERT, MUC1, CS1, RHAMM and MAGE-C1 in HMY2, the hybrid cell lines and U266

Real time PCR data analysis revealed that MAGE-C1, MUC1, h TERT and CS1 are the highest expressed antigens by the candidate cell lines. In addition, these antigens and RHAMM have high prevalence of expression in MM patients. Moreover, these antigens are potential targets for induction of antigen specific CTL responses (Minev et al., 2000; Schmitt et al., 2008; Lendvai, 2010; Kim et al., 2013; Brossart et al., 2015; Brossart et al., 1999). However, as mRNA and protein expression levels do not always correlate (Greenbaum et al., 2003), so the protein expression level of these antigens by the used cell lines were determined.

The results revealed that protein expression of the examined antigens were positive in almost all the used cell lines, in contrast to the lack of expression by PBMCs. These antigens represent candidates for stimulation of antigen specific CTL in the long-term activated T cell cultures.

Regarding h TERT, MAGE-C1 and CS1, almost all the used cell lines have a consistent order for mRNA and protein expression. HRC showed highest level of mRNA and protein expression for all of these antigens. In contrast, HIC hybrid cell line did not follow a rational order in terms of mRNA and protein expression of h TERT and MUC1. This inconsistency can occur for several reasons. Greenbaum et al. (2003) showed that there are three main reasons for such irregularity, The first reason was the complicated post-transcriptional mechanisms arising during mRNA translation into protein, which obstructs the calculation of the exact protein concentration from mRNA expression value. The second cause is the significant difference in the protein and mRNA half-lives *in vivo*. The third reason is the error that can occur in the in both mRNA and protein experiments (Greenbaum et al., 2003; Pascal et al., 2008).

In terms of MAGE-C1, the comparison of protein expression of HU266 and its parent U266 is not applicable, due to expression of the high molecular weight isoform by U266 and low molecular weight isoform by all the hybrid cell lines and HMY2. This proteomic diversity is frequently caused by alternative RNA splicing events (uniprotkb - 060732

(magc1_human), (Wu et al., 2002), and it seems that HMY2 and the hybrid cell lines can process it in the same way, producing only the lower molecular weight isoform, whilst U266 produced only the high molecular weight isoform. Both isoforms were detected in the RT PCR, and have the immunogenic peptide, which used in the subsequent long term stimulation experiments.

Concerning MUC1, HU266 showed higher levels than HMY2, and about half of MUC1 mRNA and protein expression than its parent U266. Also, U266 has the highest values of MUC1 and RHAMM mRNA and protein expression when compared with the other cell lines. However, HU266 is negative for mRNA and protein expression of RHAMM. RHAMM protein expression was positive only for the parent cell lines U266 and HMY2, and negative in all the used hybrids. This can be explained by switching off the expression of this protein during hybridisation (Tripputi et al., 1988). Due to the lack of RHAMM protein expression by the hybrid cell lines, I ruled RHAMM out of the study.

From the previous data, it is clear that the hybrids retained the phenotype of the APC (HMy2) i.e. co stimulatory molecules, HLA class I and HLA class II expression. At the same time, they have the ability to express the examined TAA at high level; however this is not a guarantee that they would have the ability to stimulate T cell responses *in vitro*. So, the functional characterisation assays of the hybrid and their parents using allogenic and antigen specific stimulation of T lymphocytes will be addressed in the next chapter.

Chapter 4 Functional characterization of the hybrid cell lines, HMY2 and U266

4.1 Introduction:

Harnessing different immune system compartments to identify and destroy malignant cells has been the main goal of tumour immunotherapy, either through vaccination of tumour bearing patients or through adoptive therapy using *ex vivo* expanded antigen specific lymphocytes (Lam et al., 2015). The use of TAA vaccines to immunize cancer patients resulted in induction of antigen specific T cells in most vaccinated patients (Timmerman et al., 2002; Smith et al., 2003). However, the magnitude of the antigen specific T cell response is usually moderate, with limited effect on cancer progression (Montes et al., 2005). On the other hand, induction of antigen specific T cell responses *in vitro* is valuable not only for screening the examined antigen immunogenicity before using it for *in vivo* vaccination, but also for generation of T cell clones, which could be used directly in adoptive immunotherapy. The use of tumour and/or antigen specific CD8+ T cells clones as an adoptive immunotherapy, based on infusion of the specific T cells into cancer patients, could represent an efficient approach for treatment of various types of tumours (Ho et al., 2002; Dudley and Rosenberg, 2003). There are numerous advantages that make adoptive T cells amenable for cancer immunotherapy, such as their antigen specificities, robust expansion after activation and their memory response (Perica et al., 2015). Also, there is increasing clinical evidence that adoptive T cell therapy is safe, feasible, well tolerated and may result in significant outcome. Also, the immune response can be optimized through adjusting the type and number of transferred cells, so high number of effector cells can be obtained *in vivo* (Montes et al., 2005; Ho et al., 2006).

For induction of antigen specific CTL clones, repeated antigen specific stimulations in long-term stimulation protocol are required (Ho et al., 2006; Wolfl and Greenberg, 2014; Perica et al., 2015). Using long-term stimulation protocols, the crucial roles of different cytokines, such as IL-2, IL-7 and IL-15, have been highlighted. The concentration and timing of adding such cytokine, will likely affect the outcome of the priming and expansion of T cells (Montes et al., 2005). IL-2 has a vital role during antigen mediated clonal expansion of T cells (Keene and Forman, 1982), as it supports the survival (Blattman et al., 2003), enhances proliferation of T cells (Bamford et al., 1994), and promotes the differentiation of naive T cells into effector cells and memory cells (Kamimura and Bevan, 2007). Also, it has a crucial rule in the generation of memory T cells, for secondary expansion after re-encounter with the antigen (Bachmann et al., 2007).

In addition, IL-7 is of crucial importance in survival and homeostatic proliferation of naive T cells (Schluns et al., 2000; Tan et al., 2001). Schluns et al. (2000) reported that IL-7 *in vivo* could mediate the homeostatic proliferation of naïve and memory CD8+ T cells. Furthermore, the cytolytic function of the effector CTLs was retained for up to sixty days by using IL-15 in their expansion and maintenance (Lu et al., 2002). IL-15 responsiveness and expression of its receptors is higher in memory T cells than in naïve T cells (Geginat et al., 2003). Therefore, the use of these cytokines could be of practical implication for induction of antigen specific T cells *in vitro.*

Some reports defined IFNγ as a tool for assessment of activation of T cells stimulated by hybrid cell lines of DC and tumour cells (Koido et al., 2007). IFNγ is a cytokine that has important physiological roles in stimulating innate and adaptive responses of the immune system, especially in preventing progress of primary and transplanted tumours. It is produced by many effector cells, for instance $CD4^+$ T cells, $CD8^+$ T cells, NK cells and NK T cells. IFNγ plays crucial roles in Th1 development. Firstly, it augments IL-12 production by APCs during $CD4^+$ T cell expansion. Also, it maintains expression of the IL-12 receptors on these cells, to ensure IL-12 responsiveness in developed $CD4^+$ T cells. Thirdly, it plays a critical role by inhibiting the development of Th2 subset through inhibition of IL-4 production (Ikeda et al., 2002b; Ikeda et al., 2002a; Schroder et al., 2004). Ghanekar et al. (2001) found a strong correlation between IFNγ expression and cytolytic activity of CD8+ T cells, and his findings demonstrated that IFNγ expression can be used as surrogate for recognition of CTLs activation (Ghanekar et al., 2001).

perforin is a degranulation protein released by CTL and NK cells, causing cell apoptosis. The killing process involves release of perforin (PFN), which binds to calcium and reacts with phospholipid membranes to form poly-PFN pores which enable delivery of granzymes (Gr) into the target cell, leading to cell apoptosis, as described in section 1.2.2.1.5 (Russell and Ley, 2002). The use of PFN ELISpot for evaluation of CTL activity could offer an attractive alternative to radioactive-labeled target cells as traditional cytotoxicity assays, for several reasons. Firstly, Elispot assays have the ability to measure the number of effector perforin or IFNγ releasing cells. Secondly, their high sensitivity allows detection of low numbers of the activated cells. Thirdly, there is no need to label the target cells, which is a cumbersome procedure (Caruso et al., 1998; Zuber et al., 2005).

In my previous chapter, I showed the ability of the hybrid cell lines and HMY2 to express HLA class I, HLA class II and the costimulatory molecules CD80 and CD86, which are

of crucial importance for induction of naïve T cells. In this chapter, I shall examine the hybrid's ability to induce allogeneic and antigen specific T cells in PBMCs isolated from healthy individuals, in the presense of the essential cytokines.

Aims and objectives of this chapter

1- To investigate the hybrid cell lines' ability to induce allogeneic T cell proliferative responses in healthy donors' PBMCs *in vitro,* using CFSE staining, IFNγ and perforin releasing responses.
2- To optimize the process of *in vitro* generation and expansion of tumour antigen specific CTL, through the addition of exogenous cytokines in the long-term stimulated cultures.
3- To address the ability of the hybrids to induce multiple myeloma antigen specific T cells in PBMCs taken from normal healthy individuals, using the optimized long-term stimulated protocols. The cultures were assessed with IF staining with HLA-A2-restricted peptide pentamers, in which the used peptides were specific to a group of antigens that are highly expressed in MM. Additionally, IFNγ and perforin release ELISpot assays were carried out, to detect the presence of activated antigen specific CTL in the produced stimulated cultures, using T2 cells pulsed with relevant and an irrelevant peptide (as negative control).
4- To use FACS sorting of the long-term activated T cell cultures, and to expand the sorted cells using Dynabeads human T activator beads (containing anti CD3, CD28 and CD137).

4.2 Result

4.2.1 Allogeneic lympho-proliferative response of stimulated PBMC to HMy2, hybrid cell lines and U266

4.2.1.1 Allogeneic lympho-proliferative response of short term stimulated PBMC using CFSE staining

The main aim of this assay was to investigate the capacity of the hybrid cell lines to induce T cell allogeneic immune responses *in vitro*, compared with the parent myeloma cell line U266 and the parent APC HMY2. PBMCs were isolated from normal healthy individuals and stained intracellularly with carboxyfluorescein diacetate succinimedyl ester (CFSE). Then the stained cells were co-cultured with hybrid cell lines, HMy2 and U266 cell lines at a ratio of 2:1, and incubated under appropriate tissue culture conditions as mentioned in Section 2.3.2.2. This stimulation was followed by flowcytometric analysis for tracking the proliferation of the short term stimulated T cell populations. Unstimulated PBMCs were used as a negative control. The assay was performed on two separate occasions with similar results, and results representative of the assays are shown as part of our pilot studies.

4.2.1.1.1 Gating strategy of flow cytometric data analysis

After five days of incubation of CFSE stained PBMCs with the hybrid cell lines, U266 and HMY2, the cells were harvested and stained with monoclonal anti-human antibodies for CD3, CD4 and CD8. Flow cytometric analyses were used to identify different subsets of T cells and their proliferation level, which has direct proportional relationship with their CFSE intensity. The gating strategy for flow cytometric analysis was as follows. Firstly, gating on the living cells was done using the forward scatter (FSS) x side scatter (SSC) dot plot, then this gate was used to identify the T cell population ($CD3^+$) and its intracellular CFSE-content by gating on CFSE x CD3 dot plot. In order to identify the sub-populations of $CD4^+$ and $CD8^+$ T cells, I used the population of $CD3^+$ T cells to derive two dot plots, one for $CD8^+$ T sub-population and the other for CD4+ T cells (Figure 4.1). Afterward, from each of $CD3^+$, $CD4^+$and $CD8^+$ T population, a histogram was derived to reflect the level of T cell proliferation, depending on their CFSE content.

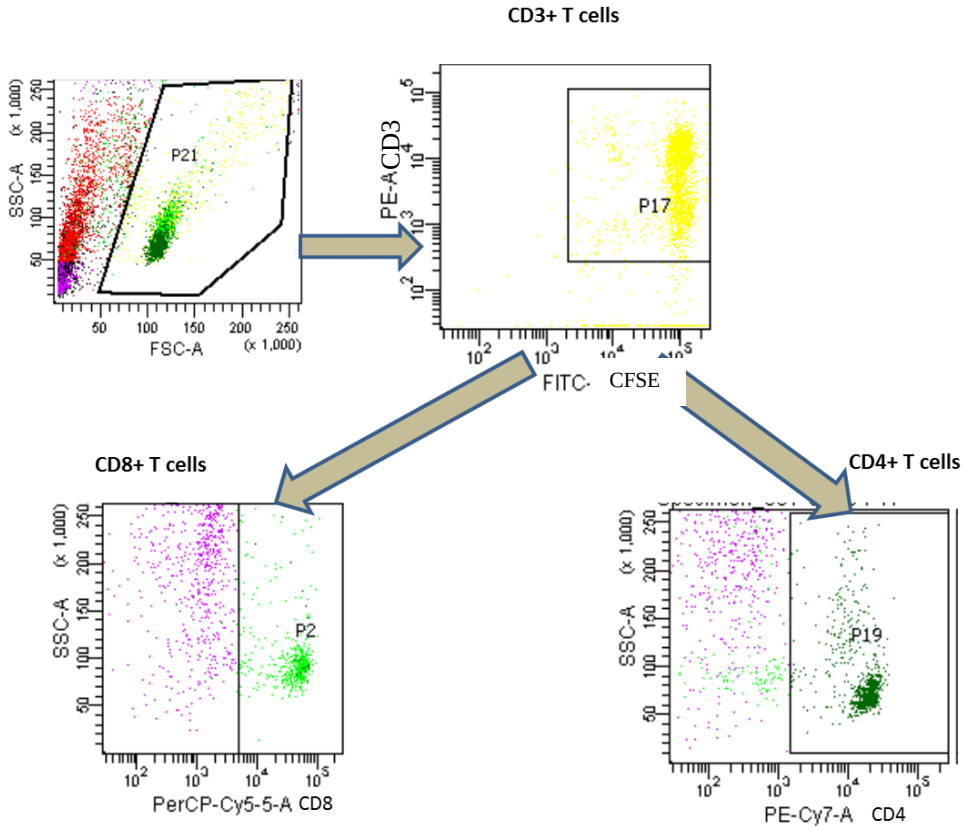


Figure 4.1: Gating process for flow cytometric data analysis. *The first dot plot was used for gating on the living cells using the FSC X SSC dot plot, in the second plot for identifying CFSE stained CD3+ T cells. In the second line, the plots are derived from P17 (CD3+ T cells): the first one was gated for CD8+ T cells and the second plot for CD4+ T cells.*

4.2.1.1.2 Phenotypic analysis of the responding T lymphocytes

The histogram plots were derived for each T cell subtype to investigate the percentage of proliferating cells stimulated by the different cell lines (Figure 4.2).

The following graphs show strong stimulation of T lymphocytes by the hybrid cell lines, HMy2 and U266, which indicates their immunogenic ability to stimulate allogeneic immune responses, and the efficacy of mismatched major histocompatibility as an immune stimulator.

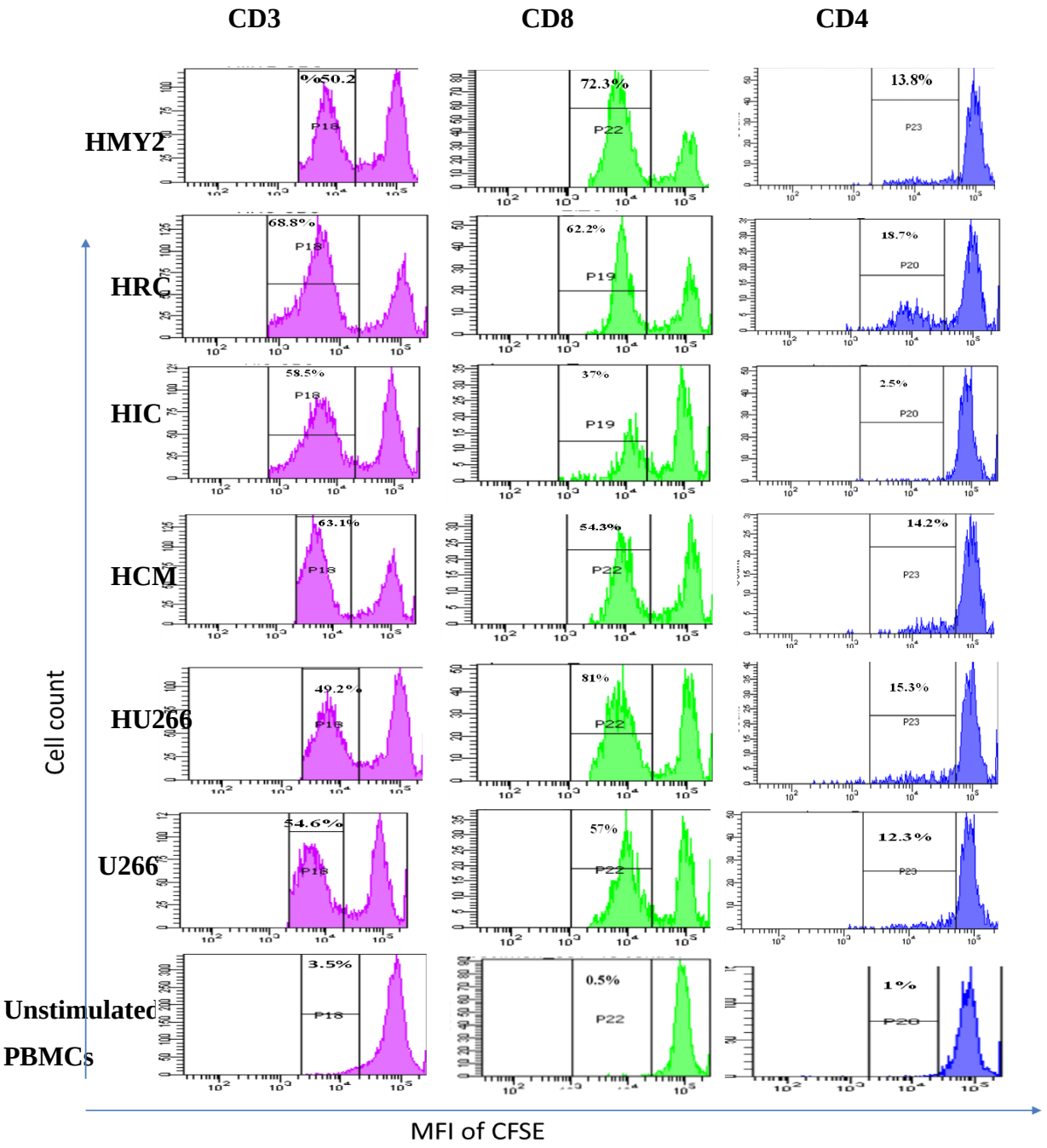


Figure 4.2: Flow cytometric analysis of proliferating T cell subtypes using CFSE staining.

Different hybrid and other cell lines used as stimulators are shown in the left legend. Unstimulated PBMCs were used as negative control. Gating on the proliferating population was made to show the percentage of the proliferating cells in the cultures. CFSE staining experiments were done only twice as part of our pilot experiments.

All of the stimulator cell lines induced stronger proliferation in CD8+ T cells than in CD4+ T cells. HRC was the highest inducer of CD3+ T cell proliferation, whilst HU266 was the highest stimulator for proliferation of $CD8^+$ T lymphocytes. HU266 showed one and half fold more stimulation of $CD8^+$ T cells (by % of proliferating cells) than its parent, U266. That may be explained by the high level of expression HLA class 1, which is required for stimulation of $CD8^+$ T cells, and also high level of costimulatory molecules CD80 and CD86 expression, by HU266, and the absence of these costimulatory molecules in U266. This is supported by high level of stimulation caused by HMy2, which expressed these costimulatory molecules at a high level, as an APC. The stimulation of $CD8^+$ T cells depends mainly on the expression of MHC class I, which was expressed at high level by all of the examined cell lines. So, all of the used cell lines showed good stimulation of $CD8^+$ T cells, however, HIC hybrid cell line was the lowest stimulator in terms of $CD8^+$ T cell stimulation. Also, HRC and HU266 were the most potent stimulator of $CD4^+$ T cells, which were slightly higher than HMY2 and HCM. U266 also, has a comparable level of stimulation of proliferation of $CD3^+$ T cell and its subsets. Figure 4.2 shows that most of the hybrids and the parent cells can induce a considerable level of allogeneic proliferation of T cells; however, there are some variations between their stimulation levels, but in all cases, CD8+ T cells showed a higher proportion of proliferating cells than CD4+ T cells. Additionally, the level of the proliferating T cell subpopulations, stimulated with all of the used cell lines, were much higher than the unstimulated PBMCs, which showed little or no proliferation in culture.

4.2.1.2 Allogeneic lympho-proliferative response of long-term stimulated PBMC using ELISpot assays

Long-term stimulated T cell cultures were carried out to investigate allogeneic stimulation of T lymphocytes, by isolating PBMCs from normal healthy $HLA\text{-}A2^+$ individuals and stimulating them with the myeloma hybrid cell lines, HMY2 and U266 at successive weekly intervals in the presence of human recombinant IL-2 and IL-7 for 3-10 weeks as described in section 2.4.2. During this time, I examined the IFNγ and perforin releasing activity of the responder cells using ELISpot assays.

ELISpot assays have the ability to assess quantitatively the functional response in the examined cultures at an individual cell level. So, after production of the long-term

activated T cells, I wanted to estimate their allogeneic activity in terms of IFNγ and perforin release using ELISpot assay.

4.2.1.2.1 Allogeneic IFNγ Enzyme Linked ImmunoSpot assay (ELISpot)

IFNγ is a member of the Th1 cytokines, which are released by different immune cells, including activated T cells, in response to *in vivo* cell infection or tumour invasion. *In vitro* detection of the frequency of responding T cells secreting IFNγ can be measured using ELISpot assays. So, IFNγ ELISpot assays were carried out to estimate the number of allogeneic activated lymphocytes following co-culture with stimulator cells.

After three weeks of stimulation of PBMCs with HMY2, HU266, HRC, HIC and U266 (HCM slowed down in the culture, in addition to its low level of expression of the examined TAA, so I ruled it out of the study), the long-term activated T cells were used as responder cells in IFNγ ELISpot assays. The responder cells were restimulated with their respective stimulator cell lines (that were used for activation of the T cell cultures) on the multiscreen PDVF filter 96 well plates for 48 hrs, for detection of the released IFNγ for quantitative assessment of the functional response of the activated T cell culture. Phytohaemaglutinin was included in the experiment as a positive stimulator, and long-term activated T lymphocytes, without any further activation in ELISpot assay, were cultured alone as a background control. After incubation and treatment with the appropriate mAb, the developing spots were counted under a dissection microscope for detection of the spots. All wells were read without knowledge of the stimulator cells. The experiment was repeated three times and the results are shown as mean of the three values ± SEM.

Number of the developing spots per 10^6 responder cells was calculated by using the following equation:

$$= \frac{\text{number of the emerged spots}}{\text{number of responder cells per well}} \text{x } 1\text{X}106$$

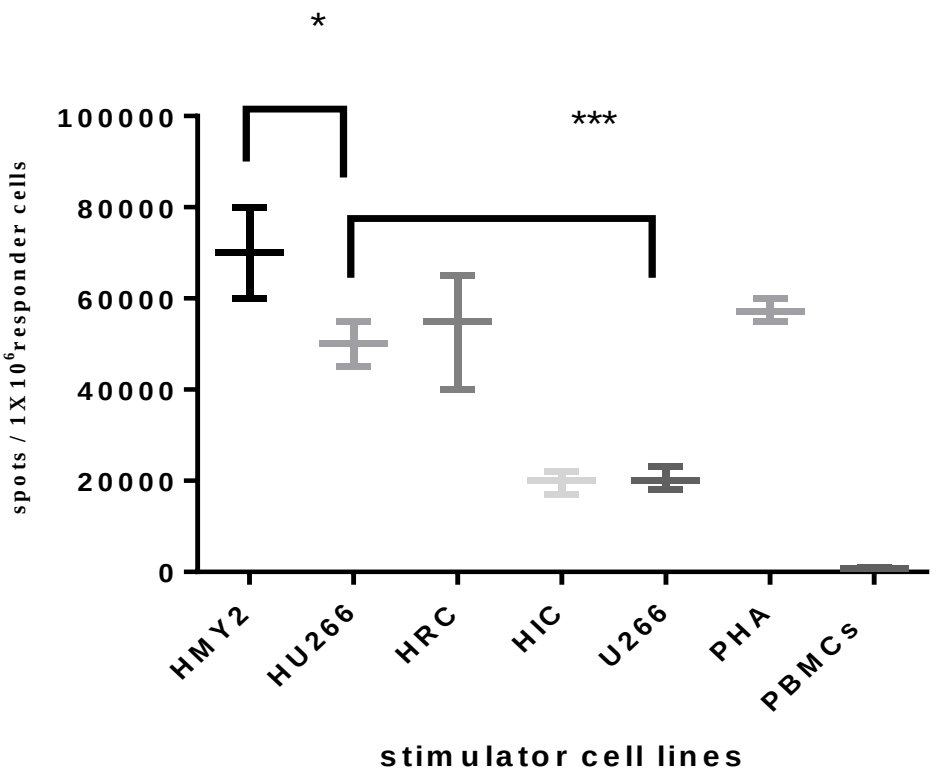


Figure 4.3: IFNγ ELISpot assay of long-term stimulated T cell cultures, using HMy2, HU266, HRC, HIC and U266. *Data are shown as mean of three experiments ± SEM. The stars show the degree of significance of difference of the response to hybrid HU266 and its parents HMY2 and U266. The levels of significances have been presented as asterisks (* <0.05, and *** ≤ 0.001).*

Figure 4.3 illustrates that elevated IFNγ releasing responses were seen by re-stimulating the long-term activated T cell cultures in ELISpot assays using HMY2, HU266 and HRC, to a greater degree than HIC and U266. Overall, all of the examined cell lines, have a significant elevation in IFNγ releasing response compared with stimulated PBMCs with no further stimulation in ELISpot assays. HMY2 was the strongest stimulator of the responder cells, as it gave significantly higher IFNγ releasing response compared with HU266, and there was no significant difference between the response induced by HMY2 and HRC. HMY2 induced significantly higher response than the response induced with HIC. This could be explained by its phenotypic characterization, as HMY2 had high level of expression of HLA class II, which is very important for stimulation of $CD4^+$ T lymphocytes. By comparing the ELISpot data of the activated cultures using the hybrid cell lines, we can see that both HU266 and HRC gave a similar increase in the number of spots, which indicates that their activation level of IFNγ release was almost the same.

Also, HU266 activated its culture about two folds more than its parent U266. It should be noted that this allogeneic response did not imply the presence of antigen and/or tumour specific responses, however, these data indicate the ability of the hybrids and HMY2 to stimulate allogeneic IFNγ releasing immune responses *in vitro*.

4.2.1.2.2 Perforin Enzyme Linked ImmunoSpot assay (ELISpot)

Perforin (PFN) is a key effector mediator of target cell apoptosis, through granule mediated cytotoxicity by T-cell activation, and acts as one of the essential cytolytic proteins which are responsible for elimination of infected and tumour cells by CTL (Zuber et al., 2005b). Perforin ELISpot assays were carried out to investigate the ability of long-term activated T cells to release perforin, which indicates the presence of cytotoxic T lymphocytes or NK cells in the activated culture.

The long-term stimulated allogeneic PBMCs from healthy individuals were harvested and used in perforin- ELISpot assays by re-stimulation with their respective stimulator cells for 5 days as described in section 2.4.2.2. In addition, responder cells without further *in vitro* stimulation were used as background controls, and PHA as a positive stimulator.

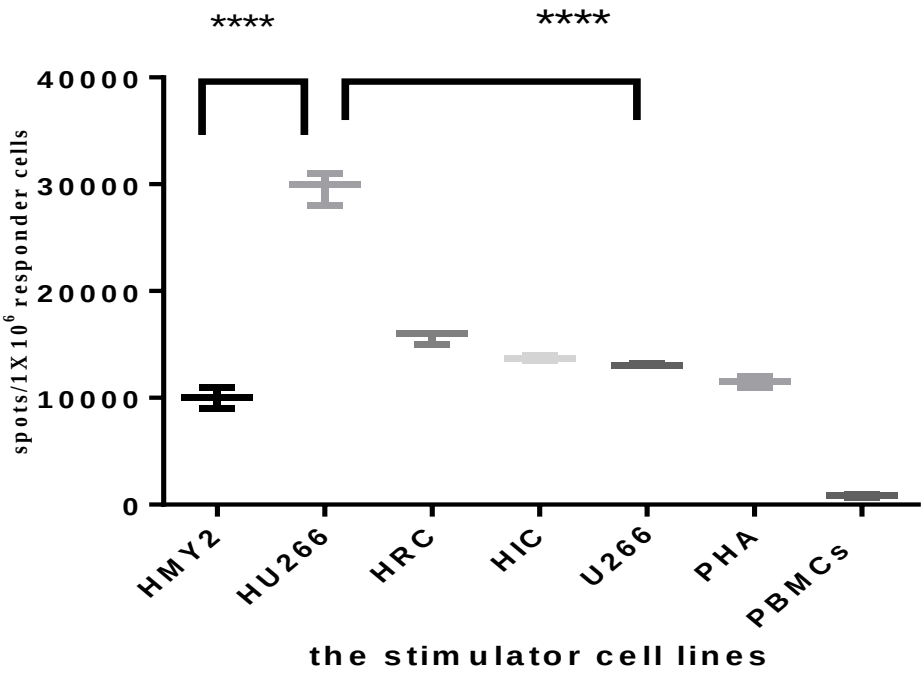


Figure 4.4: Perforin ELISpot assay of long-term activated responder cells, using HMy2, HU266, HRC, HIC and U266. *Data are shown as mean of three experiments ± SEM. The stars show the degree of significance of difference of the response to hybrid HU266 and its parents HMY2 and U266. The levels of significances were presented as asterisks (*** ≤ 0.001and **** ≤ 0.0001).*

Figure 4.4 shows that HU266 induced significantly enhanced responses of perforin release, which was around twice the level of the other hybrids and its parents HMY2 and U266. Moreover, for all of the used stimulator cell lines, a significant enhancement of perforin releasing responses of the activated T cell cultures were demonstrated, compared to that of the background PBMCs. However, the statistical significant difference of HU266 and the background PBMCs was much greater than the difference between the background PBMCs and the other used cell lines. The most interesting results in this experiment is that U266 induced perforin release at a similar level as HMY2, HRC and HIC. In addition, PHA induced a relatively lower perforin release than the used cell lines. By comparing the IFNγ and perforin ELISpot results for all the used stimulators, we can clearly see that the level of IFNγ release is higher than the level of perforin release in terms of spots per 10^6 responder cells for all the used stimuli. Which could be explained by release of IFNγ in the cultures by various cell types such as CD4+ T cells, CD8+ T cells and NK cells. However, perforin is released only by CD8+ T cells and NK cells.

To conclude, the elevated perforin releasing responses of the examined cell lines suggests stimulation of responses for allogeneic major histocompatibility (MHC) molecules expressed by the stimulators. However, the presence of antigen specific response in the long-term stimulated cultures cannot be confirmed by this assay, so further experiments were required to examine the activation of antigen specific CTL in these cultures.

4.2.2 Induction of antigen specific CTL in the long-term stimulated cultures:

Following detection of the ability of the hybrid cell lines (HU266, HRC and HIC) and their parents HMY2 and U266 to induce allogenic T lymphocyte responses in the long-term stimulated cultures, I wanted to investigate the presence of TAA specific CD8+ T cells in the stimulated cultures. Therefore, certain TAA (h TERT, MUC1, MAGE-C1 and CS1) were selected, based on their expression level by multiple myeloma patients and also by their mRNA and protein expression level in the used hybrid cell lines, as mentioned in the previous chapter.

4.2.2.1 Pilot experiment for assessment of presence of antigen specific CD8+ T cells in the long-term stimulated cultures

The evaluation of long-term stimulated cultures for the presence of antigen specific CD8+ T cells was done in a pilot experiment, by stimulating PBMCs from normal healthy HLA-

$A2^+$ individuals with the myeloma hybrid cell lines, HMY2 and U266 for four successive weeks, in the presence of human recombinant IL-2 and IL-7. After stimulation the cultures were stained with FITC-labelled anti-CD8 and PE-labelled HLA-A2-peptide pentamers specific for h TERT, MUC1, MAGE-C1 and CS1 as listed in Table 2.4.1.1. In addition, West Nile Fever Virus peptide (irrelevant peptide) pentamer, was used as a negative control. Unstimulated PBMCs stained with the same pentamers, and used as another negative control.

Figure 4.5 shows the sequential gating strategy, firstly on the live T cells using the forward scatter versus side scatter dot plot, followed by gating on the $CD8^+$ T cells using FITC stained $CD8^+$ T cells histogram, and finally determining the percentage of the $CD8^+$ pentamer$^+$ double positive T cells on the FITC versus PE dot plot. This gating strategy was applied on stimulated and unstimulated cultures (Figure 4.5).

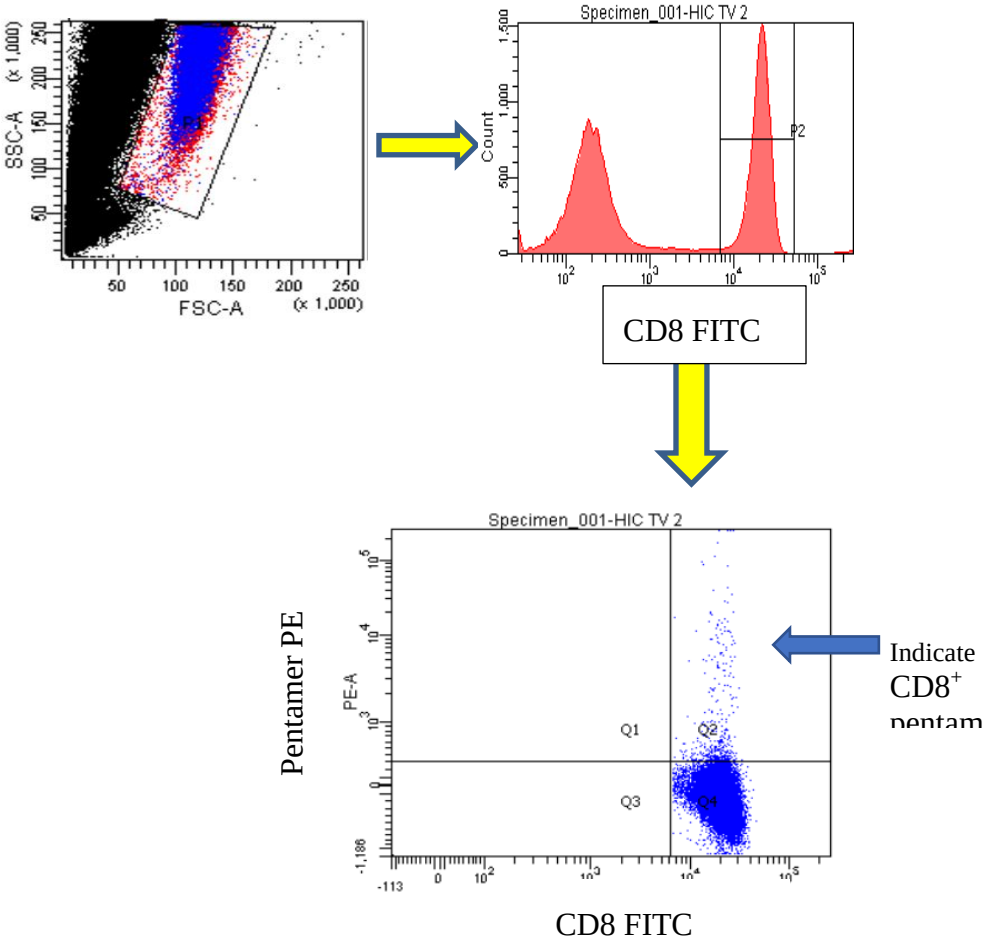


Figure 4.5: sequential gating on the live T cells using SSC X FSC dot plot, *followed by gating the positive population of CD8+ FITC histogram to represent $CD8^+$ T cells, and finally determining the percentage of the double positive $CD8^+$ pentamer$^+$ T cells on the FITC versus PE dot plot. The pentamer+ gate was defined using gating of the unstimulated PBMCs, pentamer+ CD8+ T cells, as a negative control.*

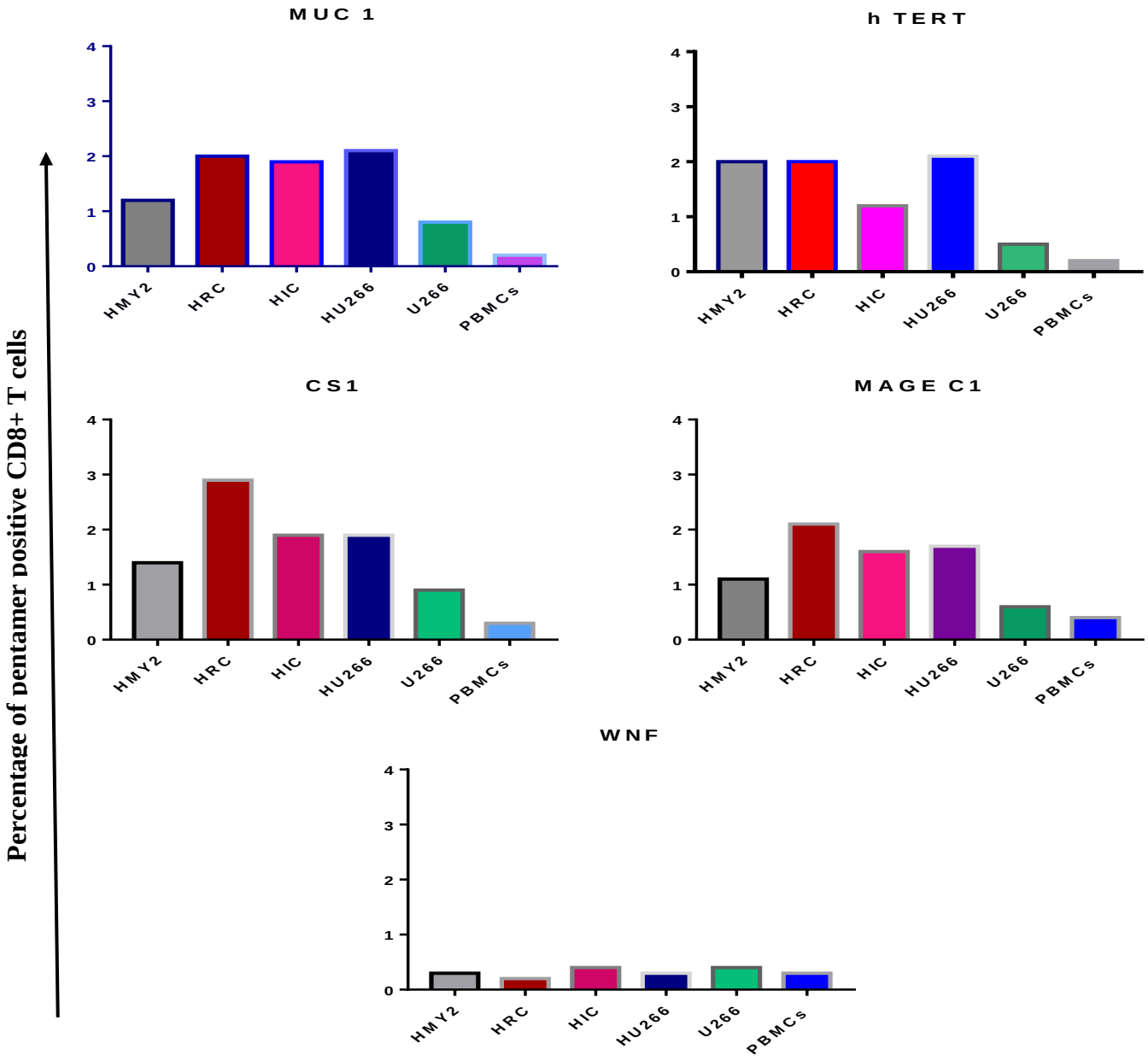


Figure 4.6: HLA-A2 peptide pentamer analysis of antigen specific CTL percentages. *After four rounds of stimulation of PBMCs isolated from healthy, HLA-A2+ donors with hybrid cell lines, HMY2 or U266, cells were stained with (MUC1, h TERT, CS1, MAGE-C1, and WNF) peptide pentamers, followed by flowcytometric analysis.*

Figure 4.6 shows the antigen specific $CD8^+$ T cells percentages stimulated by all of the used hybrid cell lines, HMY2 and U266. The hybrid cell lines HRC, HIC and HU266 induced higher percentage of MUC1, CS1 and MAGE-C1 peptide specific $CD8^+$ T

lymphocytes, compared with their parents HMY2 and U266. Regarding h TERT, HMY2 induced almost the same level of h TERT antigen specific CTL as HU266 and HRC, however, HIC induced a lower level of this antigen specific CTL, compared with the other hybrids and HMY2, but higher than U266. The level of the candidate antigen specific T cells were very low in the unstimulated PBMCs. Also, data showed no elevation of the level of WNF virus antigen specific T lymphocytes in the stimulated cultures than that of the unstimulated cultures. To conclude, most of the used hybrid cell lines appeared to induce allogenic antigen specific CTL to higher extent than their parents in PBMCs from healthy HLA-A2+ donors.

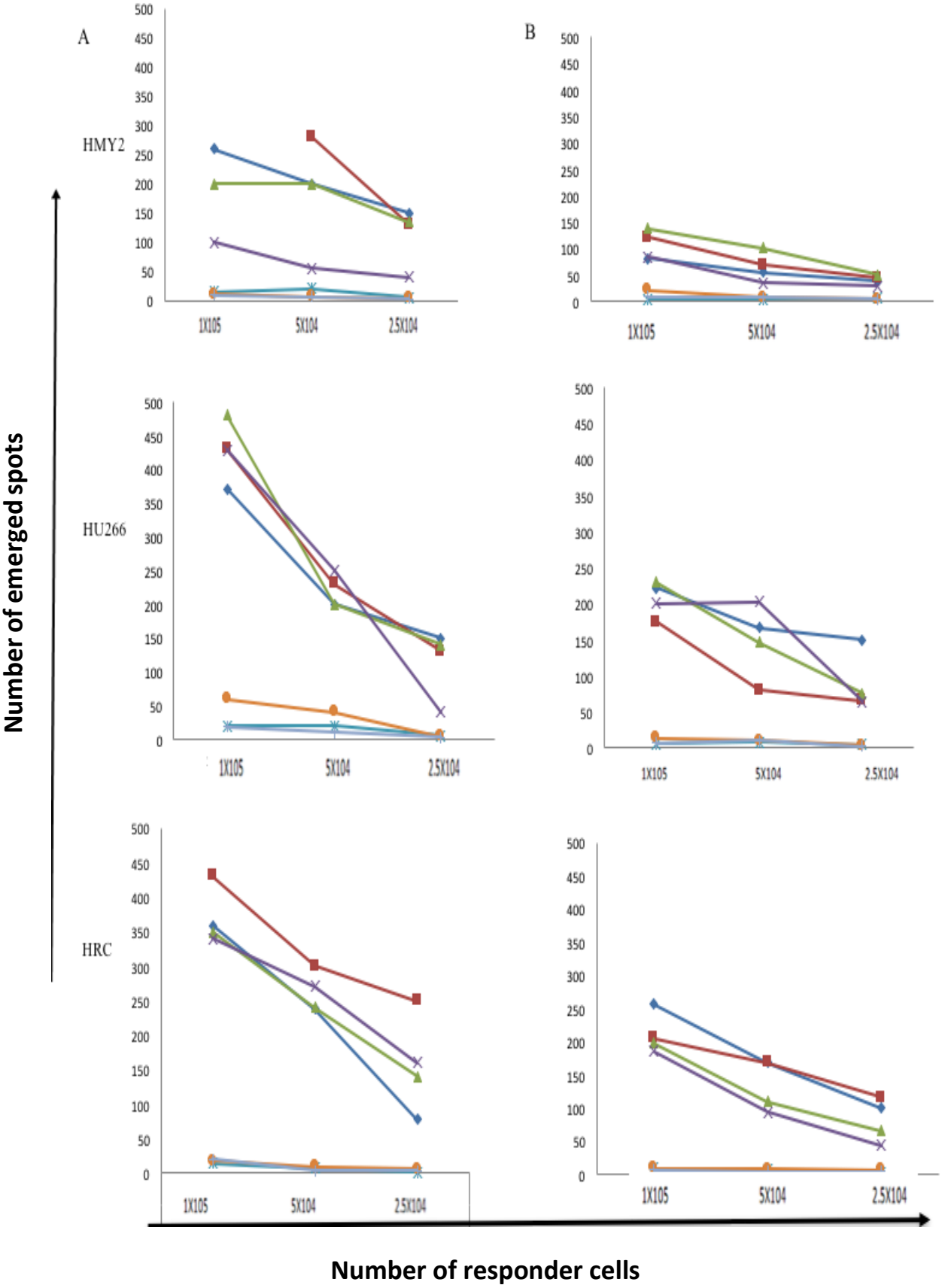
4.2.2.2 Optimization of the long-term stimulation protocol of antigen specific T lymphocytes:

After determination of the ability of the hybrids, and their parents for induction of antigen specific CD8+ T cells in the long-term activated cultures, we intended to identify the best culture protocol for efficient and rapid expansion of the activated antigen specific T cells, to obtain the highest number of functional T cells required for potential isolation and expansion of the antigen specific T cell clones. Therefore, the effect of different cytokines on the induction level of antigen specific T lymphocytes was monitored using IFN γ and perforin ELISpot assays. The protocol used previously in my supervisor's lab depended on using IL-2 as the main growth factor of T lymphocytes, and IL-7 from the third stimulation round. Based on these data, I therefore investigated the effect of some other cytokines on the activation level of the induced T lymphocytes, such as IL-7 used from the first week (to have its effect in naïve and memory T cells) (Schluns et al., 2000; Surh and Sprent, 2008), and IL-15 (due to its antiapoptotic effect) (Lu et al., 2002; Zhang et al., 1998), in the presence of IL-2 (300 u/ml).

The ELISpot assays were set up using either T2 cells pulsed with the candidate TAA-derived peptides, or with an irrelevant HLA-A2-restricted WNF virus peptide as a negative control (the used peptides were detailed previously in table 2.5. The cell line K562 was used as a target for NK cells, to estimate their activity in the stimulated cultures, and stimulated T cells without any further stimulation were used as a background control in the ELISpot assays.

4.2.2.2.1 The effect of IL-7 on the activation of antigen specific CTL using IFN - γ and Perforin ELISpot assays:

IL- 7 is of crucial importance for homeostasis and survival of naïve and memory antigen specific $CD8^+$ T lymphocytes (Schluns et al., 2000; Surh and Sprent, 2008). I therefore intended to optimize the effect of IL-7 in the produced long-term stimulated T cultures. Two long-term stimulated cultures were set up at the same time and under the same culture conditions in presence of IL-2 (300 u/ml) added from the second stimulation round. For one of these cultures, IL-7 was added during the first stimulation round at a concentration of 1ng/ml, and the same concentration was used in each repeated stimulation afterward. The other culture was done using IL-7 (1ng/ml) in the third stimulation round, and remained in the culture for one week. After three weeks of continuous weekly stimulation, the cells were harvested and used as responder cells in IFNγ and perforin ELISpot assays, as shown in Figures 4.6 and 4.7.



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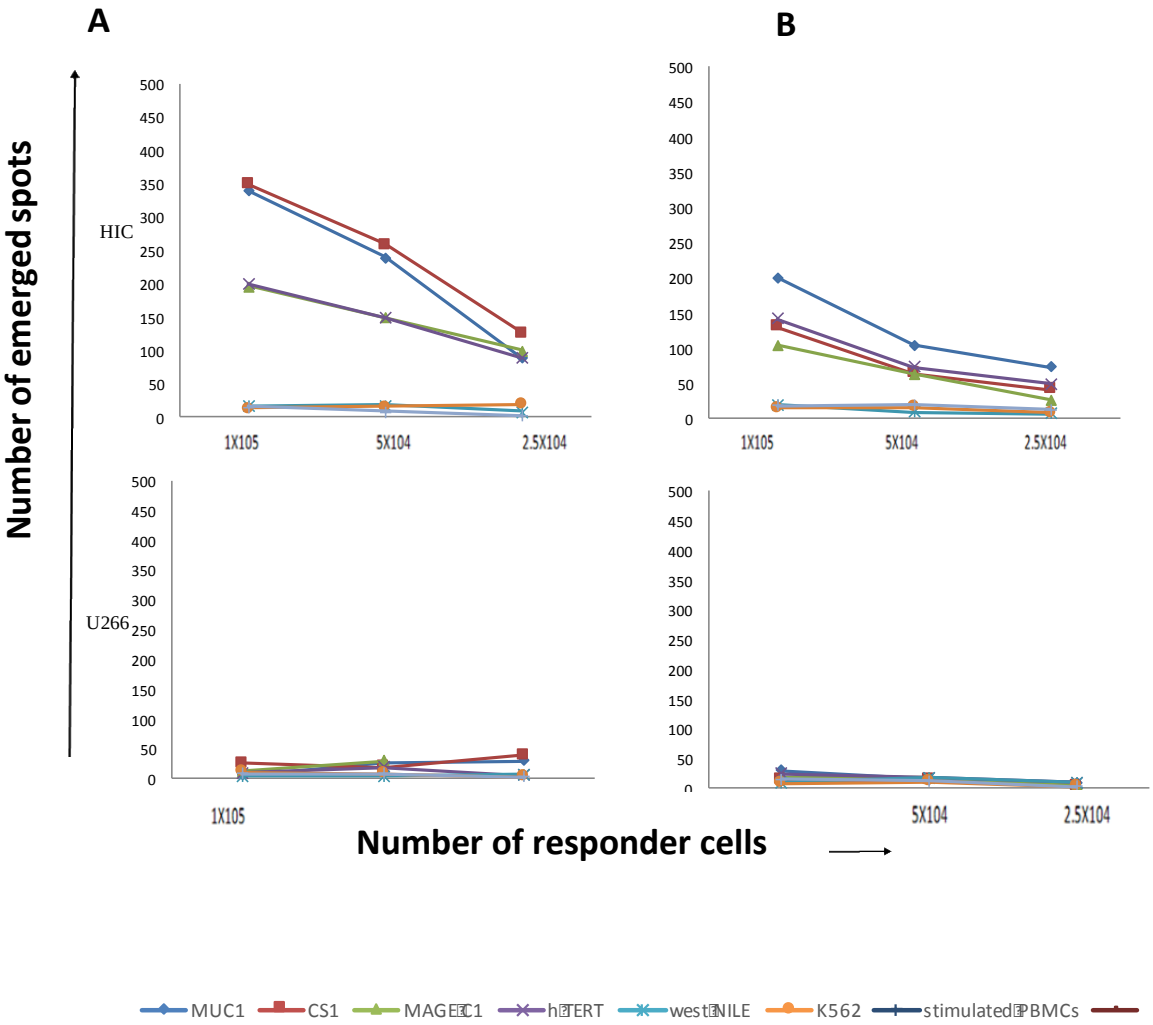
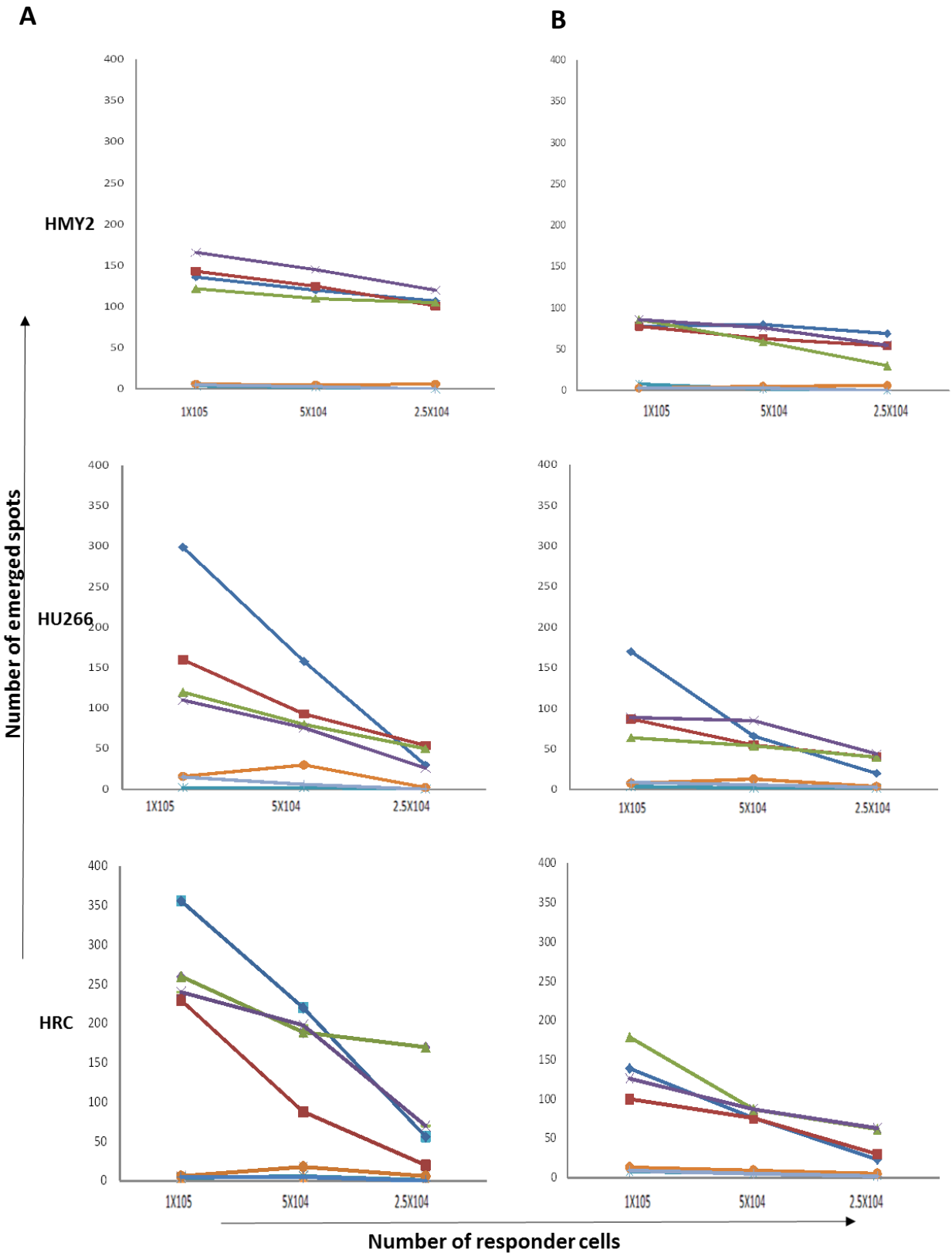


Figure 4.7: IFNγ ELISpot assays showing the effect of IL-7 in the *in vitro* expansion of antigen specific CD8+ T lymphocytes. *The used cultures were stimulated with HMY2, HU266, HRC, HIC and U266 in the presence of IL-2. Panel A were cultured in presence of IL-7 from the start of the culture, while panel B were cultured with IL-7 only from the third stimulation. The number of the emerged spots is shown in the y axis, and the number of the responder cells is shown on the X axis. For all of the examined ELISpots, 1000 stimulator cells were used. The experiment was done only once.*



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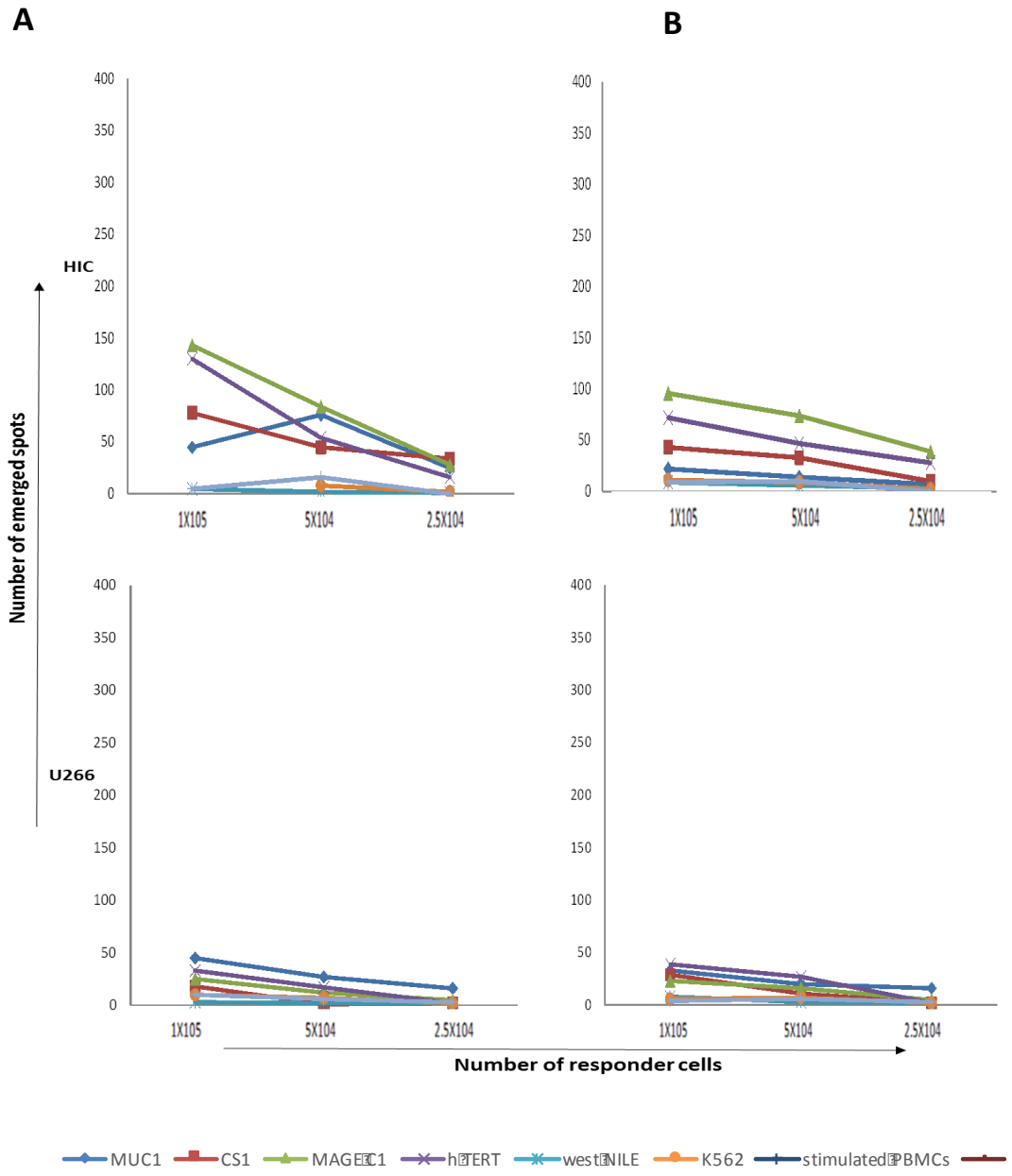


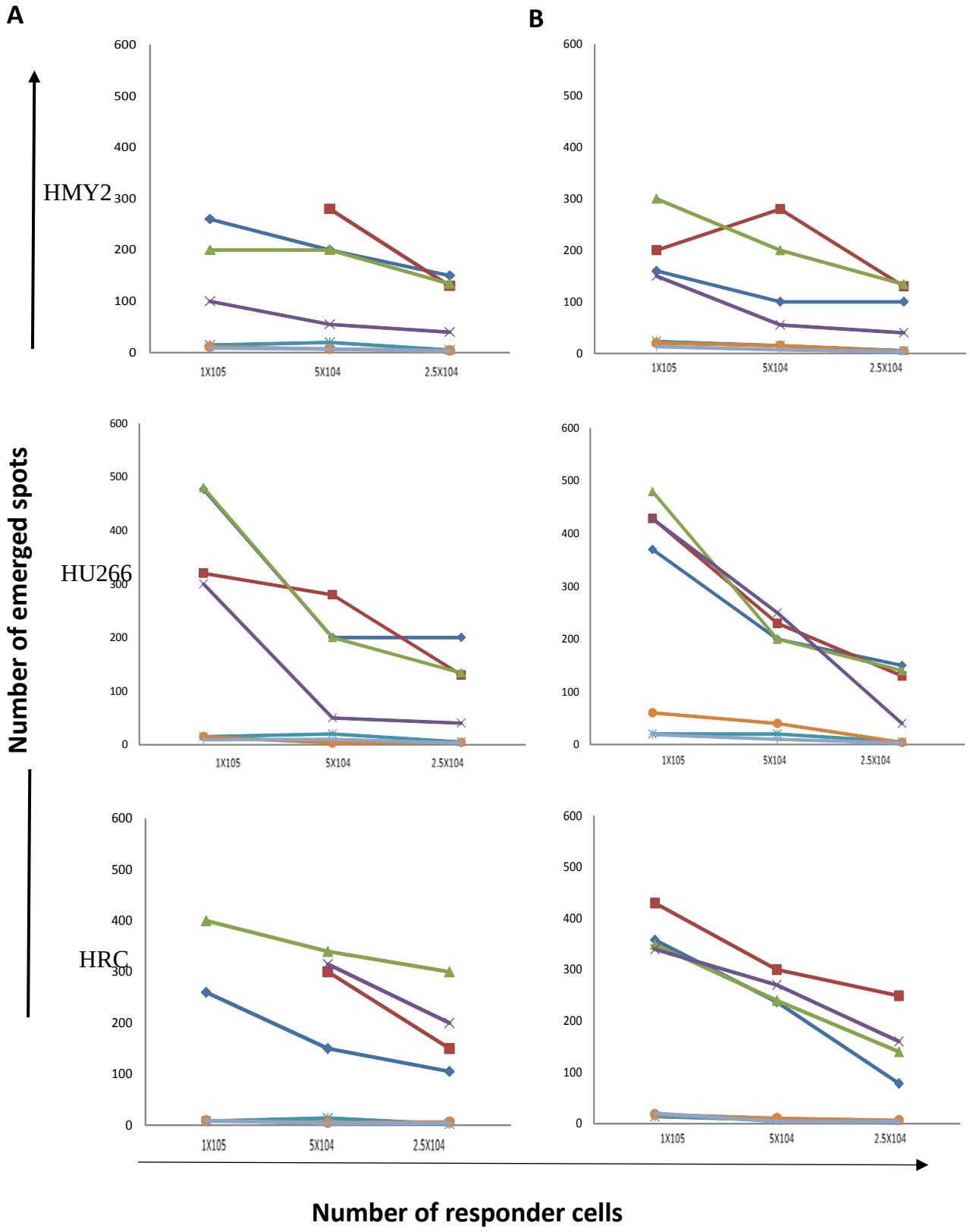
Figure 4.8: Perforin ELISpot assays showing the effect of *IL-7* in the *in vitro* expansion of CD8+ T lymphocytes. *The used cultures were stimulated with HMY2, HU266, HRC, HIC and U266 in the presence of IL-2. Panel A were cultured in presence of IL-7 from the start of the culture, while panel B were cultured with IL-7 only from the third stimulation. The number of the emerged spots is shown in the y axis, and the number of the responder cells is shown on the X axis. For all of the examined ELISpots 1000 of the stimulator cells were used. The experiment was done only once.*

The data show that HMY2, HU266 and HRC induced antigen specific IFNγ and perforin releasing responses at higher levels when IL-7 was added from the start of the culture. In the culture stimulated with HIC in presence of IL-7, both MAGE-C1 and h TERT specific IFNγ and perforin releasing responses were slightly higher than that without IL-7, while HIC stimulated MUC1 specific perforin release, slightly dropped in IL-7 contained culture in concentration of 10^5 responder cells than that without IL-7. Slight elevation of NK cells activity was observed in HU266 stimulated culture. It is of note that U266 failed to induce any antigen specific response with and without IL-7. The stimulated cultures have no induced specific response to WNF virus pulsed T2 cells, or to stimulated T cells with no further stimulation, which were used as negative controls. Also, all of the used cultures lacked NK cells activity, except HU266 stimulated culture, which showed weak NK activity.

To conclude, IL-7 induced a consistent increase of antigen specific IFNγ and perforin releasing response for all of the examined TAA when added from the start of the cultures, which reflects the effect of IL-7 on the activation of T lymphocytes.

4.2.2.2.2 The effect of IL-15 on the activation of antigen specific CTL in long-term activated cultures using IFNγ and perforin ELISpot assays

After detection of the effect of IL-7 on the enhancement of the survival, proliferation and activation of antigen specific T lymphocytes in the long-term stimulated lymphocytes, I investigated the role of another related cytokine, IL-15, as a growth and survival factor of memory $CD8^+$ T cells, as reported by many research groups (Lu et al., 2002; Zhang et al., 1998). Therefore, another two-long-term stimulated cultures were set up under the same culture conditions, but one with IL-15 (10 ng/ml) added in the second stimulation round, and the other culture without IL-15. After long-term stimulation for three weeks in presence of IL-2 (300 u/ml) and IL-7 (1ng/ml) added from the start, IFNγ and perforin ELISpot assays were set up using the long-term stimulated T lymphocytes as responder cells, and T2 cell line pulsed with either the relevant TAA or an irrelevant WNF virus peptide as target cells, as described previously.



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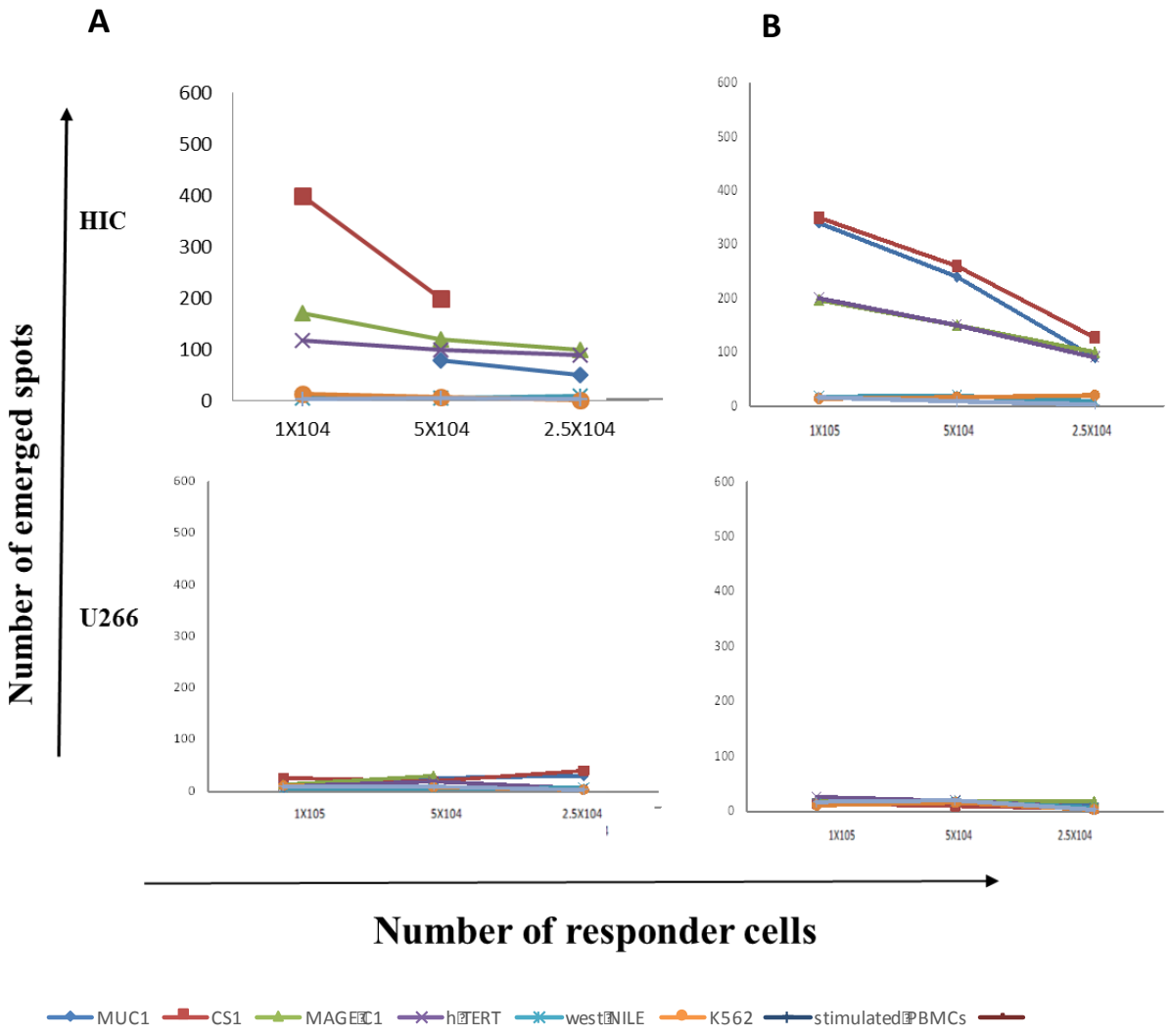


Figure 4.9: IFNγ ELISpot assays showing the effect of IL-15 on the induction of IFNγ releasing T cells. *The used cultures were stimulated with HMY2, HU266, HRC, HIC and U266 in the presence of IL-2 and IL-7. Panel A was cultured in absence of IL-15, while panel B was cultured in presence of IL-15. The number of the emerged spots was shown in the y axis, and the number of the responder cells was shown on the X axis. For all of the examined ELISpots 1000 stimulator cells were used. The experiment was done only once.*

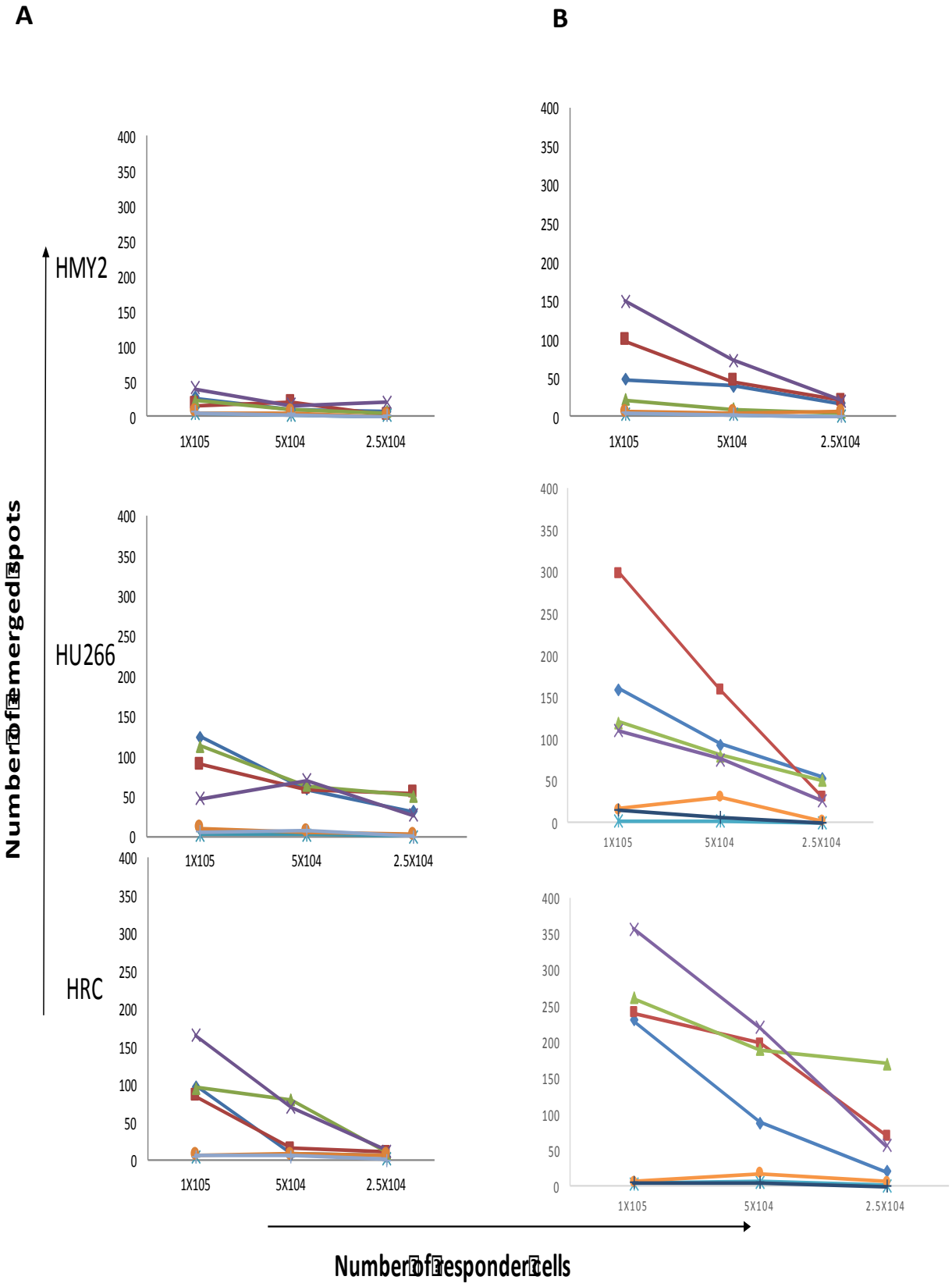
Figure 4.8 shows that HMY2 stimulated culture in the presence of IL-15 demonstrates a slight increase in the IFNγ releasing response to MAGE-C1 and h TERT peptide pulsed T2 cells, however CS1 specific IFNγ releasing response was the same with and without addition of IL-15. Also, some decrease of MUC1 specific response was observed in the IL-15 containing culture. No specific response was observed against WNF virus pulsed

T2 cells, stimulated PBMCS without further stimulation in the ELISpot assay, and no NK activity was observed in these cultures.

In the HU266 induced culture, low to moderate elevation was seen in the specific IFNγ releasing response to MUC1, CS1 and h TERT peptide pulsed T2 cells, with addition of IL-15. Regarding the used negative controls, no response was stimulated by WNF virus pulsed T2 cells, K562 or stimulated PBMCs without additional stimulation in the ELISpot assay.

In the long-term stimulated culture with HRC in presence of IL-15, enhanced IFNγ specific releasing response to CS1 and MUC1 was seen. In contrast, there is some decrease of IFNγ releasing response to MAGE-C1 in the culture containing IL-15. For HIC induced culture, MUC1, h TERT and MAGE-C1 specific IFNγ releasing response were slightly higher with addition of IL-15. However, U266 stimulated culture did not show any of the examined antigen specific IFNγ releasing response, in either of the cultures conditions. In all of the stimulated cultures with HIC, HRC and U266, no specific response was seen for the negative controls, WNF virus pulsed T2 cells, stimulated PBMCs with no further stimulation, and no stimulation of NK activity.

Overall, addition of IL-15 had only a mild impact on the induction of IFNγ releasing cells in the long-term T cell cultures



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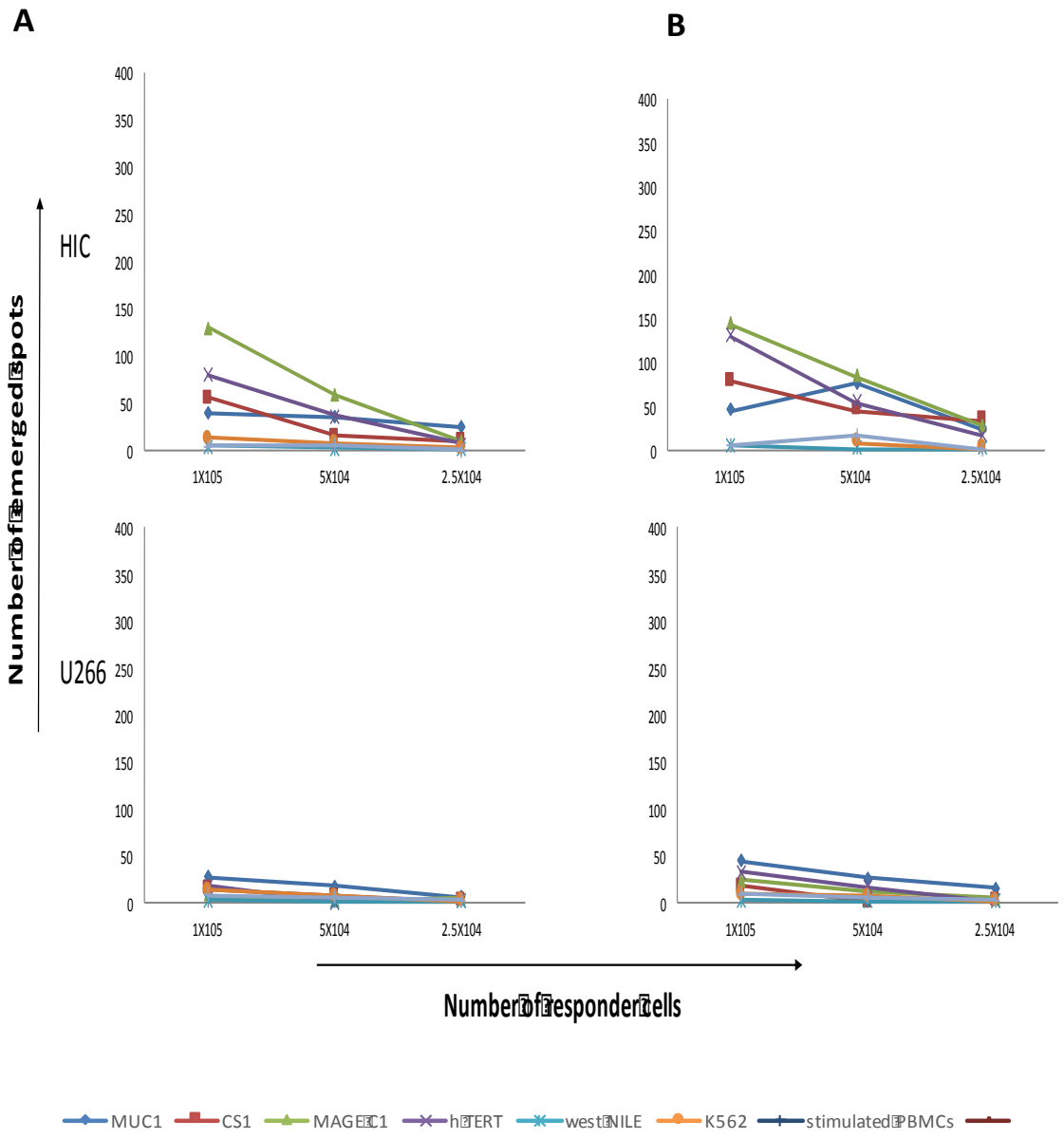


Figure 4.10: Perforin ELISpot assays showing the effect of *IL-15* in the *in vitro* expansion of perforin-releasing CD8+ T lymphocytes. *The used cultures were stimulated with HMY2, HU266, HRC, HIC and U266 in the presence of IL-2 and IL-7. Panel A was cultured in absence of IL-15, while panel B was cultured in presence of IL-15. The number of the emerged spots was shown in the y axis, and the number of the responder cells was shown on the X axis. For all of the examined ELISpots 1000 stimulator cells were used. The experiment was done only once.*

Perforin release ELISpot assay (Figure 4.9) revealed that HMY2 stimulated culture in presence of IL-15 had a slight elevation of h TERT, CS1 and MUC1 specific perforin releasing response, compared with the absence of IL-15, with no recorded elevation in the response to any of the used negative controls. In HU266 induced culture in presence of IL-15, a sharp increase of CS1 specific perforin release was observed, and slight increase

of the specific perforin releasing response to h TERT, MUC1 and MAGE-C1 peptide pulsed T2 cells was also recorded in this culture. This experiment also demonstrates low level of perforin release response to K562, only at one of the used ratios.

HRC stimulated culture showed a dramatic increase of h TERT, CS1, MAGE-C1 and MUC1 specific response in the culture containing IL-15, compared with the culture without IL-15. However, HRC stimulated cultures reported very minimal perforin releasing response to K562.

With HIC stimulation, h TERT peptide pulsed T2 cells had a slight elevation of their specific response with addition of IL-15, and no specific effect was observed in case of MAGE-C1, CS1 and MUC1 peptide pulsed T2 cells.

In contrast, U266 failed to induce any of the examined TAA specific perforin release. Moreover, in all of the examined cultures of HRC, HIC and U266, no response was detected to WNF virus pulsed T2 cell line or to PBMCs without further stimulation.

To summarize, in contrast to its minimal effect on induction of IFNγ releasing cells, IL-15 increased the antigen-specific perforin releasing response to most of the candidate antigens in the examined cultures, which indicates its effect on the survival of CD8+ T cells. However, study of the effect of IL-7 and IL-15 on the induction of antigen specific CTL experiment was done only once, so the results are an indication only, and should be treated with caution.

4.2.2.3 T cell-subsets' proliferation in long-term stimulation using different stimulator cell lines:

Responses of $CD3^+$, $CD8^+$ and $CD4^+$ T cells during the long-term stimulation with HMy2, the hybrid cell lines and U266 was investigated, in order to ensure the proliferation and increase in the number of activated T cells, and to study further the phenotype of the proliferating T cells in the long-term stimulated cultures. In addition, I wanted to show if there was a difference in the ability of the used hybrids and their parent cell lines to stimulate different subsets of T lymphocytes. The experiment was set up using the previously stated protocol for long-term activated T lymphocytes, and cells were harvested at weekly intervals up to four weeks, and stained with anti CD3+, CD8+, and CD4+ mAb using immunofluorescence technique, followed by flow cytometric analysis. The results are shown in Figure 4.10, and were consistent in two separate experiments.

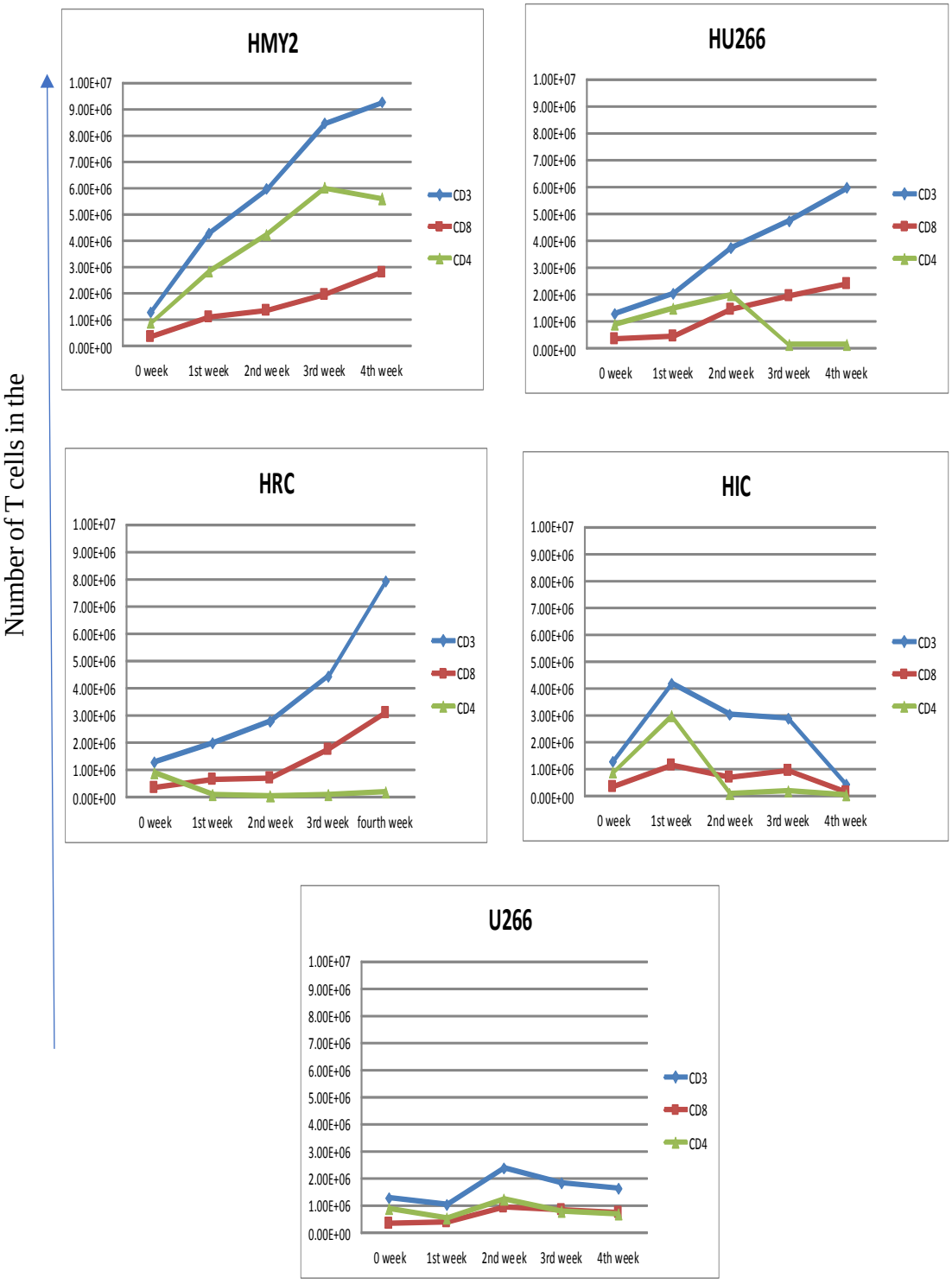


Figure 4.11: Tracking of CD3+ T cells, CD8+ T cells and CD4+ T cell populations. *In long-term stimulated culture for four weeks. The used stimulator cell lines are mentioned in the upper legend of each graph. The stimulation was done in the presence of IL-2, IL-7, and IL-15.*

The data presented in Figure 4.10 show that HMy2 stimulated a strong allogeneic T cell proliferative response, with progressive increase in CD3+ and CD8+ T cells in the culture. However, HMY2 cells supported the proliferation of CD4+ T cells to a greater degree than the proliferation of CD8+ T cells. CD4+ T cell proliferation was almost twice that of CD8+ T cells in this particular culture.

By comparing the hybrids together, HRC and HU266 showed progressive proliferation of CD3+ and CD8+ T cells. HU266 supported the proliferation of CD4+ T cells until the second week, whereas the other hybrids' cultures lacked CD4+ T cell population after the first week, which may be due to the high level of expression of HLA class II by HU266. HIC induced the proliferation of CD3+, CD4+ and CD8+ T cells during the first week only, then the number of T lymphocytes dropped gradually, most likely to be due to the low level of expression of HLA class II and CD86, leading to low level of interaction of CD80/CD86 with CD28/CTLA4. Regarding U266 cultures, it induced a weak allogeneic proliferative response of CD3+, CD4+, CD8+ T lymphocytes, which started after adding IL-2 in the culture. There was no further proliferation after the second week, which could be attributed to absence of the costimulatory molecules expression by U266. These results were consistent with the previous findings in section 4.2.1 CFSE experiment; with the exception of high proliferation of $CD4^+$ T cells in HMY2 stimulated culture.

Overall, the hybrid cell lines were able to induce an allogeneic proliferative response in CD3+ T cells and CD8+ T cells that was greater than that of U266. CD4+ T cells tended to drop down quickly, after initial proliferation. However, HMY2 induced CD4+ T cells at a higher level compared with CD8+ T cells. This could be attributed to HMY2 nature as an APCs, which are the main inducers of Th1 activity (through release of certain cytokines such as IL-12).

4.2.2.4 Estimation of antigen specific T cell induction in the long-term stimulated cultures of PBMCs from healthy HLA-A2+ individuals

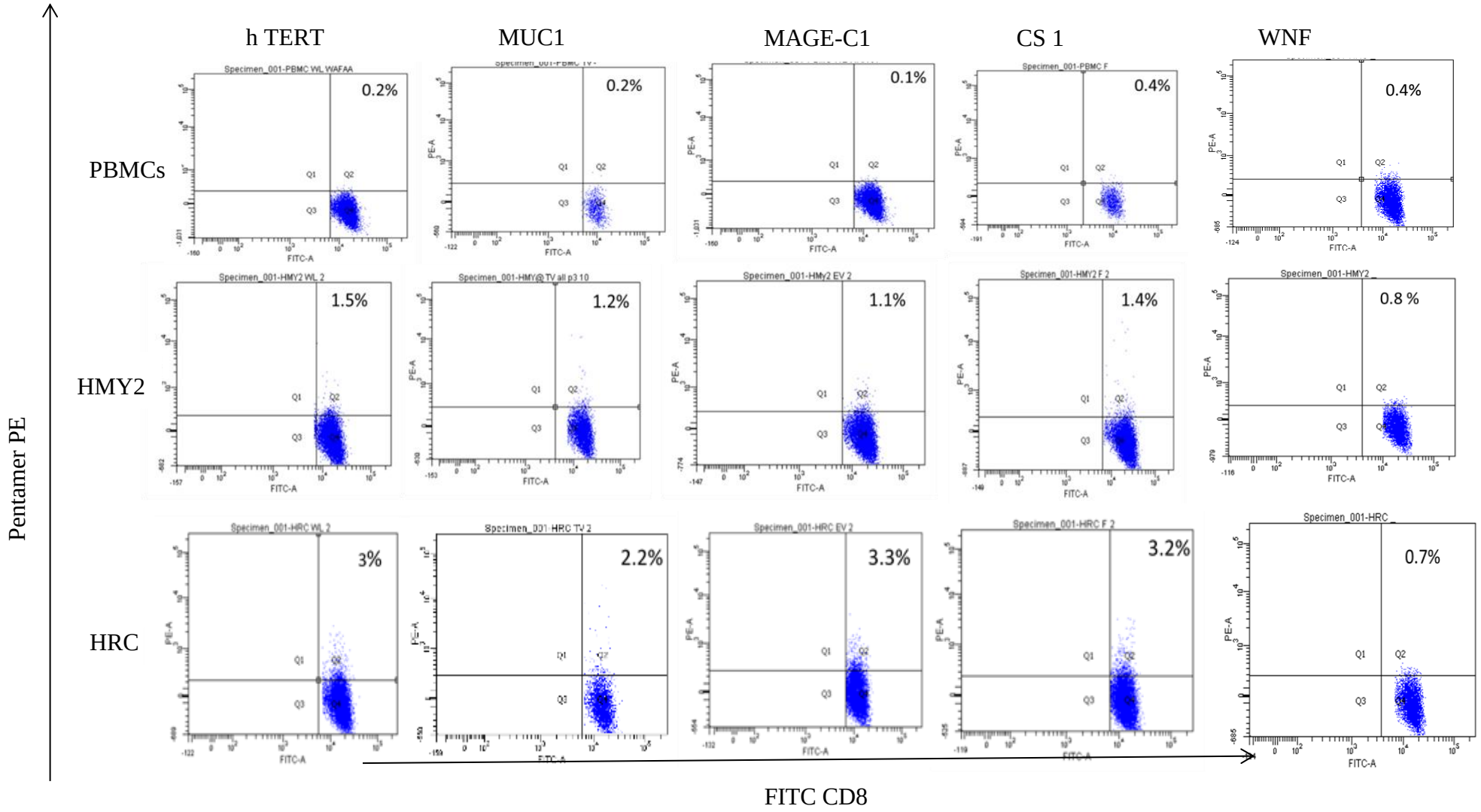
In order to confirm the ability of HMY2, the hybrid cell lines and U266 to induce antigen specific T cell immune responses *in vitro*, PBMCs isolated from allogeneic healthy individuals were co-cultured with HMY2, the hybrid cell lines and U266, and stimulated weekly by Mitomycin-C-treated stimulator cells using the optimized protocol (in presence of IL-2, IL-7, and IL-15), as mentioned in Section 2.4. After four rounds of stimulation,

the cells were harvested and used in HLA-A2 peptide pentamer staining and ELISpot assays, to assess the induction of antigen specific CTL.

4.2.2.5 HLA-A2-peptide pentamer analysis

The evaluation of long-term stimulated T cell cultures for the presence and percentage of tumour peptide specific CTLs, after using the optimized protocol, was done by staining the culture with FITC-labelled anti-CD8 and PE-labelled HLA-A2-peptide pentamers specific for h TERT, MUC1, MAGE-C1 and CS1 as described in section 4.2.2.1.

Also, two different types of negative controls were involved in the experiment: The first one was staining of the unstimulated PBMCs with all the used peptide pentamers under the same conditions as cells from the stimulated culture, and used as background to indicate the level of induction of antigen specific cell response in the long-term activated cultures. The second negative control was staining all the stimulated T cell cultures with HLA-A2 peptide pentamer incorporating a HLA-A2 binding WNF Virus peptide as an irrelevant antigen, to explore the specificity of the produced activated T lymphocytes, and to investigate the binding of the specific pentamers to the stimulated T cells. The same gating strategy was applied as mentioned in Figure 4.5.


h TERT
MUC1
MAGE-C1
CS 1
WNF
PBMCs
HMY2
HRC
0.2%
0.2%
0.1%
0.4%
0.4%
1.5%
1.2%
1.1%
1.4%
0.8 %
3%
2.2%
3.3%
3.2%
0.7%
Pentamer PE
FITC CD8

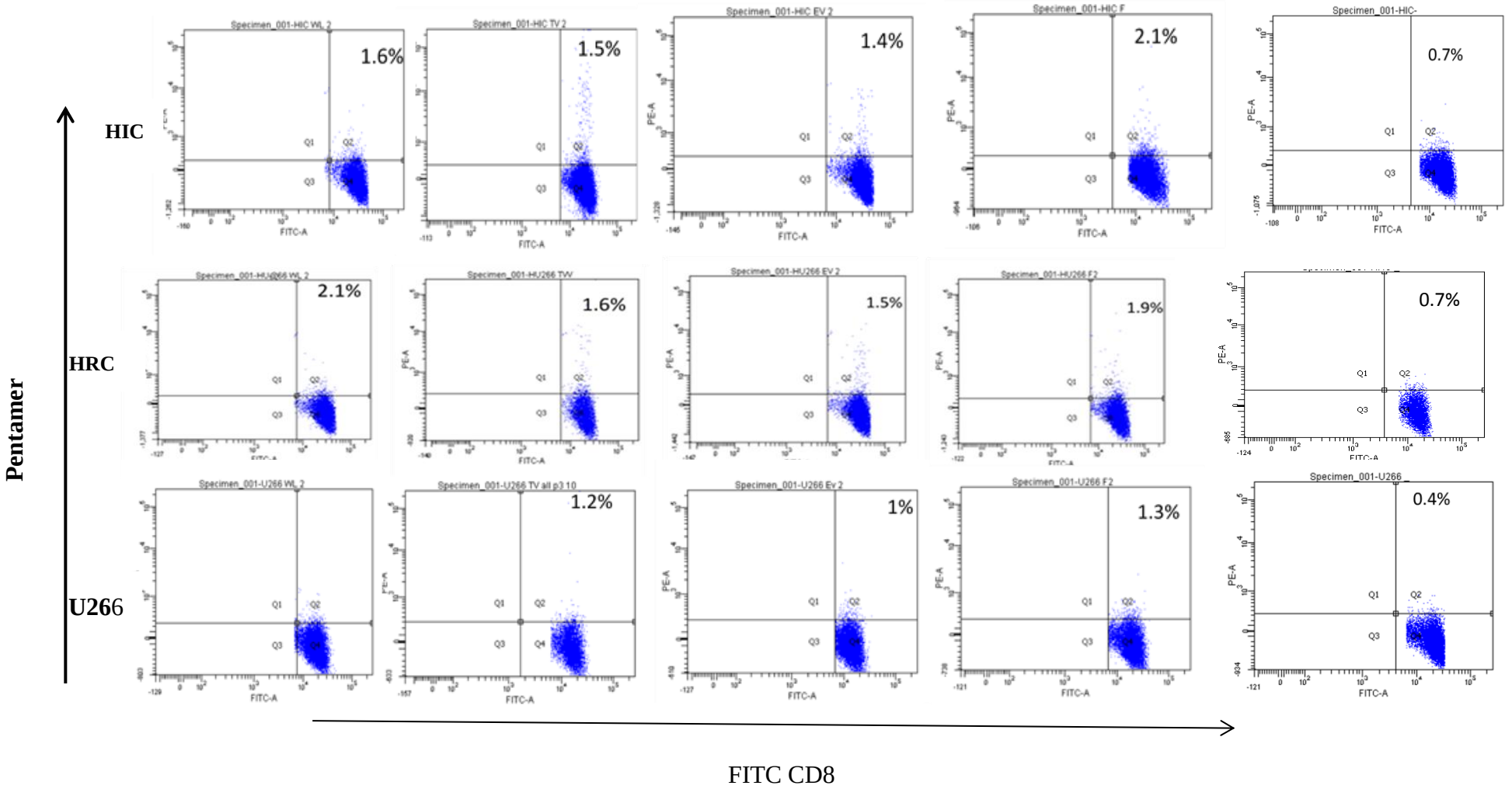


Figure 4.6: HLA-A2-peptide pentamer staining of T cell lines, stimulated by HMy2 cells, or by hybrid cells lines HU266, HRC and HIC or myeloma cell line U266.

The antigen specific CTL percentages (as a proportion of the total CD8+ T cells gated cells in the culture) are shown in the upper right quadrant. Data shown are representative of 3 experiments.

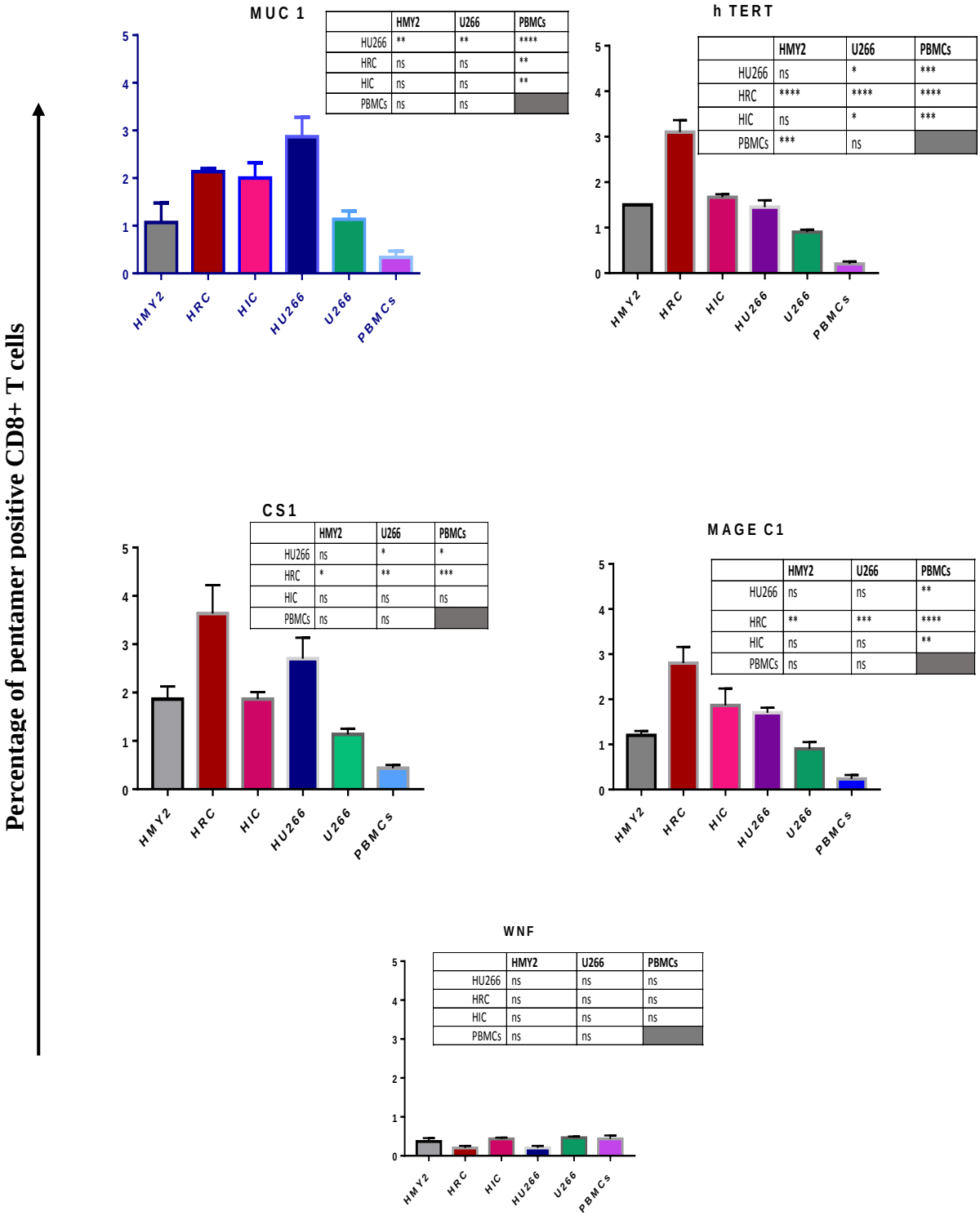


Figure 4.7: HLA-A2 peptide pentamer analysis of antigen specific CTL, as percentages of pentamer positive CD8+ T cells. *After four rounds of stimulation of PBMCs isolated from healthy, HLA-A2+ donors with hybrid cell lines, HMY2 or U266, cells were stained with (MUC1, hTERT, CS1, MAGE-C1, and WNF) peptide pentamers, followed by flowcytometric analysis. The experiment was repeated three times. Results are presented as mean ± SEM of three experiments, and stars represent the degree of significance (* <0.05, **≤ 0.01, *** ≤ 0.001and **** ≤ 0.0001).*

Figure 4.12 shows CD8 pentamer dot plots taken from one experiment, representative of three experiments. For easy interpretation, all data were summarised in Figure 4.13. The cumulative data illustrate that the percentage of tumour antigen-specific CD8+ T lymphocytes of the unstimulated PBMC gives < 0.5% of the pentamer positive cell. Therefore, I arbitrarily established the population of percent 0 - 0.5% of live pentamer positive $CD8^+$ T cells as representative of a negative response, populations with percentage higher than 1% as positive for an antigen-specific $CD8^+$ T cell response, and a population 0.5-1% was described as weak positive response (Mohamed et al., 2012b).

By following these criteria and comparing the antigen specific $CD8^+$ T cells percentages produced by all of the used hybrid cell lines, we can see that HRC gave the highest percentage as revealed by pentamer staining of h TERT, CS1 and MAGE-C1 peptide specific $CD8^+$ T lymphocytes. HU266 has significant elevation of the stimulation of antigen specific T cells compared with its parent U266, for all of the examined antigens except MAGE-C1. T cell cultures stimulated with the parent myeloma cell U266, they had no statistically significant difference compared with the unstimulated PBMCs for any of the antigens used. HMY2 did not induce the production of MUC1, CS1 and MAGE-C1 antigen specific CD8+ T lymphocytes, compared with unstimulated PBMCs, which may be attributed to the low level of the examined protein expression by HMY2 compared to the hybrid cell lines.

The unstimulated T lymphocytes were negative for all of the examined tumour antigenic peptides (< 0.5%). Also, the data showed no elevation of the level of WNF virus antigen specific T lymphocytes in the stimulated cultures or the unstimulated cultures. To conclude, most of the used hybrid cell lines have the ability to induce allogenic antigen specific CTL to higher extent than their parents in PBMCs from healthy HLA-A2+ donors.

4.2.2.6 Detection of stimulated antigen specific T Lymphocytes in long-term activated cultures using the IFN γ ELISPOT assay:

After detection of the antigen specific CD8+ T cells in the long-term stimulated culture using peptide pentamer staining, I wanted to estimate the activity of the induced antigen specific T lymphocytes in these cultures. Therefore, I assessed IFNγ and perforin release responses of the activated antigen specific T cells in the cultures, using IFNγ and perforin ELISpot assays. ELISpot assays can investigate the frequency and the activity of the

induced antigen specific CTL at a single cell level, so this assay can be used to monitor antigen specific T cell responses. The induced long-term stimulated T cells were used as responder cells in the ELISpot assay. The experiment was set up using T2 cell line pulsed with peptides of the relevant cognate antigens, the same antigen derived peptides that were used in the pentamer staining experiment, and an irrelevant antigen (WNF virus HLA-A2 restricted peptide), as a negative control. The responder cells were incubated with the peptide pulsed T2 cells on multiscreen PDVF filter 96 well plates for 48 hrs, as described in section 2.4.3. After incubation and treatment with the appropriate antibody, the emerged spots were counted using a dissection microscope. Stimulated PBMCS of each culture, without any further stimulation in the ELISpot assay, was used to control the background response, in addition to using K562 to indicate the activity of NK cells in the cultures. The experiment was repeated three times, using PBMCs of different HLA-A2+ healthy donors.

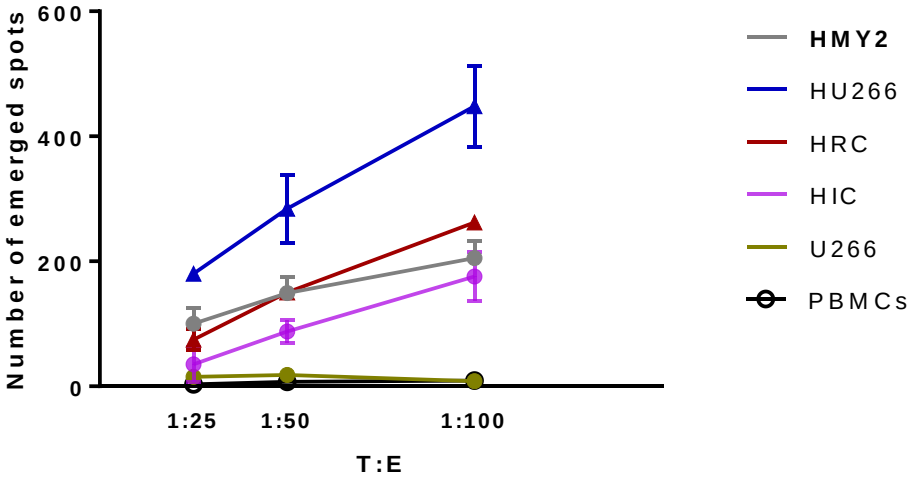
Figure 4.14 summarises data of the IFNγ ELISPOT assays. The data show that HU266 stimulated cultures induced significantly higher IFNγ releasing response to restimulation with MUC1 derived peptide pulsed T2 cell line compared with its parents HMY2 and U266, and also compared with HU266 stimulated T cells without further stimulation. There was no significant difference of U266 stimulated culture response to MUC1 compared with the response of U266 stimulated culture with no more stimulation in the ELISpot assay, which indicates absence of induction of MUC1 specific T cells in U266 stimulated cultures. Regarding MUC1 specific IFNγ releasing response, HRC stimulated cultures had a slightly lower IFNγ release response compared to HU266. HIC induced a significant enhanced response at 1:100 T:E ratio only compared with U266, and the background response of PBMCs.

The most significant IFNγ release response induced by h TERT, CS1 and MAGE-C1 derived peptide pulsed T2 cells was shown in HRC induced cultures, followed by HU266 and then HIC and HMY2. U266 stimulated cultures did not induce any significant increase of IFNγ releasing response upon further stimulation with h TERT, CS1 and MAGE-C1 peptide, compared with U266 stimulated PBMCs without further stimulation. Lack of NK activity was observed in all of the examined cultures, and no IFNγ releasing response was seen with restimulation of any of the stimulated cultures with WNF virus peptide pulsed T2 cells, which indicates the specificity of the activated antigen specific T lymphocytes.

Overall, HMY2 and the hybrid cell lines HU266 and HRC induced antigen specific T lymphocytes IFNγ releasing responses to a significantly enhanced level compared with U266. Moreover, U266 induced culture failed to induce antigen specific IFNγ responses exceeding the background levels.

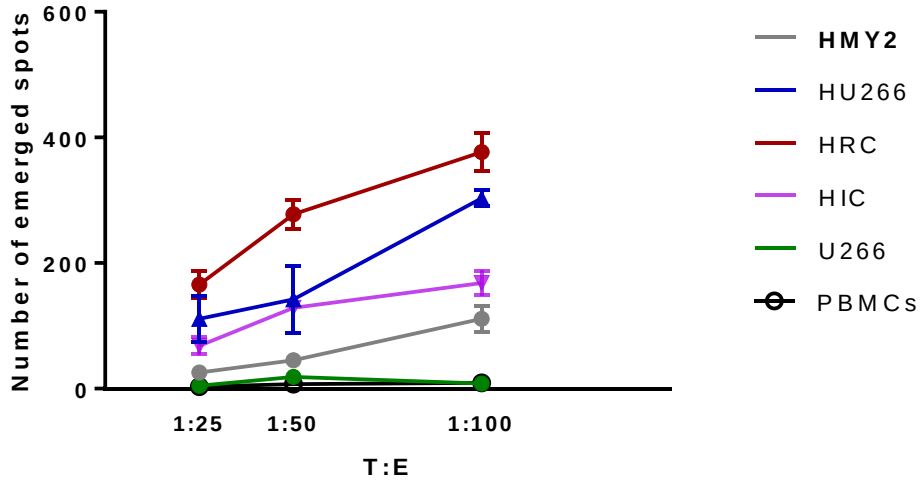
	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs
HMY2	ns	ns	ns	ns	ns	ns	*	**	ns	ns	****	****
U266	ns	ns	***	ns	*	ns	****	ns	****	***	****	ns
PBMCs	ns	ns	***		**	ns	****		****	**	****	

MUC1



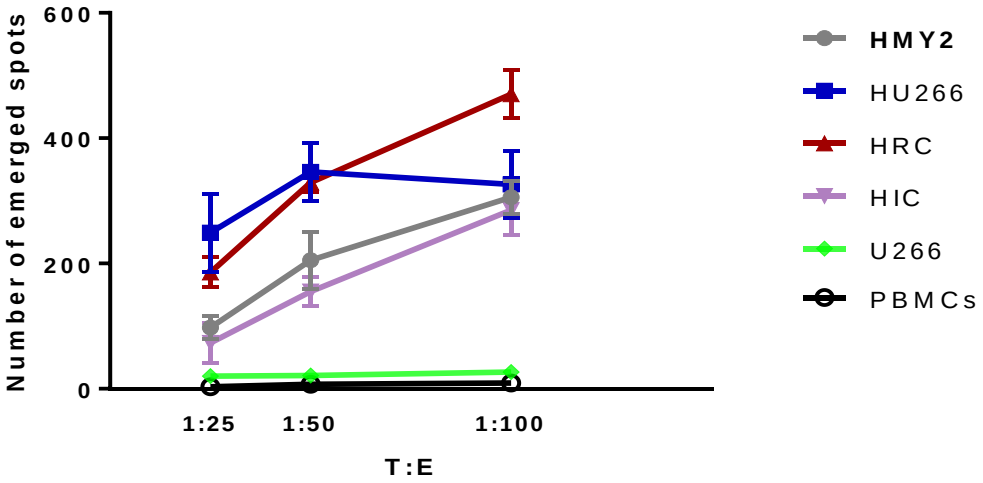
	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCS	HRC	HIC	HU266	PBMCS	HRC	HIC	HU266	PBMCS
HMY2	**	ns	ns	ns	***	ns	*	ns	****	ns	****	*
U266	***	ns	**	ns	****	*	**	ns	****	***	****	ns
PBMCS	****	ns	**		****	**	***		****	****	****	

h TERT



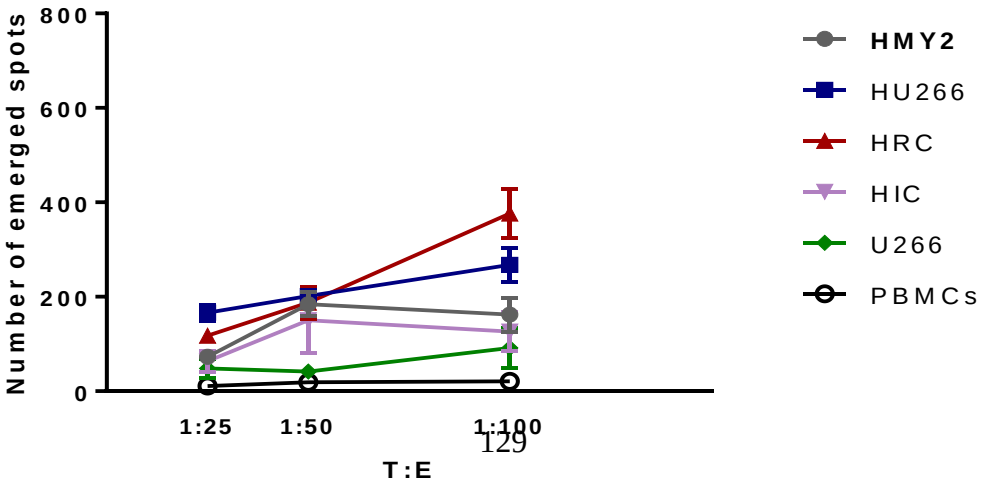
	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs
HMY2	**	ns	*	ns	ns	ns	*	***	**	ns	ns	****
U266	**	ns	***	ns	****	*	****	ns	****	***	****	ns
PBMCs	**	ns	****		****	*	****		****	****	****	

CS1

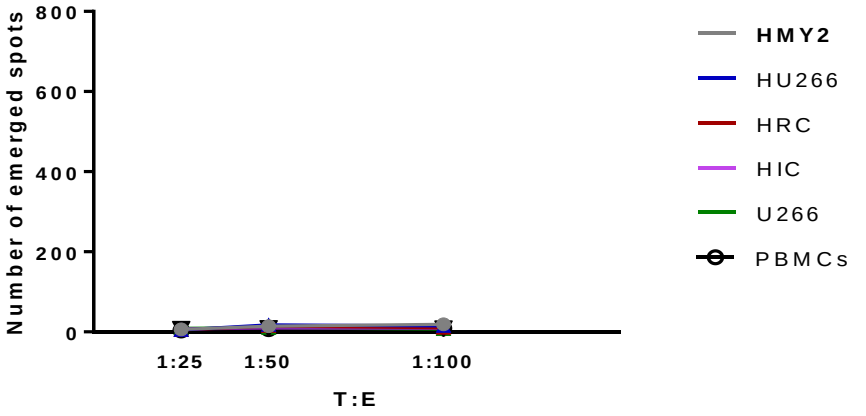


	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs
HMY2	ns	ns	ns	ns	ns	ns	ns	**	***	ns	ns	*
U266	ns	ns	ns	ns	*	ns	*	ns	****	ns	**	ns
PBMCs	ns	ns	*		**	ns	*		****	ns	****	

MAGE C1



	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs
HMY2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
U266	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
PBMCs	ns	ns	ns		ns	ns	ns		ns	ns	ns	



	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs
HMY2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
U266	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
PBMCs	ns	ns	ns		ns	ns	ns		ns	ns	ns	

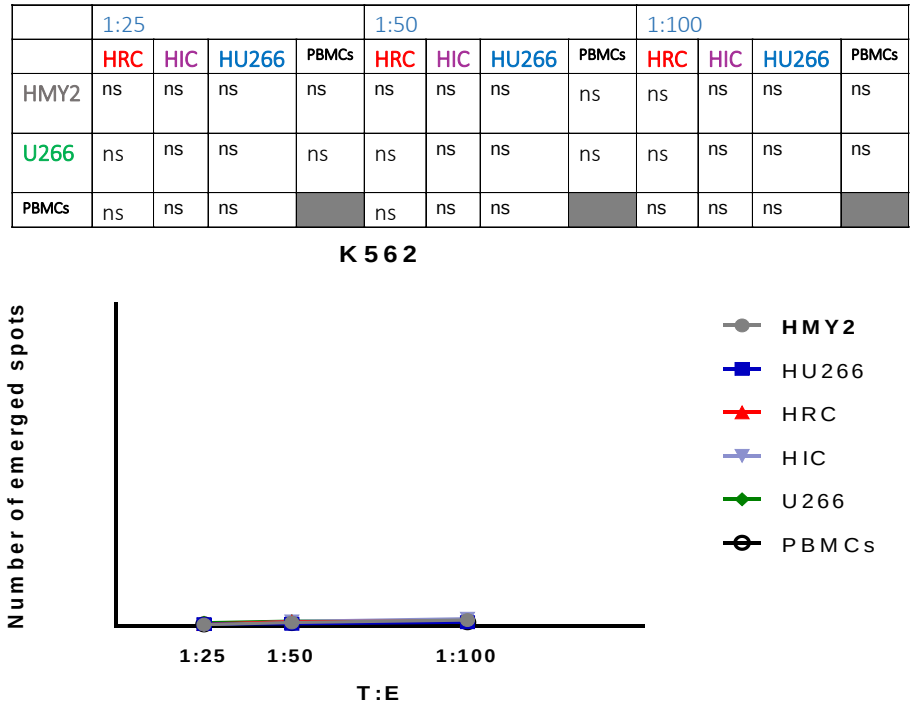


Figure 4.8: IFNγ ELISPOT assays using MUC1, h TERT, CS1, *MAGE-C1* and WNF derived peptide pulsed T2 cells. *T2 cells pulsed with the cognate peptides were mixed with long-term stimulated responder cells in a ratio 1:25 - 1:100 respectively, and incubated for two days. The emerged spots were counted. Data are presented as means of three independent experiments ± SEM. The degree of significance were presented as asterisks, and summarised in tables next to each graph (* <0.05, **≤ 0.01, *** ≤ 0.001and **** ≤ 0.0001). T:E is Target: Effector ratio.*

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4.2.2.7 Detection of stimulated antigen specific T Lymphocytes in long-term activated cultures using the perforin ELISPOT assay:

The previous experiment showed that the hybrid cell lines induced antigen specific IFNγ releasing responses in PBMCs from HLA-A2+ healthy individuals. However, perforin ELISPOT assay may represent a more direct analysis of cell-mediated cytotoxicity as compared to the IFNγ ELISPOT assay, as perforin is a key mediator of CTL induced cytotoxicity. The same experimental set up was used as for the IFNγ release ELIspot assay, but with a longer incubation time (5 days). The long-term stimulated T cells were used as responder cells, and stimulated with peptide pulsed T2 cells as target cells. The responder cells were used without further stimulation as a negative control, and K562 as an NK cell target.

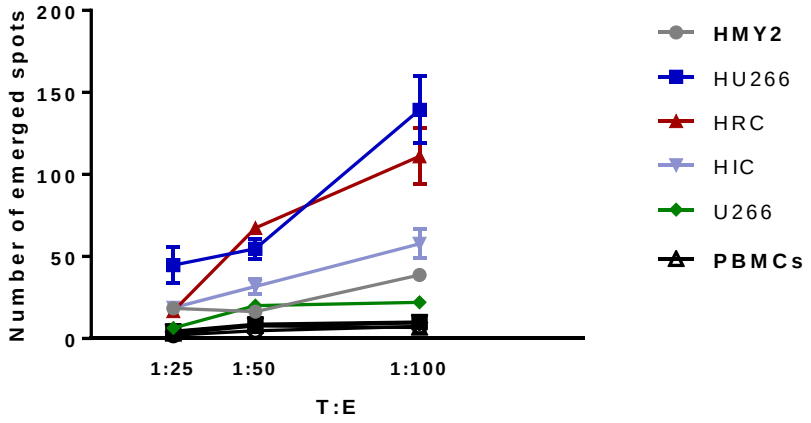
Figure 4.15 illustrates that HU266 and HRC significantly induced perforin release in response to restimulation with MUC1 derived peptide pulsed T2 cell lines, compared with the parents HMY2, U266 and unstimulated responder cells, at 1:100 and 1:50 T:E ratios. However, HIC and HMY2 induced a significant response only at 1:100 ratio compared with responder cells with no further stimulation in the ELISpot assay. U266 stimulated cultures failed to induce significant perforin release compared with its background responder cells. Regarding h TERT specific response, all of the used hybrid cell lines and HMY2 stimulated cultures showed significantly enhanced response upon restimulation with h TERT peptide pulsed T2 cells, compared with the background response of PBMCs, at 1:100 ratio. However, U266 stimulated culture showed no significant difference at this T:E ratio, between its response and the background response of the used PBMCs.

With the respect to the CS1 specific response, HRC and HU266 stimulated cultures induced the highest perforin release, followed by the response of HIC. All of the used hybrids and HMY2 induced significantly enhanced responses compared with the background response of PBMCs. However, U266 failed to induce such significant response to CS1 compared with its un-stimulated PBMCs. Restimulation with MAGE-C1 derived peptide pulsed T2 cell lines induced the lowest perforin releasing response in T cells from most of the previously stimulated cultures, compared with the other used antigens. However, perforin release induced by the hybrid cell lines and HMY2 was significantly higher than that of U266, and PBMCs background responses. No specific perforin release was induced by restimulation with WNF pulsed T2 cells or K562 in the

ELISpot assay, which indicates the specificity of the activated antigen specific T lymphocytes.

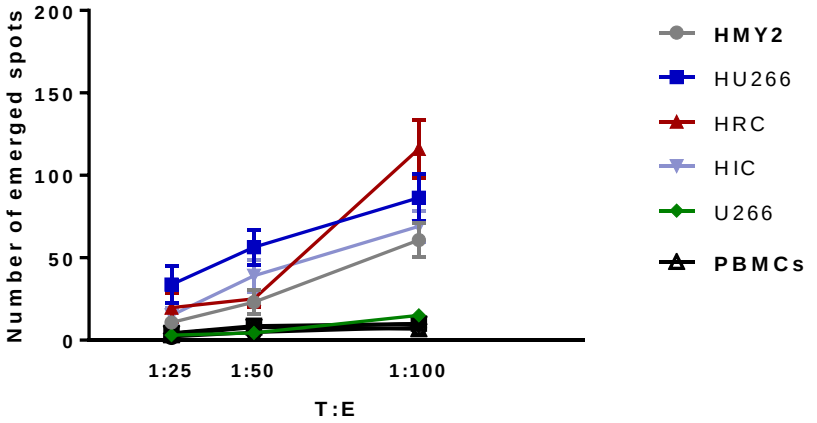
	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs
HMY2	ns	ns	ns	ns	****	ns	***	ns	****	ns	****	**
U266	ns	ns	***	ns	****	ns	**	ns	****	**	****	ns
PBMCs	ns	ns	***		****	ns	****		****	****	****	

MUC1



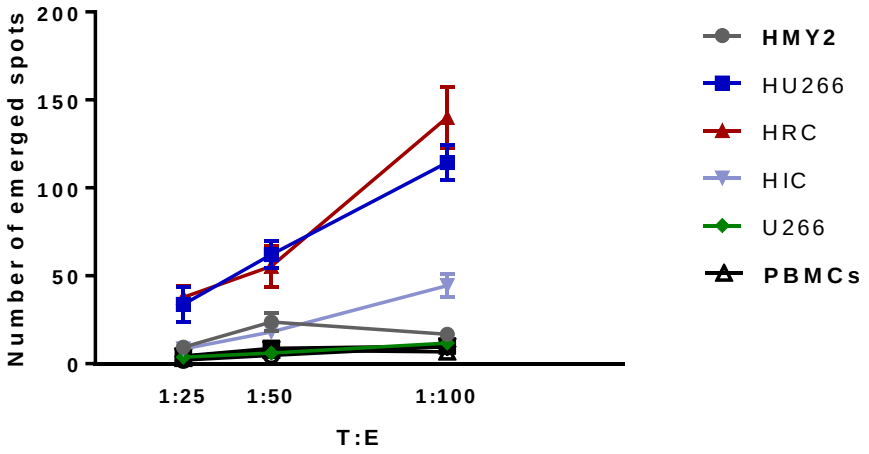
	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs
HMY2	ns	ns	ns	ns	ns	ns	*	ns	****	ns	ns	****
U266	ns	ns	*	ns	ns	*	****	ns	****	****	****	ns
PBMCs	ns	ns	*		ns	*	****		****	****	****	

h TERT



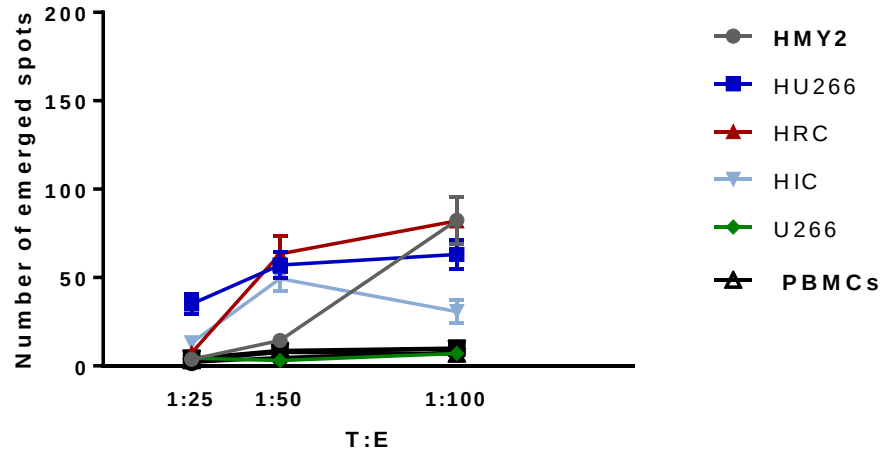
	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs
HMY2	*	ns	ns	ns	**	ns	****	ns	****	*	****	ns
U266	***	ns	**	ns	****	ns	****	ns	****	**	****	ns
PBMCs	***	ns	**		****	ns	****		****	***	****	

CS1

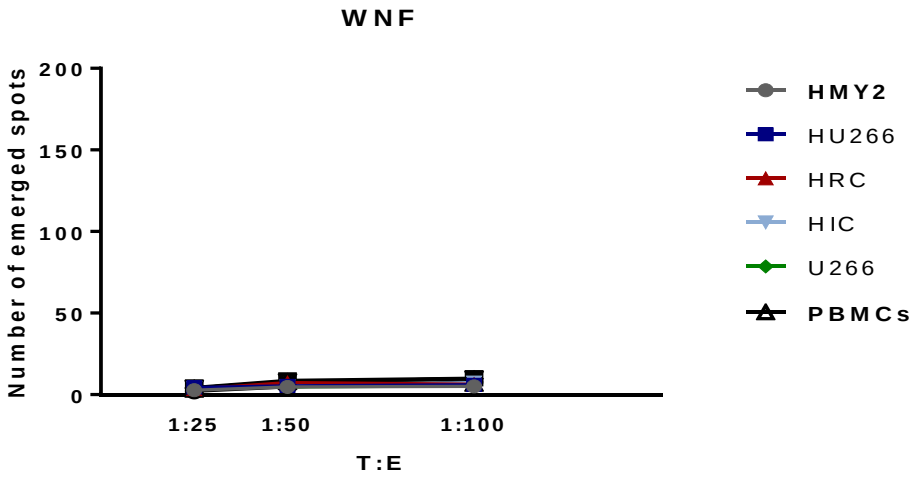


	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs
HMY2	ns	ns	***	ns	***	***	****	ns	ns	****	ns	****
U266	ns	ns	**	ns	****	****	****	ns	****	**	****	ns
PBMCs	ns	ns	**		****	****	**		****	*	****	

MAGE C1



	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs
HMY2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
U266	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
PBMCs	ns	ns	ns		ns	ns	ns		ns	ns	ns	



	1:25				1:50				1:100			
	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs	HRC	HIC	HU266	PBMCs
HMY2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
U266	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
PBMCs	ns	ns	ns		ns	ns	ns		ns	ns	ns	

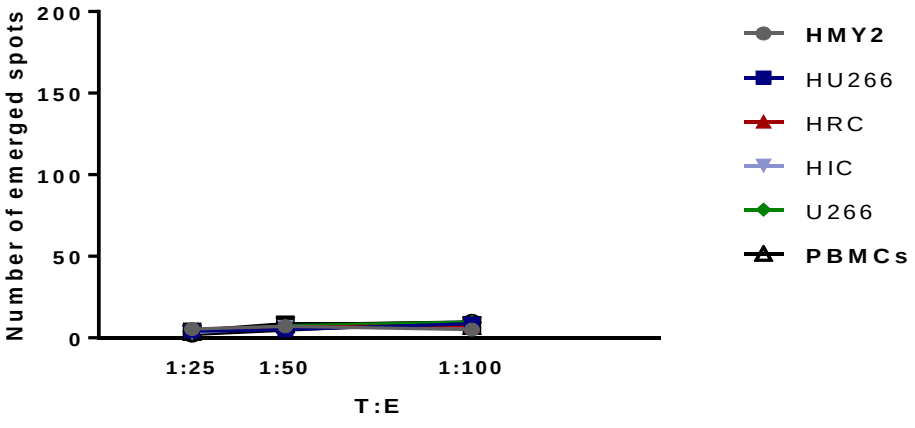


Figure 4.9: Perforin ELISpot assay, using MUC1, h TERT, CS1, MAGE-C1 and WNF derived peptide pulsed T2 cells. *T2 cells pulsed with the cognate peptides were mixed with long-term stimulated responder cells in a ratio 1:25 - 1:100 respectively, and incubated for five days. The emerged spots were counted. Data are presented as mean of three independent experiments ± SEM. The degrees of significance were presented as asterisks, and summarised in table next to each graph (* <0.05, **≤ 0.01, *** ≤ 0.001and **** ≤ 0.0001).*

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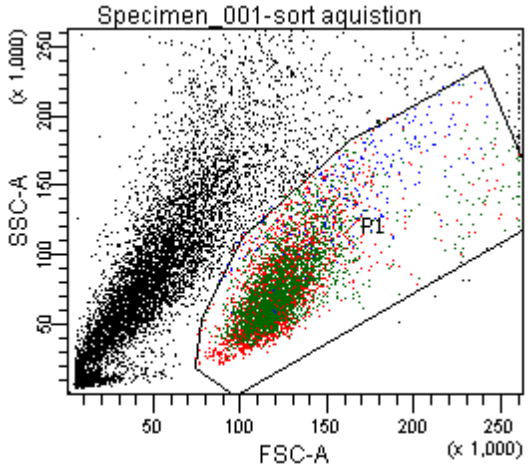
4.2.3 Attempted production of an antigen specific T cell clone

Adoptive T-cell immunotherapy is a promising potential treatment for cancer. Using this approach, T cell clones of known specificity are infused into cancer patients, aiming to recognize and destroy tumour cells (Perica et al., 2015). Accordingly, I attempted to isolate and selectively expand *in vitro* antigen specific T cell lines, from the bulk long-term stimulated T lymphocytes cultures, for potential use in adoptive immunotherapy as antigen specific T cell clones. These cultures were not done to GMP standard, therefore could not be used to treat patients, so this is therefore only a feasibility study. The induced activated T cells had first to be isolated from the stimulated culture and expanded *in vitro*, for production of adequate numbers of the desired antigen specific T cells for infusion into tumour patients.

In an attempt to isolate the tumour antigen specific $CD8^+$ T cells from the bulk cultures, I used FACS-sorting of the produced long-term stimulated T cell culture using FACSAriaII cell sorter, and the data analysis was done using FACSDiva software Version 6.1.3 (BD Biocsiences, San Jose, USA). The FACS sorting was done for two antigens (MUC1 and CS1), using T lymphocytes stimulated with HU266 and HRC respectively (Figure 4.16). The selection of these antigen specific T cells was done based on the level of induction of antigen specific T cells in the long-term stimulated T cell cultures used in this experiment (results not shown). I was able to isolate 320,000 CS1 specific T cells (3% of the live PBMCs in this culture), and 180,000 MUC1 specific T lymphocytes (identified as 2.1% of the live PBMCs in the culture), as shown in Figure 4.16. Following sorting, CS1 and MUC1 specific T cells were activated using Dynabeads human T activator beads (containing anti CD3, CD28 and CD137) in presence of IL-2, IL-7 and IL-15, as described in section 2.4.4.1.

Following the manufacturer's instructions, using 1:10 beads: cells ratio, and restimuation after 7 days, the cells remained alive in the culture for a few days, followed which they died out (Figure 4.17). Consequently, I tried to find out the cause of this observed result. Firstly, I tried three different beads: cells ratios (1:10, 1:5 and 1:3), using HU266 activated cultures (bulk, unsorted culture). Additionally, three different experiments were set up using different stimulation time points (after 3 days, one week and 10 days). Figure 4.18 shows the proliferation of the cell cultures at different time points, following stimulation with different ratios of T activator Dynabeads.

A



B

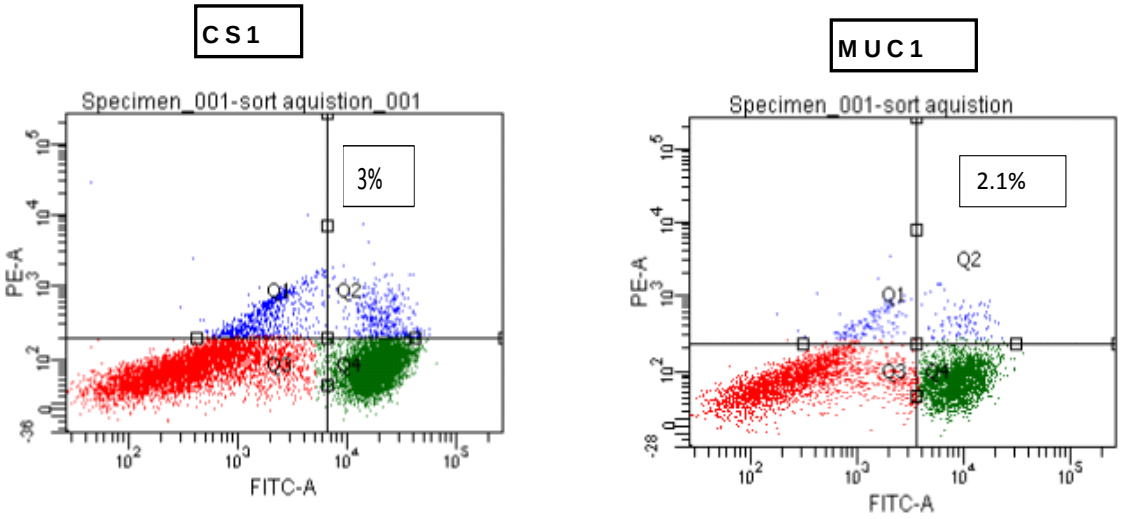


Figure 4.10: (A) Gating on the living cells using FSC X SSC dot plot. (B) The double positive populations were FACS sorted, to isolate *CS1 and MUC1 specific T cells from long-term cultures stimulated by HRC and HU266 respectively. The sorting was done using a plate sort in single cell mode, which has the most stringent settings for sorting. Therefore in theory purity was considered to be 100%.*

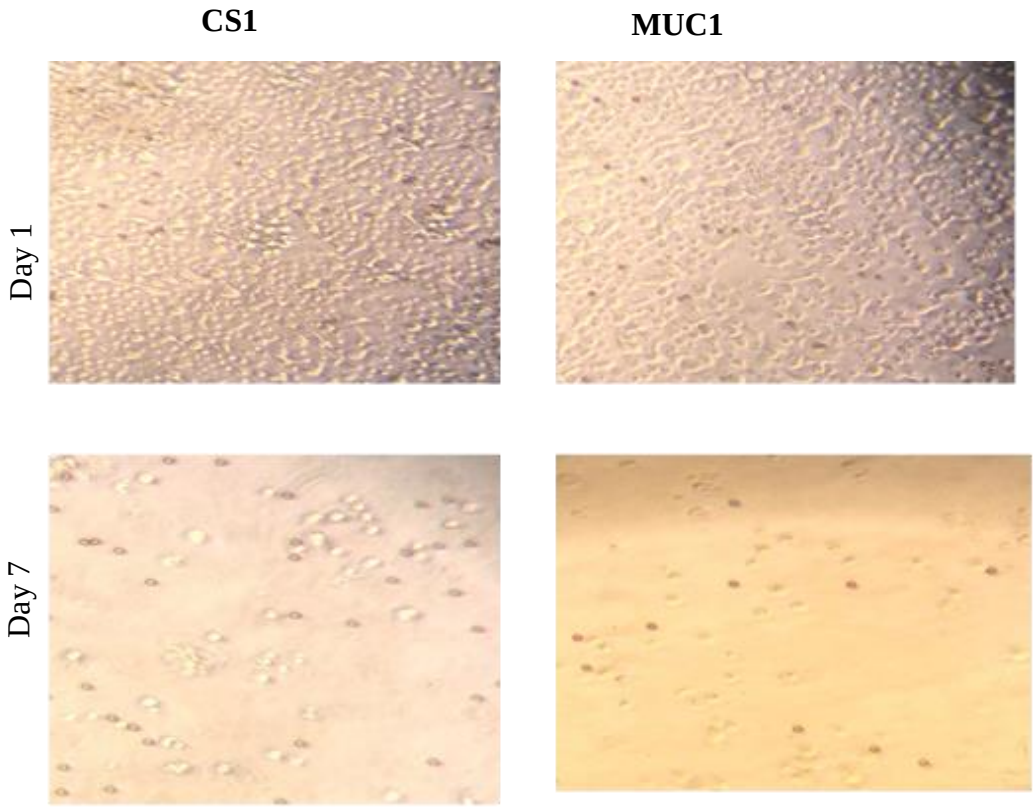


Figure 4.11: The long-term stimulated cultures after cell sorting.

The first row shows CS1 and MUC1 specific T lymphocytes with high cell density, one day after sorting. The second row shows that CS1 and MUC1died out seven days after sorting. The dark dots are the Dynabeads in the cultures.

Figure 4.18 shows that the highest proliferation rate was obtained by using the beads: cells ratio of 1:3 at weekly repeated stimulation, whereas using the ratio of 1:10 (which used for the previously sorted cells), leads to a drop in cell numbers after approximately three days, which might be explained by insufficient number of the beads used in the restimulation experiment. Restimulation after three days and ten days led to decrease of the cell number at all of the used ratios compared to restimlation after one week, under the revised culture conditions. I was unable to repeat the experiment with FACS-sorted antigen specific T cells due to time constraints.

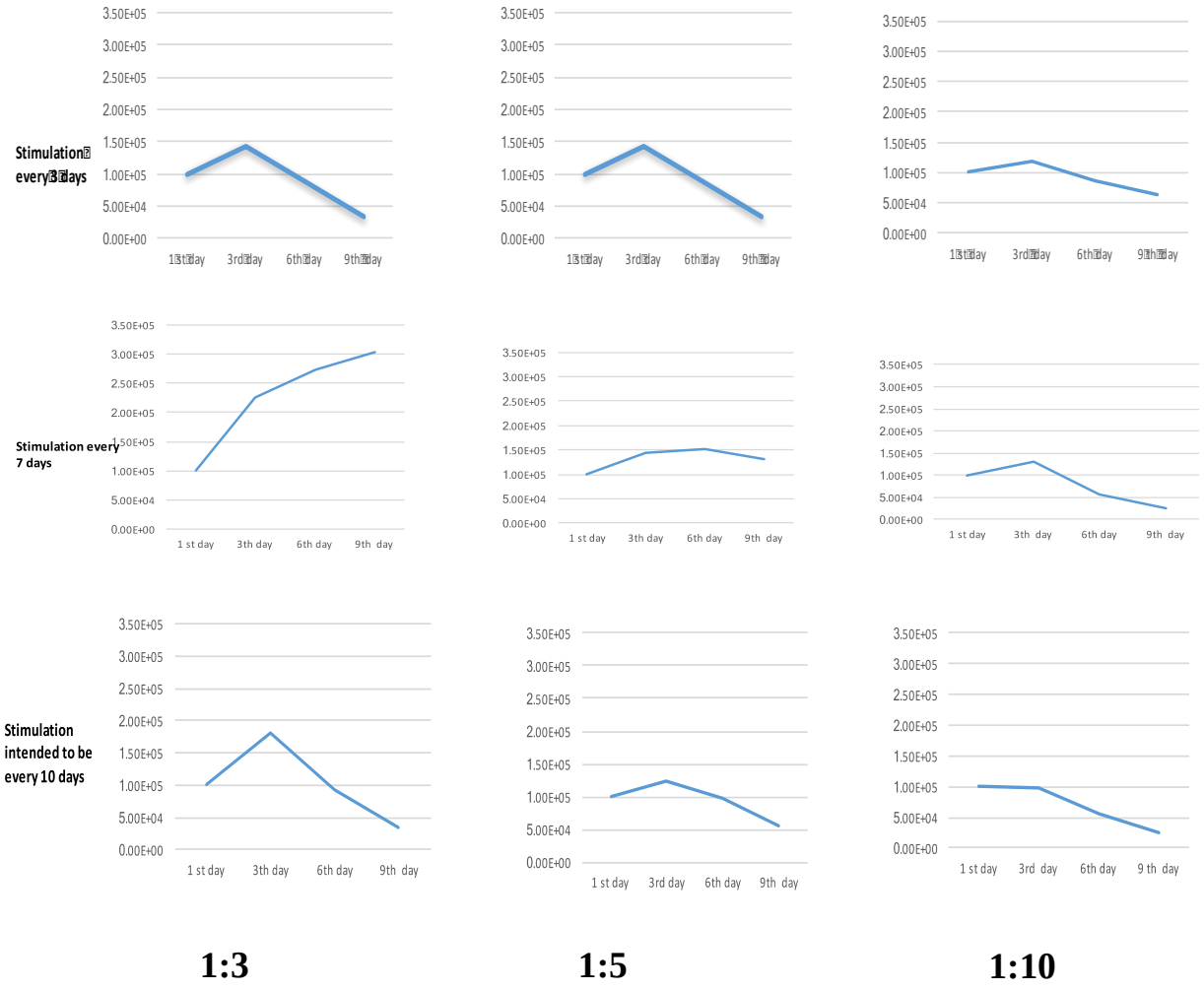


Figure 4.12: setting up restimulation experiment using the dynabeads, *for stimulation of the activated antigen specific T cells after sorting.*

4.3 Discussion

The heightened immunogenicity of the hybrid cells compared with their parent tumour cells is the principal reason of use the hybrid cells in various types cancer immunotherapy. In the previous chapter that dealt on with the phenotypic characterisation of the hybrid cell lines, the parent myeloma cell line U266 and HMY2, I showed that the hybrid cell lines and HMY2 expressed costimulatory molecules, such as CD80 and CD86, and antigen presenting molecules as HLA class I and HLA class II, in addition to expression of various TAAs; by contrast U266 lacked the expression of the costimulatory molecules, and HLA class II. This raises the question, does the difference of expression levels for costimulatory molecules between hybrids and parent tumour cell lines associated with increased immunogenicity of the hybrid cell lines compared with unfused tumour cells? Dunnion et al. (1999) showed that the hybrid cell lines that expressed the costimulatory molecules had markedly enhanced immuno-stimulatory ability compared with the parent tumour cells, whereas hybrids that lacked the expression of these costimulatory molecules had a diminished immunostimulatory capacity compared with the stimulatory hybrids. Basically, the enhanced immunostimulatory ability of the hybrids is attributed to the activation of naïve, effector and memory T cells. Stimulation of naïve T cells requires the presence of two essential signals. The first one is the recognition of the antigen specific peptide in the context of MHC by TCR, and the second signal is the additional stimulation mediated by the costimulatory signals such as CD80 and CD86 (Flynn and Müllbacher, 1996; Mohamed et al., 2012b).

Taking this further, Cywinski et al. (2006) reported enhanced lymphoproliferative T cell responses of allogeneic PBMCs induced by using HMy2 x haematological tumour hybrids (which expressed CD80 and CD86), and by HMy2 x solid tumours hybrids (which lacked CD80 and CD86 expression), compared with the parent tumour cells. The enhanced lymphoproliferative T cell responses were reduced by using CTLA-4 Ig blocking effects, even with the hybrids that lacked the costimulatory molecules expression. To explain these results, she removed the non-T cell fraction from the allogeneic PBMCs before stimulation by the HMy2 x solid tumours hybrids (which lacked the expression of CD80 and CD86) and as a result, she noticed reduction of the lymphoproliferative response to the background level, which was restored by re-addition of the isolated fraction. She concluded that the presence of bystander APCs in the removed non-T cell fraction acted as a “third party” in providing the co-stimulation, in a phenomenon named “bystander co-

stimulation" (Cywinski et al., 2006). In addition, Walewska et al. (2007) reinforced the role of the costimulatory molecules, HLA class I, class II in the induction of allogeneic and autologous T-cell responses *in vitro*, using the same hybrids as in my study, in a mixed lymphocytic reaction. She used monoclonal antibody (mAb) blocking experiments, and showed that the enhanced lymphoproliferative responses produced by the hybrid cells were dependent on their expression of CD80, CD86, HLA class I and HLA class II (Walewska et al., 2007). In a similar way, Mohamed et al. (2012a) demonstrated the crucial role CD80 and CD86, HLA class I and HLA class II in the induction of the lymphoproliferative responses in naïve T cells in MLR, using CTLA-4 Ig fusion protein (soluble ligand of CD80 and CD86), W6/32 (HLA class I mAb) and L243 (HLA class II mAb) in a group of haematological hybrid cell lines. Based on these results, the described hybrids were used for induction of antigen specific CTL in PBMCs isolated from healthy individuals, *in vitro* in long-term stimulated cultures. The induced T cells showed enhanced antigen specific activity and cytotoxicity (Mohamed et al., 2012b; Mohamed et al., 2016).

Induction of primary immune response of naïve cells is generally difficult due to its stringent activation and costimulatory molecule requirements. Most of the target antigens in cancer are self-antigens, therefore most of the potentially reactive high affinity T cells specific to these antigens have been removed during the thymic selection process, so the remaining naïve cells are rare and have lower avidity (Ho et al., 2006). Therefore, the major limitation of such therapy is the low frequency of precursor antigen-specific T cells repertoire in naïve individuals, which results in difficulties in priming and expansion of the antigen-specific T cells. Repeated antigen specific stimulations in long-term stimulation protocol are required to obtain the antigen specific T cell clones (Ho et al., 2006; Wolfl and Greenberg, 2014; Perica et al., 2015). In the long-term stimulation protocol, the priming efficiency of the naïve cells is affected by many factors, such as the type of a stimulant and method of stimulation and T cell preparation, and effector to target ratio at the time of priming (Wolfl and Greenberg, 2014). Ho et al. (2006) described the use of peptide-loaded monocyte-derived DC in expanding high-avidity HLA-A2 restricted WT1-reactive CTL clones, in nearly all the healthy HLA-A2 positive individuals involved in this study. These clones have the ability to recognize and destroy leukemic cells (Ho et al., 2006). Another study demonstrated the availability of using allogeneic WT1 specific CD8+ T cells in leukemia patients relapsed after HSCT. The

used clones were produced from an HLA-matched donor, and repeated stimulation with WT peptide pulsed autologous DC. The transferred lymphocytes were detectable in the patient's blood for a long time, with stable disease in 3 out of 11 patients, and transient response in 2/11 patients (Chapuis et al., 2013). Oelke et al. (2000) successfully induced HLA-A2-restricted Melan-A-specific CD8+ T cells, using Melan-A-pulsed DCs, and expanded these cells non-specifically up to 600 fold without losing their specificity using anti-CD3 and anti-CD28 antibodies. Additionally, adoptively transferred lymphocytes were applied in patients with ovarian cancer and melanoma (Muul et al., 1987; Rosenberg et al., 1988; Vignard et al., 2005; Chapuis et al., 2012; Perica et al., 2015) and multiple myeloma (Rapoport,et al., 2011).

In keeping with these features, to investigate the competence of the currently used hybrids as potential candidate immunotherapeutic agents, various immunostimulatory and functional characterization assays were carried out as part of this project.

4.3.1 Allogeneic lymphoproliferative T cell responses induced by the hybrids

Firstly, I assessed the allogeneic activation ability of the hybrid cell lines and their parents through their mismatched HLAs, which is considered a strong inducer of T lymphocyte proliferation and differentiation. Co-culturing of the cell lines with PBMCs from normal healthy individuals in CFSE staining experiments, and in IFNγ and perforin ELISpot assays, resulted in induction of T cell proliferative immune responses, when compared to unstimulated lymphocytes. CFSE is an intracellular dye, which divides equally between proliferating daughter cells. CFSE labelling experiments (Fig 4.2) were used to assess the proliferation levels of T cell subsets in short term stimulated cultures, which reflect the level of immunogenicity of the stimulator cell lines. The results showed that HU266 was the highest stimulator of CD8+ T cell proliferation, followed by HMY2. However, their parent myeloma cells U266, gave slightly lower values. This may be due to high expression level of HLA class I, HLA class II, CD80 and CD86 in HU266 and HMY2, which led to higher stimulation of CD8+ T cells. However, U266 the parent myeloma cell lines showed an almost comparable level of allogeneic activation of T cells to the other used hybrid cell lines (HRC, HCM and HIC) in spite of absence of the costimulatory molecules expression, which suggests that the responding T cells were derived from the memory T cell pool that could be activated in absence of CD80 and CD86, whereas naïve

T cells require the presence of these costimulatory molecules for activation and induction of T cell response (Flynn and Müllbacher, 1996). Another possible explanation is the presence of the bystander APCs in PBMCs, which provide the appropriate second signals (costimulatory molecules) required for T cell activation as previously mentioned (Cywinski et al., 2006). Also, U266 stimulated culture had almost the same percentage of proliferation of CD4+ T cells, in spite of the absence of HLA class II expression by U266. This may be explained by the presence of the bystander APCs in PBMCs, which express HLA class II (Cywinski et al., 2006). Also, Swain (2000) reported that CD4+ memory T-cell can survive in MHC class II deficient host for a short time. The CD8+ T cells were stimulated, in all cases, at higher levels than the CD4+ T cell population. This may be due to the higher level of HLA class I expression compared with the level of HLA class II expression by the used cell lines. In addition, Abbas et al. (2012) attributed the higher number of CD8+ T cells after activation than CD4+ T cells to their function, as CD4+ T cells act as cytokine producers, which can be performed by low number of the cells, while CTL require higher numbers of the cells to perform their cytotoxic function. Overall, these results agreed with Mohamed (2011), who detected the presence of activated T cells in short term stimulated cultures using similar hybrid cell lines, in CFSE staining experiments. Whether or not these allogeneic activated T cells are functional (cytotoxicity and/or cytokine release) could not be answered in this experiment.

The long-term activation of allogeneic PBMCs from healthy individuals with the hybrid cell lines and their parents for several successive rounds induced T cell cultures with augmented IFNγ releasing activity, which were detected by IFNγ releasing ELISpot assays (Fig 4.3). The results showed that the hybrid cell line, HU266, had significantly greater ability to stimulate IFNγ releasing responder cells than its parent, U266, which can be explained by the lack of the costimulatory molecules expression by U266, and the presence of the APC costimulatory effect in the hybrid culture. Also, APC are the main inducers of Th1 activity (through release of certain cytokines such as IL-12), which are the principal producers of IFNγ (Wakil et al., 1998). This suggestion is supported by the elevated IFNγ release in HMY2 (pure APC) stimulated culture. Also, HMY2, HU266 and HRC induced almost the same level of allogeneic IFNγ releasing responses as PHA, which indicate the strength of the allogenic responses induced by these cell lines. However, U266 and HIC showed the lowest level of IFNγ releasing response. This may be related to their level of costimulatory molecules expression, (HIC almost lacked CD86

expression, and had a very low expression level of CD80 and HLA class II; also U266 lacked the expression of the costimulatory molcules and HLA class II), but their IFNγ releasing responses were still significantly higher than the background PBMCs. The responding population in HIC and U266 stimulated cultures suggests the presence of memory CD8+ T cells and CD4+ T cells in U266 and HIC cultures, which can remain in the absence or low expression of the costimulatory molecules and MHC class II (Flynn and Müllbacher, 1996; Swain, 2000). In addition, all of the used hybrids induced significantly elevated IFNγ releasing responses than PBMCs. This result supports Mohamed et al. (2012b) who showed successful induction of allogeneic long-term activated IFNγ releasing T cell cultures using these hybrids. The importance of detection of IFNγ releasing response in T cell cultures has been demonstrated previously in different studies. Ghanekar et al. (2001) found a strong correlation between IFNγ expression and cytolytic activity of CD8+ T cells in the CMV system, and his findings demonstrate that IFNγ expression can be used as surrogate for recognition of CTL precursor cells. Also, IFNγ was defined as a tool for assessment of activation of T cells stimulated by hybrid cell lines of DC and tumour cells (Koido et al., 2007).

Additionally, perforin release by the induced allogeneic long-term activated T lymphocytes, as a marker of cytolytic activity, was detected using perforin releasing ELISpot assays (Fig 4.4). Several studies have shown the significance of perforin release as a tool for assessment of activation of CTL (Zuber et al., 2005a; Hersperger et al., 2008; Ranieri et al., 2014). For the long-term stimulated cultures, the data demonstrated that HU266 induced significantly elevated perforin release responses, around twice the response of its parents, HMY2 and U266. This may be because HU266 has higher expression of HLA class I, CD80 and CD86, the essential cell surface markers for CD8+ T cell stimulation, than the other cell lines. Also, for all of the used cell lines, there was a significant elevation in the perforin release compared with that of PBMCs background control. The most interesting result here is that U266 induced about the same level of perforin release as HMY2, HRC and HIC, which could be attributed to absence of HLA class II expression by U266, this results in their inability to stimulate CD4+ T cells, consequently this led to activation of a higher proportion of CD8+ T cells. Another plausable explanation for induction of perforin release by U266 is the moderate level of expression of HLA class I by U266, which was almost the same for HIC and HRC. However, HMY2 has higher MHC class I expression level, but it seems that HMY2, due

to its nature as APC, supports the antigen presentation using HLA class II, and growth of CD4+ T cells, as discussed in the IFN-γ releasing experiment, and supported by the results of T cell subsets tracking expermint, which will be discussed shortly.

The elevated IFNγ and perforin releasing responses to the examined stimulator cell lines suggests stimulation of responses to allogeneic major histocompatibility (MHC) molecules expressed by the stimulators. The possibility that some TAA specific responses were stimulated through presentation by MHC cannot be confirmed by these assays, so further experiments were required to examine the presence of induction of tumour antigen specific CTL in these long-term activated cultures.

4.3.2 Optimization of the long-term stimulation protocol for induction of antigen specific CD8+ T lymphocytes:

The allogeneic immunostimulatory ability of the hybrid cell lines and their parents, HMY2 and U266, to induce activated T cells, of PBMC isolated from healthy individuals in short and long-term activated cultures was demonstrated using CFSE and ELISpot assays respectively. The induction of HLA-A2 restricted tumour antigen specific CD8+ T lymphocytes in PBMC from allogeneic HLA-$A2^+$ normal healthy donors *in vitro* was assessed in the long-term stimulated cultures, using HLA-A2-peptide pentamers incorporating known epitopes of the cognate TAAs (MUC1, h TERT, CS1 and MAGE-C1). The data (Fig 4.5) showed an enhanced ability of the hybrid cell lines and HMY2 to expand antigen (MUC1, h TERT, CS1 and MAGE-C1) specific CTL *in vitro* in PBMCs from healthy donors, compared with the parent myeloma cell U266, which could be attributed to the expression of the costimulatory molecules by the hybrid cell lines.

Then, I wanted to demonstrate and optimize the process of antigen specific CTL generation and expansion *in vitro* using the hybrid cell lines, in the presence of different cytokines. Recently, numerous reports demonstrated the proficiency of IL-2, IL-7 and IL-15 in enhancement of activation and proliferation of induced antigen specific cytotoxic T lymphocytes in cell cultures, thus suggesting the use of these cytokines in the long-term stimulated cultures of my study. Based on the previous protocol used by Mohamed et al (2012), using IL-2 as the main growth factor of T lymphocytes, and IL-7 from the third stimulation round (Mohamed et al., 2012b), I therefore intended to investigate the effect of selected other cytokines on the activation level of the induced T lymphocytes, such as IL-7 used from the first week (to have its effect in naïve and memory T cells) (Schluns et

al., 2000; Surh and Sprent, 2008), and IL-15 based on its anti-apoptotic effect (Zhang et al., 1998; Lu et al., 2002).

Adding IL-7 in the first stimulation round of the long-term stimulated cultures enhanced IFNγ (Fig 4.6) and perforin (Fig 4.7) releasing antigen specific T cells, compared with adding it in the third stimulation round. This may be due to the crucial effect of IL-7 on the proliferation and survival of naïve and memory T cells, as reported by previous studies (Tan et al., 2001; Schluns et al., 2000; Seddon and Zamoyska, 2002; Surh and Sprent, 2002; Osborne et al., 2007). However, unlike naïve T cells, memory T cells require the presence of IL-15 in addition to IL-7 for survival (Boyman et al., 2009).

Using IL-15 in the long-term stimulated cultures demonstrated much clearer difference in perforin ELIspot (Fig 4.9) than in IFNγ ELISpot (Fig 4.8), suggesting that IL-15 has a greater effect on CD8+ T cells and/or T cell mediated cytotoxicity. This could be attributed to multiple effects of IL-15 on CD8+ T cells in the long-term stimulated cultures. Several studies have demonstrated that IL-15 induced the activation and proliferation of naïve and memory CD8+ T cells, and memory CD4+ T cells (Kanegane and Tosato, 1996; Berard et al., 2003). However, IL-15 failed to induce the proliferation of naïve CD4+ T cells (Kanegane and Tosato, 1996). Also, Lu et al. (2002) reported that the cytolytic function of effector CTLs can be retained for up to sixty days by using IL-15 in their expansion and maintenance. They explained that IL-15 has an effect on CD8+ T cells, but not on CD4+ T cells, due to the expression of IL-15 alpha chain on the surface of CD8+ T cells; by contrast CD4+ T cells lack the expression of this chain (Lu et al., 2002). Some studies demonstrated the enhancement of cytokine secretion and promotion of survival and proliferation of antigen specific CD8+ T cells in the presence of IL-15 (Schluns and Lefrançois, 2003; Brincks and Woodland, 2010). In addition, Mueller et al. (2005) reported enhanced effect of memory CD8+ T lymphocytes and NK cells in response to human immunodeficiency virus in the presence of IL-15. However, regarding my ELISpot assays, the use of K562 as stimulator indicated absence or very low activity of NK cells in the long-term stimulated cultures. The experiments investigating the effect of IL-7 and IL-15 were carried out only once, to identify the trend of the effect of these cytokines on the induced antigen specific T cells in long-term stimulated culture, and need to be repeated to confirm the findings. If I had more time I would repeat these experiments. However, based on these data, I used the combination of IL-2/IL-7/IL-15 in the subsequent experiments.

4.3.3 Immunostimulatory proliferative responses of T cell subsets in the long-term stimulated cultures

Tracking of T cell subtypes in long-term stimulated cultures (Fig 4.10) revealed that the hybrid cell lines HRC and HU266 induced the proliferation of CD8+ T cells at a much higher level than CD4+ T cells, which could be attributed to high level of expression of HLA class I and the costimulatory molecules (CD80 and CD86), in addition to the use of IL-7 and IL-15 in the long-term stimulated cultures, as these cytokines enhance the survival and proliferation of naïve and memory CD8+ T cell as previously discussed. IL-7 and IL-15 have a synergistic effect on the homeostatic proliferation of memory phenotype of CD8+ T cells, but not on memory CD4+ T cells (Tan et al., 2002). Also, administration of recombinant IL-7 *in vivo*, decreased CD4/CD8 ratio, and increased CD8+ T cells proliferation (Komschlies et al., 1994; Faltynek et al., 1992). In contrast to CD8+ T cell increase in number, CD4+ T cells tended to drop down quickly, after initial proliferation. However, CD4+ T cells proliferated in HU266 stimulated cultures for the first two weeks before waning, which may be due to the high level of expression of HLA class II by HU266. Another possible explanation of low numbers of CD4+ T cells in the long-term activated cultures, is the presence of IL-2, in high concentration, as Blattman et al. (2003) showed that the use of IL-2, during T cell expansion *in vivo* resulted in inhibition of CD4+ T cells expansion. In contrast, HMY2 stimulated cultures supported the proliferation of CD4+ T cells, which have the upper hand in this particular culture over $CD8^+$ T cells. This may be due to the nature of HMY2 as an APC, as it may support the antigen presentation using MHC class II. The T cell population stimulated by U266 showed unresponsiveness to further stimulation rounds, which may be attributed to suboptimal co-stimulation by U266, which lead to tolerance (Lenschow et al., 1996; Schwartz et al., 1989). In HIC stimulated cultures, all the T cell subsets' proliferation diminished dramatically after the first week, and died out by the fourth week, which could be attributed to very low level of CD86 expression, leading to low level of interaction of CD80/CD86 with CD28, as CD86 is of crucial importance in the initiation of TCR dependent T cell proliferation (Hathcock et al., 1994). Alternatively, this may be due to a low ability of HIC hybrid to process and present peptides in the context of its HLA class I and HLA class II, or due to the low level of expression of HLA class II, which led to diminished CD4+ T cell proliferation. Overall, all of the hybrids and HMY2 induced

immunostimulatory proliferation of CD3+ T cells and CD8+ T cells to a greater or lesser degree, while U266 stimulated cultures lacked this stimulation.

4.3.4 Induction of MUC1, h TERT, CS1 and MAGE-C1 specific CD8+ T in the long-term stimulated cultures:

The long-term stimulated culture in presence of IL-2/IL-7/IL-15, was assessed for induction of HLA-A2 restricted tumour antigen (MUC1, h TERT, CS1 and MAGE-C1) specific CD8+ T lymphocytes in PBMC from HLA-$A2^+$ healthy donors, using HLA-A2-peptide pentamers staining and IFNγ and perforin ELISpot assays.

The HLA-A2-peptide pentamers data (Fig 4.13), showed increased ability of the hybrid cell lines and HMY2 to induce antigen specific CTL *in vitro* in PBMCs from healthy donors, in most cases, compared with the parent myeloma cell U266. It was noteworthy that HRC mostly induced the highest antigen specific CTL percentage (in the cases of h TERT, CS1 and MAGEC1), compared with the other hybrids, HMY2 and U266. This can be attributed to the high level of expression of these TAAs at protein level by HRC. In addition, this reflects the ability of HRC to process and present peptides of the mentioned TAAs in the context of HLA-A2. HRC, also, expressed the costimulatory molecules, CD80 and CD86. Regarding MUC1, although HIC, HRC and U266 had higher protein expression level of MUC1 antigens compared with HU266, HU266 induced significantly higher MUC1 peptide-specific CD8+ T cell percentage compared with the other hybrid cell lines, HMY2 and U266. This may be explained by the proficiency of HU266 to process and present MUC1 peptide better than the other hybrids. Also, the parent cell lines HMY2 and U266 induced lower MUC1-specific CD8+ T cell populations than the hybrid cell line HU266, which can be rationalized by their incompetence to present the examined peptide of MUC1 in the context of HLA-A2, or the low level of expression of MUC1 at protein level by HMY2, and absence of the costimulatory molecules in case of U266. Overall, all of the hybrid cell lines induced significantly higher levels of the examined antigen specific CD8+ T cells, as measured by HLA-A2 peptide pentamers, compared with the myeloma parent cell line U266, except in case of MAGE-C1. A possible explanation of this will be discussed in the next chapter. In addition, all of the used hybrid cell lines induced significantly elevated responses, compared with unstimulated PBMCs. However, the parent cell lines, HMY2 and U266, failed to induce such significant responses, except with HMY2 in the case of h TERT, which may be

explained by high level of protein expression of h TERT by HMY2. Furthermore, Mohammad et al. (2012b) successfully used these hybrids previously to induce MAGE-A1, PRAME, Survivin and WT-1 specific CTL (Mohamed et al., 2012b).

There was a clear correlation between the TAA protein expression level by the used cell lines and their ability to stimulate the relevant CTL, as detected by HLA-A2 peptide pentamers. There were 20 cases where the used cell lines expressed the related TAAs at protein level, and induced the pentamer positive $CD8^{+}$CTL populations, whilst the level of the candidate TAA at protein expression level had a direct relationship with the level of induction of antigen specific CD8+ T cells in 13 out of the 20 occasions (65%). The remaining (35%) had no such direct relationship, which may be result from the other factors that can affect the level of activation of T cells, such as the levels of protein processing by the used cell lines, and the level of presentation of the peptides in the context of their MHCs molecules. Additionally, the mRNA expression of the examined TAA was positive only for 17 occasions out of 20, whereas the remaining occasions (15%), the mRNA expression was absent or very weak. In spite of this, the protein expression and the pentamer staining assay were positive. Hence, this reflects the significance of determination of protein expression level more than that of mRNA expression level (Greenbaum et al., 2003; Pascal et al., 2008).

However, the main limitation of HLA peptide pentamer assay is the absence of any indication of activity of the present antigen specific T lymphocytes. So, IFNγ and perforin ELISpot assays were carried out to reflect the presence of functional activated antigen specific T cells in the cultures.

4.3.5 Antigen specific IFNγ and perforin release in the long-term stimulated cultures:

The results of IFNγ (Fig 4.14) and perforin ELISpot assays (Fig 4.15) reflect the presence of functional activated antigen specific T cells in the long-term stimulated cultures, and go in agreement with the pentamer staining data. For MUC1, HU266 was the highest cell line in terms of induction of MUC1 specific IFNγ and perforin release, as well as in MUC1 specific pentamer staining, in spite of its low MUC1 protein expression level as previously disscussed. On the other hand, HRC had the highest h TERT, CS1 and MAGE-C1 specifc IFNγ and perforin release, which reflects its high protein expression level compared with the other cell lines of the study. HMY2 showed increased MAGE-C1 specific perforin release only at 1:100 (target: effector) ratio, which quicky diluted and

disappeared at the other used ratios, thus reflecting low functional ability of HMY2 stimulated T cells to induce MAGE-C1 specific perforin release at the lower used ratios. In terms of U266 stimulated cultures, in most cases, they lacked the induction of activated antigen specific T cells above the background level, in terms of IFNγ and perforin specific responses, in spite of production of comparable levels of antigen specific T cells in the pentamer staining assays, which may be explained by presense of the cells in non functional (anergic) state due to the absense of the costimulation by U266 (Schwartz, 2003; Bachmann and Oxenius, 2007). However, U266 induced a low level of MUC1 specific perforin release. This release could come from MHC unrestricted response to MUC1 (Takahashi and Imai, 1994; Wright et al., 2008). However, for unknown reasons, there is some MAGE-C1 specific IFNγ release in U266 stimulated culture, which may be due to the presence of low level of MAGE-C1 specific T cells in normal individuals (Nuber et al., 2010), as they detected MAGE- C1 specific T cells in 1 out of 10 normal individuals (Nuber et al., 2010).

In summary, the level of IFNγ and perforin releasing responses was proportional to the responder/ stimulator cell ratios, and in all cases the responses achieved with the hybrid cell lines HU266 and HRC were significantly higher than the responses obtained with the parent stimulation by HMY2 and U266. Also, I decided not to investigate the immunostimulatory properties of HIC any further, because it has the lowest immunostimulatory effect amongst the used hybrids. This was slightly higher than U266, but similar to HMY2 effect. The hybrid must have higher immunostimulatory effect than the parent cell line, HMY2, to have any advantages over the use of the parent.

4.3.6 FACS sorting of the induced antigen specific T cells:

Finally, I attempted to isolate the produced antigen specific T lymphocytes using HLA-A2 peptide pentamer staining and FACS sorting, and to expand the sorted T cells using Dynabeads human T activator (containing anti CD3, CD28 and CD137). Unfortunately, the FACS sorted cells stopped growing in the cultures after a few days. The possibility that pentamer binding to TCR on the sorted cells led directly or indirectly to cell death can not be excluded. Therefore, I undertook an experiment to optimize the culture conditions to expand activated T lymphocytes (not FACS sorted cells) (Fig 4.18). The result demonstrated that excessive stimulation of the isolated culture with Dynabeads (every three days) did not work, possibly due to activation induced cell death in the culture, and stimulation every 10 days was not enough to keep the cells alive in the culture.

The best stimultion condition was obtained with stimulation every week, using 1:3 ratio of beads: cells. However, because of the time constraint, I could not repeat the experiment using the optimized conditions.

According to these data, we can see that all of the examined hybrid cell lines have the capability to induce the production of antigen specific CD8+ T cells to all the examined TAAs, to variable extents, in the long-term stimulated cultures of PBMCs from normal healthy individuals. So did (to a lesser extent) HMY2, while U266 was constantly a poor stimulator in the long-term stimulated cultures. In addition, it is clear that there was a strong correlation between the level of protein expression of the TAA by hybrid cell lines, and their ability to stimulate relevant CD8+ T cell populations, as shown by HLA-A2 pentamer staining and ELISpot assays.

Chapter 5 Induction of antigen specific CTL in PBMCs isolated from patients with multiple myeloma

5.1 Introduction

Multiple myeloma (MM) represents a malignant tumour of plasma cells, that includes many complications, such as monoclonal paraprotein produced in excess by abnormal proliferation of plasma cells, haematological disorders (bone marrow failure, anaemia), renal dysfunction, and bone erosion (Bladé and Rosiñol, 2007). Additionally, the major cause of morbidity and mortality caused by MM is the immune dysfunction, which is a crucial feature of this disease (Pratt et al., 2007). Various types of immunodeficiency have been described in MM. The most pronounced reason of immunoparesis in MM is the depression in primary antibody responses (Pilarski et al., 1986). On the other hand, multiple myeloma associated immunodeficiency can result from the alteration of both quantity and functional ability of the T lymphocytes in the tumour microenvironment of MM. So, there has been a considerable interest in the principal role of T lymphocytes in MM. Dosani et al. (2015) reported a decrease in $CD4^+$ $CD8^+$ T cell ratio, which was caused by decrease of the absolute and relative number of $CD4^+$ T cells and decrease of the relative number of $CD8^+$ T lymphocytes. The decrease in this ratio is mostly associated with the progression of the disease and poor prognosis (Dosani et al., 2015). The majority of T cell expansion during MM is represented in $CD8^+$ T cells. However, the expansion of $CD8^+$ T cells is more frequent in the precursor disease patients (MGUS) than in MM patients, in both peripheral blood and bone marrow (Halapi et al., 1997). Reduction in $CD8^+$ T cell numbers in MM, compared with MGUS, could be explained by increase of the cytotoxic T cell dysfunction and decrease in their proliferation with the progression of MM (Halapi et al., 1997). T cell tolerance and unresponsiveness against autologous tumor cells have been reported in a variety of tumors and especially with MM. Despite the presence of tumor specific memory T cells in blood or in lymphoid organs, their capacity to react with the tumor cells is very low (Horna and Sotomayor, 2007). There are various reasons described for the T lymphocytes' unresponsiveness *in vivo*. Firstly, the MM microenvironment *in vivo* inhibits DC maturation, and also causes DC dysfunction (Pratt et al., 2007). This DC dysfunction could lead to T cell inactivation due to absence of the costimulatory molecules. Neither DC depletion nor its dysfunction has been seen in the MGUS patients, which reflects the existence of T cell mediated cytotoxicity in those patients (Pratt et al., 2007). However, Ratta et al. (2002) described that the DC unresponsiveness of MM patients can be circumvented through using *in vitro* culture conditions (Ratta et al., 2002). Secondly, it was reported that some adhesion

molecules, such as several carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) expressed on the cell surface of freshly isolated myeloma cells, upon the interaction with the T cells, can result in inhibition of T cell activation and cause their unresponsiveness (Witzens-Harig et al., 2013). Thirdly, the observed immunoparesis in MM could be attributed to boosted cellular regulatory elements, such as regulatory T cells (Treg), myeloid-derived suppressor cells (MDSC), or a T-helper cell imbalance (Murakami et al., 2004). The fourth described reason for the existence of nonfunctional T cells is the interaction of PD-1 on the surface of T cells with PDL1 on the surface of MM cells (Fichtner et al., 2015). These are some respectable explanations of the poor ability of $CD8^+$ T cell to react against tumour cells *in vivo*. These are the reasons why successful immunotherapy, using the conventional $CD8^+$ T cell subset for treatment of MM, has to overcome T cell and DC dysfunctions.

In spite of their reaction with the tumour *in vivo* being very low, the lymphocytes, specifically $CD8^+$ T cells, represent the key effectors of anti-tumour immunity, hence, they have been used widely in tumour immunotherapy. Kolb et al. (1990) reported compelling graft versus leukemia effect after allogenic stem cell transplantation, due to the action of effector lymphocytes. So, donor lymphocyte infusion (DLI) can be used, in the case of relapse after allogenic stem cell transplantation. However, a major complication of DLI is the graft-versus-host disease (GVHD) (Kolb et al., 1990).

Additionally, an allogeneic T cell immunotherapy based approach was done by Drobyski et al. (1992). They tried to treat a chronic myelogenous leukemia patient with donor leukocyte infusion, after two successive failed HLA matched bone marrow transplantation. The patient had an antileukemic response, which occurred initially in the absence of GVHD, but complete disappearance of the leukemic clone did not happen until after the onset of GVHD (Drobyski et al., 1992). Also, Biorkstrand et al. (1996) reported low survival rate of MM patients after allogenic bone marrow transplantation, which could be attributed to GVHD (Bjorkstrand et al., 1996). However, this drawback could be circumvented by using identified T cells that target specific tumour antigens (Kolb et al., 1990).

Numerous advantages have been reported for using autologous cancer antigen induced specific T lymphocytes for adoptive tumour immunotherapy. Firstly, using T lymphocytes collected from the patient minimizes the graft versus host disease across MHC barriers (Kolb et al., 1990; Bjorkstrand et al., 1996). Secondly, the isolated T

lymphocytes can be stimulated *in vitro* using a number of identified antigen-derived HLA-restricted epitopes, to accelerate production of antigen specific cytotoxic T lymphocytes (Wang et al., 2014). Numerous reports described successful treatment of the solid and haematological malignancies using autologous and allogeneic T cell adoptive immunotherapy (Aqui and June, 2008; Nelson and Paulos, 2015; Ghosh et al., 2014). Furthermore, adoptive immunotherapy, using patient derived myeloma cell / DC fusion vaccine, was feasible in most of the patients and resulted in a potent antitumour immune effect, which led to disease stabilization (Rosenblatt et al., 2011).

The main limitation of using adoptive autologous T lymphocytes has been the difficulties of the development of culture systems for efficient expansion of the functional T cells, to obtain a large number of antigen specific T cells for intravenous infusion in those patients. Developing an *in vitro* culture depends on the fact that both antigen stimulation and secondary stimulus are necessary for optimal T cell activation, and adequate expansion of the effector T lymphocytes *in vitro* (Turin et al., 2007). Using proficient antigen presenting cells as a partner in the fusion with the tumour cells provides the required costimulatory molecules, such as CD80 and CD86. These secondary stimuli are a principal requirement for activation of T lymphocytes and prevent their anergy, through their interaction with CD28 expressed on the surface of T cells. Three evidences ensure the role of the costimulatory molecules-CD28 interaction in T cell stimulation. The first one is that inhibition of these costimuli by CTLA-4-Ig *in vivo* leads to massive reduction of T cell activation (Grossi et al., 1992). In addition to that, it was shown that activation of TCR signalling in the absence of the costimulatory molecules leads to activation induced cell death (Groux et al., 1993). The third evidence, described by Boise et al. (1995), who demonstrated that intrinsic signals that protect the activated lymphocytes are produced upon the interaction of the accessory molecules and CD28. They found that CD28–B7 interaction leads to accumulation of Bcl-xL intracellulary, which protects from extrinsic and intrinsic death signals (Boise et al., 1995). Furthermore, overexpression of Bcl-xL inhibits T cell apoptosis caused by anti- CD3, FAS, or cytokine withdrawal (Watts, 2010).

Using different cytokines that have a critical role in the proliferation of T lymphocytes in *in vitro* cultures, such as IL-2, IL-7 and IL-15, maintains the proliferation and activities of these cells in the cultures and prevents their unresponsiveness (Vella et al., 1998; Montes et al., 2005). All these cytokines belong to the same family, as their receptors

share the common gamma chain of IL-2R. IL-2 is known as the lymphocyte growth factor as it maintains the proliferation of T lymphocytes. Also, IL-7 maintains the cell survival *in vivo* and *in vitro* (Vella et al., 1998). In addition to that, IL-15 has a considerable role in activation and proliferation of T lymphocytes to produce long-term memory T cells (Lugli et al., 2010). So, the expansion protocol used so far was further optimized to obtain a large number of the antigen specific T cells, and this is described in chapter 4.

In chapter 4, I have shown the ability of the hybrid cell lines to induce antigen specific cytotoxic T lymphocytes using long-term stimulated cultures (4-6 weeks) of PBMCs isolated from healthy (HLA-A2+) individuals. Moreover, I have examined the activated cultures by pentamer staining, IFNγ release and perforin release ELISpot assays, to detect the presence of activated antigen specific CTL in the produced stimulated cultures.

Aims and objectives of this chapter

1- In this chapter, I addressed the ability of the hybrid cell lines to elicit antigen specific cytotoxic T lymphocytes in PBMCs taken from patients with MM, to investigate the efficiency of the hybrid cell lines to induce multiple myeloma antigen specific cytotoxic T lymphocytes using long-term stimulated culture of the patients' PBMCs, with potential to use such antigen specific T cells as an adoptive tumour immunotherapy of multiple myeloma after production to GMP standards.

2- I assessed the activated cultures with the HLA-A2-peptide pentamer staining, IFNγ release and perforin release ELISpot assays, to detect the presence of activated antigen specific CTL in the produced stimulated cultures.

3- T-cell responses were confirmed by CTL-mediated cytotoxicity assays, to ensure their cytotoxic activity and absence of tolerance and unresponseveness in the long-term activated cultures.

4- Lastly, I wanted to ensure the adequate immunogenicity of the hybrids to induce antigen/tumour specific cytotoxic response of CTL in PBMCs of MM patients, given previous reports of antigen-specific T cell dysfunction in patients with MM (Murakami et al., 2004; Witzens-Harig et al., 2013).

5.2 Results:

In this chapter, two selected myeloma hybrid cell lines (HU266 and HRC), were used as stimulators in long-term activated T cell cultures, by co-culturing *in vitro* with PBMCs isolated from MM bearing patient, in the presence of IL-2, IL-7 and IL-15. The long-term stimulated culture was carried out to activate antigen specific cytotoxic T cells, with the purpose of potential use of the produced antigen specific CTL cell line after production at GMP standard as an autologous adoptive immunotherapy of MM (not for my cultures as we do not have GMP facilities). The selected hybrid cell lines were chosen based on their efficiency in the induction of antigen specific CTL in PBMCs isolated from HLA-$A2^+$ healthy donors, as described in the previous chapter, and their level of expression of the examined antigens. Nine different MM patient samples were collected, to estimate the effect of the used hybrids on the activation of T cells isolated from different MM patients. Details of those patients are shown in Table 2.1.2 (five of these patients were HLA-$A2^+$, while four patients were HLA-$A2^-$).

5.2.1 Estimation of allogeneic activation of MM patients' T cell subsets, using CFSE staining:

Various reports demonstrated the unresponsiveness of T cells isolated from MM patients (Pratt et al., 2007; Witzens-Harig et al., 2013). However, other reports illustrated the ability of T cells isolated from tumours of myeloma patients to be activated *in vitro* using DC, to enhance the cytotoxic response to the autologous tumour (Dhodapkar et al., 2002; Ratta et al., 2002). So, prior to trying to induce antigen specific CTL in MM patients' PBMCs, I wanted to examine the *in vitro* allogeneic activation of patients' T lymphocytes, after recognition of mismatched HLAs of the hybrid cell lines, to ensure the ability of the patients' T cells to respond to the *in vitro* allogeneic activation, and the absence of their unresponsiveness. The second aim of this experiment was to confirm the ability of the hybrid cell lines as immunogens, which have the ability to activate the patients' T cells. The allogeneic stimulation was done using the selected hybrid cell lines, HU266 and HRC, in addition to their parents, HMY2 and the parent myeloma cell line U266. The experiment was set up using the same method as in section 4.2.1.1. After isolation of PBMCs from MM patients, the cells were stained with CFSE and stimulated for five days with HMY2, HU266, HRC and U266. Afterwards, the cells were stained with relevant monoclonal antibodies for CD3, CD8, and CD4, and subjected to flow cytometric

analysis, to indicate T cell subset proliferation after short term stimulation. Unstimulated PBMCs were stained in parallel with the stimulated ones, and used as negative controls. During the flow cytometric analysis, the same gating strategy was used as described in section 4.2.1.1.1. Figure 5.1 shows that the hybrid cell lines and the parent APC (HMY2) had the capability to induce proliferation of $CD3^+$ T cells, $CD8^+$ T cells and $CD4^+$ T cells. However, the parent myeloma cell line U266 induced very little proliferation of these different subsets, which could be attributed to the absence of expression of the costimulatory molecules by U266 cell line. HMY2 showed the highest allogeneic stimulation of $CD8^+$ T cell proliferation, whereas HU266 demonstrated the most enhanced proliferation of CD4+ T lymphocytes and CD3+ T lymphocytes.

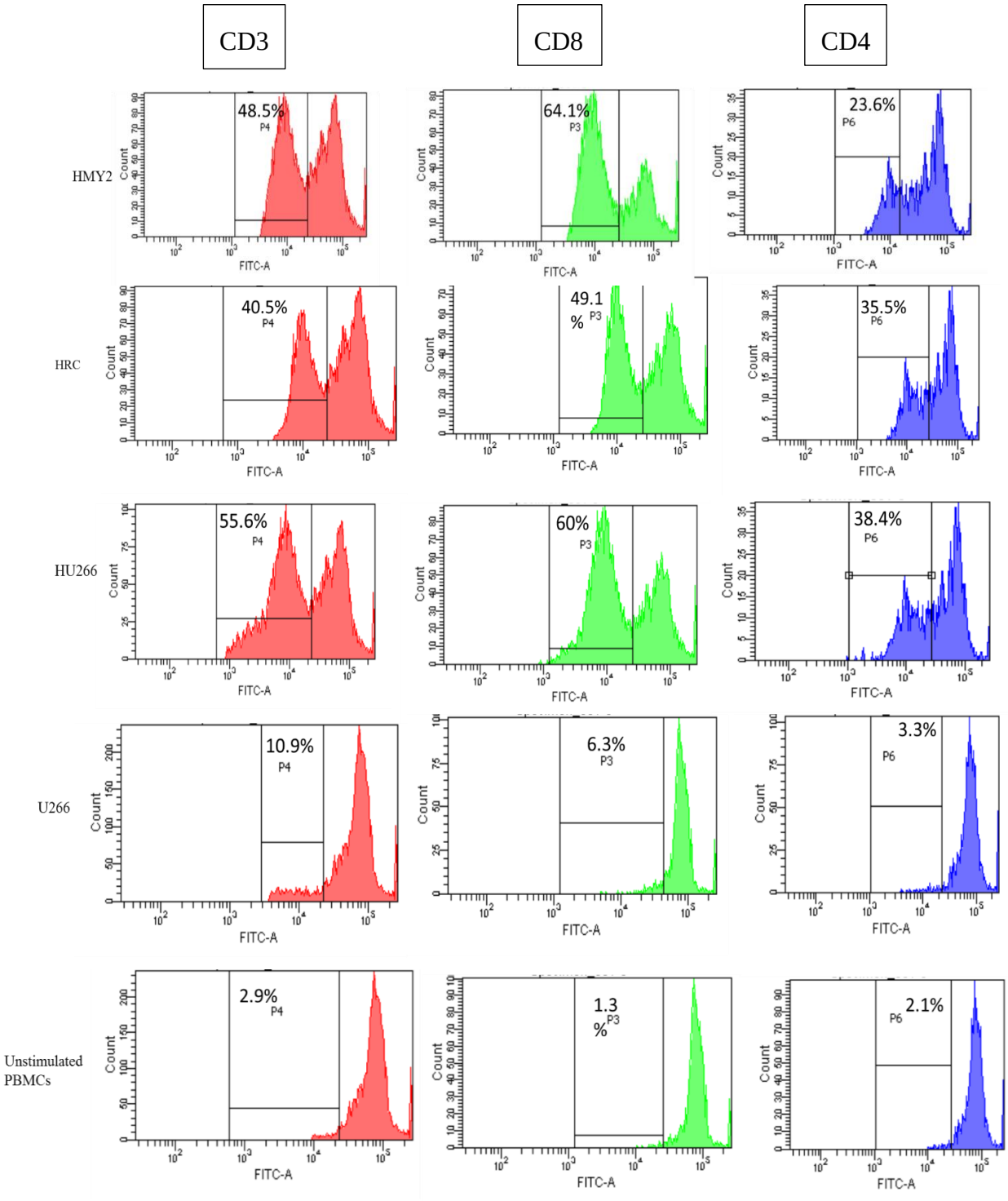


Figure 5.1: The percentage of proliferating T cells, isolated from MM patient. *After CFSE staining and allogeneic stimulation with HMY2, HRC, HU266, and U266, for five days. The unstimulated PBMCs were used as a negative control. The experiment was done only once, due to limited number of patient samples.*

5.2.2 Induction of allogeneic long-term activated antigen-specific CTL in PBMCs of MM patients

Multiple myeloma patients' PBMCs were stimulated in long-term, weekly activated cultures (4-7 weeks), using the hybrid cell lines HU266 and HRC, and their parent cells HMY2 and U266. The produced long-term stimulated cultures were investigated for the presence and percentage of certain antigen specific cytotoxic T lymphocytes. The candidate tumour associated antigens (h TERT, MUC1, MAGE-C1 and CS1) were used to examine the induction of the antigen specific CTL in the long-term stimulated cultures. These antigens were chosen because of their high prevalence of expression in MM patients, and also high level of expression of their mRNA and protein by the used cell lines, as shown in chapter 3. The specificities of the induced CTL were investigated using IF staining by HLA-$A2^+$ restricted peptide pentamers, specific for the previously mentioned antigens, followed by flow cytometric analysis. Furthermore, the specificity and activity of the produced antigen specific CTL was demonstrated using IFNγ and perforin ELISpot assays, and the cytotoxic function was measured using DELFIA® EuTDA cytotoxicity assays.

5.2.2.1 HLA-A2-peptide pentamer analysis

The produced long-term stimulated cultures were examined, after four weeks of continuous weekly stimulation, for the presence and percentage of the induced antigen specific CTL using FITC-labelled anti-CD8 and PE-labelled HLA-A2-peptide pentamers specific for the previously mentioned antigens. Following staining, the cells were analysed by flow cytometry. The same sequential gating strategy was applied as described in section 4.2.2.5. Figure 5.2 shows representative examples of the examined HLA-$A2^+$ antigen specific CTL in different cultures, and Figure 5.3 shows the percentage of the examined HLA-$A2^+$ antigen specific CTLs, in the total CD8+ T cells in each culture.

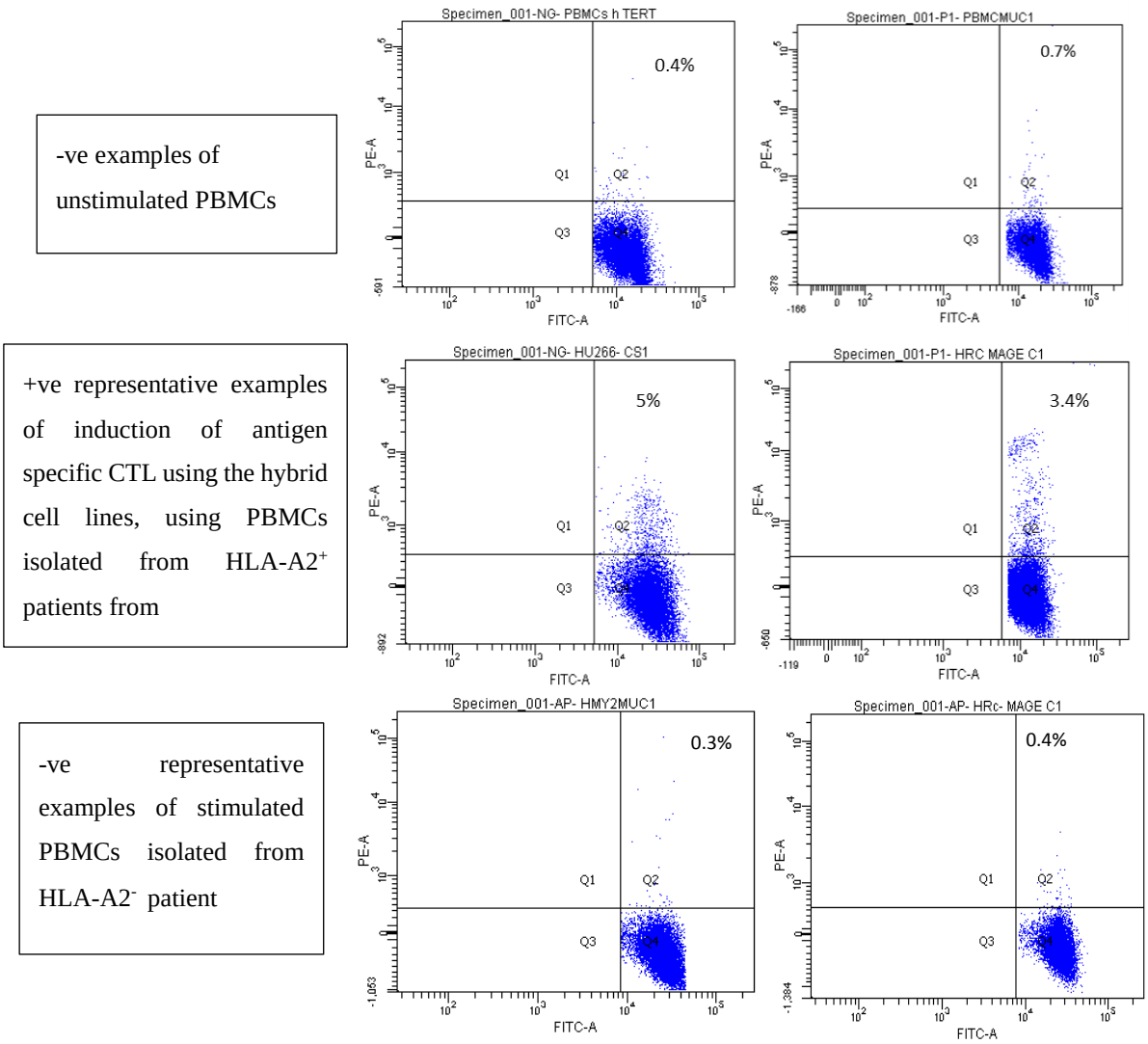
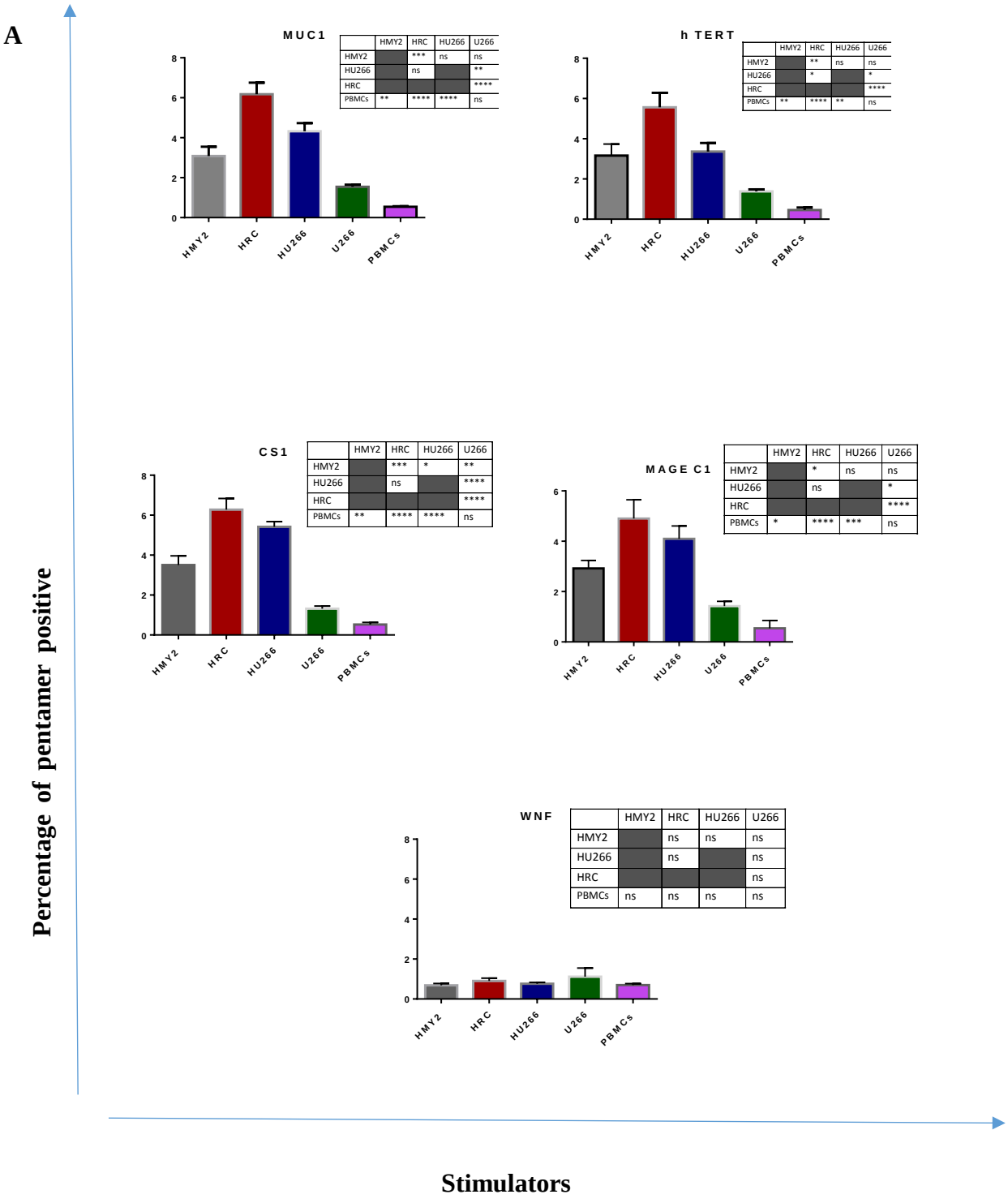


Figure 5.2: Representative examples of the HLA-A2 restricted peptide pentamer staining of different antigen specific CD8+ T cell populations, in different cultures. *The upper row represents two negative control plots of the unstimulated PBMCs of different patients, and the second row represents HU266 and HRC stimulated HLA-A2 restricted CS1 and MAGE-C1 specific CD8+ T cell population respectively. The lower row represents negative examples of HLA-A2 restricted antigen specific stimulation in PBMCs isolated from HLA-A2- patients.*

Three sets of negative controls were used in this experiment, to ensure the antigen specificity and HLA-A2 restriction of responses. The first one was using PBMCs from HLA-A2$^-$ patients, as a negative control for the HLA-A2$^+$ restricted responses. The second negative control was an irrelevant peptide antigen, West Nile Fever virus peptide, to ensure the antigen-specific T cell response against each tumour specific peptide. The third negative control was the staining of the unstimulated PBMCs (the same PBMCs used in

stimulation experiment) in parallel with the same peptide pentamers as the stimulated T lymphocytes. These PBMCs were used as a background, to indicate the induction of the antigen specific T lymphocyte in the stimulated cultures.



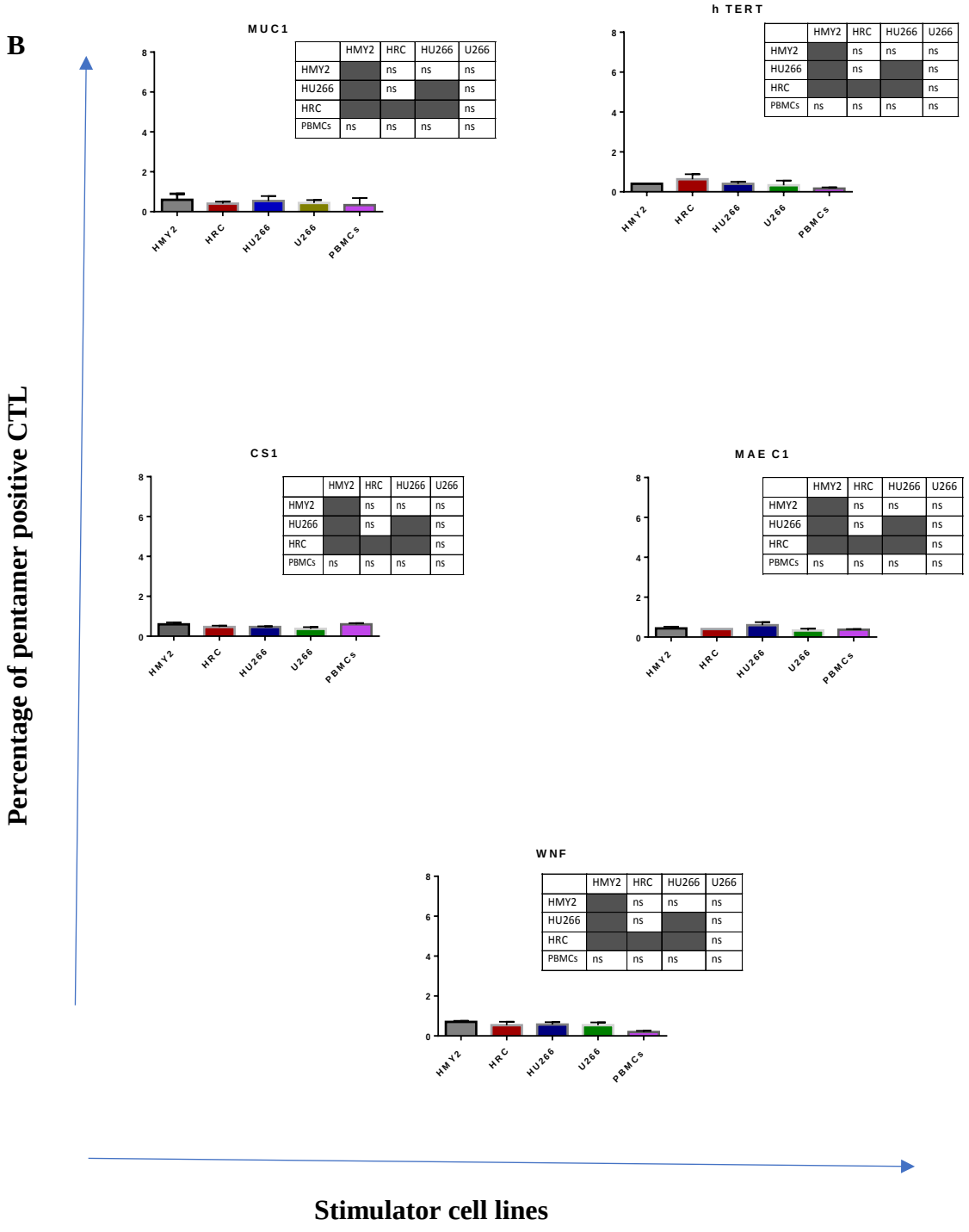


Figure 5.3: Antigen specific CTL percentages of HLA-A2 peptide pentamer analysis, using MUC1, h TERT, CS1, MAGE-C1 and WNF peptide pentamers, *After four rounds of stimulation of PBMCs isolated from patients with MM using the hybrid cell lines HRC and HU266, or the parent cell lines HMY2 and U266 as stimulators, the cultures were stained with HLA-A2 peptide pentamers incorperating cognate peptide antigens, followed by flowcytometric analysis. A- Represents the results of five HLA-A 2 positive patients, and figure B- shows the data of three HLA-A2 negative patients. Results are presented as mean ± SEM. The levels of significances have been summarized in the table next to each chart and presented as asterisks (* <0.05, **≤ 0.01, *** ≤ 0.001and **** ≤ 0.0001).*

Figure 5.3 (A) shows that HMY2 and the two hybrid cell lines HRC and HU266, but not the parent myeloma cell line, U266, induced HLA-A2 restricted MUC1 antigen specific CD8+ T cell populations to significantly higher level than that of the unstimulated PBMCs. Additionally, both hybrid cell lines (HU266 and HRC) caused a significant induction of MUC1 specific response compared with U266. Also, HRC hybrid cell line produced a significantly higher antigen specific response to MUC1 compared with HMY2, but no significant difference was seen between the level of induction of HLA-A2 restricted MUC1 antigen specific CD8+ T cell populations by HMY2 and U266. Also, the difference between the both hybrid cell lines was not significant.

The response against h TERT was similar to that of MUC1. The highest HLA-A2 restricted h TERT antigen specific CD8+ T cell populations were produced by the hybrid cell line HRC. HU266 elicited antigen specific T cells more strongly than the unstimulated PBMCs, and its parent cell line U266, whilst HMY2 induced a greater response to h TERT than was seen in unstimulated PBMCs, but not compared with U266. With regard to U266, there was no significant difference between the presence of h TERT specific T cell in their culture and that of the unstimulated PBMCs.

Concerning CS1, the level of induction of HLA-A2 restricted CS1 antigen specific CD8+ T cell populations was highly significant with HU266 and HRC, compared with the parent myeloma cell U266 and the unstimulated PBMCs. In addition, HRC and HU266 induced higher percentages of pentamer positive CD8+ T cells than HMY2. All of the used cell lines showed significant induction of CS1 specific CD8+ T cells compared with the unstimulated PBMCs, except for U266.

With respect to MAGE-C1, we can see a similar pattern of induction of MAGE-C1 specific CD8+ T cell populations, with HRC showing the strongest induction, followed by HU266, and then HMY2. There was no significant difference between HMY2 and HU266, but HU266 had a significant elevation compared with its parent U266, which was not seen with HMY2. U266 stimulation failed to induce a significant increase in MAGE-C1 specific CD8+ T cells compared with unstimulated PBMCs.

As regards the negative control, West Nile fever virus peptide pentamer, there was no significant difference in the presence of WNF antigen specific $CD8^+$ T cell populations between any of the stimulated cultures and the unstimulated PBMCs, confirming the specificity of the produced $CD8^+$ T cell population in the other cultures.

The examination of the presence of HLA-A2 restricted antigen specific CD8+ T cell populations in long-term induced cultures of PBMCs isolated from HLA-A2 negative patients (Figure 5.3 B), showed that there was no significant elevation of HLA-A2 restricted antigen specific CD8+ T cell percentages compared with that of the background of the ustimulated PBMCs, or compared with that of the irrelevant antigen (WNF), indicating that the elevation levels of antigen specific CD8+ T cells in PBMCs of HLA-$A2^+$ patients was restricted to the presence of the HLA-A2 molecule.

Overall, the examined hybrid cell lines have the capability to induce increased numbers of antigen specific CD8+ T cells to all of the examined TAAs. HMY2 also induced antigen specific $CD8^+$ T cell, but to a lesser extent, whilst U266 failed to induce antigen specific CD8+ T cell populations to any of the candidate antigens.

5.2.2.2 Detection of induced antigen specific CTL using IFNγ ELISPOT assays

Following the detection of antigen specific $CD8^+$ T lymphocytes in the long-term stimulated MM patients' PBMCs using Pro5 MHC Class I Pentamers, I wanted to estimate the activity of the induced antigen specific CTL in the stimulated cultures, using the IFNγ enzyme-linked immunospot assay (ELISPOT). This assay has the ability to assess both frequency and functional ability of the induced antigen specific CTL, so this assay can effectively be used to monitor the T cell response, as discussed in chapter 4. In this experiment, I examined the IFNγ releasing response of long-term stimulated CTL, co-cultured with HMY2, HU266, HRC and U266, to T2 cells pulsed with the same candidate antigens as delineated (MUC1, h TERT, CS1 and MAGE-C1). Moreover, the experiment was set up using an irrelevant peptide loaded T2 cell (WNF peptide), as a negative control. Additional negative controls that were used were K562 (to examine NK cells activation), and stimulated T cells without further stimulation during the ELISpot assay, to assess the background response. In addition to that, the same assay was done using PBMCs isolated from HLA-$A2^-$ MM patients, to assess the restriction of CTL response to specific antigens presented in the context of HLA-A2.

The results illustrated (in Figure 5.4 A) summarise IFNγ release (ELISPOT) assays using data collected from five different HLA-$A2^+$ MM patients. The data show that the hybrid cell lines (HU266 and HRC) stimulated significant elevation of IFNγ releasing cells in response to restimulation with MUC1 derived peptide-pulsed T2 cell line, compared with their parent HMY2, the parent myeloma cell line U266, and cultured PBMCs without

further re-stimulation. There was no significant difference of this response between HMY2 and U266 simulated cultures, at any of the used ratios. Regarding the IFNγ releasing response induced by h TERT derived peptide pulsed T2 cells, HRC induced culture showed the highest IFNγ releasing response for this antigen, followed by HU266 and HMY2, with no significant difference between them at the ratio of 1:100, whilst the response of HMY2 stimulated culture was diluted very quickly at the ratio of 1:25. The parent myeloma cell line, U266 stimulated culture illustrated the lowest response with regard to the h TERT specific IFNγ release. Regarding CS1 specific IFNγ release, both HU266 and HRC induced a significant increase of CS1 specific IFNγ releasing response, more than that of the parents HMY2 and U266. There was no statistically significant difference between the responses induced by HRC and HU266. In addition, U266 stimulated culture failed to induce an antigen specific CS1 IFNγ releasing response. HMY2 induced a significant CS1 specific IFNγ releasing response only at the ratio of 1:100. Regarding the MAGE-C1 specific response, HMY2, HU266 and HRC stimulated cultures induced significantly enhanced responses to restimulation with MAGE-C1 derived peptide pulsed T2 cell line, but U266 failed to induce a significant IFNγ response. There was no significant difference between the hybrid stimulated cultures and the parent APC (HMY2) stimulated culture for MAGE-C1 induced IFNγ release.

The use of stimulated PBMCs from each culture, with no further stimulation in the ELISPOT assay, acted as a negative control, indicating the antigen specific IFNγ releasing response induced by further stimulation with the relevant antigenic peptides. Also, restimulation of the long-term stimulated T lymphocytes using WNF derived peptide pulsed T2 cells failed to induce any IFNγ releasing response. These data showed that the hybrid cell lines and HMY2 induced MM antigen specific responses in long-term stimulated T lymphocytes cultures, but U266 failed to induce a significant response. Additionally, K562 (NK cell target) stimulation showed a lack of NK cell activity in all stimulated cultures.

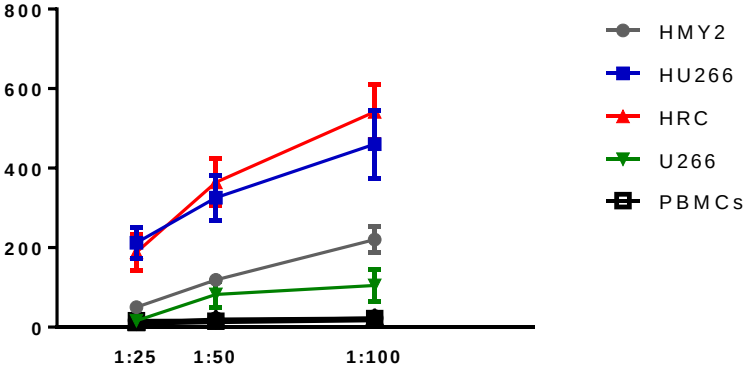
Finally, PBMCs from three different HLA-$A2^-$ MM patients were stimulated in parallel with the HLA-$A2^+$ MM samples, and used as negative controls (Figure 5.4B) in IFNγ releasing ELISpot assays. The produced long-term stimulated lymphocytes were used as responders to T2 cells pulsed with the same, previously mentioned antigenic peptides in IFNγ ELISpot assays.

The data collected from two of HLA-A2 negative patients showed no elevation of any of the examined antigen specific responses. However, one of HLA-A2$^-$ patients' samples showed apparent weak to moderate antigen specific IFNγ releasing responses, to the examined antigens in all the stimulated cultures. I tried to explain this result in the next section.

A

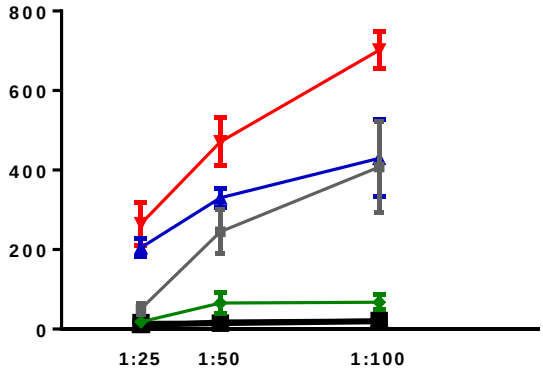
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		**	*	ns		***	***	ns		****	***	ns
HU266			ns	***			ns	****			ns	****
HRC				**				****				****
PBMCs	ns	***	**	ns	ns	****	****	ns	****	****	****	ns

MUC 1



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	**	ns		ns	**	*		ns	****	****
HU266			ns	**			ns	***			***	****
HRC				**				****				****
PBMCs	ns	**	****	ns	***	****	****	ns	****	****	****	ns

h TERT

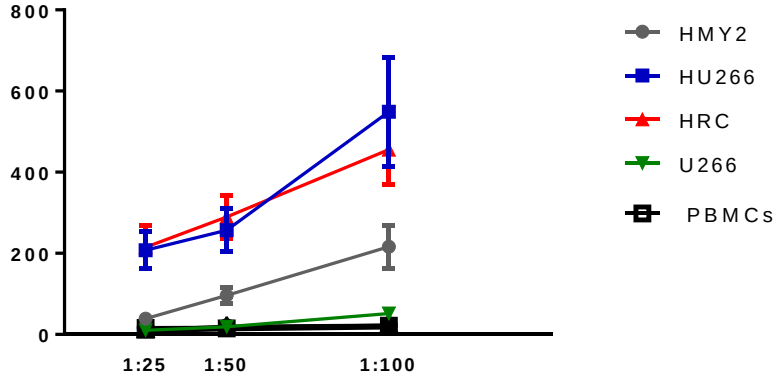


Number of the emerged spots

T:E ratio

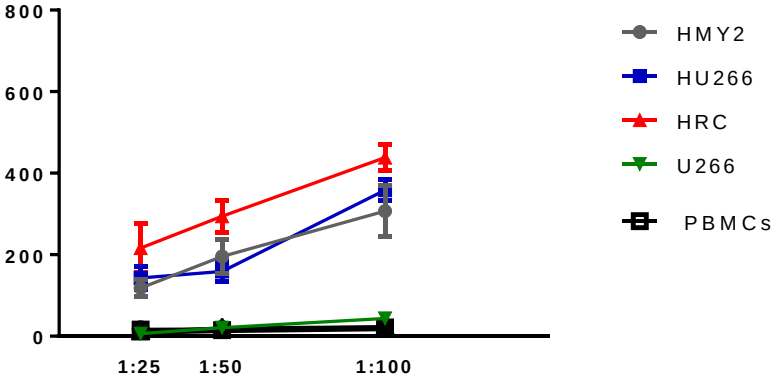
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		*	*	ns		*	**	ns		****	**	*
HU266			ns	*			ns	**			ns	****
HRC				**				***				****
PBMCs	ns	***	****	ns	ns	****	****	ns	**	****	****	ns

CS1



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	*		ns	ns	***		ns	**	****
HU266			ns	*			*	**			ns	****
HRC				****				****				****
PBMCs	*	**	****	ns	****	***	****	ns	****	****	****	ns

MAGE C1

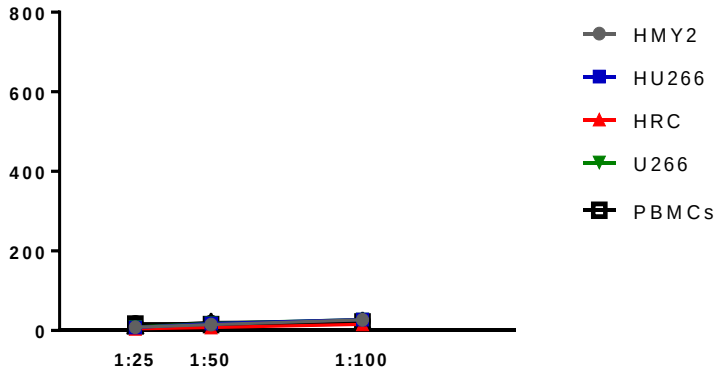


Number of the emerged spots

T:E ratio

	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
PBMCs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

WNF



	1:25				1:50				1:100			
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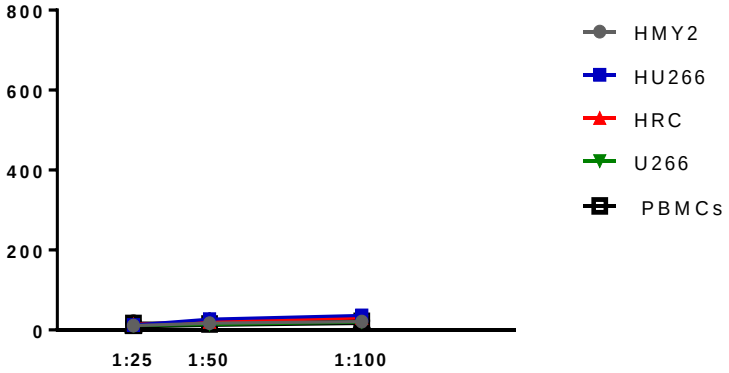
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
PBMCs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

K562



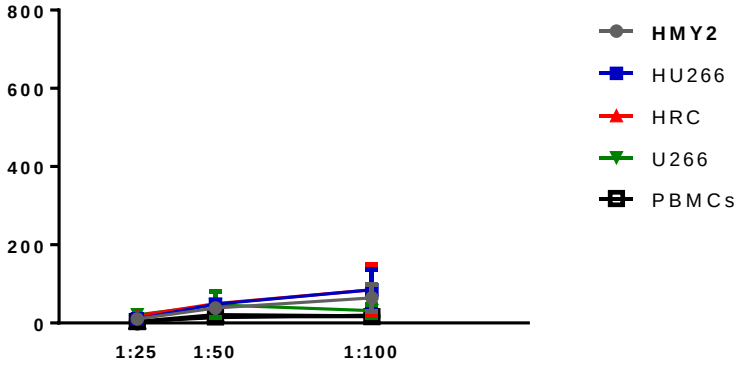
Number of the emerged spots

T:E ratio

B

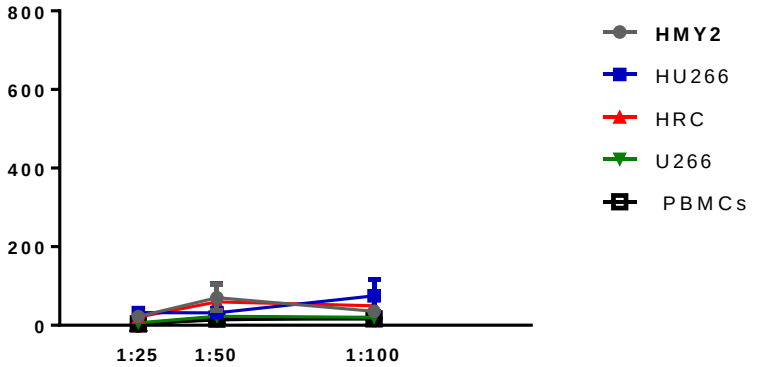
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
PBMCs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

MUC 1



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
PBMCs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

h TERT

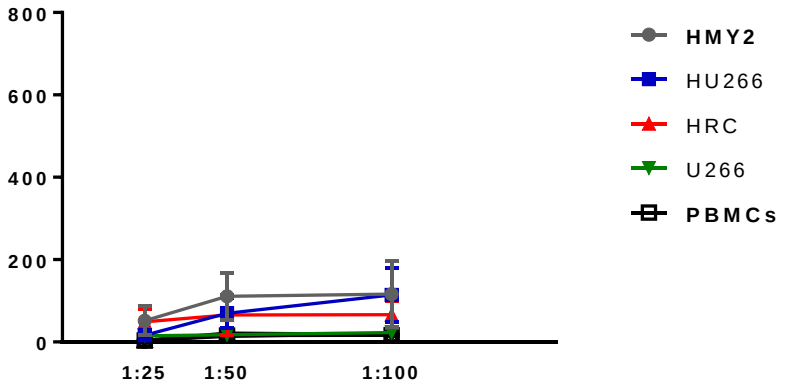


Number of the emerged spots

T:E ratio

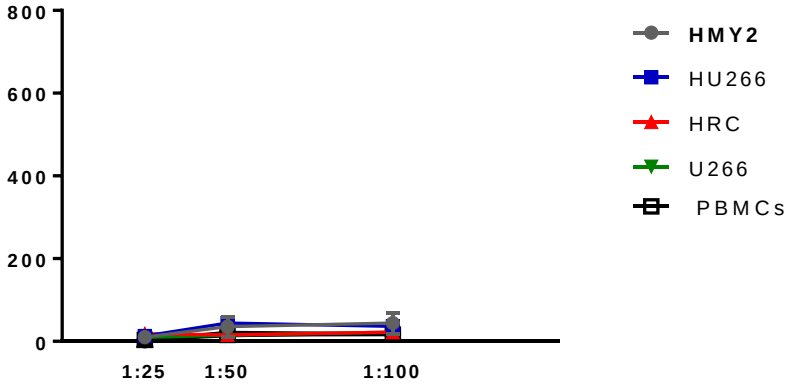
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
PBMCs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

CS1



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
PBMCs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

MAGE C1

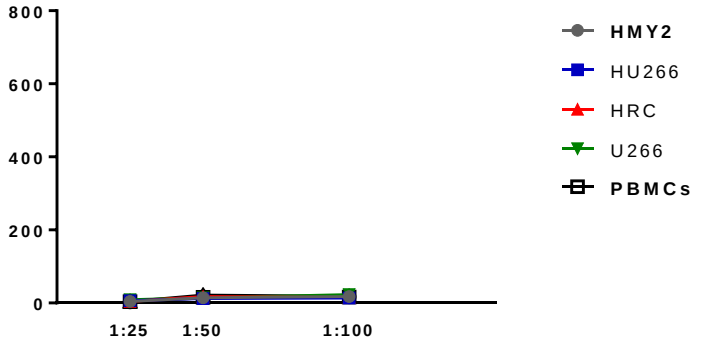


Number of the emerged spots

T:E ratio

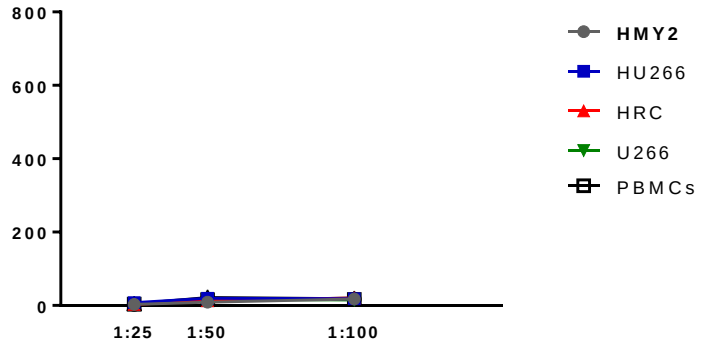
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
PBMCs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

WNF



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
PBMCs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

K562



Number of the emerged spots

T:E ratio

Figure 5.4: IFNγ ELISpot assays. *Responder cells (allogeneic PBMCs, isolated from MM patients and stimulated with HMY2, HU266, HRC and U266) were mixed with T2 stimulator cell lines (after pulsing with MUC1, h TERT, CS1, MAGE-C1 and WNF derived peptides) in IFNγ ELISpot assays. Chart A represents responses of five HLA-A2^{+} MM patients, and B represents responses of three different HLA-A2^{-} MM patients. Data are presented as mean value of the patients' responses ± SEM. Stimulated PBMCS in the long-term culture of the used cell lines without further stimulation were used as a negative control in each corresponding ELISpot assays of the same cell lines. The levels of significances have been summarized in the table next to each chart and presented as asterisks (* <0.05, **≤ 0.01, *** ≤ 0.001and **** ≤ 0.0001).*

5.2.2.2.1 Explanation of the presence of IFNγ releasing response in one of the HLA A2-patients:

In one of the HLA-A2 negative patient samples, some IFNγ releasing response was observed in the IFNγ ELISpot assays, in response to the tumour antigenic peptides, but not to WNF peptide (Figure 5.5). Absence of this response to WNF pulsed T2 cells suggested a lack of allogeneic (mismatched HLA) response to T2 cells. In addition, absence of HLA-A2 expression in this patient (Figure 5.6) suggests that the response observed was an allogeneic response to HLA-A2, rather than an antigen-specific HLA-A2 restricted response (Darrow et al., 1989; Crowley et al., 1990).

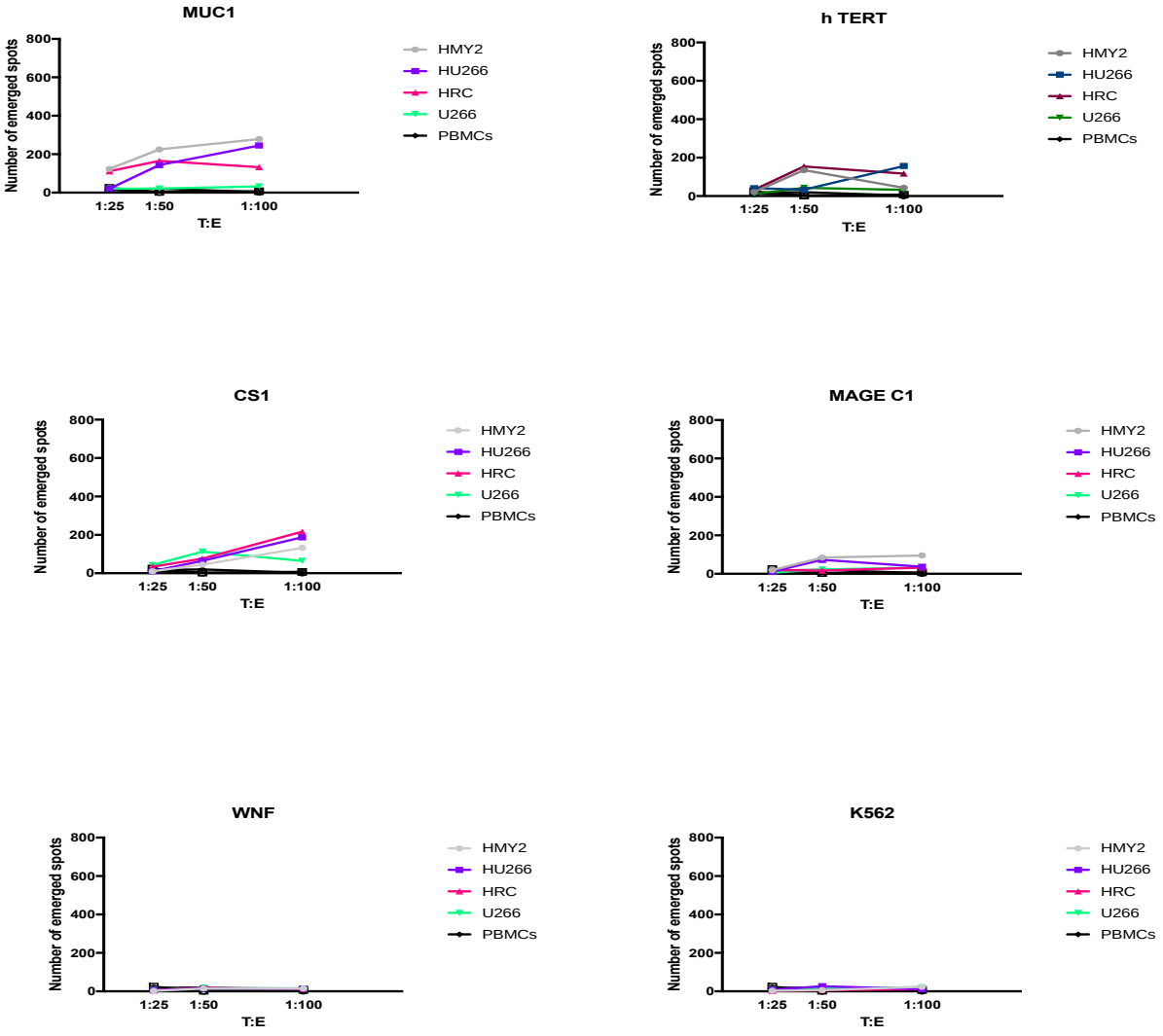


Figure 5.5: IFNγ releasing ELISpot assay in one of HLA-A2 negative patient. *There are observed increases of IFNγ releasing responses in the case of MUC1 (in all of the used ratios), h TERT (in 1:100 and 1:50), and CS1 only in 1:100 ratio.*

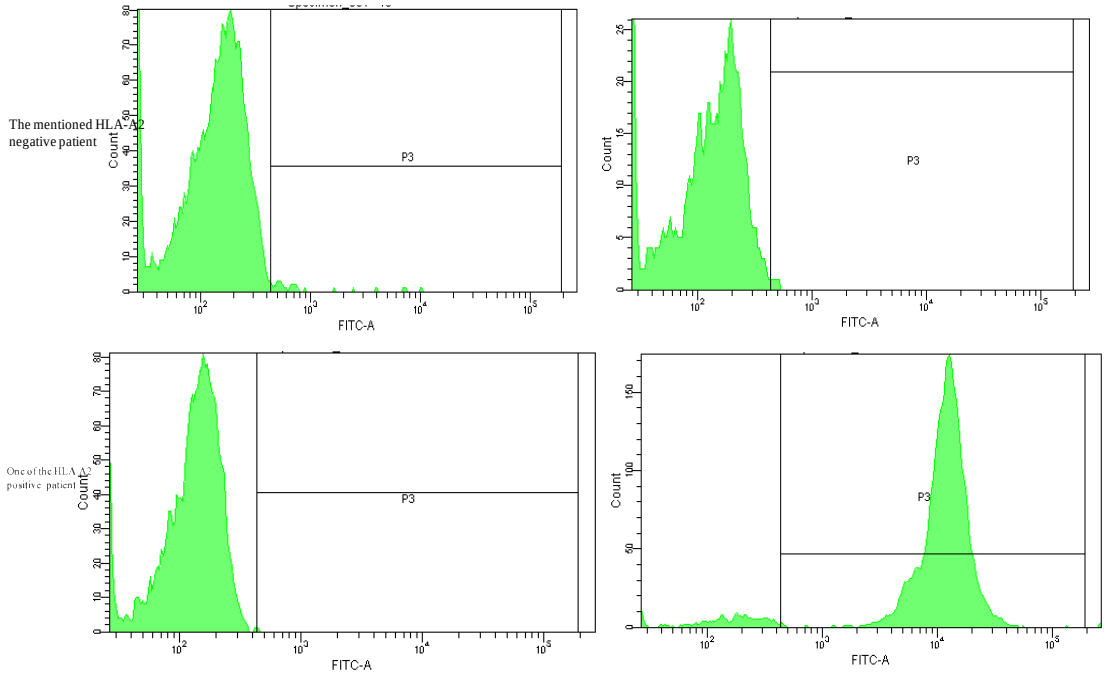


Figure 5.6: Flowcytometric histograms of HLA-A2 expression by the mentioned HLA-A2 negative patient *(in the upper row), and one of the HLA-A2 positive patients in the lower row. The left hand histogram of each line represents the isotype control staining of this patient, and the right hand histogram represents cells stained with the HLA-A2 specific mAb BB7.2.*

One possible explanation for the IFNγ ELISpot results was that the presence of some antigenic peptides stabilized the HLA-A2 molecule on the surface of T2 cells more effectively than the others, which may lead to upregulation of HLA-A2 expression at the cell surface, and induction of an HLA-A2- specific allogeneic response. For this reason, I examined the actual stability of HLA-A2 expression of the T2 cell line after pulsing with all the candidate peptides, using the flow cytometric analysis (Figure 5.7). Unpulsed T2 cells were used as a negative control. The results showed that MUC1 and h TERT derived peptides caused the highest level of HLA-A2 stabilization, followed by CS1and MAGE-C1, whilst, WNF virus derived peptide caused lowest HLA-A2 stabilization level. Moreover, I used Syfpeithi score software (www.syfpeithi.de) to estimate the binding avidity of each HLA-A2 loaded peptide of the used MM antigen derived peptides and WNF derived peptide. Table 5.1 gives the MFI of HLA-A2 expression on T2 pulsed peptide, and the syfpeithi scores. The results indicate that the TAA have a relatively high binding avidity for HLA-A2, compared with WNF virus peptide by both level of HLA-A2 expression and Syfpeithi score. The predicted HLA-A2 binding avidity of MUC1was the highest followed by h TERT, CS1, MAGEC1, and WNF in a descending order. Accordingly, these data suggest that the increase of HLA-A2 stability caused by the TAA

peptide could lead to some HLA-A2 specific allogeneic response in these particular cultures, using PBMCs from the HLA-A2$^-$ myeloma patients.

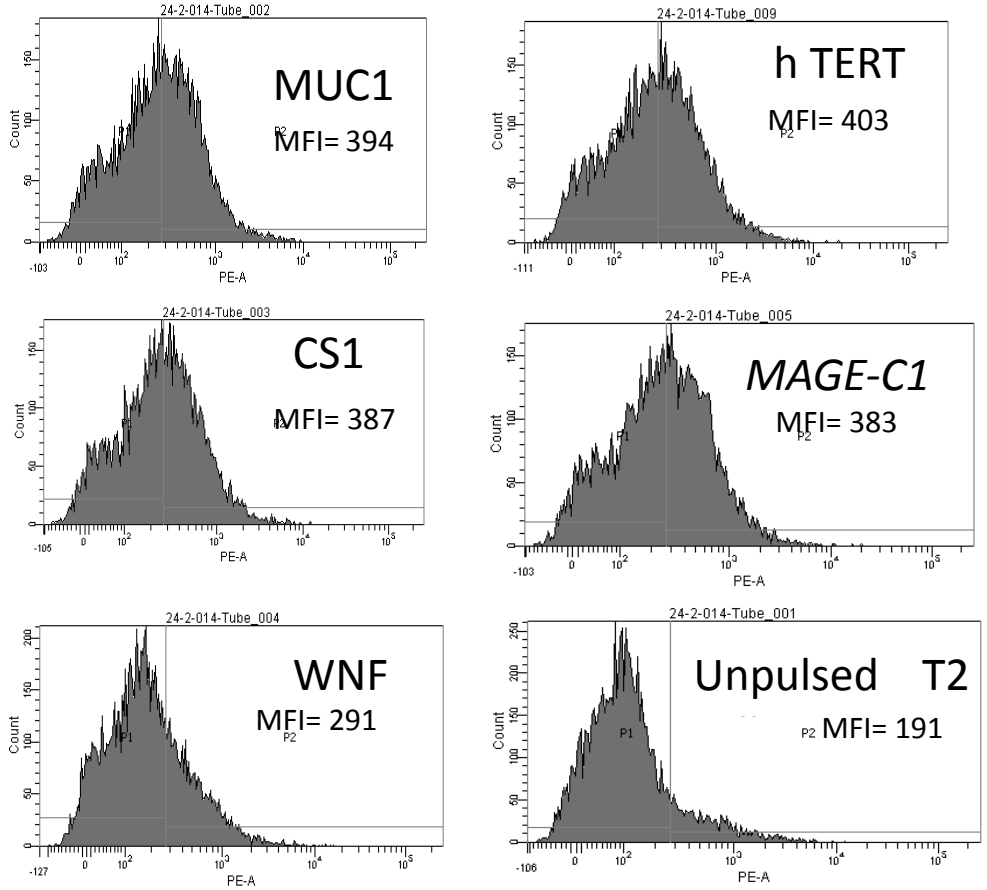


Figure 5.7: Flow cytometric analysis of the stability of HLA-A2 molecule *of T2 cell line pulsed with the examined MM antigens, irrelevant WNF peptide antigen, or unpulsed T2 cell line.*

Table 5.1: Stability of HLA-A2 pulsed with WNF, MUC1, h TERT, CS1 and MAGE-C1 *using flow cytometric analysis and Syfpeithi score.*

Antigen	**Peptide sequence**	**MFI after loading on T2 cells**	**Binding prediction score (Syfpeithi score)**
MUC1	LLLLTVLTV	394	31
h TERT	ILAKFLHWL	403	30
CS1	SLFVLGLFL	387	25
MAGE-C1	ILFGISLREV	383	18
WNF	LGMSNRDFL	291	13
Unpulsed T2 cells	N/A	191	N/A

5.2.2.3 Detection of antigen specific CTL in long-term activated cultures using the perforin ELISPOT assay:

The previous experiment showed the suitability of the IFNγ ELISPOT assay for monitoring T cell responses in long-term stimulated cultures, and the ability of the hybrid cell lines to stimulate functional T cell responses. However, perforin ELISPOT assay may represent a more direct analysis of cell-mediated cytotoxicity, as compared to the IFNγ ELISPOT, since perforin is a key mediator of target cell death via the granule-mediated pathway. The same experimental set up was used as for IFNγ release ELIspot assay. However, due to shortage of patients' PBMCs, the experiment was done using three different patient samples from HLA-$A2^+$ MM patients only. There were insufficient cells in the cultures of PBMCs from HLA-A2- MM patients to use these as controls in this experiment.

Figure 5.8 shows that the hybrid cell lines HU266 and HRC stimulated strong perforin releasing responses to MUC1 derived peptide pulsed T2 cell line, significantly higher than HMY2 (not in 1:25 ratio), the parent myeloma cell line U266, and the background response of stimulated PBMCs with no more stimulation in ELISpot. U266 stimulation did not induce a significant increase of the specific perforin release to this TAA compared with the background PBMCs. The difference between the hybrid cell lines (HRC and HU266) and their parent APC (HMY2) was significant at most of the used ratios.

The hybrid cell line HRC stimulated culture caused the most significant elevation of h TERT specific perforin release, compared with the other used cell lines and the background PBMCs. Also, the hybrid cell line HU266 induced a significant increase in perforin release in response to h TERT, when compared with its parent myeloma cell line and the background PBMCs. However, no significant difference was seen between HU266 and its parent APC, HMY2, regarding h TERT specific perforin release. Also, the difference between the responses of U266 stimulated culture and PBMCs was not significant.

The stimulation effect of the hybrid cell lines HU266 and HRC to induce CS1 specific perforin releasing cells was almost the same, and their CS1 specific perforin release effects were significantly higher than that of the parent myeloma cell line U266 and the background PBMCs. Furthermore, the difference between this specific induction level of these hybrids and HMY2 was significant at 1:100 (target cells: effectors) ratio. HMY2

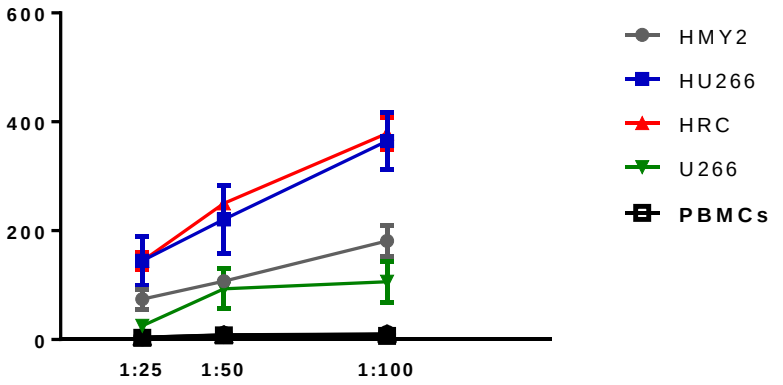
activated cells showed higher stimulation of perforin release than PBMCs without further stimulation, but only induced stronger perforin release in response to CS1 than U266 at the highest T: E ratio (1:100).

It is of note that almost all of the stimulated cultures showed a weaker perforin releasing response to MAGE-C1 derived peptide pulsed T2 cells than the other candidate antigens. However, it follows the same order, as the hybrid cell lines HRC and HU266 stimulated cultures were the highest producers of the perforin releasing response specific to peptide derived MAGE-C1 pulse T2 cells, followed by HMY2, and the weakest effect was shown by the U266 stimulated cultures.

The negative controls used in the experiments were stimulated PBMCs taken from each culture without further stimulation in ELISpot assays,T2 pulsed with an irrelevant peptide (WNF), and K562 (NK cell control). All these negative controls did not induce any perforin release, using different long-term stimulated T cells.

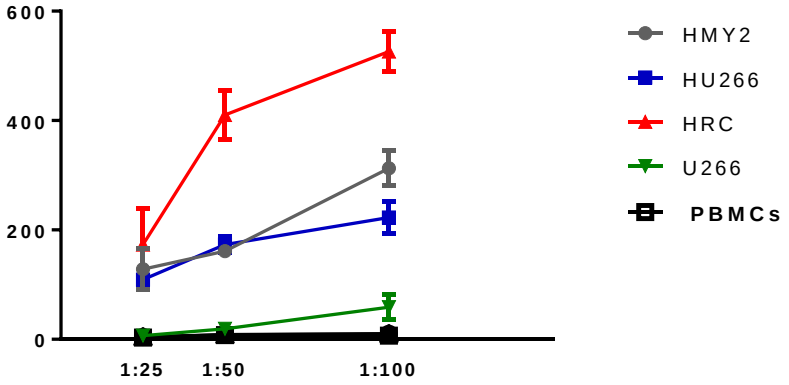
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		*	**	ns		***	***	ns
HU266			ns	*			ns	*			ns	****
HRC				*				**				****
PBMCs	ns	**	**	ns	ns	****	****	ns	***	****	****	ns

MUC1



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	*		ns	****	**		ns	****	****
HU266			ns	*			****	**			****	**
HRC				**				****				****
PBMCs	**	*	****	ns	****	****	****	ns	****	****	****	ns

h TERT

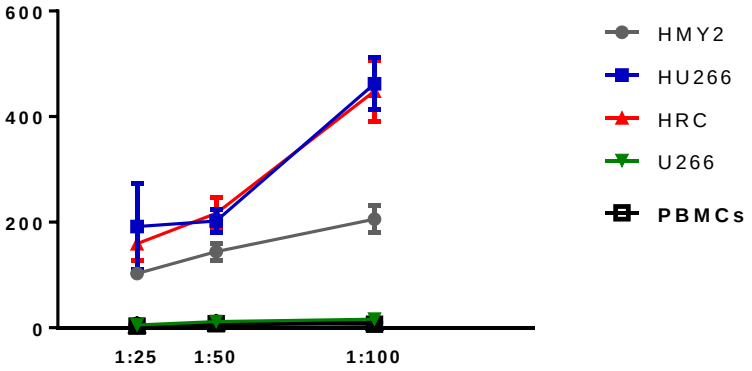


Number of the emerged spots

T:E ratio

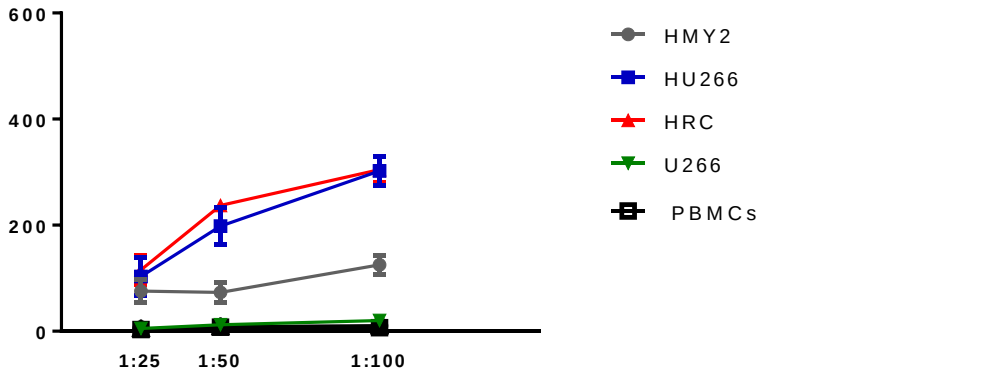
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		**	**	*
HU266			ns	*			ns	*			ns	****
HRC				*				**				****
PBMCs	ns	***	**	ns	*	***	****	ns	***	****	****	ns

CS1



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		**	****	ns		****	****	**
HU266			ns	*			ns	****			ns	****
HRC				**				****				****
PBMCs	*	***	***	ns	ns	****	****	ns	****	****	****	ns

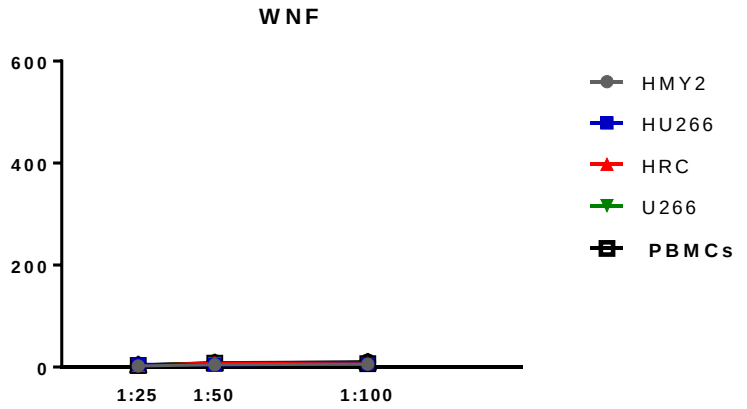
MAGE C1



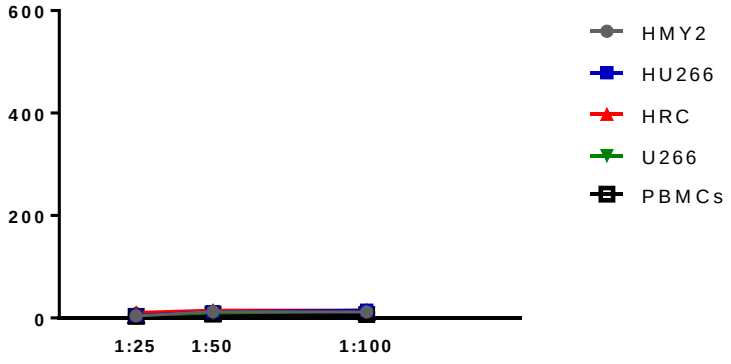
Number of the emerged spots

T:E ratio

	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
PBMCs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
PBMCs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns



Number of the emerged spots

T:E ratio

Figure 5.8: Perforin release ELISpot assays. *Long-term stimulated responder cells (PBMCs, isolated from MM patients and stimulated with HMY2, HU266, HRC and U266) were cocultured with T2 stimulator cell lines (after pulsing with MUC1, h TERT, CS1, MAGE-C1 and WNF derived peptides) in perforin ELISpot assays. Charts represent responses of three HLA-A2^{+} different MM patients. Data are presented as mean value of the patients' response ± SEM. Level of significance were indicated as asterisks, and summarised in the tables next to each graph.*

5.2.2.4 Detection of cytotoxic effect of antigen specific CTL in long-term stimulated cultures using DELFIA® EuTDA Cytotoxicity assays:

In the previous experiments, detection of antigen specific CTL in the long-term stimulated MM patients' PBMCs was carried out using Pro5® MHC Class I Pentamer analysis, and IFNγ and perforin ELISpot assays. Afterward, I wanted to assess the cytotoxic ability of the activated antigen specific $CD8^+$ CTL produced in the long-term stimulated cultures, to ensure their activity, using DELFIA® EuTDA Cytotoxicity assays. The experiment was set up using T2 cell line pulsed with either the relevant TAA- derived peptides, or with an irrelevant, HLA-A2 restricted (WNF) virus peptide, as a negative control, as described previously. The cell line K562 was used as a target cells to determine the killing activity of NK cells produced in the cultures. Moreover, to examine the antitumour and allogeneic activation of the induced T lymphocytes, U266 was used as target cell line in the cytotoxicity experiment.

The cytotoxicity data are shown in Figure 5.9, which summarises the cytotoxicity results of long-term stimulated T lymphocytes *in vitro*, isolated from five HLA-$A2^+$ MM and three HLA-$A2^-$ patients. The following formula was used to calculate the cytotoxic activity observed.

Regarding the MUC1 specific cytotoxic response, HRC and HU266 induced cultures showed the highest cytotoxic response against MUC1 peptide pulsed T2 cell line compared with unpulsed T2 cells. HU266 induced CTL exhibited a strong statistically significant response at the entire used target: effector ratios compared with its parent U266. However, the HU266 induced cultures showed a significantly increased response only at 1:100 (target: effector cells) ratio compared with its APC parent, HMY2. The hybrid cell line HRC elicited a significant MUC1 specific cytolytic response compared with U266. HRC, also, induced a moderate significantly increased cytotoxic response compared with HMY2. However, U266 failed to induce MUC1 specific cytotoxic response compared with unpulsed T2 cell line.

Concerning the h TERT specific CTL response, HRC stimulated culture showed the highest specific cytotoxic response to T2 cell line pulsed with HLA-A2 restricted h TERT peptide. There was no significant difference of the cytotoxic response between the cultures induced by HMY2 and HU266. Also, both of the used hybrids (HU266 and HRC) activated cultures induced strongly significant cytotoxic responses, compared with U266

stimulated culture and with unpulsed T2 cell line. HMY2 also induced significant response at all T: E, compared with U266 and unpulsed T2 cells. Regarding U266, no significant difference was observed between h TERT derived peptide pulsed T2 cells and unpulsed T2 cell line, induced by U266.

The hybrid cell line HU266 induced the most significantly elevated cytotoxic response to CS1 peptide-pulsed T2 cell line compared with unpulsed T2 cells. The response of HU266 was followed by that of HRC and HMY2. Both of the hybrid cell lines induced a significantly elevated response compared with the response of U266 stimulated cells, whereas the HMY2 induced cytotoxic effect diluted quickly, at the ratio of 1:25 (target: responder cell ratio). Also, the difference between HMY2 and HRC stimulated cultures was significant only at the T: E ratio 1:100. U266 did not induce any CS1 specific cytotoxic response compared with unpulsed T2 cell line.

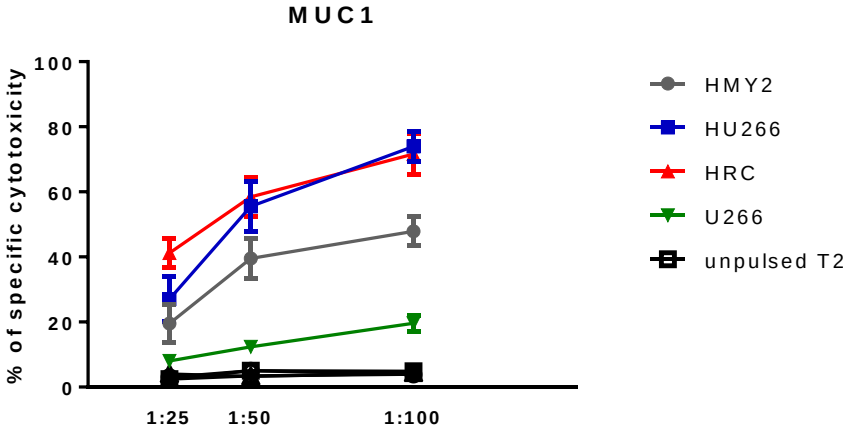
HU266 induced the most significantly upregulated response to MAGE-C1 peptide-loaded T2 cells, compared with the other cell lines, except with HRC at 1:25 and 1:100 ratio, where there was no significant difference between HU266 and HRC. In addition to that, there was a significant increase of the cytotoxic response between the used hybrid cell lines stimulated cultures and that of the used myeloma cell line, U266. HMY2 induced significantly higher cytotoxic response than that of U266, however, its response was significantly lower than that of HU266. No significant difference was seen between HMY2 and HRC induced cultures at 1:25 and 1:100 ratios.

The cytotoxicity experiments were also set up using different negative controls, such as WNF virus peptide- pulsed T2 cell line, and K562 (control of NK cells) as a target cells. None of the used negative controls induced any cytotoxic response, using any of the stimulated cultures. Moreover, the stimulated HLA-$A2^-$ PBMCs of three patients did not upregulate any cytotoxic response against any of the examined antigens using all of the used stimulators, as shown in Figure 5.9 B.

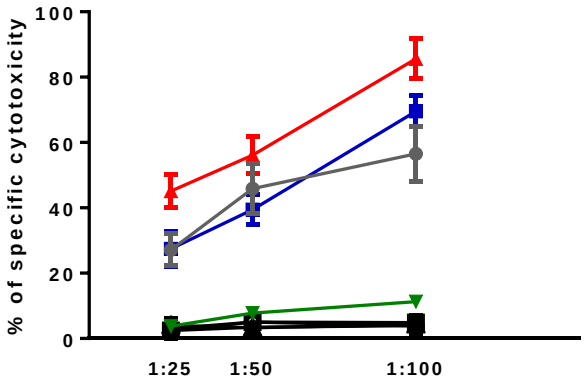
Overall, both HRC and HU266 hybrid cell lines induced significant HLA-A2 restricted antigen-specific cytotoxicity against all of the candidate TAAs. HMY2 also induced tumour antigen specific cytotoxicity, but at lower level than the two hybrid cell lines. The parent myeloma tumour cell lines U266 failed of induce any significant TAA specific cytotoxic response to peptide pulsed T2 cells compared with unpulsed T2 cells.

A

	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	*	ns		ns	*	**		**	**	**
HU266			ns	*			ns	****			ns	****
HRC				***				****				****
Unpulsed T2 cells	ns	*	****	ns	****	****	****	ns	****	****	****	ns



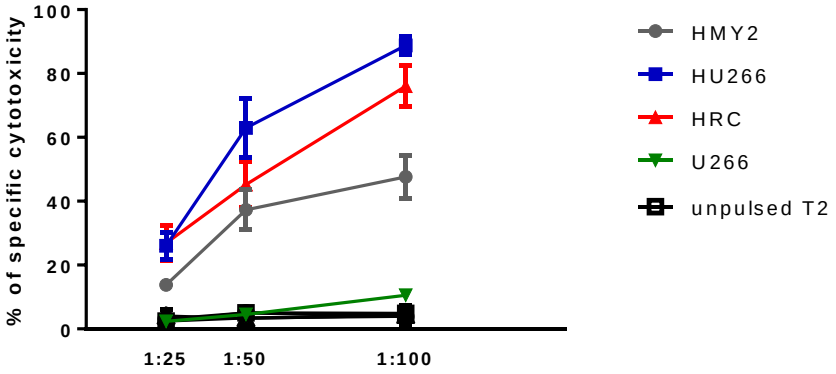
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	*	**		ns	ns	****		ns	***	****
HU266			*	**			ns	***			ns	****
HRC				****				****				****
Unpulsed T2 cells	**	**	****	ns	****	****	****	ns	****	****	****	ns



T:E ratio

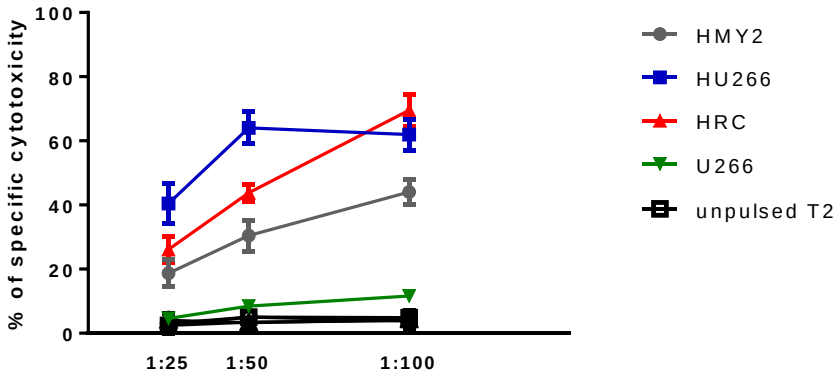
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		*	ns	**		****	**	***
HU266			Ns	*			ns	****			ns	****
HRC				*				****				****
Unpulsed T2 cells	ns	*	**	ns	****	****	****	ns	****	****	****	ns

CS1



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		**	ns	ns		****	ns	**		*	***	****
HU266			ns	****			**	****			ns	****
HRC				**				****				****
Unpulsed T2 cells	*	****	***	ns	****	****	****	ns	****	****	****	ns

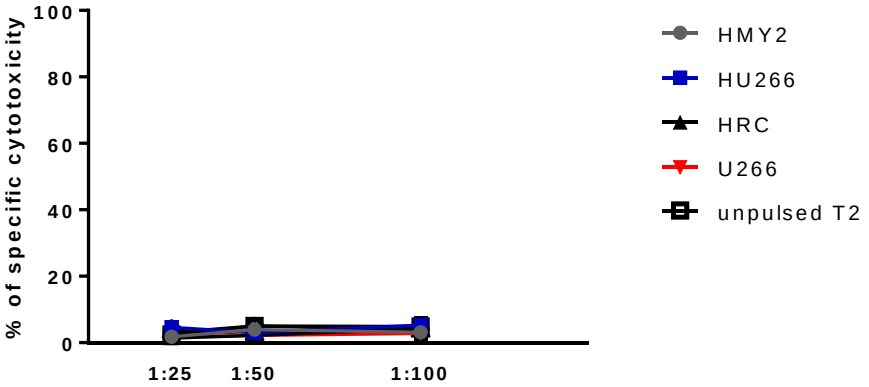
MAGE C1



T:E ratio

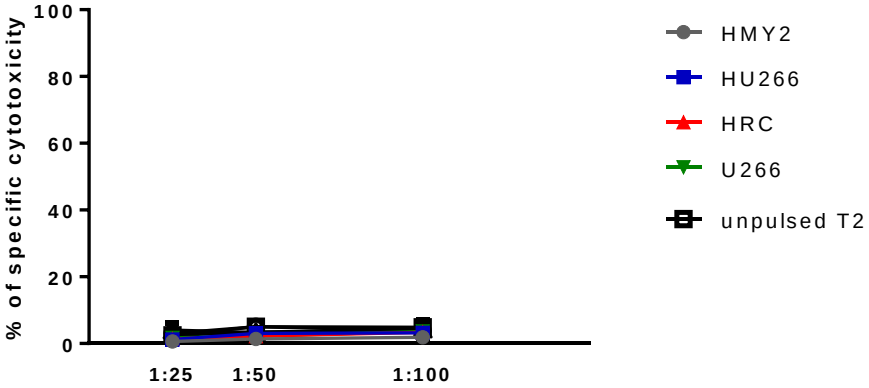
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
Unpulsed T2 cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

WNF



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
Unpulsed T2 cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

k562

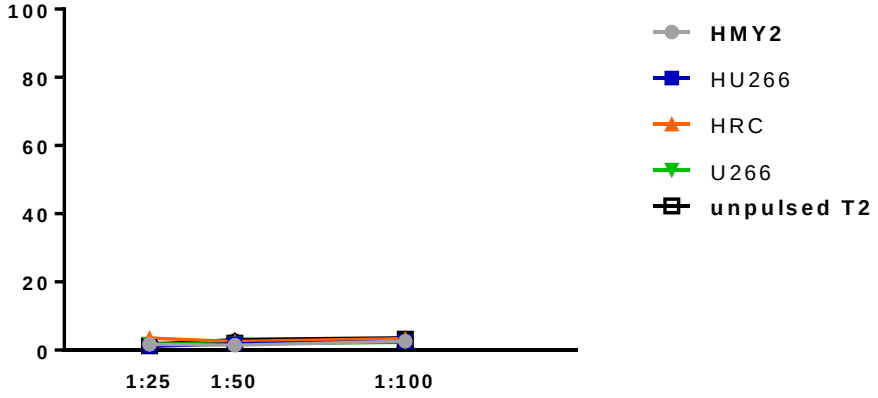


T:E ratio

B

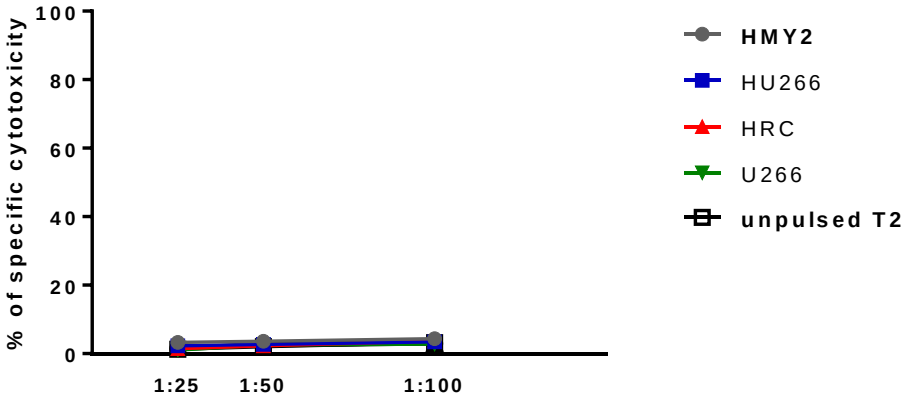
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
Unpulsed T2 cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

MUC 1



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
Unpulsed T2 cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

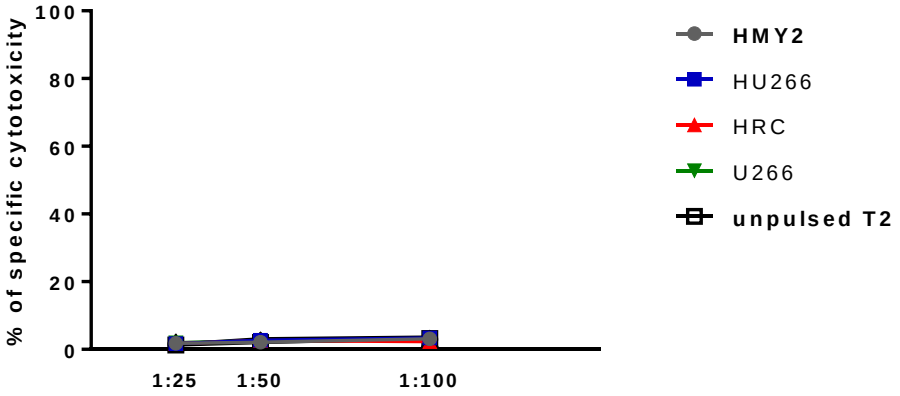
h TERT



T:E ratio

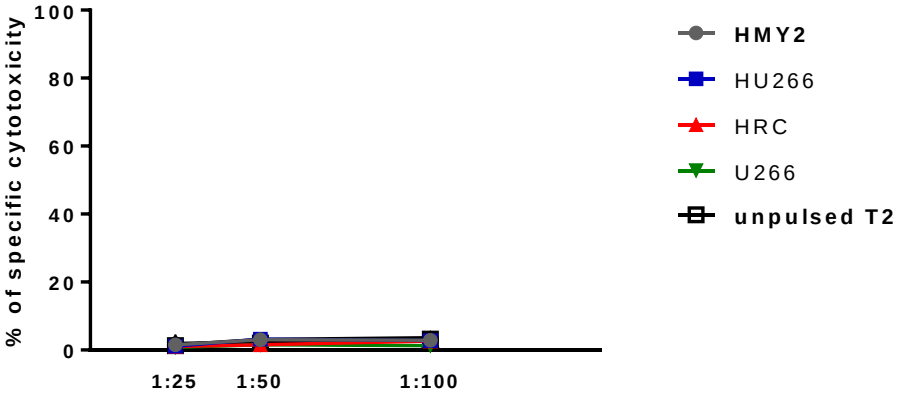
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
Unpulsed T2 cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

cs1



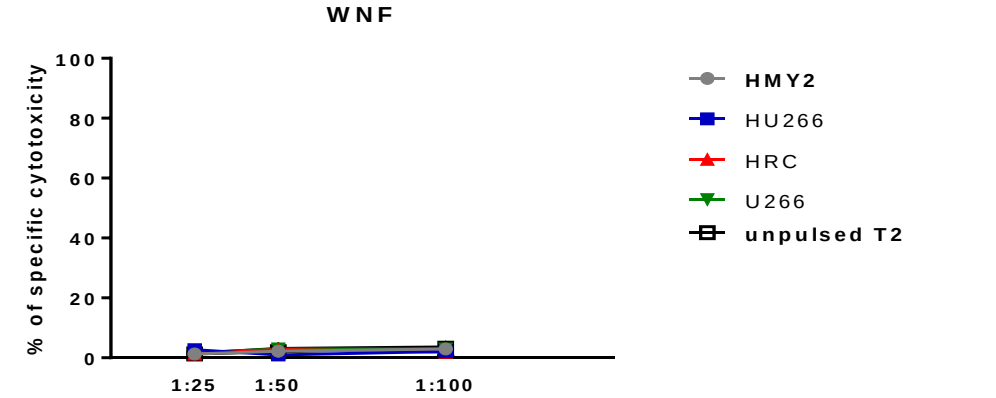
	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
Unpulsed T2 cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

MAGE C1

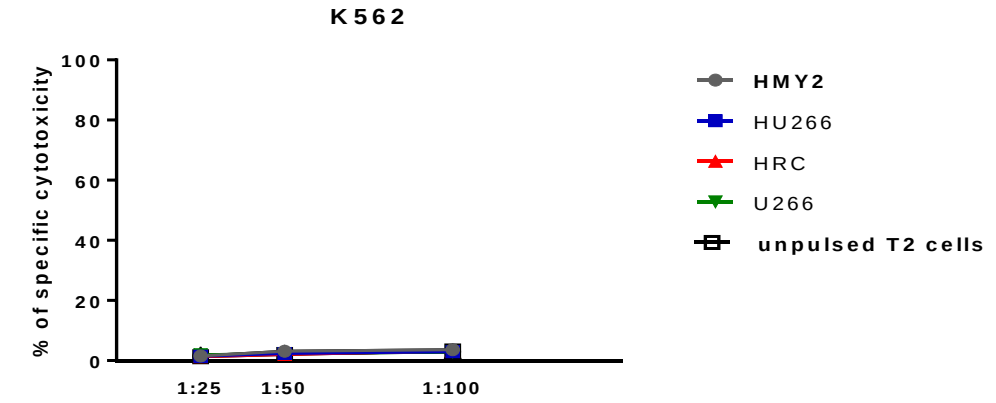


T:E ratio

	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
Unpulsed T2 cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns



	1:25				1:50				1:100			
	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266	HMY2	HU266	HRC	U266
HMY2		ns	ns	ns		ns	ns	ns		ns	ns	ns
HU266			ns	ns			ns	ns			ns	ns
HRC				ns				ns				ns
Unpulsed T2 cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns



T:E ratio

Figure 5.9: The cytotoxic activity of the long-term stimulated antigen specific cytotoxic T lymphocytes. *(A) isolated from HLA-A2+ MM patients, and directed against T2 cell line pulsed with the relevant HLA-A2 restricted TAA derived peptides of MUC1, h TERT, CS1 and MAGE-C1, and an irrelevant antigen peptide WNF virus). K562 and WNF pulsed T2 cells were used as negative controls. The hybrid cell lines, HU266, HRC and the parent cell lines HMy2 and U266, were used for stimulation of the induced antigen specific T cell line. Data are presented as mean ± SEM of % specific lysis of five different HLA-A2+ MM patients. Statistically significant differences were indicated as asterisks, and summarised in the table next to each graph (* <0.05, **≤ 0.01, *** ≤ 0.001and **** ≤ 0.0001). (B) The cytotoxic effect of long-term stimulated T lymphocytes isolated from three different HLA-A2$^-$ MM patients, using the same experimental set up as that of the HLA-A2$^+$ samples.*

5.2.2.5 *Allogeneic cytotoxic response of the produced long-term stimulated antigen specific CTL:*

To examine the presence of allospecific cytotoxic response rather than the antigen specific cytotoxic response of the produced long-term stimulated cultures, I used U266 in the cytotoxicity assay as a target cells. This experiment would detect both allogeneic cytotoxic effect, due to mismatched HLAs, as well as the antigen specific response, as U266 can express high level of specific TAAs as mentioned in section 3.2.4. Unfortunately, due to the shortage of the used patient's PBMCs, this experiment was done using the long-term stimulated PBMCs of two different HLA-$A2^+$ patient's samples only. The results, shown in Figure 5.10, illustrated that HRC induced the most upregulated cytotoxic response to U266, followed by HU266 and HMY2. The parent myeloma cell line U266 induced a lowest cytotoxic response. The difference of the induction level of the cytotoxity is highly significant between the used hybrid cell lines and U266, and the difference of HMY2 effect was of low significance, compared with U266. The enhanced responses to U266 may imply the presence of allospecific response or shared tumour antigen which lead to specific T cell responses. However, which of these responses caused the cytotoxic effect could not be answered by this experiment. The levels of killing of U266 cells induced by the different stimulator cell lines broadly reflected the hierarchy of responses that was seen for the antigen-specific CTL induction.

To summarise, the efficiency of the used hybrid cell lines to induce antigen specific CTL effectors in PBMCs from (HLA-$A2^+$) MM patients was confirmed using the cytotoxicity assay. In addition to that, HMY2 induced a moderate cytotoxic response in MM patient's PBMCs, while U266 failed to induce such response in the cultures.

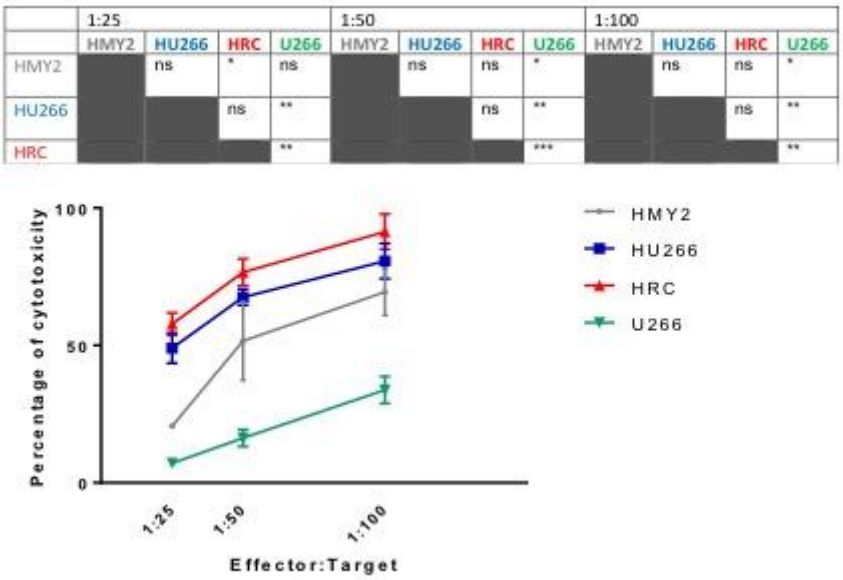


Figure 5.10: Cytotoxic response of the long-term stimulated CTL to U266. *The induced CTL were activated by HMY2, HU266, HRC and U266. Data are presented as mean ± SEM of % specific tumour lysis of two different HLA-A2^{+}patients, and the significant differences were summarised in the table next to the graph, and shown as asterisks (* <0.05, **≤ 0.01, *** ≤ 0.001and **** ≤ 0.0001).*

5.3 Discussion:

Immunosuppressive activity of MM micro-environment targets cytotoxicity of T cells through different mechanisms: presence of cellular regulatory elements, e.g. T reg (Murakami et al., 2004), upregulation of cell adhesion-mediated immune resistance (that take place through adhesion of myeloma cells to accessory cells, such as bone marrow stromal cells or vascular endothelial cells, which lead to downregulation of Fas, and upregulation of survivin) (de Haart et al., 2013), or DC dysfunction (Brimnes et al., 2006; Pratt et al., 2007), as delineated in section 5.1. Accordingly, various reports demonstrated the unresponsiveness of T cells isolated from MM patients to tumour cells (Pratt et al., 2007; Witzens-Harig et al., 2013). However, other reports have illustrated the ability to enhance the cytotoxic response of T cells to autologous tumour *in vivo* using tumour antigen vaccination or *in vitro* through activation of T lymphocytes (Dhodapkar et al., 2002; Hayashi et al., 2003; Rosenblatt et al., 2013).

Vaccination with tumour antigens has the advantage that screening of the antigen immunogenicity and generation of the effector immune cells is done by the patient's own immune system (Ho et al., 2006). Vaccination with self-antigens usually results in low memory response and a time limited effect. In a trial to break tolerance to self-antigens, Hofmann et al. (2015) used analogue peptides produced by modification of the peptides that bind to the anchor residues of MHC, thus enhancing the peptide binding and leading to better exposure of the peptide to T cells. Use of these analogue peptide in vaccination of patients with acute myeloid leukemia showed a notable success rate with enhancement the immunogenicity of these peptides (Hofmann et al., 2015). Rosenblatt et al. (2013) reported the efficiency of cellular immunotherapy in MM patients, using DC/MM cell fusion vaccine, after transplantation. They showed that this vaccination resulted in expansion of MM-specific CD8+ T cells, and reduction of minimal residual disease (Rosenblatt et al., 2013).

The use of tumour antigen based vaccines in tumour immunotherapy induced a moderate magnitude of antigen specific T cells, with limited effect on the cancer progression (Montes et al., 2005). Moreover, in case of failure to induce a significant tumour-specific immune response, it would be difficult to identify reasons of the failure, and how to circumvent them (Ho et al., 2006). The presence of dysfunctional antigen presenting cells and regulatory T cells in tumour bearing patients limit vaccine efficacy (Rosenblatt et al.,

2010). This delivers a good rationale for trying to induce antigen specific T cell responses *in vitro,* not only for screening the examined antigen immunogenicity before using it for *in vivo* vaccination, but also for generation of T cell clones, which could be used directly in adoptive immunotherapy. There are numerous forms of antigens (such as tumour cell lysate or antigen derived peptides), and stimulator cells (APCs) that have been employed *in vitro,* and showed effective expansion of *ex vivo* T cells that have been primed *in vivo* (Ho et al., 2006). For instance, *ex vivo* PBMCs were stimulated weekly using autologous DCs, pulsed with apoptotic MM bodies, and the stimulated T cells showed significantly greater proliferation, compared with PBMCs stimulated with MM cells only, and they also showed significant cytotoxicity against autologous MM cells (Hayashi et al., 2003).

Another study showed that T cells isolated from MM patients at various stages of the disease could not induce any response to autologous MM tumour cells. However, after stimulation with tumour loaded DCs, specific cytotoxicity and IFNγ release responses to autologous tumour cells, was seen in six out of seven patients (Dhodapkar et al., 2002). The effector T cells isolated from myeloma patients' blood and bone marrow tumour environment were of similar efficiency after stimulation (Dhodapkar et al., 2002). In addition, adoptive cellular transfer of autologous, *ex vivo* stimulated TIL have been used in treatment of melanoma patients, with a success rate of 50%, and lymphodepletion of the patients before infusion of TIL resulted in improvement of the rate to 70% (Rosenberg and Dudley, 2009). DC/ myeloma cell hybrid cell lines induced *ex vivo* T cells that had a predominantly activated phenotype, showing high percentages of T cells expressing CD69 and IFNγ, and high ability to lyse autologous myeloma tumour cells (Rosenblatt et al., 2011). In terms of induction of antigen specific responses, the stimulated cultures exhibited expansion of T cells with MUC1 specificity (Rosenblatt et al., 2010).

The ability of multiple myeloma hybrid cell lines (HRC, HU266), and HMY2 to induce allogeneic lymphoproliferative responses, and TAA-specific IFNγ and perforin releasing responses, using PBMCs isolated from healthy HLA-A2+ donors was shown *in vitro,* and the data were presented in Chapter 4. These cultures induced HLA-A2 restricted CD8+ T cells specific for MUC1, h TERT, CS1, and MAGE-C1.

Consequently, in this chapter I have examined the hybrids' ability to induce allogeneic and TAA specific CTL responses *in vitro,* in PBMCs isolated from myeloma bearing

patients. There were three possible outcomes from these experiments. The first possibility was the presence of T cell responses to allogeneic and antigen specific stimulation, thus demonstrating the absence of unresponsiveness of T cells isolated from MM patients to the hybrid cells stimulation. The second possibility was the occurrence of allogeneic responses, but not antigen specific responses. This would indicate that the antigens are not sufficiently immunogenic to activate the antigen specific response. The third possibility was to obtain negative responses in both allogeneic and antigen specific stimulation. This would suggest the unresponsiveness of the T cells from myeloma patients, as we knew from the previous data that the hybrids are antigenically strong enough to stimulate allogeneic and antigen specific responses in PBMCs from healthy donors.

5.3.1 Induction of allogeneic stimulation of T cell subsets in MM patients' PBMCs samples:

The hybrid cell lines (HU266 and HRC), and HMY2, induced activated allogeneic lymphoproliferative T cell responses in CFSE staining experiments, using PBMCs from MM patients. In all of the stimulated cultures, the level of induction of CD8+ T cells was higher than CD4 + T cells, which could be attributed to the fact that the level of HLA class I expression was higher than the level of expression of HLA class II (Figure 3.2), or due to their functions (Abbas et al., 2012), as discussed earlier (section 4.3.1). HU266 demonstrated the most enhanced proliferation of $CD3^+$ T cells and CD4+ T lymphocytes, in addition to high level of stimulation CD8+ T lymphocytes. This may be explained by its high level of expression of CD80, CD86, HLA class I and HLA class II.

The parent myeloma cell line, U266, stimulated cultures almost lacked the proliferation of these different T cell subsets, which could be attributed to many reasons. Firstly, the absence of the costimulatory molecules expression by U266 cell line. Secondly, Horna and Sotomayor (2007) described that, in spite of the presence of tumor specific T cells in blood or in lymphoid organs of tumor bearing patients, their capacity to react with the tumor cells was very low. The third possible explanation is that inactivation of bone marrow derived APCs, such as DCs in MM patients (Ratta et al., 2002), could lead to deactivation of T lymphocytes due to the absence of bystander antigen presenting cells (Cywinski et al., 2006) in the patients' PBMCs. This could address the differences between U266 induced

allogeneic stimulation of MM patients' PBMCs and normal healthy donors' PBMCs in CFSE staining, as U266 induced a high level of allogeneic T cell subsets proliferation in healthy individuals' PBMCs, but not with patient samples. Another difference between these two experiments is that CD4+ T cells in MM patients' PBMCs have been proliferated to a higher degree, compared with CD4+ T cells in healthy donors PBMCs, which could be explained by the increased level of T reg cells in patients with MM, whilst the other types of $CD4^+$ T cells are usually decreased in MM (Brimnes et al., 2010).

Overall, the presence of allogeneic lymphoproliferative responses in MM patients' PBMCs after stimulation with the hybrid cell lines (HU266 and HRC), and HMY2, demonstrates that T cells from MM patients are not anergic. However, this experiment was done once only, due to limited number of patient samples, so these results should be treated with caution.

5.3.2 Induction of antigen specific T lymphocytes in MM patients' PBMCs:

Long-term stimulated T cell cultures were carried out for induction of antigen specific CTL in PBMCs from MM patients. The produced long-term stimulated cultures were examined for the presence and percentage of the antigen specific CTL using FITC-labelled anti-CD8 and PE-labelled HLA-A2-peptide pentamers specific for MUC1, h TERT, CS1, and MAGE-C1. In HLA-A2+ patients, the hybrid cell line HRC induced the highest significant elevation of MUC1, h TERT, CS1, and MAGE-C1 specific CTL, compared with U266, and the background PBMCs. This could be explained by the high level of the protein expression of these antigens by HRC, or its ability to process and present the examined peptides in the context of its HLA-A2. There was no significant difference between HU266 and HRC, in terms of induction of antigen specific CTLs, except for h TERT, which reflects the difference in their levels of expression of h TERT at a protein level. The difference between HU266 and the parent myeloma cell line, U266, was significant in all of the examined cultures. This may be due to the expression of the costimulatory molecules in HU266, or to appropriate presentation of TAA derived peptides by HLA-A2. U266 did not induce any significant elevation of the examined antigens specific CTL, compared with the unstimulated PBMCs, which may be due to the absence of the expression of the co-stimulatory molecules, as stimulation of T lymphocytes in absence of the secondary stimulation signals could lead to tolerance

(Bour-Jordan et al., 2011). For all of the examined antigens, HMY2 also induced significantly higher antigen specific CD8+ T lymphocytes than the unstimulated PBMCs. However, HRC induced responses were significantly higher than the responses induced by HMY2, for all of the candidate TAAs. Also, HU266 induced CS1 antigen specific CD8+ T cells responses significantly higher than HMY2. This may also reflect the protein expression levels in these cell lines, and the higher ability of the hybrids to process and present these antigens.

For each of the candidate antigens, the long-term stimulated cultures induced proportional pentamer positive CD8+ T cell populations that correlated with the level of protein antigens expressed by each of the stimulating cell lines, except h TERT. Although the protein expression level of h TERT in HU266 is much lower than HMY2, there was no significant difference between their levels of induction of h TERT antigen specific CTLs, as revealed by pentamer staining assays. This contradiction might be due to the stability of this peptide, as shown in Table 5.1. So, HU266 was able to induce h TERT specific population even at a low protein expression level. Another possible explanation might be the proficiency of HU266 in processing and presentation of h TERT, compared with HMY2.

Absence of induction of HLA-A2 restricted antigen specific $CD8^+$ T cells in PBMCs isolated from HLA-A2 negative MM patients, confirms the HLA-A2 restricted response of the produced antigen specific CD8+ T cells in HLA-A2 positive patients. The specificity of the induced antigen specific T lymphocytes was confirmed by the absence of induction of irrelevant antigen (WNF) specific T lymphocytes, in both HLA-A2+, and HLA-A2- patients' PBMCs.

The significant elevation of all of the hybrid stimulated antigen specific T lymphocytes, compared with the unstimulated T lymphocytes and U266 stimulated antigen specific T lymphocytes, demonstrates the expansion (increase of percentages) of the pentamer positive antigen specific CD8+ T cells in the hybrids long-term stimulated cultures. This can be explained by high level of expression of the candidate TAAs and the required costimulatory molecules in the hybrid cell lines.

The main limitation of the pentamer staining experiment is its ability only to detect the presence and percentages of the antigen specific CD8+ T lymphocytes in the cultures, but

does not indicate their activity. So, the presence of functional antigen specific CTLs could not be guaranteed by these experiments.

To answer the question of whether activated, functional antigen specific CTLs were generated in the long-term stimulated cultures or not, antigen-specific IFNγ and perforin releasing ELISpot assays, and cytotoxic responses were detected, using T2 cell line pulsed with the cognate antigen derived peptides.

5.3.3 Enhancement of IFNγ release of the induced antigen specific T cells in clinical samples (myeloma patients' PBMCs):

In HLA-A2+ patients' PBMCs IFNγ releasing ELISpot assays, the hybrid cell lines (HRC and HU266) induced a significant increase of the IFNγ releasing response, followed by HMY2 (with significantly lower response in case of MUC1, h TERT compared with HRC, but not HU266; CS1, and MAGE-C1 compared with HRC only at 1:100 ratio, but not HU266). These data showed good correlation with the pentamer staining experiments data. HMY2 stimulated T cells lacked MUC1, h TERT and CS1 specific IFNγ releasing responses at the lower ratio (1:25), whilst the hybrid cell lines still showed enhanced responses at this ratio, in spite of h TERT having a high protein expression level in HMY2. This indicated a lower ability of HMY2 to process and present these antigens, and to induce IFNγ releasing T cell responses, compared with the hybrid cell lines HRC and HU266. The U266 cell line did not induce any significant antigen specific elevation of IFNγ releasing responses above the background control, which might be explained by presence of T cells in an unresponsiveness state due to the absence of the costimulatory molecules, as previously discussed (Bour-Jordan et al., 2011).

In IFNγ releasing ELISpot assays, three HLA-A2$^-$ patients' samples were used as negative controls. Two of them showed no elevation of any of the examined antigen specific IFNγ releasing responses. However, one of the used HLA-A2$^-$ patients' samples showed weak IFNγ releasing response against HLA-A2 restricted TAA peptides pulsed T2 cells, but not WNF virus peptide pulsed T2 cells. For explanation of these results, we kept in mind that the observed responses were unlikely to be antigen specific responses, due to absence of HLA-A2 expression in this patient (Figure 5.6) (Darrow et al., 1989; Crowley et al., 1990). In addition, absence of this response to WNF virus peptide pulsed T2 cells suggested a lack of allogeneic (mismatched HLA) response to T2 cells. However, the

response observed might have been an allogeneic response to HLA-A2 itself, which had been stabilized by pulsing the TAA peptides on the surface of T2 cells. This stabilization led to upregulation of the expressed HLA-A2, and induction of an HLA-A2 allogeneic response. Accordingly, I measured the expression of HLA-A2 on T2 cells pulsed with the examined antigenic peptides, (and un-pulsed T2 cell lines as a negative control) using flow-cytometric analysis. In addition, I used Syfpeithi score software to estimate the binding avidity of the TAA peptides and WNF virus peptide for HLA-A2. The results indicated that the TAA derived peptides induced a relatively high binding stability and avidity for HLA-A2, compared with WNF virus peptide, by both flow cytometry and Syfpeithi score. The actual stability of peptide binding HLA-A2 and predicted HLA-A2 binding avidity of MUC1 and h TERT were higher than CS1, MAGEC1. The WNF was the lowest in both flow cytometric and Syfpeithi score data (Table 5.1). Therefore, it was suggested that an increase of HLA-A2 stability caused by loading of the TAA peptide led to some anti-HLA-A2 allogeneic response in these particular cultures. However, this response was not seen in the cytotoxicity assays, as will be discussed later. This implies that IFNγ release in this particular patient might come from mismatched allogeneic activation of CD4+ T lymphocytes recognising HLA-A2 as an alloantigen (Amir et al., 2012). Further experiments would be required, however, to confirm that this was the case.

However, using IFNγ ELISpot assay does not indicate the source of the released IFNγ, which could be $CD4^+$ T cells, $CD8^+$ T cells or NK cells. Importantly the use of K562, as a control, indicated absence of IFNγ released by NK in the stimulated cultures. Furthermore, absence of responses to an irrelevant WNF virus peptide, suggested that the released IFNγ came from antigen specific T-lymphocytes.

5.3.4 Enhancement of perforin release responses of the hybrid stimulated antigen specific T lymphocytes in MM patients' PBMCs:

The antigen specific perforin release induced by the hybrids (HU266 and HRC), compared with their parents (HMY2 and U266) was determined *in vitro* using perforin ELISpot assays. For each of the candidate antigens, HU266 and HRC induced significantly higher antigen specific perforin release responses, compared with U266, and the unstimulated PBMCs. Additionally, at the ratio of 1:100, the hybrid cell lines, induced significantly elevated antigen specific perforin release responses, compared with the level

of antigen specific perforin release induced by HMY2. The exception was that h TERT specific T lymphocytes induced by HU266 were not significantly different, compared with HMY2 induced h TERT specific T lymphocytes. This may be explained by the high level of h TERT protein expression in HMY2, or HU266 is proficient in processing and presentation of this protein, in addition to high level of stability of this protein, as discussed in pentamer staining experiments. HMY2 stimulated cultures induced antigen specific perforin release responses at significantly higher levels than the responses against PBMCs background control, which might be attributed to its expression level of the costimulatory molecules and the examined TAAs. To identify which T-cells are making the perforin, flow cytometric analysis could be used.

Absence of perforin release responses to K562 indicated absence of NK activity, and suggested that CTLs were the only producers of the perforin in these stimulated cultures. Additionally, absence of WNF specific perforin release responses demonstrated the antigenic specificity of the induced perforin releasing responses.

However, assessment of the perforin release in the induced antigen specific T lymphocytes does not necessarily show the actual killing ability of these stimulated lymphocytes to the target cells expressing these antigens. So, cytotoxicity assays were required to prove the cytotoxic ability of the induced antigen specific T lymphocytes.

5.3.5 Cytotoxicity of the induced antigen specific CTL:

The antigen specific cytotoxic effects of the hybrids induced CTLs, compared with their parents (HMY2 and U266), was determined *in vitro* using DELFIA® EuTDA Cytotoxicity assays. The environmental concerns about using radioisotopes, such as Cr release assays, for detection of T cell cytotoxicity, suggested using a nonradioactive method. Europium (Eu) release assay is a nonradioactive assay, introduced many years ago as an alternative to Cr release assays (Blomberg et al., 1986; Bohlen et al., 1994), with several advantages. The Eu assay is rapid and easy to carry out, due to the higher rate of EU release compared with Cr release; this higher rate of release is because the Eu is an inert chemical material, which does not bind to any of the cellular proteins, resulting in rapid release from the lysed cells. In contrast, Cr is present in an ionic form, which forms an ionic bond with the cellular proteins, leading to difficulty in the release from the injured cells (Hemmilä et al., 1984). Von Zons et al. (1997) found that even with reduced

incubation time, Eu assays' results were comparable to standard 51Cr release assays (Von Zons et al., 1997). In addition, it eliminates the problem of radioactive waste and health risk.

The Eu release assay showed a significant enhancement of the ability of the hybrid cell lines to induce antigen specific CTL responses, compared with the parent myeloma cell line U266, and the background control of T2 cells without peptide, or pulsed with WNF negative control peptide. In addition, the differences of the cytotoxic response induced by HMY2 and the hybrid cell lines were significant at the higher T:E ratio of 1:100, with the exception of the cytotoxic response induced by HU266 and HMY2 against h TERT peptide pulsed T2 cells, which was not significant. This may be due to the proficiency in processing and presentation of h TERT by HU266, in spite of low protein expression level of h TERT in HU266, which is supported by pentamer staining and IFNγ and perforin releasing data, as mentioned previously.

The activation of the cytotoxic effect induced by the hybrid cell lines may be rationalized by their high level of expression of CD80 and CD86, which are of crucial importance for stimulation of CTLs (Boesteanu and Katsikis, 2009). The parent myeloma cell line U266 failed to induce any significant cytotoxic response, which could be attributed to the suboptimal activation of CTLs, due to the absence of the costimulatory molecules expression. Additionally, absence of the antigen specific cytotoxicity in the stimulated HLA-A2 negative patients' PBMCs, indicated that the cytotoxic response seen was restricted to HLA-A2.

There was a good correlation between perforin release assay and the Europium (Eu) release cytotoxicity assay, as the level of perforin release response was proportional to the level of the cytotoxicity in 17 out of 20 cases (85%). This confirms the possibility of using perforin release ELISpot assay as a surrogate for the cytotoxicity assays (Malyguine et al., 2012), as discussed in section 4.3. The miscorrelation of the remaining three instances might be attributed to the presence of other cytotoxicity mechanisms rather than perforin mediated cytotoxicity, such as receptor-ligand binding of Fas /Fas L molecules (Groscurth and Filgueira, 1998; Janeway et al., 2001).

However, a very weak response of U266 long-term stimulated culture was observed against MUC1 peptide pulsed T2 cell line in all of the used assays. This release might be explained by MHC unrestricted response to MUC1, which leads to activation of numerous

T cell receptors (Takahashi and Imai, 1994; Wright et al., 2008). The unrestricted response to hypo- glycosylated MUC1 antigens expressed on tumour cells leads to exposure of previously unidentified repeated antigenic sites on each MUC1 molecule, leading to triggering of large number of TCRs, and activation of CTL. This effect can be blocked by TCR antibody, but not by MHC antibody (McKolanis and Finn, 2000; Roulois et al., 2012). However, I could not confirm if this was the case in my cultures, as data from the other HLA-A2 negative patients did not support this explanation. Another explanation is that these weak responses might be caused by the high stability of this peptide on HLA-A2 (Brossart et al., 1999), and mentioned in Table 5.1, in addition to high MUC1 protein expression level in U266, which could led to activation of some antigen specific T lymphocytes. The most important finding of all of these experiments was that the difference between the hybrid responses and U266 stimulated responses was highly significant.

Additionally, we can see that MAGE-C1 peptide was the lowest TAA peptide, in terms of its ability to enhance the stability and expression of HLA-A2 on T2 cells (Table 5.1). This may be a possible explanation of why the hybrid cell lines induced relatively low responses of MAGE-C1 specific T lymphocytes, in most of the used assays.

Using patients' myeloma cells as a target in the ELISpot assays and cytotoxicity assays was included in our original plan at the beginning of this study, but we were not able to obtain bone marrow patients' samples for the isolation of autologous tumour cells. Use of autologous tumour cells may give different results of activation of T cells, than what were seen using T2 cell line pulsed with the antigens derived peptides, for a number of reasons. Firstly, presentation of endogenous antigens may be different, compared with pulsed exogenous antigens. Secondly, the use of patients' own myeloma cells enhances the antigen specific effect, rather than the allogeneic effect. Thirdly, the myeloma cells may express other antigens at higher level (rather than using specific peptides), which may be present and activated in the hybrid stimulated cultures, leading to higher responses. The cytotoxic responses of the long-term stimulated cultures, induced by the hybrid cell lines, and HMY2 was confirmed against U266, in PBMCs of two HLA A2+ MM patients. The tumour cell line U266 would act as a source of alloantigens, as well as shared tumour antigens, which lead to specific T cell responses. This experiment could not determine which of these responses, allogeneic or antigen specific, caused this cytotoxic effect. However, the killing responses against U266 induced by the different stimulator cell lines

broadly reflected the hierarchy of responses that was seen for the antigen-specific CTL induction.

It is worth noting that PBMCs from MM patients gave higher levels of antigen specific CTLs than PBMCs from healthy donors. Therefore, it is likely that patients with myeloma are immunologically primed to the examined TAAs expressed by the tumour cells (Thurber et al., 2002). However, we planned to collect bone marrow samples from these MM patients, as mentioned previously, to ensure that these patients' tumours expressed the examined antigens, but it was difficult to have such samples during the time of the study. Also, the higher responses obtained by stimulation of the patients' PBMCs samples using the hybrid cell lines may be attributed to stimulation of both naïve and memory T cells, whilst in normal PBMCs, the stimulated cells were most likely to be only naïve cells.

The number of the patient samples (five HLA-A2 positive, and three HLA-A2 negative), was enough to confirm the enhanced effect of the hybrid compared with the parent myeloma cell line U266, given the highly consistent results. However, more patients' samples will be required in the future to examine the ability to induce different antigen specific T lymphocytes, and examine the imunostimulatory effect of the hybrids on other haematological or solid cancers.

To conclude, the hybrid cell lines (HRC and HU266) induced antigen specific CTLs to the four selected TAAs in long-term stimulated cultures *in vitro*, using PBMCs from HLA-A2+ MM patients. So did HMY2, but to a lesser extent. In contrast, the myeloma cell line, U266, failed to activate antigen specific CTLs in the cultures. These data support using these hybrid cell lines as a potential therapeutic agent to induce antigen specific CTL, for use in adoptive immunotherapy.

Chapter 6 General discussion

6.1 Introduction:

Adoptive cellular immunotherapy, using autologous or allogeneic antigen specific CTL, has been of clinical importance in treatment of patients with various haematological malignancies, including multiple myeloma (Rapoport et al., 2005; Rosenblatt et al., 2010; Rapoport et al., 2011). MM is an ideal target disease for trials of treatment using adoptive T cell immunotherapy, for a number of reasons: 1) MM is an incurable disease by using conventional therapy regimes. 2) The low toxicity, long lasting activity and better specificity of immunotherapy compared with the conventional therapies. 3) The use of allogeneic SCT with myeloablative conditioning has a good therapeutic effect on patients with MM (Bruno et al., 2009), due to graft-versus-myeloma effects, however, it is associated with a high mortality rate. 4) Allogeneic T cell infusions have been used in patients with myeloma in case of relapse after allogeneic SCT, or in partial remission, which resulted in complete remissions in a number of patients (van de Donk et al., 2006; Levenga et al., 2007; Kroger et al., 2009).

The possibility of generating antigen specific CTL to GMP standards, *in vitro*, and using them in treatment of myeloma patients has been shown previously (Rapoport et al., 2011). Rapoport et al. (2011) showed that, in the context of SCT, adoptive transfer of tumour antigen vaccine primed and costimulated autologous T cells led to acceleration of cellular and humoral immune responses in myeloma patients. Additionally, in *in vitro* studies Rosenblatt et al. (2010) showed that hybrid cell lines (made by fusion of APC and MM cells) could induce tumour antigen-specific CTL responses in *ex vivo* PBMCs, with highly activated phenotype, and marked ability to lyse autologous tumour cells.

The use of hybrid cell lines in immunotherapy is based on their expression of, and ability to process and present, TAAs in the context of their HLA class I and HLA class II molecules, which are required for stimulation of $CD8^+$ T cells, and $CD4^+$ T cells respectively (Browning, 2013; Koido, 2016). There are several advantages of using the hybrids in immunotherapy: firstly, the hybrid cell lines express most of the antigen spectrum expressed on the parent tumour cells. Therefore, there is no need to identify tumour antigens, as the hybrid cell line should express both dominant and subdominant antigens. This results in reducing the risk of an epitope loss variant arising. Secondly, any defect in T cell costimulation by the tumour cells may be amended through fusion with

the professional APC. Thirdly, the use of allogeneic APCs in the hybrid formation may lead to expression of allogeneic MHC class I, and MHC class II by the hybrid cells, which may have an adjuvant effect on stimulation of T lymphocytes (Chouaib et al., 1988). Fourthly, for using the hybrids in immunotherapy, they must have strong immunogenic ability to induce tumour specific immune responses, at a greater level than the responses induced by unfused tumour cells (Cywinski et al., 2006; Koido, 2016).

Gao et al. (1994) showed that immunization using activated B-cells fused with hepatocarcinoma cells resulted in eradication of pre-established tumour in rat models, and protected against further tumour challenge. Then, the professional APCs (DCs) have been used as a fusion partner in hybrid formation, and showed therapeutic effectiveness in a number of phase I/II clinical studies of tumour immunotherapy of different tumours, including renal cell carcinoma (Kugler et al., 1998; Avigan et al., 2004; Zhou et al., 2009), breast cancer (Avigan et al., 2004), metastatic melanoma (Trefzer et al., 1998; Krause et al., 2002; Trefzer et al., 2005), and multiple myeloma (Raje et al., 2004; Rosenblatt et al., 2011; Rosenblatt et al., 2013).

However, some limitations had been shown for using DC as an APC partner in hybrid formation. These limitations include short-lived nature, low fusion efficiency, low ability to replicate in culture, the need for hybrids to be tailor made for each patient, with limited standardized characterization after fusion, in addition to the low number of DCs in PBMC (Phan et al., 2003; Dunnion et al., 1999; Cywinski et al., 2006; Walewska et al., 2007; Koido, 2016).

The EBV B-lymphoblastoid cell line HMY2, has the same features of DCs, in terms of HLA-class I and class II, and co-stimulatory molecules expression (Thorley-Lawson and Mann, 1985; Gregory et al., 1988). In addition, its immortalised growth in the cell culture (Dunnion et al., 1999), and its sensitivity to HAT, in addition to sensitivity of tumour cells to ouabain, allow for double chemical selection of the hybrid cells after fusion (Dunnion et al., 1999; Cywinski et al., 2006; Walewska et al., 2007). Consequently, it provides a suitable alternative to DCs, as APC partner in the hybrid cell formation (Edwards et al., 1982). Therefore, a group of solid and haematological tumour cell/ cell lines were fused with HMY2, forming hybrids which have been used in several studies done previously in my supervisor's lab (Dunnion et al., 1999; Cywinski et al., 2006; Walewska et al., 2007; Mohamed, 2011; Mohamed et al., 2012b; Mohamed et al., 2012a), and formed the basis of my study. The central aim of the present study was to demonstrate that hybrid cell lines

produced (previously) by fusion of HMy2 cells and myeloma cells are capable of inducing HLA-A2-restricted tumour antigen specific cytotoxic T cells (CTL) *in vitro*, in long-term stimulated PBMCs isolated from both HLA-A2-positive multiple myeloma patients and healthy donors. The second aim of my project was to optimize the process of antigen specific CTL generation and expansion *in vitro*. To achieve these objectives, I started my study by phenotypic characterisation of the previously made myeloma hybrids, the parent APC (HMY2) and the parent myeloma cell line U266.

6.2 Phenotypic Characterisation of the Hybrids, HMY2 and U266:

The data presented in Chapter 3 relies on flow cytometric analysis of the expression of a range of important costimulatory molecules, including CD80, CD86, certain cell surface markers, CD19, CD138, and antigen presenting molecules HLA class I, class II, and HLA-A2 by the hybrid cell lines, HMy2, and U266. This analysis showed that, like the APC parent (HMY2), all the hybrid cell lines expressed CD80, CD86, and HLA class II, in spite of their absence in the parent myeloma cell U266. In addition, all the hybrid cell lines and HMY2 expressed all the candidate cell surface markers at much higher level than the parent tumour cell U266, with the exception of the myeloma marker CD138 (plasma cell marker). These expressions were stable over culture time and freeze-thaw cycles as documented previously (Walewska et al., 2007, Mohamed et al., 2012).

However, the potential use of the hybrid cell lines as immunogens, for induction of antigen specific immune response in tumour immunotherapy, is determined not only by the APC-derived phenotype, but also by the immunogenicity and effective presentation of the TAAs' peptide in the context of MHC molecules.

Accordingly, a group of TAAs (MAGE-A1, PRAME, Survivin, MAGE-A3, MUC1, RHAMM, DKK-1, CS1, MAGE-C1 and h TERT) were selected for study. These antigens are tumour specific, highly immunogenic, of essential importance in tumourigenesis, and potent targets for tumour immunotherapy, especially for antigen specific CD8+ T cells (Cloosen et al., 2006, Tai et al., 2008b, Meklat et al., 2007, Schmitt et al., 2008, Yin et al., 2012b). The expression levels of these candidate antigens by HMy2, the hybrid cell lines (HU266, HRC, HCM and HIC) and U266 were assessed using RT PCR, qRT PCR (to estimate the level of mRNA expression of these tumour antigens in the examined cell lines), and by western blot or flow cytometry (to estimate protein expression).

The relative mRNA expression level of most of the candidate TAAs by HMy2 was lower than most of the hybrid cell lines, which indicates that hybridization may cause stabilisation and elevation of the expression of these antigens than their expression level by the parent APC. The myeloma cell line U266 showed the highest values of almost all of these TAAs expression. Normal PBMCs lacked the expression of all of the examined antigens, with the exception of PRAME and survivin. A surprising result of this study was the positive expression of most of the candidate antigens by HMY2. That can be attributed to the original nature of HMy2 (EBV transfected B lymphoblastoid cell line). So, it may resemble myeloma cells (malignant plasma cells) in its antigen expression profile, as both of them were originate from B-lymphocytes. However, the mRNA and protein expression level are not colinear in most case, because of complex regulation of transcription and translation process (Greenbaum et al., 2003), so the protein abundance of the examined antigens by the used cell lines was determined.

Taking the q PCR data into consideration, it showed that MUC1, h TERT, CS1, MAGE-C1, and RHAMM were the highest expressed antigens by the hybrid cell lines of interest. Additionally, these tumour associated antigens have high prevalence in multiple myeloma patients (Cloosen et al., 2006; Meklat et al., 2007; Schmitt et al., 2008; Tai et al., 2008; de Carvalho and Vettore, 2012; Yin, 2012). Therefore, the protein expression level of these antigens by HMY2, the hybrid cell lines, and U266 was done, with the view to use these antigens for detection of presence of antigen specific CTL in cultures stimulated with these cell lines (Brossart et al., 1999; Minev et al., 2000; Lendvai, 2010; Kim et al., 2013). As a result, the level of protein expression of the examined antigens were positive for almost all the candidate cell lines compared to level of expression by PBMCs.

These results suggest that the hybrids have unequivocal phenotypes, as they retain the parent HMY2 phenotypic characterization, as well as the tumour cells' ability to express all of the candidate TAAs at the protein level, with the exception of RHAMM, which was not expressed by the hybrid cell lines. However, this doesn't guarantee their functional immunogenic ability to stimulate antigen specific T cells in the cultures.

6.3 Functional characterization of the hybrid cell lines

Increased hybrid cell lines' immunogenicity, compared with the parent tumour cell (U266), and their ability to induce allogeneic and antigen specific CTL in PBMCs isolated from normal healthy HLA-A2+ donors, were demonstrated in chapter 4. The allogeneic

activation was confirmed in short-term cultures using CFSE staining, and in long-term activated cultures using IFNγ and perforin ELISpot assay. The short-term cultures showed induction of T cell proliferative immune responses upon stimulation with the hybrid cell lines, compared to unstimulated lymphocytes. Additionally, in all cases, the CD8+ T cells were stimulated at higher levels than CD4+ T cell population. For induction of antigen specific CTLs, the long-term stimulated cultures were done by co culturing PBMCs from normal healthy HLA-A2+ individuals with the myeloma hybrid cell lines, HMY2 and U266 in the presence of IL-2 and IL-7 for 3-10 weeks.

Perforin and IFNγ release assays have been used by many investigators as a tool for assessment of activation of T cells (Malyguine et al., 2012; Koido et al., 2007; Zuber et al., 2005; Hersperger et al., 2008; Ranieri et al., 2014). The results of allogeneic IFNγ and perforin ELISpot assays indicated that the cell lines of interest induced elevated IFNγ and perforin releasing, compared with that of PBMCs background control, suggesting stimulation of responses to allogeneic major histocompatibility (MHC) molecules expressed by the stimulators. The possibility that some TAA specific responses were stimulated through presentation by MHC, cannot be confirmed by these assays.

A pilot HLA-A2 peptide pentamer staining experiment investigated the presence of TAA specific CD8+ T cells in the long-term stimulated cultures. Certain TAA (h TERT, MUC1, MAGE-C1 and CS1) were selected, based on their expression level by multiple myeloma patients, and also by their mRNA and protein expression level in the used hybrid cell lines, as mentioned previously.

The second aim of the present study was to identify the best culture protocol for efficient and rapid expansion of the activated antigen specific CTLs. Therefore, the effect of different cytokines on the induction level of antigen specific T lymphocytes was investigated, using IFNγ and perforin ELISpot assays. In my study, I added IL-7 at the beginning of the cultures (to have its effect in naïve and memory T cells) (Schluns et al., 2000; Surh and Sprent, 2008), and IL-15 (due to its antiapoptotic effect) (Lu et al., 2002; Zhang et al., 1998).

IL-7 induced a consistent increase in antigen specific IFNγ and perforin release responses for all of the examined TAA when added from the start of the cultures, which reflects the effect of IL-7 on the activation of naïve T lymphocytes (Schluns et al., 2000; Surh and Sprent, 2008). Regarding IL-15, in contrast to its minimal effect on induction of IFNγ

releasing cells, IL-15 increased the antigen-specific perforin releasing response to most of the candidate antigens in the examined cultures, which supports its effect on the survival of CD8+ T cells (Schluns and Lefrançois, 2003; Brincks and Woodland, 2010; Lu et al., 2002; Zhang et al., 1998). IL-7 and IL-15 have been shown to have a synergistic effect on the homeostatic proliferation of memory phenotype of CD8+ T cells, but not on the memory CD4+ T cells (Tan et al., 2002).

The long-term stimulated cultures, in presence of IL-2/IL-7/IL-15, were assessed for induction of HLA-A2 restricted tumour antigen (MUC1, h TERT, CS1 and MAGE-C1) specific CD8+ T lymphocytes in PBMC from HLA-$A2^+$ healthy donors, using HLA-A2-peptide pentamer staining, and IFNγ and perforin ELISpot assays. Most of the hybrid cell lines induced significantly higher levels of antigen specific CD8+ T cells, as measured by HLA-A2 peptide pentamers staining, compared with the myeloma parent cell line U266. In addition, all of the used hybrid cell lines induced significantly elevated responses, compared with unstimulated PBMCs. The hybrid cell line HRC induced significantly higher responses, compared with HMY2 in most cases, whilst HU266 induced significantly higher levels of MUC1 specific CD8+ T cells, compared with the APC (HMY2). The results of IFNγ and perforin ELISpot assays reflected the presence of functional, activated antigen-specific T cells in the cultures, and go in agreement with the pentamer staining data. The IFNγ and perforin release induced by the hybrid cell lines, with the exception of HIC, was significantly higher than that of U266, and PBMCs background responses. The hybrid cell lines HRC, and HU266 induced significantly elevated responses compared with HMY2 in most cases, whilst HIC, did not induce any significant elevation of the response, compared with HMY2, in nearly all of the cases. So, I ruled HIC out of the study.

6.4 Induction of antigen specific CTL in PBMCs isolated from patients with multiple myeloma:

The ability of the hybrids to induce allogeneic and TAA specific CTL responses in PBMCs isolated from myeloma bearing patients was demonstrated in chapter 5. The hybrid cell lines and HMY2 were capable of inducing the allogeneic activation in PBMCs isolated from MM patients, in CFSE staining experiment. This indicates the responsiveness of T cells isolated from MM patients, and the immunogenic ability of the

hybrid cell lines and HMY2, to activate them, whilst, U266 induced much lower proliferation of these T cells subsets.

In long-term stimulated cultures, the presence of antigen specific T cells in PBMCs isolated from MM patients, stimulated with the hybrid cell lines was confirmed by the peptide pentamer assays. The presence of functional antigen specific CTLs in the long-term activated cultures, was confirmed using antigen-specific IFNγ and perforin releasing ELISpot assay, and cytotoxic responses. T2 cell line pulsed with the cognate antigen derived peptides were used as stimulators in these assays. The examined hybrid cell lines have the capability to significantly enhance the production of antigen specific CTLs to all of the examined TAAs, compared with the parent myeloma cell line U266, the background control, and HMY2 in most cases, whilst U266 failed to induce antigen specific CTL populations to any of the candidate antigens compared with the unstimulated PBMCs. This may be due to the absence of the expression of the co-stimulatory molecules, as stimulation of T lymphocytes in absence of the secondary stimulation signals, could leads to tolerance (Bour-Jordan et al., 2011).

Importantly the use of K562, as a control, indicated absence of NK cells activity in the stimulated cultures. Furthermore, the absence of responses to an irrelevant WNF virus peptide, suggested that the observed responses obtained from antigen specific T-lymphocytes. Furthermore, there was a proportional relationship between the level of perforin release response and the level of the cytotoxicity in 85% of the cases, which indicates the possibility of using perforin release ELISpot assay as a surrogate for the cytotoxicity assay (Malyguine et al., 2012).

Use of autologous tumour cells to activate T cells, may give different results than using T2 cell line pulsed with the antigens derived peptides. However, it was difficult to have such samples during the time of the study.

To conclude, these data indicated that HMy2-derived hybrids had the ability to induce activated antigen specific CTL in MM patient PBMCs. This suggests that these candidate hybrid cell lines, after production under GMP protocols, represent a good target for potential use in adoptive T cell immunotherapy. Further *in vivo* clinical studies are necessary as next step to prove hybrid cell competence in tumour immunotherapy,

Interestingly, some of the examined TAA, such as h TERT and MUC1 are widely expressed in various tumours, suggesting the use of its antigen specific CTL may not be

exclusive to MM, but may be used in treatment of other tumours that express these shared tumour antigens.

7- Future plan

Adoptive tumour immunotherapy has a great scope for treatment of various haematological cancers, given the data shown in this study, and other literature studies. The hybrid cell lines have a good potential for use in immunotherapy, and warrant further investigations. To continue the work on the current project, I would

- Recruit more myeloma patients' samples (including, bone marrow samples, myeloma infiltrating lymphocytes and PBMCs), to determine the highest expressed myeloma associated antigens by the myeloma cells, and see if there are differences in stimulating PBMCs and/or MIL, using these antigen-positive hybrid cell lines.
- Cell sorting of antigen specific T cells from the hybrids stimulated cultures, and using the optimized protocol, using the Dynabeads, for expansion of the antigen specific CTL clones.
- Use an immune deficient, humanized mouse model to see the efficiency of the induced antigen specific CTL in rejection of the previously established tumours.
- Follow the GMP standards for production of antigen specific T cells *in vitro,* for trying the adoptive transfer of the induced and expanded antigen specific clinical grade CTL clones.
- Use the hybrid cell lines in long-term stimulation of PBMCs or TIL isolated from other haematological and solid tumour bearing patients.
- Determine if semi autologous (allogenic HMY2 x autologous tumour cells) hybrid cells are superior in stimulating T lymphocytes, to allogeneic hybrid cell lines. Semi autologous HMY2 x tumour hybrid cell may lead to expression of the relevant tumour-antigens, which are presented in the context of allo-MHC, leading to an adjuvant effect, and may be more efficacious therapeutically.

References

Abbas, A. K., Lichtman, A. H. & Pillai, S. 2012. *Basic immunology: functions and disorders of the immune system*: Elsevier Health Sciences.

Abbas, A. K., Lichtman, A. H. & Pillai, S. 2014. *Cellular and Molecular Immunology: with student consult Online Access*: Elsevier Health Sciences.

Ahmadi, T., Flies, A., Efebera, Y. & Sherr, D. H. 2008. CD40 Ligand-activated, antigen-specific B cells are comparable to mature dendritic cells in presenting protein antigens and major histocompatibility complex class I-and class II-binding peptides. *Immunology,* 124(1)**,** pp 129-140.

Amir, A. L., Hagedoorn, R. S., van Luxemburg-Heijs, S. A., Marijt, E. W., Kruisselbrink, A. B., Frederik Falkenburg, J. H. & Heemskerk, M. H. 2012. Identification of a coordinated CD8 and CD4 T cell response directed against mismatched HLA Class I causing severe acute graft-versus-host disease. *Biol Blood Marrow Transplant,* 18(2)**,** pp 210-9.

Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E., Fili, L., Ferri, S., Frosali, F., Giudici, F., Romagnani, P., Parronchi, P., Tonelli, F., Maggi, E. & Romagnani, S. 2007. Phenotypic and functional features of human Th17 cells. *J Exp Med,* 204(8)**,** pp 1849-61.

Aqui, N. A. & June, C. H. 2008. Post-transplant adoptive T-cell immunotherapy. *Best Pract Res Clin Haematol,* 21(3)**,** pp 503-19.

Arber, C., Feng, X., Abhyankar, H., Romero, E., Wu, M. F., Heslop, H. E., Barth, P., Dotti, G. & Savoldo, B. 2015. Survivin-specific T cell receptor targets tumor but not T cells. *J Clin Invest,* 125(1)**,** pp 157-68.

Arrenberg, P., Halder, R. & Kumar, V. 2009. Cross-regulation between distinct natural killer T cell subsets influences immune response to self and foreign antigens. *J Cell Physiol,* 218(2)**,** pp 246-50.

Atanackovic, D., Luetkens, T., Hildebrandt, Y., Arfsten, J., Bartels, K., Horn, C., Stahl, T., Cao, Y., Zander, A. R. & Bokemeyer, C. 2009. Longitudinal analysis and prognostic effect of cancer-testis antigen expression in multiple myeloma. *Clinical Cancer Research,* 15(4)**,** pp 1343-1352.

Attal, M., Moreau, P., Avet-Loiseau, H. & Harousseau, J.-L. 2007. Stem cell transplantation in multiple myeloma. *ASH Education Program Book,* 2007(1)**,** pp 311-316.

Autissier, P., Soulas, C., Burdo, T. H. & Williams, K. C. 2010. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. *Cytometry A,* 77(5)**,** pp 410-9.

Avigan, D., Vasir, B., Gong, J., Borges, V., Wu, Z., Uhl, L., Atkins, M., Mier, J., McDermott, D. & Smith, T. 2004. Fusion cell vaccination of patients with

metastatic breast and renal cancer induces immunological and clinical responses. *Clinical Cancer Research,* 10(14)**,** pp 4699-4708.

Bachmann, M. F. & Oxenius, A. 2007. Interleukin 2: from immunostimulation to immunoregulation and back again. *EMBO Rep,* 8(12)**,** pp 1142-8.

Bachmann, M. F., Wolint, P., Walton, S., Schwarz, K. & Oxenius, A. 2007. Differential role of IL-2R signaling for CD8+ T cell responses in acute and chronic viral infections. *European journal of immunology,* 37(6)**,** pp 1502-1512.

Bamford, R. N., Grant, A. J., Burton, J. D., Peters, C., Kurys, G., Goldman, C. K., Brennan, J., Roessler, E. & Waldmann, T. 1994. The interleukin (IL) 2 receptor beta chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. *Proceedings of the National Academy of Sciences,* 91(11)**,** pp 4940-4944.

Barbuto, J. A. M., Ensina, L. F., Neves, A. R., Bergami-Santos, P. C., Leite, K. R., Marques, R., Costa, F., Martins, S. C., Camara-Lopes, L. H. & Buzaid, A. C. 2004. Dendritic cell–tumor cell hybrid vaccination for metastatic cancer. *Cancer Immunology, Immunotherapy,* 53(12)**,** pp 1111-1118.

Barth, R. J., Mulé, J. J., Asher, A. L., Sanda, M. G. & Rosenberg, S. A. 1991. Identification of unique murine tumor associated antigens by tumor infiltrating lymphocytes using tumor specific secretion of interferon-γ and tumor necrosis factor. *Journal of immunological methods,* 140(2)**,** pp 269-279.

Baxevanis, C. N., Perez, S. A. & Papamichail, M. 2009. Combinatorial treatments including vaccines, chemotherapy and monoclonal antibodies for cancer therapy. *Cancer immunology, immunotherapy,* 58(3)**,** pp 317-324.

Berard, M., Brandt, K., Paus, S. B. & Tough, D. F. 2003. IL-15 Promotes the Survival of Naive and Memory Phenotype CD8+ T Cells. *The Journal of Immunology,* 170(10)**,** pp 5018-5026.

Bjorkman, P. J. & Parham, P. 1990. Structure, function, and diversity of class I major histocompatibility complex molecules. *Annual review of biochemistry,* 59(1)**,** pp 253-288.

Bjorkstrand, B., Ljungman, P., Svensson, H., Hermans, J., Alegre, A., Apperley, J., Blade, J., Carlson, K., Cavo, M. & Ferrant, A. 1996. Allogeneic bone marrow transplantation versus autologous stem cell transplantation in multiple myeloma: a retrospective case-matched study from the European Group for Blood and Marrow Transplantation. *Blood,* 88(12)**,** pp 4711-4718.

Bladé, J. & Rosiñol, L. 2007. Complications of multiple myeloma. *Hematology/oncology clinics of North America,* 21(6)**,** pp 1231-1246.

Blattman, J. N., Grayson, J. M., Wherry, E. J., Kaech, S. M., Smith, K. A. & Ahmed, R. 2003. Therapeutic use of IL-2 to enhance antiviral T-cell responses in vivo. *Nature medicine,* 9(5)**,** pp 540-547.

Blomberg, K., Granberg, C., Hemmilä, I. & Lövgren, T. 1986. Europium-labelled target cells in an assay of natural killer cell activity: I. A novel non-radioactive method based on time-resolved fluorescence. *Journal of immunological methods,* 86(2)**,** pp 225-229.

Blum, J. S., Wearsch, P. A. & Cresswell, P. 2013. Pathways of antigen processing. *Annu Rev Immunol,* 31, pp 443-73.

Boesteanu, A. C. & Katsikis, P. D. Memory T cells need CD28 costimulation to remember. Seminars in immunology, 2009. Elsevier, 69-77.

Bohlen, H., Manzke, O., Engert, A., Hertel, M., Hippler-Altenburg, R., Diehl, V. & Tesch, H. 1994. Differentiation of cytotoxicity using target cells labelled with europium and samarium by electroporation. *Journal of immunological methods,* 173(1)**,** pp 55-62.

Boise, L. H., Minn, A. J., Noel, P. J., June, C. H., Accavitti, M. A., Lindsten, T. & Thompson, C. B. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-x L. *Immunity,* 3(1)**,** pp 87-98.

Bonilla, F. A. & Oettgen, H. C. 2010. Adaptive immunity. *J Allergy Clin Immunol,* 125(2 Suppl 2)**,** pp S33-40.

Boon, T. a. B., P. 1996. Human Tumor Antigens Recognized by T Lymphocytes. *J. Exp. Med.,* 183, pp 725-729.

Bossi, G., Gerry, A. B., Paston, S. J., Sutton, D. H., Hassan, N. J. & Jakobsen, B. K. 2013. Examining the presentation of tumor-associated antigens on peptide-pulsed T2 cells. *Oncoimmunology,* 2(11)**,** pp e26840.

Bour-Jordan, H., Esensten, J. H., Martinez-Llordella, M., Penaranda, C., Stumpf, M. & Bluestone, J. A. 2011. Intrinsic and extrinsic control of peripheral T-cell tolerance by costimulatory molecules of the CD28/ B7 family. *Immunol Rev,* 241(1)**,** pp 180-205.

Boyman, O., Létourneau, S., Krieg, C. & Sprent, J. 2009. Homeostatic proliferation and survival of naive and memory T cells. *European journal of immunology,* 39(8)**,** pp 2088-2094.

Brayman, M., Thathiah, A. & Carson, D. D. 2004. MUC1: a multifunctional cell surface component of reproductive tissue epithelia. *Reproductive Biology and Endocrinology,* 2(1)**,** pp 1.

Bremers, A. J. & Parmiani, G. 2000. Immunology and immunotherapy of human cancer: present concepts and clinical developments. *Critical reviews in oncology/hematology,* 34(1)**,** pp 1-25.

Brenchley, J., Douek, D., Ambrozak, D., Chatterji, M., Betts, M., Davis, L. & Koup, R. 2002. Expansion of activated human naïve T-cells precedes effector function. *Clinical & Experimental Immunology,* 130(3)**,** pp 432-440.

Brennan, P. J., Brigl, M. & Brenner, M. B. 2013. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol,* 13(2)**,** pp 101-17.

Brimnes, M. K., Svane, I. M. & Johnsen, H. E. 2006. Impaired functionality and phenotypic profile of dendritic cells from patients with multiple myeloma. *Clin Exp Immunol,* 144(1)**,** pp 76-84.

Brimnes, M. K., Vangsted, A. J., Knudsen, L. M., Gimsing, P., Gang, A., Johnsen, H. E. & Svane, I. 2010. Increased Level of both CD4+ FOXP3+ Regulatory T Cells and CD14+ HLA-DR−/low Myeloid-Derived Suppressor Cells and Decreased Level of Dendritic Cells in Patients with Multiple Myeloma. *Scandinavian journal of immunology,* 72(6)**,** pp 540-547.

Brincks, E. L. & Woodland, D. L. 2010. Novel roles for IL-15 in T cell survival. *F1000 Biol Rep,* 2(67.

Brossart, P., Heinrich, K. S., Stuhler, G., Behnke, L., Reichardt, V. L., Stevanovic, S., Muhm, A., Rammensee, H.-G., Kanz, L. & Brugger, W. 1999. Identification of HLA-A2–restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies. *Blood,* 93(12)**,** pp 4309-4317.

Brossart, P., Schneider, A., Dill, P., Schammann, T., Grünebach, F., Wirths, S., Kanz, L., Bühring, H.-J. & Brugger, W. 2001. The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. *Cancer Research,* 61(18)**,** pp 6846-6850.

Brossart, P., Wirths, S., Stuhler, G., Reichardt, V. L., Kanz, L. & Brugger, W. 2000. Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. *Blood,* 96(9)**,** pp 3102-3108.

Brown, R., Ho, P. J., Gibson, J. & Joshua, D. 2013. T Cell Responses in Myeloma. *Advances in Biology and Therapy of Multiple Myeloma.* Springer.

Browning, M. J. 2013. Antigen presenting cell/ tumor cell fusion vaccines for cancer immunotherapy. *Hum Vaccin Immunother,* 9(7)**,** pp 1545-8.

Bruno, B., Giaccone, L., Sorasio, R. & Boccadoro, M. 2009. Role of allogeneic stem cell transplantation in multiple myeloma. *Semin Hematol,* 46(2)**,** pp 158-65.

Bubeník, J. 2004. MHC class I down-regulation: tumour escape from immune surveillance?(review). *International journal of oncology,* 25(487-492.

Burmester, G. a. P., A. 2003. Colour Atlas Of Immunology.

Burnet, M. 1957. Cancer—a biological approach: I. The processes of control. II. The significance of somatic mutation. *British medical journal,* 1(5022)**,** pp 779.

Cao, X., Zhang, W., Wang, J., Zhang, M., Huang, X., Hamada, H. & Chen, W. 1999. Therapy of established tumour with a hybrid cellular vaccine generated by using granulocyte-macrophage colony-stimulating factor genetically modified dendritic cells. *immunology-oxford-,* 97. pp616-625.

Caruso, A., Licenziati, S., Morelli, D., Fiorentini, S., Ricotta, D., Malacarne, F., Sfondrini, L. & Balsari, A. 1998. Segregation of type 1 cytokine production in human peripheral blood lymphocytes: phenotypic differences between IFN-γ and IL-2-producing cells in the CD 8+ T cell subset. *European journal of immunology,* 28(11)**,** pp 3630-3638.

Chan, D. V., Gibson, H. M., Aufiero, B. M., Wilson, A. J., Hafner, M. S., Mi, Q.-S. & Wong, H. K. 2014. Differential CTLA-4 expression in human CD4+ versus CD8+ T cells is associated with increased NFAT1 and inhibition of CD4+ proliferation. *Genes and immunity,* 15(1)**,** pp 25-32.

Chapuis, A. G., Ragnarsson, G. B., Nguyen, H. N., Chaney, C. N., Pufnock, J. S., Schmitt, T. M., Duerkopp, N., Roberts, I. M., Pogosov, G. L., Ho, W. Y., Ochsenreither, S., Wolfl, M., Bar, M., Radich, J. P., Yee, C. & Greenberg, P. D. 2013. Transferred WT1-reactive CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients. *Sci Transl Med,* 5(174)**,** pp 174.

Chapuis, A. G., Thompson, J. A., Margolin, K. A., Rodmyre, R., Lai, I. P., Dowdy, K., Farrar, E. A., Bhatia, S., Sabath, D. E. & Cao, J. 2012. Transferred melanoma-specific CD8+ T cells persist, mediate tumor regression, and acquire central memory phenotype. *Proceedings of the National Academy of Sciences,* 109(12)**,** pp 4592-4597.

Cheever, M. A., Allison, J. P., Ferris, A. S., Finn, O. J., Hastings, B. M., Hecht, T. T., Mellman, I., Prindiville, S. A., Viner, J. L. & Weiner, L. M. 2009. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clinical Cancer Research,* 15(17)**,** pp 5323-5337.

Chien, Y. H., Meyer, C. & Bonneville, M. 2014. gammadelta T cells: first line of defense and beyond. *Annu Rev Immunol,* 32, pp121-55.

Chlewicki, L. K., Holler, P. D., Monti, B. C., Clutter, M. R. & Kranz, D. M. 2005. High-affinity, peptide-specific T cell receptors can be generated by mutations in CDR1, CDR2 or CDR3. *J Mol Biol,* 346(1)**,** pp 223-39.

Chomez, P., De Backer, O., Bertrand, M., De Plaen, E., Boon, T. & Lucas, S. 2001. An overview of the MAGE gene family with the identification of all human members of the family. *Cancer research,* 61(14)**,** pp 5544-5551.

Chouaib, S., Bensussan, A., Termijtelen, A.-M., Andreeff, M., Marchiol-Fournigault, C., Fradelizi, D. & Dupont, B. 1988. Allogeneic T cell activation triggering by MHC class I antigens. *The Journal of Immunology,* 141(2)**,** pp 423-429.

Clark, H. 1991. Tumour progression and the nature of cancer. *British Journal of Cancer* 64, pp 631-44.

Cloosen, S., Gratama, J., van Leeuwen, E. B., Senden-Gijsbers, B. L., Oving, E. B., von Mensdorff-Pouilly, S., Tarp, M. A., Mandel, U., Clausen, H., Germeraad, W. T. & Bos, G. M. 2006. Cancer specific Mucin-1 glycoforms are expressed on multiple myeloma. *Br J Haematol,* 135(4)**,** pp 513-6.

Coico, R. & Sunshine, G. 2015. *Immunology: a short course*: John Wiley & Sons.

Comoli, P., De Palma, R., Siena, S., Nocera, A., Basso, S., Del Galdo, F., Schiavo, R., Carminati, O., Tagliamacco, A. & Abbate, G. 2004. Adoptive transfer of allogeneic Epstein–Barr virus (EBV)-specific cytotoxic T cells with in vitro antitumor activity boosts LMP2-specific immune response in a patient with EBV-related nasopharyngeal carcinoma. *Annals of Oncology,* 15(1)**,** pp 113-117.

Condomines, M., Veyrune, J.-L., Larroque, M., Quittet, P., Latry, P., Lugagne, C., Hertogh, C., Kanouni, T., Rossi, J.-F. & Klein, B. 2010. Increased plasma-immune cytokines throughout the high-dose melphalan-induced lymphodepletion in patients with multiple myeloma: a window for adoptive immunotherapy. *The journal of immunology,* 184(2)**,** pp 1079-1084.

Cook, G., Campbell, J., Carr, C. E., Boyd, K. S. & Franklin, I. M. 1999. Transforming growth factor beta from multiple myeloma cells inhibits proliferation and IL-2 responsiveness in T lymphocytes. *Journal of leukocyte biology,* 66(6)**,** pp 981-988.

Cooper, M. A., Fehniger, T. A. & Caligiuri, M. A. 2001. The biology of human natural killer-cell subsets. *Trends in immunology,* 22(11)**,** pp 633-640.

Coulie, P. G., Van den Eynde, B. J., van der Bruggen, P. & Boon, T. 2014. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nat Rev Cancer,* 14(2)**,** pp 135-46.

Crainie, M., Belch, A. R., Mant, M. J. & Pilarski, L. M. 1999. Overexpression of the receptor for hyaluronan-mediated motility (RHAMM) characterizes the malignant clone in multiple myeloma: identification of three distinct RHAMM variants. *Blood,* 93(5)**,** pp 1684-1696.

Crowley, N. J., Slingluff, C. L., Darrow, T. L. & Seigler, H. F. 1990. Generation of human autologous melanoma-specific cytotoxic T-cells using HLA-A2-matched allogeneic melanomas. *Cancer research,* 50(3)**,** pp 492-498.

Curtsinger, J. S., C.; Mondino, A.; Lins, D., Kedl, R., Jenkins, M. and Mescher, M. 1999. Inflammatory Cytokines Provide a Third Signal for Activation of Naive CD4 + and CD8 + T Cells. *The Journal of Immunology,,* 162, pp 3256-62.

Cywinski, A. L., Dunnion, D. J., Teobald, I., Tucker, V. C. & Browning, M. J. 2006. Hybrid cells formed by fusion of Epstein-Barr virus-associated B-lymphoblastoid cells and either marrow-derived or solid tumour-derived cell lines display different co-stimulatory phenotypes and abilities to activate allogeneic T-cell responses in vitro. *Tissue Antigens,* 68(2)**,** pp 115-26.

Dahlberg, C. I., Sarhan, D., Chrobok, M., Duru, A. D. & Alici, E. 2015. Natural Killer Cell-Based Therapies Targeting Cancer: Possible Strategies to Gain and Sustain Anti-Tumor Activity. *Front Immunol,* 6(605).

Danylesko, I., Beider, K., Shimoni, A. & Nagler, A. 2012. Novel strategies for immunotherapy in multiple myeloma: previous experience and future directions. *Clinical and Developmental Immunology,* 2012.

Darrow, T. L., Slingluff, C. & Seigler, H. 1989. The role of HLA class I antigens in recognition of melanoma cells by tumor-specific cytotoxic T lymphocytes. Evidence for shared tumor antigens. *The Journal of Immunology,* 142(9)**,** pp 3329-3335.

Davis, M. B., P. 1988. T cell antigen receptor genes and T cell recognition. *Nature,* 334. pp395-402.

de Carvalho, F. & Vettore, A. L. C., G. W. 2012. Cancer/Testis Antigen MAGE-C1/CT7: new target for multiple myeloma therapy. *Clin Dev Immunol,* 2012, pp257695-7.

de Haart, S. J., van de Donk, N. W., Minnema, M. C., Huang, J. H., Aarts-Riemens, T., Bovenschen, N., Yuan, H., Groen, R. W., McMillin, D. W., Jakubikova, J., Lokhorst, H. M., Martens, A. C., Mitsiades, C. S. & Mutis, T. 2013. Accessory cells of the microenvironment protect multiple myeloma from T-cell cytotoxicity through cell adhesion-mediated immune resistance. *Clin Cancer Res,* 19(20)**,** pp 5591-601.

Dhodapkar, M. V., Krasovsky, J. & Olson, K. 2002. T cells from the tumor microenvironment of patients with progressive myeloma can generate strong, tumor-specific cytolytic responses to autologous, tumor-loaded dendritic cells. *Proc Natl Acad Sci U S A,* 99(20)**,** pp 13009-13.

Dosani, T., Carlsten, M., Maric, I. & Landgren, O. 2015. The cellular immune system in myelomagenesis: NK cells and T cells in the development of myeloma [corrected] and their uses in immunotherapies. *Blood Cancer J,* 5(306).

Drobyski, W., Roth, M., Thibodeau, S. & Gottschall, J. 1992. Molecular remission occurring after donor leukocyte infusions for the treatment of relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation. *Bone marrow transplantation,* 10(3)**,** pp 301-304.

Dudley, M. E. & Rosenberg, S. A. 2003. Adoptive-cell-transfer therapy for the treatment of patients with cancer. *Nature Reviews Cancer,* 3(9)**,** pp 666-675.

Dudley, M. E., Wunderlich, J. R., Yang, J. C., Hwu, P., Schwartzentruber, D. J., Topalian, S. L., Sherry, R. M., Marincola, F. M., Leitman, S. F. & Seipp, C. A. 2002. A phase I study of nonmyeloablative chemotherapy and adoptive transfer of autologous tumor antigen-specific T lymphocytes in patients with metastatic melanoma. *Journal of immunotherapy (Hagerstown, Md.: 1997),* 25(3)**,** pp 243-51.

Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. & Schreiber, R. D. 2002. Cancer immunoediting: from immunosurveillance to tumor escape. *Nature immunology,* 3(11)**,** pp 991-998.

Dunnion, D., Cywinski, A., Tucker, V., Murray, A., Rickinson, A., Coulie, P. & Browning, M. 1999. Human antigen-presenting cell-tumour cell hybrids stimulate strong allogeneic.pdf. *Immunology,* 98, pp 541-550.

Edwards, P. A., Smith, C. M., Neville, A. M. & O'hare, M. J. 1982. A human-human hybridoma system based on a fast-growing mutant of the ARH-77 plasma cell leukemia-derived line. *European journal of immunology,* 12(8)**,** pp 641-648.

Ellis, J. H., Burden, M. N., Vinogradov, D. V., Linge, C. & Crowe, J. S. 1996. Interactions of CD80 and CD86 with CD28 and CTLA4. *The Journal of Immunology,* 156(8)**,** pp 2700-2709.

Epping, M. T. & Bernards, R. 2006. A causal role for the human tumor antigen preferentially expressed antigen of melanoma in cancer. *Cancer Res,* 66(22)**,** pp 10639-42.

Escors, D. 2014. Tumour immunogenicity, antigen presentation and immunological barriers in cancer immunotherapy. *New J Sci,* 2014.

Fabre, J. W. 2001. The allogeneic response and tumor immunity. *Nature medicine,* 7(6)**,** pp 649-652.

Faltynek, C. R., Wang, S., Miller, D., Young, E., Tiberio, L., Kross, K., Kelley, M. & Kloszewski, E. 1992. Administration of human recombinant IL-7 to normal and irradiated mice increases the numbers of lymphocytes and some immature cells of the myeloid lineage. *The Journal of Immunology,* 149(4)**,** pp 1276-1282.

Fichtner, S., Hose, D., Engelhardt, M., Meissner, T., Neuber, B., Krasniqi, F., Raab, M., Schonland, S., Ho, A. D., Goldschmidt, H. & Hundemer, M. 2015. Association of antigen-specific T-cell responses with antigen expression and immunoparalysis in multiple myeloma. *Clin Cancer Res,* 21(7)**,** pp 1712-21.

Finke, J. H., Rayman, P., Hart, L., Alexander, J. P., Edinger, M. G., Tubbs, R. R., Klein, E., Tuason, L. & Bukowski, R. M. 1994. Characterization of Tumor-Infiltrating Lymphocyte Subsets from Human Renal Cell Carcinoma: Specific Reactivity Defined by Cytotoxicity, Interferon-[gamma] Secretion, and Proliferation. *Journal of immunotherapy,* 15(2)**,** pp 91-104.

Flynn, K. & Müllbacher, A. 1996. Memory alloreactive cytotoxic T cells do not require costimulation for activation in vitro. *Immunology & Cell Biology,* 74(5)**,** pp 413-420.

Fukuda, S. 2006. Survivin, a cancer target with an emerging role in normal adult tissues. *Molecular Cancer Therapeutics,* 5(5)**,** pp 1087-1098.

Galson, D. L., Silbermann, R. & Roodman, G. D. 2012. Mechanisms of multiple myeloma bone disease. *Bonekey Rep,* 1(8)**,** pp 135.

Gao, Y., Wu, M., Chen, H., Wang, X., Liu, G., Li, G., Ma, J. & Sy, M.-S. 1994. Effective tumor vaccine generated by fusion of hepatoma cells with activated B cells. *Science,* 263(5146)**,** pp 518-521.

Gaponova, V. T. M., L. S.; . Misurin, A.V.; Varlamova, E.; Parovichnikova,E.N.; Savchenko, V.G. 2009. Expression of tumor-associated genes in multiple myeloma patients during high-dose chemotherapy and auto-SCT. *Cellular Therapy and Transplantation (CTT),,* 2(5)**,** pp 17-20.

Gavriatopoulou, M., Dimopoulos, M.-A., Christoulas, D., Migkou, M., Iakovaki, M., Gkotzamanidou, M. & Terpos, E. 2009. Dickkopf-1: a suitable target for the management of myeloma bone disease. *Expert opinion on therapeutic targets,* 13(7)**,** pp 839-848.

Geginat, J., Lanzavecchia, A. & Sallusto, F. 2003. Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood,* 101(11)**,** pp 4260-66.

Gerdemann, U., Katari, U., Christin, A. S., Cruz, C. R., Tripic, T., Rousseau, A., Gottschalk, S. M., Savoldo, B., Vera, J. F. & Heslop, H. E. 2011. Cytotoxic T lymphocytes simultaneously targeting multiple tumor-associated antigens to treat EBV negative lymphoma. *Molecular Therapy,* 19(12)**,** pp 2258-68.

Ghanekar, S. A., Nomura, L. E., Suni, M. A., Picker, L. J., Maecker, H. T. & Maino, V. C. 2001. Gamma interferon expression in CD8(+) T cells is a marker for circulating cytotoxic T lymphocytes that recognize an HLA A2-restricted epitope of human cytomegalovirus phosphoprotein pp65. *Clin Diagn Lab Immunol,* 8(3)**,** pp 628-31.

Ghosh, A., Holland, A. M. & van den Brink, M. R. 2014. Genetically engineered donor T cells to optimize graft-versus-tumor effects across MHC barriers. *Immunol Rev,* 257(1)**,** pp 226-36.

Goldrath, W. a. B., M. 1999. Selecting and maintaining a diverse T-cell repertoire. *Nature,* 402, pp255-261.

Gong, J., Apostolopoulos, V., Chen, D., Chen, H., Koido, S., Gendler, S. J., McKenzie, I. & Kufe, D. 2000. Selection and characterization of MUC1-specic CD8+ T cells from MUC1 transgenic mice immunized with dendritic-carcinoma fusion cells. *Immunology* 2000(101)**,** pp 316-324.

Gool, S. W., Vandenberghe, P., Boer, M. d. & Ceuppens, J. L. 1996. CD80, CD86 and CD40 provide accessory signals in a multiple-step T-cell activation model. *Immunological reviews,* 153(1)**,** pp 47-83.

Greenbaum, D., Colangelo, C., Williams, K. & Gerstein, M. 2003. Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol,* 4(9)**,** pp 117.

Greene, J. L., G .; Emswiler, J.; Peach, R.; Bajorath, J.; & Cosand, W. a. L., P. 1996. Covalent Dimerization of CD28/CTLA-4 and Oligomerization of CD80/CD86 Regulate T Cell Costimulatory Interactions. *the journal of biological chemistry,* 271(26762-26771.

Gregory, C. D., Murray, R. J., Edwards, C. & Rickinson, A. 1988. Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underlies tumor cell escape from virus-specific T cell surveillance. *Journal of Experimental Medicine,* 167(6)**,** pp 1811-1824.

Greiner, J., Ringhoffer, M., Taniguchi, M., Schmitt, A., Kirchner, D., Krähn, G., Heilmann, V., Gschwend, J., Bergmann, L., Döhner, H., Schmitt, M., 2002. Receptor for hyaluronan acid–mediated motility (RHAMM) is a new immunogenic leukemia-associated antigen in acute and chronic myeloid leukemia. *Experimental Hematology*. 30, pp1029-1035.

Groscurth, P. & Filgueira, L. 1998. Killing mechanisms of cytotoxic T lymphocytes. *Physiology,* 13(1)**,** pp 17-21.

Groscurth, P. F., L. 1998. Killing Mechanisms of Cytotoxic T Lymphocytes. *Physiology,* 13, pp 17-21.

Grossi, J. A., Raulet, D. H. & Allison, J. P. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature,* 356, pp 607-9.

Groux, H., Monte, D., Plouvier, B., Capron, A. & Ameisen, J. C. 1993. CD3-mediated apoptosis of human medullary thymocytes and activated peripheral T cells: Respective roles of interleukin-1, interleukin-2, interferon-γ and accessory cells. *European journal of immunology,* 23(7)**,** pp 1623-1629.

Grube, M., Moritz, S., Obermann, E. C., Rezvani, K., Mackensen, A., Andreesen, R. & Holler, E. 2007. CD8+ T cells reactive to survivin antigen in patients with multiple myeloma. *Clin Cancer Res,* 13(3)**,** pp 1053-60.

Guckel, B., Stumm, S., Rentzsch, C., Marme, A., Mannhardt, G. & Wallwiener, D. 2005. A CD80-transfected human breast cancer cell variant induces HER-2/neu-specific T cells in HLA-A*02-matched situations in vitro as well as in vivo. *Cancer Immunol Immunother,* 54(2)**,** pp 129-40.

Guinn, B.-A., Mohamedali, A., Thomas, N. S. B. & Mills, K. I. 2007. Immunotherapy of myeloid leukaemia. *Cancer Immunology, Immunotherapy,* 56(7)**,** pp 943-957.

Halapi, E., Werner, Å., Wahlström, J., Österborg, A., Jeddi-Tehrani, M., Yi, Q., Janson, C. H., Wigzell, H., Grunewald, J. & Mellstedt, H. 1997. T cell repertoire in patients with multiple myeloma and monoclonal gammopathy of undetermined significance: clonal CD8+ T cell expansions are found preferentially in patients with a low tumor burden. *European journal of immunology,* 27(9)**,** pp 2245-2252.

Hao, S., Bi, X., Xu, S., Wei, Y., Wu, X., Guo, X., Carlsen, S. & Xiang, J. 2004. Enhanced antitumor immunity derived from a novel vaccine of fusion hybrid between dendritic and engineered myeloma cells. *Experimental Oncology,* 26(4)**,** pp 300-306.

Hardwick, N., Buchan, S., Ingram, W., Khan, G., Vittes, G., Rice, J., Pulford, K., Mufti, G., Stevenson, F. & Guinn, B.-a. 2013. An analogue peptide from the Cancer/Testis antigen PASD1 induces CD8+ T cell responses against naturally processed peptide. *Cancer Immunity Archive,* 13(3)**,** pp 16.

Hathcock, K. S., Laszlo, G., Pucillo, C., Linsley, P. & Hodes, R. J. 1994. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. *The Journal of experimental medicine,* 180(2)**,** pp 631-640.

Haughton, G. & Amos, D. 1968. Immunology of carcinogenesis. *Cancer research,* 28(9)**,** pp 1839-1848.

Hayashi, T., Hideshima, T., Akiyama, M., Raje, N., Richardson, P., Chauhan, D. & Anderson, K. C. 2003. Ex vivo induction of multiple myeloma-specific cytotoxic T lymphocytes. *Blood,* 102(4)**,** pp 1435-42.

He, L., Ji, J.-n., Liu, S.-q., Xue, E., Liang, Q. & Ma, Z. 2014. Expression of cancer-testis antigen in multiple myeloma. *Journal of Huazhong University of Science and Technology [Medical Sciences],* 34(181-185.

Hemmilä, I., Dakubu, S., Mukkala, V.-M., Siitari, H. & Lövgren, T. 1984. Europium as a label in time-resolved immunofluorometric assays. *Analytical biochemistry,* 137(2)**,** pp 335-343.

Henderson, R. N., M.; Watkins, M.; Robbins, P.; Ball, E. and Finn, O. 1996. Human Dendritic Cells Genetically Engineered to Express High Levels of the Human Epithelial Tumor Antigen Mucin (MUC-1). *Immunology,* 56(16)**,** pp 3763-70.

Hersperger, A. R., Makedonas, G. & Betts, M. R. 2008. Flow cytometric detection of perforin upregulation in human CD8 T cells. *Cytometry Part A,* 73(11)**,** pp 1050-1057.

Ho, W. Y., Nguyen, H. N., Wolfl, M., Kuball, J. & Greenberg, P. D. 2006. In vitro methods for generating CD8+ T-cell clones for immunotherapy from the naive repertoire. *Journal of immunological methods,* 310(1)**,** pp 40-52.

Ho, W. Y., Yee, C. & Greenberg, P. D. 2002. Adoptive therapy with CD8+ T cells: it may get by with a little help from its friends. *The Journal of clinical investigation,* 110(10)**,** pp 1415-1417.

Hofmann, S., Mead, A., Malinovskis, A., Hardwick, N. R. & Guinn, B. A. 2015. Analogue peptides for the immunotherapy of human acute myeloid leukemia. *Cancer Immunol Immunother,* 64(11)**,** pp 1357-67.

Homma, S., Kikuchi, T., Ishiji, N., Ochiai, K., Takeyama, H., Saotome, H., Sagawa, Y., Hara, E., Kufe, D. & Ryan, J. 2005. Cancer immunotherapy by fusions of dendritic and tumour cells and rh-IL-12. *European journal of clinical investigation,* 35(4)**,** pp 279-286.

Hori, S. N., T. and Sakaguchi, S. 2003. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science,* 299(1057-1061.

Horna, P. & Sotomayor, E. M. 2007. Cellular and molecular mechanisms of tumor-induced T-cell tolerance. *Current cancer drug targets,* 7(1)**,** pp 41-53.

Horst, A., Hunzelman, N., Arce, S., Herber, M., Manz, R. & and Assenmacher, M. 2002. Detection and characterization of plasma cells in peripheral blood: correlation of IgE+ plasma cell frequency with IgE serum titre. *Clin Exp Immunol,* 130(3)**,** pp 8.

Houet, L. & Veelken, H. 2006. Active immunotherapy of multiple myeloma. *European Journal of Cancer,* 42(11)**,** pp 1653-1660.

Hudolin, T., Kastelan, Z., Ilic, I., Levarda-Hudolin, K., Basic-Jukic, N., Rieken, M., Spagnoli, G. C., Juretic, A. & Mengus, C. 2013. Immunohistochemical analysis of the expression of MAGE-A and NY-ESO-1 cancer/testis antigens in diffuse large B-cell testicular lymphoma. *Journal of translational medicine,* 11(123)**,** pp 1-6.

Hunder, N. N., Wallen, H., Cao, J., Hendricks, D. W., Reilly, J. Z., Rodmyre, R., Jungbluth, A., Gnjatic, S., Thompson, J. A. & Yee, C. 2008. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *New England Journal of Medicine,* 358(25)**,** pp 2698-2703.

Iannello, A., Debbeche, O., Samarani, S. & Ahmad, A. 2008. Antiviral NK cell responses in HIV infection: I. NK cell receptor genes as determinants of HIV resistance and progression to AIDS. *Journal of leukocyte biology,* 84(1)**,** pp 1-26.

Ichim, C. V. 2005. Revisiting immunosurveillance and immunostimulation: Implications for cancer immunotherapy. *Journal of translational medicine,* 3(1)**,** pp 8.

Ikeda, H., Old, L. J. & Schreiber, R. D. 2002b. The roles of IFNγ in protection against tumor development and cancer immunoediting. *Cytokine & growth factor reviews,* 13(2)**,** pp 95-109.

Jäger, D. J., E.; Knuth, A. 2001. Immune responses to tumour antigens: implications for antigen specific immunotherapy of cancer. *Clin Pathol,* 54(54)**,** pp 669–674.

Janeway, C. 1992. The T cell receptor as a multi-component sign machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annual Review o f Immunology.,* 10 pp 654-674.

Janeway Jr, C., Travers, P. & Walport, M. 2012. Immunobiology. 8th Edition. *New York: Garland Science*.

Janeway, j. r. C. A., Travers, P., Walport, M. & Shlomchik, M. J. 2001. Immunobiology: The Immune System in Health and Disease. 5th edition. *New York: Garland Science*.

Jensen, P. E. 2007. Recent advances in antigen processing and presentation. *Nature immunology,* 8(10)**,** pp 1041-1048.

Jerome, K. R., Domenech, N. & Finn, O. J. 1993. Tumor-specific cytotoxic T cell clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin complementary DNA. *The Journal of Immunology,* 151(3)**,** pp 1654-1662.

Jungbluth, A. A. M. D., S.; Niesvizky,R.; Williamson,B.; Frosina, D.; Chen,Y.; Bhardwaj,N.; & Chen-Kiang, S. O., L.; and Cho, H, J. 2005. The cancer-testis antigens CT7 (MAGE-C1) and MAGE-A3/6 are commonly expressed in multiple myeloma and correlate with plasma-cell proliferation. *The American society of haematology,* 106(1)**,** pp 167-174.

Kamimura, D. & Bevan, M. J. 2007. Naive CD8+ T cells differentiate into protective memory-like cells after IL-2–anti–IL-2 complex treatment in vivo. *Journal of Experimental Medicine,* 204(8)**,** pp 1803-1812.

Kanegane, H. & Tosato, G. 1996. Activation of naive and memory T cells by interleukin-15. *Blood,* 88(1)**,** pp 230-235.

Kaplan, M. H., Hufford, M. M. & Olson, M. R. 2015. The development and in vivo function of T helper 9 cells. *Nat Rev Immunol,* 15(5)**,** pp 295-307.

Kawano, T. C., J.; Koezuka,Y.; Toura, I.; Kaneko,Y.; Sato, H.; Kondo, E.; Harada, M.; Koseki, H.; Nakayama, T.; Tanaka, Y. and Taniguchi, M. 1998. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Vα14 NKT cells. *Proc Natl Acad Sci U S A.,* 12, pp 5690-93.

Keene, J. & Forman, J. 1982. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. *Journal of Experimental Medicine,* 155(3)**,** pp 768-782.

Kelly, R. M., Chen, J., Yauch, L. E. & Levitz, S. M. 2005. Opsonic requirements for dendritic cell-mediated responses to Cryptococcus neoformans. *Infection and immunity,* 73(1)**,** pp 592-598.

Kerr, B. M., Lear, A. L., Rowe, M., Croom-Carter, D., Young, L. S., Rookes, S. M., Gallimore, P. H. & Rickinson, A. B. 1992. Three transcriptionally distinct forms

of Epstein-Barr virus latency in somatic cell hybrids: cell phenotype dependence of virus promoter usage. *Virology,* 187(1)**,** pp 189-201.

Kershaw, M. H., Westwood, J. A. & Darcy, P. K. 2013. Gene-engineered T cells for cancer therapy. *Nature Reviews Cancer,* 13(8)**,** pp 525-541.

Kikuchi, T., Akasaki, Y., Abe, T., Fukuda, T., Saotome, H., Ryan, J. L., Kufe, D. W. & Ohno, T. 2004. Vaccination of glioma patients with fusions of dendritic and glioma cells and recombinant human interleukin 12. *Journal of Immunotherapy,* 27(6)**,** pp 452-459.

Kim, G. Y., Chae, H. J., Kim, K. H., Yoon, M. S., Lee, K. S., Lee, C. M., Moon, D. O., Lee, J. S., Jeong, Y. I., Choi, Y. H. & Park, Y. M. 2007. Dendritic cell-tumor fusion vaccine prevents tumor growth in vivo. *Biosci Biotechnol Biochem,* 71(1)**,** pp 215-21.

Kim, J. R., Horton, N. C., Mathew, S. O. & Mathew, P. A. 2013. CS1 (SLAMF7) inhibits production of proinflammatory cytokines by activated monocytes. *Inflamm Res,* 62(8)**,** pp 765-72.

Kisielow, P. T., H.; Blüthmann, H. and Boehmer, H. 1988. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature,* 335, pp 730 - 733.

Kocoglu, M. & Badros, A. 2016. The role of immunotherapy in multiple myeloma. *Pharmaceuticals,* 9(1)**,** pp 1-13.

Koido, S. 2016. Dendritic-Tumor Fusion Cell-Based Cancer Vaccines. *Int J Mol Sci,* 17(6)**,** pp 1-16.

Koido, S., Yasuhiro, T., Tajiri, H., a., Gong, J. 2007. Generation and Functional Assessment of Antigen-specific T Cells Stimulated by Fusions of Dendritic Cells and Allogeneic Breast Cancer Cells. *Vaccine,* 25(14)**,** pp 9.

Koido, S., Homma, S., Hara, E., Mitsunaga, M., Namiki, Y., Takahara, A., Nagasaki, E., Komita, H., Sagawa, Y. & Ohkusa, T. 2008. In vitro generation of cytotoxic and regulatory T cells by fusions of human dendritic cells and hepatocellular carcinoma cells. *Journal of Translational Medicine,* 6(1)**,** pp 51.

Koido, S., Homma, S., Hara, E., Namiki, Y., Takahara, A., Komita, H., Nagasaki, E., Ito, M., Ohkusa, T. & Gong, J. 2010. Regulation of tumor immunity by tumor/dendritic cell fusions. *Clinical and Developmental Immunology,* 2010(1-14.

Kolb, H.-J., Schattenberg, A., Goldman, J. M., Hertenstein, B., Jacobsen, N., Arcese, W., Ljungman, P., Ferrant, A., Verdonck, L. & Niederwieser, D. 1995. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia [see comments]. *Blood,* 86(5)**,** pp 2041-2050.

Kolb, H., Mittermuller, J., Clemm, C., Holler, E., Ledderose, G., Brehm, G., Heim, M. & Wilmanns, W. 1990. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood,* 76(12)**,** pp 2462-2465.

Komlos, L. H., J.; Kleine, T.; Livni, E.; Notmann, J.; Vardimon, D. ; Ben-rafael, Z and Halbrecht, I. 1995. Contribution of Class I HLA-A2 Antigen in Immune Reactions. *Medical Hypotheses (1995) 45, 54-58,* 45(54-58).

Komschlies, K. L., Gregorio, T. A., Gruys, M. E., Back, T. C., Faltynek, C. R. & Wiltrout, R. H. 1994. Administration of recombinant human IL-7 to mice alters the composition of B-lineage cells and T cell subsets, enhances T cell function, and induces regression of established metastases. *The Journal of Immunology,* 152(12)**,** pp 5776-5784.

Korngold, R. & Sprent, J. 1978. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. *Journal of Experimental Medicine,* 148(6)**,** pp 1687-1698.

Krausa, p. & Browning M. 1996. Genetic diversity of HLA-A2: evolutionary and functional significance. *Immunology Today,* Volume 17(4)**,** pp 165–170.

Krause, S. W., Neumann, C., Soruri, A., Mayer, S., Peters, J. H. & Andreesen, R. 2002. The treatment of patients with disseminated malignant melanoma by vaccination with autologous cell hybrids of tumor cells and dendritic cells. *Journal of Immunotherapy,* 25(5)**,** pp 421-428.

Krishnadas, D. K., Bai, F. & Lucas, K. G. 2013. Cancer testis antigen and immunotherapy. *Immunotargets Ther,* 2, pp11-9.

Kroger, N., Badbaran, A., Lioznov, M., Schwarz, S., Zeschke, S., Hildebrand, Y., Ayuk, F., Atanackovic, D., Schilling, G., Zabelina, T., Bacher, U., Klyuchnikov, E., Shimoni, A., Nagler, A., Corradini, P., Fehse, B. & Zander, A. 2009. Post-transplant immunotherapy with donor-lymphocyte infusion and novel agents to upgrade partial into complete and molecular remission in allografted patients with multiple myeloma. *Exp Hematol,* 37(7)**,** pp 791-8.

Kugler, A., Seseke, F., Thelen, P., Kallerhoff, M., Müller, G., Stuhler, G., Müller, C. & Ringert, R. 1998. Autologous and allogenic hybrid cell vaccine in patients with metastatic renal cell carcinoma. *British journal of urology,* 82, pp 487-493.

Kunzmann, V., Bauer, E., Feurle, J., Weißinger, F., Tony, H.-P. & Wilhelm, M. 2000. Stimulation of γδ T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. *Blood,* 96(2)**,** pp 384-392.

Kyle, R. A. & Vincent Rajkumar, S. 2006. Monoclonal gammopathy of undetermined significance. *British journal of haematology,* 134(6)**,** pp 573-589.

Lam, S. S., Zhou, F., Hode, T., Nordquist, R. E., Alleruzzo, L., Raker, J. & Chen, W. R. 2015. Advances in strategies and methodologies in cancer immunotherapy. *Discovery medicine,* 19(105)**,** pp 293-301.

Lee, J.-J., Choi, B.-H., Kang, H.-K., Park, M.-S., Park, J.-S., Kim, S.-K., Pham, T.-N. N., Cho, D., Nam, J.-H. & Kim, Y.-J. 2007. Induction of multiple myeloma-specific cytotoxic T lymphocyte stimulation by dendritic cell pulsing with purified and optimized myeloma cell lysates. *Leukemia & lymphoma,* 48(10)**,** pp 2022-2031.

Leibson, P. J. 1997. Signal transduction during natural killer cell activation: inside the mind of a killer. *Immunity,* 6(6)**,** pp 655-661.

Lendvai, N. G., S.; Ritter,E.; Mangone, M.; Austin1, W.; Reyner,k.; Jayabalan, D. 2010. Cellular immune responses against CT7 (MAGE-C1) and humoral responses against other cancer-testis antigens in multiple myeloma patients. *Cancer Immunity,* 10(4).

Lenschow, D. J., Walunas, L. T. & and Bluestone, J. 1996. CD28/B7 SYSTEM OF T CELL COSTIMULATION. *Immunol. 1996.,* 14(233–58).

Levenga, H., Levison-Keating, S., Schattenberg, A. V., Dolstra, H., Schaap, N. & Raymakers, R. A. 2007. Multiple myeloma patients receiving pre-emptive donor lymphocyte infusion after partial T-cell-depleted allogeneic stem cell transplantation show a long progression-free survival. *Bone Marrow Transplant,* 40(4)**,** pp 355-9.

Li, Y., Yin, Y. & Mariuzza, R. A. 2013. Structural and biophysical insights into the role of CD4 and CD8 in T cell activation. *Front Immunol,* 4(206)**,** pp 1-14.

Linley, A. J., Ahmad, M. & Rees, R. C. 2011. Tumour-associated antigens: considerations for their use in tumour immunotherapy. *Int J Hematol,* 93(3)**,** pp 263-73.

Lizée, G., Radvanyi, L. G., Overwijk, W. W. & Hwu, P. 2006. Improving antitumor immune responses by circumventing immunoregulatory cells and mechanisms. *Clinical Cancer Research,* 12(16)**,** pp 4794-4803.

Lu, J., Giuntoli, R. L., Omiya, R., Kobayashi, H., Kennedy, R. & Celis, E. 2002. Interleukin 15 promotes antigen-independent in vitro expansion and long-term survival of antitumor cytotoxic T lymphocytes. *Clinical cancer research,* 8(12)**,** pp 3877-3884.

Luckheeram, R. V., Zhou, R., Verma, A. D. & Xia, B. 2012. CD4(+)T cells: differentiation and functions. *Clin Dev Immunol,* 2012(925135).

Lugli, E., Goldman, C. K., Perera, L. P., Smedley, J., Pung, R., Yovandich, J. L., Creekmore, S. P., Waldmann, T. A. & Roederer, M. 2010. Transient and persistent effects of IL-15 on lymphocyte homeostasis in nonhuman primates. *Blood,* 116(17)**,** pp 3238-3248.

Mackensen, A., Meidenbauer, N., Vogl, S., Laumer, M., Berger, J. & Andreesen, R. 2006. Phase I study of adoptive T-cell therapy using antigen-specific CD8+ T cells for the treatment of patients with metastatic melanoma. *Journal of Clinical Oncology,* 24(31)**,** pp 5060-5069.

Malmstrom, V., Trollmo, C. & Klareskog, L. 2005. Modulating co-stimulation: a rational strategy in the treatment of rheumatoid arthritis? *Arthritis Res Ther,* 7 Suppl 2(15-20).

Malyguine, A. M., Strobl, S., Dunham, K., Shurin, M. R. & Sayers, T. J. 2012. ELISPOT Assay for Monitoring Cytotoxic T Lymphocytes (CTL) Activity in Cancer Vaccine Clinical Trials. *Cells,* 1(2)**,** pp 111-26.

Mantovani, A., Romero, P., Palucka, A. K. & Marincola, F. M. 2008. Tumour immunity: effector response to tumour and role of the microenvironment. *The Lancet,* 371(9614)**,** pp 771-783.

Manzo, T., Heslop, H. E. & Rooney, C. M. 2015. Antigen-specific T cell therapies for cancer. *Human molecular genetics,* 24(1)**,** pp 67-73.

Marincola, F. M., Jaffee, E. M., Hicklin, D. J. & Ferrone, S. 1999. Escape of human solid tumors from T–cell recognition: molecular mechanisms and functional significance. *Advances in immunology,* 74, pp 181-273.

Martin-Orozco, N., Muranski, P., Chung, Y., Yang, X. O., Yamazaki, T., Lu, S., Hwu, P., Restifo, N. P., Overwijk, W. W. & Dong, C. 2009. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity,* 31(5)**,** pp 787-98.

Maxwell, C. A., Rasmussen, E., Zhan, F., Keats, J. J., Adamia, S., Strachan, E., Crainie, M., Walker, R., Belch, A. R., Pilarski, L. M., Barlogie, B., Shaughnessy, J., Jr. & Reiman, T. 2004. RHAMM expression and isoform balance predict aggressive disease and poor survival in multiple myeloma. *Blood,* 104(4)**,** pp 1151-8.

McKolanis, J. R. & Finn, O. J. 2000. Analysis of the Frequency of MHC-Unrestricted MUC1-Specific CytotoxicT-Cells in Peripheral Blood by Limiting Dilution Assay. *Glycoprotein Methods and Protocols: The Mucins,* 463-470.

Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nature Reviews Immunology,* 1(2)**,** pp 135-145.

Meklat, F., Li, Z., Wang, Z., Zhang, Y., Zhang, J., Jewell, A. & Lim, S. H. 2007. Cancer-testis antigens in haematological malignancies. *Br J Haematol,* 136(6)**,** pp 769-76.

Melief, C. J. 2003. Mini-review: Regulation of cytotoxic T lymphocyte responses by dendritic cells: peaceful coexistence of cross-priming and direct priming? *Eur J Immunol,* 33(10)**,** pp 2645-54.

Miceli, M. & Parnes, J. The roles of CD4 and CD8 in T cell activation. Seminars in immunology, 1991. 133-141.

Milazzo, C., Reichardt, V. L., Müller, M. R., Grünebach, F. & Brossart, P. 2003. Induction of myeloma-specific cytotoxic T cells using dendritic cells transfected with tumor-derived RNA. *Blood,* 101(3)**,** pp 977-982.

Mimura, K., Ando, T., Poschke, I., Mougiakakos, D., Johansson, C. C., Ichikawa, J., Okita, R., Nishimura, M. I., Handke, D., Krug, N., Choudhury, A., Seliger, B. & Kiessling, R. 2011. T cell recognition of HLA-A2 restricted tumor antigens is impaired by the oncogene HER2. *Int J Cancer,* 128(2)**,** pp 390-401.

Min, B., Prout, M., Hu-Li, J., Zhu, J., Jankovic, D., Morgan, E. S., Urban, J. F., Jr., Dvorak, A. M., Finkelman, F. D., LeGros, G. & Paul, W. E. 2004. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J Exp Med,* 200(4)**,** pp 507-17.

Minev, B., Hipp, J., Firat, H., Schmidt, J. D., Langlade-Demoyen, P. & Zanetti, M. 2000. Cytotoxic T cell immunity against telomerase reverse transcriptase in humans. *Proc Natl Acad Sci U S A,* 97(9)**,** pp 4796-801.

Mittal, D., Gubin, M. M., Schreiber, R. D. & Smyth, M. J. 2014. New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. *Curr Opin Immunol,* 27, pp 16-25.

Mohamed, Y. S., Bashawri, L. A., Vatte, C., Abu-Rish, E. Y., Cyrus, C., Khalaf, W. S. & Browning, M. J. 2016. The in vitro generation of multi-tumor antigen-specific cytotoxic T cell clones: Candidates for leukemia adoptive immunotherapy following allogeneic stem cell transplantation. *Mol Immunol,* 77, pp79-88.

Mohamed, Y. S., Dunnion, D., Teobald, I., Walewska, R. & Browning, M. J. 2012a. In vitro evaluation of human hybrid cell lines generated by fusion of B-lymphoblastoid cells and ex vivo tumour cells as candidate vaccines for haematological malignancies. *Vaccine,* 30(46)**,** pp 6578-87.

Mohamed, Y. S., Dunnion, D., Teobald, I., Walewska, R. & Browning, M. J. 2012b. Long-lived fusions of human haematological tumour cells and B-lymphoblastoid cells induce tumour antigen-specific cytotoxic T-cell responses in vitro. *Immunobiology,* 217(7)**,** pp 719-29.

Mohamed, Y. S. A. 2011. *The use of antigen presenting cell/tumour cell hybrids for the in vitro induction of tumour-specific T cells.* PhD thesis, University of Leicester.

Montes, M., Rufer, N., Appay, V., Reynard, S., Pittet, M. J., Speiser, D. E., Guillaume, P., Cerottini, J. C., Romero, P. & Leyvraz, S. 2005. Optimum in vitro expansion of human antigen-specific CD8 T cells for adoptive transfer therapy. *Clin Exp Immunol,* 142(2)**,** pp 292-302.

Morgan, R. A., Chinnasamy, N., Abate-Daga, D. D., Gros, A., Robbins, P. F., Zheng, Z., Feldman, S. A., Yang, J. C., Sherry, R. M. & Phan, G. Q. 2013. Cancer regression and neurologic toxicity following anti-MAGE-A3 TCR gene therapy. *Journal of immunotherapy (Hagerstown, Md.: 1997),* 36(2)**,** pp 133.

Morton, D. L., Foshag, L. J., Hoon, D., Nizze, J. A., Famatiga, E., Wanek, L., Chang, C., Davtyan, D., Gupta, R. & Elashoff, R. 1992. Prolongation of survival in metastatic melanoma after active specific immunotherapy with a new polyvalent melanoma vaccine. *Annals of surgery,* 216(4)**,** pp 463-82.

Mosmann, T. R. & Coffman, R. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual review of immunology,* 7(1)**,** pp 145-173.

Moviglia, G. A., Carrizo, A. G., Varela, G., Gaeta, C. A., de Lima, A. P., Farina, P. & Molina, H. 2008. Preliminary report on tumor stem cell/B cell hybridoma vaccine for recurrent glioblastoma multiforme. *Hematology/oncology and stem cell therapy,* 1(1)**,** pp 3-13.

Mueller, S. N., Gebhardt, T., Carbone, F. R. & Heath, W. R. 2013. Memory T cell subsets, migration patterns, and tissue residence. *Annu Rev Immunol,* 31,pp137-61.

Mueller, Y. M., Petrovas, C., Bojczuk, P. M., Dimitriou, I. D., Beer, B., Silvera, P., Villinger, F., Cairns, J. S., Gracely, E. J., Lewis, M. G. & Katsikis, P. D. 2005. Interleukin-15 increases effector memory CD8+ t cells and NK Cells in simian immunodeficiency virus-infected macaques. *J Virol,* 79(8)**,** pp 4877-85.

Murakami, H., Ogawara, H. & Hiroshi, H. 2004. Th1/Th2 cells in patients with multiple myeloma. *Hematology,* 9(1)**,** pp 41-5.

Muul, L. M., Spiess, P. J., Director, E. P. & Rosenberg, S. A. 1987. Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma. *The Journal of Immunology,* 138(3)**,** pp 989-995.

Nelson, M. H. & Paulos, C. M. 2015. Novel immunotherapies for hematologic malignancies. *Immunol Rev,* 263(1)**,** pp 90-105.

Nguyen-Pham, T.-N., Lee, Y.-K., Lee, H.-J., Kim, M.-H., Yang, D.-H., Kim, H.-J. & Lee, J.-J. 2012b. Cellular immunotherapy using dendritic cells against multiple myeloma. *The Korean journal of hematology,* 47(1)**,** pp 17-27.

Nishikawa, H. M., Y; Ishida, T. ; Gnjatic S.; Sato,E.; Mori, F.; Sugiyama,D.; & Ito, A. F., Y., Utsunomiya,A.; Inagaki,H.; Old,L.; UedaR.; and Sakaguchi, S. 2012. Cancer/testis antigens are novel targets of immunotherapy for adult T-cell leukemia/lymphoma. *Immunobiology,* 119(13)**,** pp 3097-104.

Nishimura, M., Mitsunaga, S., Akaza, T., Mitomi, Y., Tadokoro, K. & Juji, T. 1994. Protection against natural killer cells by interferon-gamma treatment of K562 cells cannot be explained by augmented major histocompatibility complex class I expression. *Immunology,* 83(1)**,** pp 75-80.

Noonan, K. A., Huff, C. A., Davis, J., Lemas, M. V., Fiorino, S., Bitzan, J., Ferguson, A., Emerling, A., Luznik, L., Matsui, W., Powell, J., Fuchs, E., Rosner, G. L., Epstein, C., Rudraraju, L., Ambinder, R. F., Jones, R. J., Pardoll, D. & Borrello, I. 2015. Adoptive transfer of activated marrow-infiltrating lymphocytes induces

measurable antitumor immunity in the bone marrow in multiple myeloma. *Sci Transl Med,* 7(288)**,** pp 288ra78.

Noto, H., Takahashi, T., Makiguchi, Y., Hayashi, T., Hinoda, Y. & Imai, K. 1997. Cytotoxic T lymphocytes derived from bone marrow mononuclear cells of multiple myeloma patients recognize an underglycosylated form of MUC1 mucin. *International Immunology,* 9(5)**,** pp 791-798.

Novellino, L., Castelli, C. & Parmiani, G. 2005. A listing of human tumor antigens recognized by T cells: March 2004 update. *Cancer Immunol Immunother,* 54(3)**,** pp 187-207.

Nuber, N., Curioni-Fontecedro, A., Matter, C., Soldini, D., Tiercy, J. M., von Boehmer, L., Moch, H., Dummer, R., Knuth, A. & van den Broek, M. 2010. Fine analysis of spontaneous MAGE-C1/CT7-specific immunity in melanoma patients. *Proc Natl Acad Sci U S A,* 107(34)**,** pp 15187-92.

O'Connell, F. P., Pinkus, J. L. & Pinkus, G. S. 2004. CD138 (Syndecan-1), a Plasma Cell Marker. *American Journal of Clinical Pathology,* 121(2)**,** pp 254-263.

Ocadlikova, D., Kryukov, F., Mollova, K., Kovarova, L., Buresdova, I., Matejkova, E., Penka, M., Buchler, T., Hajek, R. & Michálek, J. 2009. Generation of myeloma-specific T cells using dendritic cells loaded with MUC1-and hTERT-drived nonapeptides or myeloma cell apoptotic bodies. *Neoplasma,* 57(5)**,** pp 455-464.

Oelke, M., Moehrle, U., Chen, J.-L., Behringer, D., Cerundolo, V., Lindemann, A. & Mackensen, A. 2000. Generation and purification of CD8+ melan-A-specific cytotoxic T lymphocytes for adoptive transfer in tumor immunotherapy. *Clinical Cancer Research,* 6(5)**,** pp 1997-2005.

Osborne, L. C., Dhanji, S., Snow, J. W., Priatel, J. J., Ma, M. C., Miners, M. J., Teh, H. S., Goldsmith, M. A. & Abraham, N. 2007. Impaired CD8 T cell memory and CD4 T cell primary responses in IL-7R alpha mutant mice. *J Exp Med,* 204(3)**,** pp 619-31.

Palena, Z., M.; Schlom, J. and Tsang, K. 2004. Human B cells that hyperexpress a triad of costimulatory molecules via avipox-vector infection: an alternative source of efficient antigen-presenting cells. *Blood,* 2004(104)**,** pp 192-199.

Palumbo, A. A., K. 2011. Multiple Myeloma. *The New Engl and journal of medicine,* 364, pp1046-60.

Parker, D. C. 1993. T cell-dependent B cell activation. *Annual review of immunology,* 11(1)**,** pp 331-360.

Parkhurst, M. R., Yang, J. C., Langan, R. C., Dudley, M. E., Nathan, D.-A. N., Feldman, S. A., Davis, J. L., Morgan, R. A., Merino, M. J. & Sherry, R. M. 2011. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Molecular Therapy,* 19(3)**,** pp 620-626.

Pascal, L. E., True, L. D., Campbell, D. S., Deutsch, E. W., Risk, M., Coleman, I. M., Eichner, L. J., Nelson, P. S. & Liu, A. Y. 2008. Correlation of mRNA and protein levels: cell type-specific gene expression of cluster designation antigens in the prostate. *BMC Genomics,* 2008(9)**,** pp 246.

Perica, K., Varela, J. C., Oelke, M. & Schneck, J. 2015. Adoptive T cell immunotherapy for cancer. *Rambam Maimonides Med J,* 6(1).

Phan, V., Errington, F., Cheong, S. C., Kottke, T., Gough, M., Altmann, S., Brandenburger, A., Emery, S., Strome, S., Bateman, A., Bonnotte, B., Melcher, A. & Vile, R. 2003. A new genetic method to generate and isolate small, short-lived but highly potent dendritic cell-tumor cell hybrid vaccines. *Nat Med,* 9(9)**,** pp 1215-9.

Pilarski, L. M., Andrews, E. J., Mant, M. J. & Ruether, B. A. 1986. Humoral immune deficiency in multiple myeloma patients due to compromised B-cell function. *Journal of clinical immunology,* 6(6)**,** pp 491-501.

Player, M. A., Barracchini, K. C., Simonis, T. B., Rivoltini, L., Arienti, F., Castelli, C., Mazzocchi, A., Belli, F. & Parmiani, G. 1996. Differences in frequency distribution of HLA-A2 subtypes between North American and Italian white melanoma patients: relevance for epitope specific vaccination protocols. *Journal of Immunotherapy,* 19(5)**,** pp 357-363.

Pozzi, S., Fulciniti, M., Yan, H., Vallet, S., Eda, H., Patel, K., Santo, L., Cirstea, D., Hideshima, T., Schirtzinge, L., Kuhstoss, S., Anderson, K. C., Munshi, N., Scadden, D., Kronenberg, H. M. & Raje, N. 2013. In vivo and in vitro effects of a novel anti-Dkk1 neutralizing antibody in multiple myeloma. *Bone,* 53(2)**,** pp 487-96.

Pratt, G., Goodyear, O. & Moss, P. 2007. Immunodeficiency and immunotherapy in multiple myeloma. *British journal of haematology,* 138(5)**,** pp 563-579.

Pule, M. A., Savoldo, B., Myers, G. D., Rossig, C., Russell, H. V., Dotti, G., Huls, M. H., Liu, E., Gee, A. P. & Mei, Z. 2008. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nature medicine,* 14(11)**,** pp 1264-1270.

Qian, J., Wang, S., Yang, J., Xie, J., Lin, P., Freeman, M. E. & Yi, Q. 2005. Targeting heat shock proteins for immunotherapy in multiple myeloma: generation of myeloma-specific CTLs using dendritic cells pulsed with tumor-derived gp96. *Clinical Cancer Research,* 11(24)**,** pp 8808-8815.

Qian, J., Xie, J., Hong, S., Yang, J., Zhang, L., Han, X., Wang, M., Zhan, F., Shaughnessy, J. D., Jr., Epstein, J., Kwak, L. W. & Yi, Q. 2007. Dickkopf-1 (DKK1) is a widely expressed and potent tumor-associated antigen in multiple myeloma. *Blood,* 110(5)**,** pp 1587-94.

Qiang, Y.-W., Barlogie, B., Rudikoff, S. & Shaughnessy, J. D. 2008. Dkk1-induced inhibition of Wnt signaling in osteoblast differentiation is an underlying mechanism of bone loss in multiple myeloma. *Bone,* 42(4)**,** pp 669-680.

Raez, L. E., Cassileth, P. A., Schlesselman, J. J., Sridhar, K., Padmanabhan, S., Fisher, E. Z., Baldie, P. A. & Podack, E. R. 2004. Allogeneic vaccination with a B7.1 HLA-A gene-modified adenocarcinoma cell line in patients with advanced non-small-cell lung cancer. *J Clin Oncol,* 22(14)**,** pp 2800-7.

Raje, N., Hideshima, T., Davies, F. E., Chauhan, D., Treon, S. P., Young, G., Tai, Y. T., Avigan, D., Gong, J. & Schlossman, R. L. 2004. Tumour cell/dendritic cell fusions as a vaccination strategy for multiple myeloma. *British journal of haematology,* 125(3)**,** pp 343-352.

Ramanathan, R. K., Lee, K. M., McKolanis, J., Hitbold, E., Schraut, W., Moser, A. J., Warnick, E., Whiteside, T., Osborne, J., Kim, H., Day, R., Troetschel, M. & Finn, O. J. 2005. Phase I study of a MUC1 vaccine composed of different doses of MUC1 peptide with SB-AS2 adjuvant in resected and locally advanced pancreatic cancer. *Cancer Immunol Immunother,* 54(3)**,** pp 254-64.

Ranieri, E., Popescu, I. & Gigante, M. 2014. CTL ELISPOT Assay. *Cytotoxic T-Cells: Methods and Protocols,* 75-86.

Rapoport, A. P., Aqui, N. A., Stadtmauer, E. A., Vogl, D. T., Fang, H.-B., Cai, L., Janofsky, S., Chew, A., Storek, J. & Akpek, G. 2011. Combination immunotherapy using adoptive T-cell transfer and tumor antigen vaccination on the basis of hTERT and survivin after ASCT for myeloma. *Blood,* 117(3)**,** pp 788-797.

Rapoport, A. P., Stadtmauer, E. A., Aqui, N., Badros, A., Cotte, J., Chrisley, L., Veloso, E., Zheng, Z., Westphal, S. & Mair, R. 2005. Restoration of immunity in lymphopenic individuals with cancer by vaccination and adoptive T-cell transfer. *Nature medicine,* 11(11)**,** pp 1230-1237.

Ratta, M., Fagnoni, F., Curti, A., Vescovini, R., Sansoni, P., Oliviero, B., Fogli, M., Ferri, E., Della Cuna, G. R. & Tura, S. 2002. Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. *Blood,* 100(1)**,** pp 230-237.

Redeker, A. & Arens, R. 2016. Improving Adoptive T Cell Therapy: The Particular Role of T Cell Costimulation, Cytokines, and Post-Transfer Vaccination. *Frontiers in Immunology,* 7(345)**,** pp 1-17.

Restifo, N. P., Dudley, M. E. & Rosenberg, S. A. 2012. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol,* 12(4)**,** pp 269-81.

Robertson, F. C., Berzofsky, J. A. & Terabe, M. 2014. NKT cell networks in the regulation of tumor immunity. *Front Immunol,* 5(543)**,** pp 1-12.

Röllig, C., Knop, S. & Bornhäuser, M. 2015. Multiple myeloma. *The Lancet,* 385(9983)**,** pp 2197-2208.

Rosenberg, S. A. & Dudley, M. E. 2009. Adoptive cell therapy for the treatment of patients with metastatic melanoma. *Curr Opin Immunol,* 21(2)**,** pp 233-40.

Rosenberg, S. A., Packard, B. S., Aebersold, P. M., Solomon, D., Topalian, S. L., Toy, S. T., Simon, P., Lotze, M. T., Yang, J. C. & Seipp, C. A. 1988. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. *New England Journal of Medicine,* 319(25)**,** pp 1676-1680.

Rosenberg, S. A. & Restifo, N. P. 2015. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science,* 348(6230)**,** pp 62-68.

Rosenberg, S. A., Yannelli, J. R., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Weber, J. S., Parkinson, D. R., Seipp, C. A., Einhorn, J. H. & White, D. E. 1994. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *Journal of the National Cancer Institute,* 86(15)**,** pp 1159-1166.

Rosenblatt, J., Avivi, I., Vasir, B., Uhl, L., Munshi, N. C., Katz, T., Dey, B. R., Somaiya, P., Mills, H. & Campigotto, F. 2013. Vaccination with dendritic cell/tumor fusions following autologous stem cell transplant induces immunologic and clinical responses in multiple myeloma patients. *Clinical Cancer Research,* 19(13)**,** pp 3640-3648.

Rosenblatt, J., Vasir, B., Uhl, L., Blotta, S., Macnamara, C., Somaiya, P., Wu, Z., Joyce, R., Levine, J. D., Dombagoda, D., Yuan, Y. E., Francoeur, K., Fitzgerald, D., Richardson, P., Weller, E., Anderson, K., Kufe, D., Munshi, N. & Avigan, D. 2011. Vaccination with dendritic cell/tumor fusion cells results in cellular and humoral antitumor immune responses in patients with multiple myeloma. *Blood,* 117(2)**,** pp 393-402.

Rosenblatt, J., Wu, Z., Vasir, B., Zarwan, C., Stone, R., Mills, H., Friedman, T., Konstantinopoulos, P. A., Spentzos, D., Ghebremichael, M., Stevenson, K., Neuberg, D., Levine, J. D., Joyce, R., Tzachanis, D., Boussiotis, V., Kufe, D. & Avigan, D. 2010. Generation of tumor-specific T lymphocytes using dendritic cell/tumor fusions and anti-CD3/CD28. *J Immunother,* 33(2)**,** pp 155-66.

Roulois, D., Grégoire, M. & Fonteneau, J.-F. 2012. MUC1-specific cytotoxic T lymphocytes in cancer therapy: induction and challenge. *BioMed research international,* 2013(871936)**,** pp 1-10.

Ruffini, P. A. & Kwak, L. W. Immunotherapy of multiple myeloma. Seminars in hematology, 2001. Elsevier, 260-267.

Russell, J. H. & Ley, T. J. 2002. Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol,* 2002(20)**,** pp 323-70.

Sallusto, F., Lenig, D., Forster, R., Lipp, M. and Lanzavecchia, A. 1999. Two subsets of memory T lymphocytes with distinct homing potential and effector functions. *Nature.,* 401(708-712.

Schluns, K. S., Kieper, W. C., Jameson, S. C. & Lefrançois, L. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nature immunology,* 1(5)**,** pp 426-432.

Schluns, K. S. & Lefrançois, L. 2003. Cytokine control of memory T-cell development and survival. *Nature Reviews Immunology,* 3(4)**,** pp 269-279.

Schmidt, S.M., Schag, K., Muller, M.R., Weck, M.M., Appel, S., Kanz, L., Grunebach, F., Brossart, P., 2003. Survivin is a shared tumor-associated antigen expressed in a broad variety of malignancies and recognized by specific cytotoxic T cells. Blood. 102, 571-576.

Schmitt, M., Schmitt, A., Rojewski, M. T., Chen, J., Giannopoulos, K., Fei, F., Yu, Y., Gotz, M., Heyduk, M., Ritter, G., Speiser, D. E., Gnjatic, S., Guillaume, P., Ringhoffer, M., Schlenk, R. F., Liebisch, P., Bunjes, D., Shiku, H., Dohner, H. & Greiner, J. 2008. RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome, and multiple myeloma elicits immunologic and clinical responses. *Blood,* 111(3)**,** pp 1357-65.

Schroder, K., Hertzog, P. J., Ravasi, T. & Hume, D. A. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol,* 75(2)**,** pp 163-89.

Schwartz, R., Mueller, D., Jenkins, M. & Quill, H. T-cell clonal anergy. Cold Spring Harbor Symposia on Quantitative Biology, 1989. Cold Spring Harbor Laboratory Press, 605-610.

Schwartz, R. H. 2003. T cell anergy. *Annual review of immunology,* 21(1)**,** pp 305-334.

Seddon, B. & Zamoyska, R. 2002. TCR and IL-7 receptor signals can operate independently or synergize to promote lymphopenia-induced expansion of naive T cells. *The Journal of Immunology,* 169(7)**,** pp 3752-3759.

Sette, A. & Sidney, J. 1999. Nine major HLA class I supertypes account for the vast preponderance of HLA-A and-B polymorphism. *Immunogenetics,* 50(3-4)**,** pp 201-212.

Shortman, K. a. W., L. 1996. Early T lymphocyte progenitors. *Annu. Rev. Immunol.,* 14,PP 29-47.

Sigal, L. H. 2012. Basic Science for the Clinician 56: Inducible T-cell Costimulator—The World of Costimulation Gets More Complicated… and Interesting. *JCR: Journal of Clinical Rheumatology,* 18(4)**,** pp 212-216.

Simpson, A. J., Caballero, O. L., Jungbluth, A., Chen, Y.-T. & Old, L. J. 2005. Cancer/testis antigens, gametogenesis and cancer. *Nature Reviews Cancer,* 5(8)**,** pp 615-625.

Smith, J. W., Walker, E. B., Fox, B. A., Haley, D., Wisner, K. P., Doran, T., Fisher, B., Justice, L., Wood, W. & Vetto, J. 2003. Adjuvant immunization of HLA-A2–

positive melanoma patients with a modified gp100 peptide induces peptide-specific CD8+ T-cell responses. *Journal of clinical oncology,* 21(8)**,** pp 1562-1573.

Smith, S. N., Sommermeyer, D., Piepenbrink, K. H., Blevins, S. J., Bernhard, H., Uckert, W., Baker, B. M. & Kranz, D. M. 2013. Plasticity in the contribution of T cell receptor variable region residues to binding of peptide-HLA-A2 complexes. *J Mol Biol,* 425(22)**,** pp 4496-507.

Souberbielle, B., Westby, M., Ganz, S., Kayaga, J., Mendes, R., Morrow, W. & Dalgleish, A. 1998. Comparison of four strategies for tumour vaccination in the B16-F10 melanoma model. *Gene therapy,* 5(11)**,** pp 1447-1454.

Southam, C. M., Brunschwig, A., Levin, A. G. & Dizon, Q. S. 1966. Effect of leukocytes on transplantability of human cancer. *Cancer,* 19(11)**,** pp 1743-1753.

Spanoudakis, E., Hu, M., Naresh, K., Terpos, E., Melo, V., Reid, A., Kotsianidis, I., Abdalla, S., Rahemtulla, A. & Karadimitris, A. 2009. Regulation of multiple myeloma survival and progression by CD1d. *Blood,* 113(11)**,** pp 2498-2507.

Starr, T. K., Jameson, S. C. & Hogquist, K. A. 2003. Positive and negative selection of T cells. *Annu Rev Immunol,* 21,pp139-76.

Stuhler, G., Trefzer, U. & Walden, P. 1998. Hybrid Cell Vaccination in Cancer Immunotherapy. *Gene Therapy of Cancer.* Springer.451, pp 277-282.

Surh, C. D. & Sprent, J. 2002. Regulation of naive and memory T-cell homeostasis. *Microbes and infection,* 4(1)**,** pp 51-56.

Surh, C. D. & Sprent, J. 2008. Homeostasis of naive and memory T cells. *Immunity,* 29(6)**,** pp 848-62.

Swain, S. L. 2000. CD4 T-cell memory can persist in the absence of class II. *The Royal society,* 355(407-411).

Tai, Y.-T., Dillon, M., Song, W., Leiba, M., Li, X.-F., Burger, P., Lee, A. I., Podar, K., Hideshima, T. & Rice, A. G. 2008. Anti-CS1 humanized monoclonal antibody HuLuc63 inhibits myeloma cell adhesion and induces antibody-dependent cellular cytotoxicity in the bone marrow milieu. *Blood,* 112(4)**,** pp 1329-1337.

Tai, Y.-T., Soydan, E., Song, W., Fulciniti, M., Kim, K., Hong, F., Li, X.-F., Burger, P., Rumizen, M. J. & Nahar, S. 2009. CS1 promotes multiple myeloma cell adhesion, clonogenic growth, and tumorigenicity via c-maf–mediated interactions with bone marrow stromal cells. *Blood,* 113(18)**,** pp 4309-4318.

Tajima, K., Obata, Y., Tamaki, H., Yoshida, M., Chen, Y.-T., Scanlan, M. J., Old, L. J., Kuwano, H., Takahashi, T., Takahashi, T. & Mitsudomi, T. 2003. Expression of cancer/testis (CT) antigens in lung cancer. *Lung Cancer,* 42(1)**,** pp 23-33.

Takahashi, T. M., Y.; Hinoda, Y.; Kakiuchi, H.; Nakagawa, N.; & Imai, K. Y., A. 1994. Expression of MUC1 on Myeloma Cells and Induction of HLA-Unrestricted CTL Against MUC1 from a Multiple Myeloma Patient. *The Journal of Immunology,*, 153(5), pp 2102-09.

Tan, J. T., Dudl, E., LeRoy, E., Murray, R., Sprent, J., Weinberg, K. I. & Surh, C. D. 2001. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci U S A,* 98(15)**,** pp 8732-7.

Tan, J. T., Ernst, B., Kieper, W. C., LeRoy, E., Sprent, J. & Surh, C. D. 2002. Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. *Journal of Experimental Medicine,* 195(12)**,** pp 1523-1532.

Terabe, M. & Berzofsky, J. A. 2008. Chapter 8 The Role of NKT Cells in Tumor Immunity. 101(277-348.

Thorley-Lawson, D. A. & Mann, K. P. 1985. Early events in Epstein-Barr virus infection provide a model for B cell activation. *J Exp Med,* 162(1)**,** pp 45-59.

Thurber, S. E., Khong, H. T., Kammula, U. S. & Rosenberg, S. A. 2002. Identification of Endogenous HLA-A2–Restricted Reactivity Against Shared Melanoma Antigens in Patients Using the Quantitative Real-Time Polymerase Chain Reaction. *Journal of immunotherapy (Hagerstown, Md.: 1997),* 25(1)**,** pp 63-71.

Timmerman, J. M., Czerwinski, D. K., Davis, T. A., Hsu, F. J., Benike, C., Hao, Z. M., Taidi, B., Rajapaksa, R., Caspar, C. B. & Okada, C. Y. 2002. Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. *Blood,* 99(5)**,** pp 1517-1526.

Titzer, S., Christensen, O., Manzke, O., Tesch, H., Wolf, J., Emmerich, B., Carsten, C., Diehl, V. & Bohlen, H. 2000. Vaccination of multiple myeloma patients with idiotype-pulsed dendritic cells: Immunological and clinical aspects. *British journal of haematology,* 108(4)**,** pp 805-816.

Topham, N. J. & Hewitt, E. W. 2009. Natural killer cell cytotoxicity: how do they pull the trigger? *Immunology,* 128(1)**,** pp 7-15.

Trefzer, U., Herberth, G., Wohlan, K., Milling, A., Thiemann, M., Sharav, T., Sparbier, K., Sterry, W. & Walden, P. 2005. Tumour-dendritic hybrid cell vaccination for the treatment of patients with malignant melanoma: immunological effects and clinical results. *Vaccine,* 23(17)**,** pp 2367-2373.

Trefzer, U. & Walden, P. 2003. Hybrid-cell vaccines for cancer immune therapy. *Molecular biotechnology,* 25(1)**,** pp 63-69.

Trefzer, U., Weingart, G., Chen, Y., Adrian, K., Audring, H., Winter, H., Guo, Y., Sterry, W. & Walden, P. 1998. A phase I trial with a hybrid cell vaccine in patients with metastatic melanoma. *Gene Therapy of Cancer.* Springer.(451), pp519-525.

Trefzer, U., Weingart, G., Chen, Y., Herberth, G., Adrian, K., Winter, H., Audring, H., Guo, Y., Sterry, W. & Walden, P. 2000. Hybrid cell vaccination for cancer immune therapy: first clinical trial with metastatic melanoma. *International Journal of Cancer,* 85(5)**,** pp 618-626.

Tricot, G. J. 2002. New insights into role of microenvironment in multiple myeloma. *International journal of hematology,* 76(1)**,** pp 334-336.

Tripputi, P., Guerin, S. L. & Moore, D. D. 1988. Two mechanisms for the extinction of gene expression in hybrid cells. *Science,* 241(4870)**,** pp 1205.

Turin, I., Pedrazzoli, P., Tullio, C., Montini, E., La Grotteria, M. C., Schiavo, R., Perotti, C., Locatelli, F., Carretto, E., Maccario, R., Siena, S. & Montagna, D. 2007. GMP production of anti-tumor cytotoxic T-cell lines for adoptive T-cell therapy in patients with solid neoplasia. *Cytotherapy,* 9(5)**,** pp 499-507.

Turtle, C. J., Brown, R. D., Joshua, D. E. & Hart, D. N. 2004. DC in multiple myeloma immunotherapy. *Cytotherapy,* 6(2)**,** pp 128-37.

van Baren, N., Brasseur, F., Godelaine, D., Hames, G., Ferrant, A., Lehmann, F., Andre, M., Ravoet, C., Doyen, C., Spagnoli, G.C., Bakkus, M., Thielemans, K., Boon, T., 1999. Genes encoding tumor-specific antigens are expressed in human myeloma cells. *Blood.* 94, pp 1156-1164.

van de Donk, N. W., Kroger, N., Hegenbart, U., Corradini, P., San Miguel, J. F., Goldschmidt, H., Perez-Simon, J. A., Zijlmans, M., Raymakers, R. A., Montefusco, V., Ayuk, F. A., van Oers, M. H., Nagler, A., Verdonck, L. F. & Lokhorst, H. M. 2006. Prognostic factors for donor lymphocyte infusions following non-myeloablative allogeneic stem cell transplantation in multiple myeloma. *Bone Marrow Transplant,* 37(12)**,** pp 1135-41.

van der Bruggen, P. & Traversari, C. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science,* 254(5038)**,** pp 1643-7.

Van Eynde, B. D., Hainaut, P., Hérin, M., Knuth, A., Lemoine, C., Weynants, P., Van Bruggen, P. D., Fauchet, R. & Boon, T. 1989. Presence on a human melanoma of multiple antigens recognized by autologous CTL. *International journal of cancer,* 44(4)**,** pp 634-640.

Van Parijs, L., Biuckians, A., Ibragimov, A., Alt, F. W., Willerford, D. M. & Abbas, A. K. 1997. Functional responses and apoptosis of CD25 (IL-2R alpha)-deficient T cells expressing a transgenic antigen receptor. *The Journal of Immunology,* 158(8)**,** pp 3738-3745.

Varela-Rohena, A., Molloy, P. E., Dunn, S. M., Li, Y., Suhoski, M. M., Carroll, R. G., Milicic, A., Mahon, T., Sutton, D. H. & Laugel, B. 2008. Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. *Nature medicine,* 14(12)**,** pp 1390-1395.

Vasir, B., Borges, V., Wu, Z., Grosman, D., Rosenblatt, J., Irie, M., Anderson, K., Kufe, D. & Avigan, D. 2005. Fusion of dendritic cells with multiple myeloma cells

results in maturation and enhanced antigen presentation. *British journal of haematology,* 129(5)**,** pp 687-700.

Vella, A. T., Dow, S., Potter, T. A., Kappler, J. & Marrack, P. 1998. Cytokine-induced survival of activated T cells in vitro and in vivo. *Proceedings of the National Academy of Sciences,* 95(7)**,** pp 3810-3815.

Vergati, M., Intrivici, C., Huen, N.-Y., Schlom, J. & Tsang, K. Y. 2010. Strategies for cancer vaccine development. *BioMed Research International,* 2010(596432)**,** pp 1-13.

Vignard, V., Lemercier, B., Lim, A., Pandolfino, M.-C., Guilloux, Y., Khammari, A., Rabu, C., Echasserieau, K., Lang, F. & Gougeon, M.-L. 2005. Adoptive transfer of tumor-reactive Melan-A-specific CTL clones in melanoma patients is followed by increased frequencies of additional Melan-A-specific T cells. *The Journal of Immunology,* 175(7)**,** pp 4797-4805.

Villunger, A., Egle, A., Marschitz, I., Kos, M., Böck, G., Ludwig, H., Geley, S., Kofler, R. & Greil, R. 1997. Constitutive expression of Fas (Apo-1/CD95) ligand on multiple myeloma cells: a potential mechanism of tumor-induced suppression of immune surveillance. *Blood,* 90(1)**,** pp 12-20.

Vivier, E., Raulet, D. H., Moretta, A., Caligiuri, M. A., Zitvogel, L., Lanier, L. L., Yokoyama, W. M. & Ugolini, S. 2011. Innate or adaptive immunity? The example of natural killer cells. *Science,* 331(6013)**,** pp 44-49.

Von Boehmer, H. & Kisielow, P. 1990. Self-nonself discrimination by T cells. *Science,* 248(4961)**,** pp 1369-1373.

Von Zons, P., Crowley-Nowick, P., Friberg, D., Bell, M., Koldovsky, U. & Whiteside, T. L. 1997. Comparison of europium and chromium release assays: cytotoxicity in healthy individuals and patients with cervical carcinoma. *Clinical and diagnostic laboratory immunology,* 4(2)**,** pp 202-207.

Wakil, A. E., Wang, Z.-E., Ryan, J. C., Fowell, D. J. & Locksley, R. M. 1998. Interferon γ derived from CD4+ T cells is sufficient to mediate T helper cell type 1 development. *Journal of Experimental Medicine,* 188(9)**,** pp 1651-1656.

Walewska, R., Teobald, I., Dunnion, D., Abdulmajed, H., Aldred, M., Sadler, J., Chapman, C. & Browning, M. 2007. Preclinical development of hybrid cell vaccines for multiple myeloma. *Eur J Haematol,* 78(1)**,** pp 11-20.

Walzer, T., Dalod, M., Robbins, S. H., Zitvogel, L. & Vivier, E. 2005. Natural-killer cells and dendritic cells: "l'union fait la force". *Blood,* 106(7)**,** pp 2252-8.

Wang, K. W., G.; & and Liu, D. 2012. CD19: a biomarker for B cell development, lymphoma diagnosis and therapy. *Experimental Hematology & Oncology,* 1(1)**,** pp 1-36.

Wang, M., Yin, B., Wang, H. Y. & Wang, R.-F. 2014. Current advances in T-cell-based cancer immunotherapy. *Immunotherapy,6(12),pp 1265-78.*

Watanabe, K., Goodrich, J., Li, C., Agha, M. & Greenberg, P. 1992. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science,* 257(5067)**,** pp 238-241.

Watts, T. H. 2010. Staying alive: T cell costimulation, CD28, and Bcl-xL. *J Immunol,* 185(7)**,** pp 3785-7.

Watzl, C. & Long, E. O. 2010. Signal transduction during activation and inhibition of natural killer cells. *Current Protocols in Immunology,* 11.9 B. 1-11.9 B. 17.

Weaver, C. T., Harrington, L. E., Mangan, P. R., Gavrieli, M. & Murphy, K. M. 2006. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity,* 24(6)**,** pp 677-88.

Weber, G., Caruana, I., Rouce, R. H., Barrett, A. J., Gerdemann, U., Leen, A. M., Rabin, K. R. & Bollard, C. M. 2013a. Generation of tumor antigen-specific T cell lines from pediatric patients with acute lymphoblastic leukemia--implications for immunotherapy. *Clin Cancer Res,* 19(18)**,** pp 5079-91.

Weber, G., Gerdemann, U., Caruana, I., Savoldo, B., Hensel, N. F., Rabin, K. R., Shpall, E. J., Melenhorst, J. J., Leen, A. M., Barrett, A. J. & Bollard, C. M. 2013b. Generation of multi-leukemia antigen-specific T cells to enhance the graft-versus-leukemia effect after allogeneic stem cell transplant. *Leukemia,* 27(7)**,** pp 1538-47.

Weiden, P. L., Flournoy, N., Thomas, E. D., Prentice, R., Fefer, A., Buckner, C. D. & Storb, R. 1979. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. *New England Journal of Medicine,* 300(19)**,** pp 1068-1073.

Weiss, C., Uziel, O., Wolach, O., Nordenberg, J., Beery, E., Bulvick, S., Kanfer, G., Cohen, O., Ram, R., Bakhanashvili, M., Magen-Nativ, H., Shilo, N. & Lahav, M. 2012. Differential downregulation of telomerase activity by bortezomib in multiple myeloma cells-multiple regulatory pathways in vitro and ex vivo. *Br J Cancer,* 107(11)**,** pp 1844-52.

Welniak, L. A., Blazar, B. R. & Murphy, W. J. 2007. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu. Rev. Immunol.,* 25(139-170.

Westbrook, V. A., Schoppee, P. D., Diekman, A. B., Klotz, K. L., Allietta, M., Hogan, K. T., Slingluff, C. L., Patterson, J. W., Frierson, H. F. & Irvin, W. P. 2004. Genomic organization, incidence, and localization of the SPAN-X family of cancer-testis antigens in melanoma tumors and cell lines. *Clinical cancer research,* 10(1)**,** pp 101-112.

Whiteside, T. L. 2008. The tumor microenvironment and its role in promoting tumor growth. *Oncogene,* 27(45)**,** pp 5904-12.

Whiteside, T. L. 2015. The role of regulatory T cells in cancer immunology. *Immunotargets Ther,* 4, pp159-71.

Wilson, N. S., El-Sukkari, D. & Villadangos, J. A. 2004. Dendritic cells constitutively present self antigens in their immature state in vivo and regulate antigen presentation by controlling the rates of MHC class II synthesis and endocytosis. *Blood,* 103(6)**,** pp 2187-2195.

Witzens-Harig, M., Hose, D., Jünger, S., Pfirschke, C., Khandelwal, N., Umansky, L., Seckinger, A., Conrad, H., Brackertz, B. & Rème, T. 2013. Tumor cells in multiple myeloma patients inhibit myeloma-reactive T cells through carcinoembryonic antigen-related cell adhesion molecule-6. *Blood,* 121(22)**,** pp 4493-4503.

Wolfl, M. & Greenberg, P. D. 2014. Antigen-specific activation and cytokine-facilitated expansion of naive, human CD8+ T cells. *Nat Protoc,* 9(4)**,** pp 950-66.

Wright, S. E., Rewers-Felkins, K. A., Quinlin, I. S., Fogler, W. E., Phillips, C. A., Townsend, M., Robinson, W. & Philip, R. 2008. MHC-unrestricted lysis of MUC1-expressing cells by human peripheral blood mononuclear cells. *Immunol Invest,* 37(3)**,** pp 215-25.

Wu, J. I., Reed, R. B., Grabowski, P. J. & Artzt, K. 2002. Function of quaking in myelination: regulation of alternative splicing. *Proc Natl Acad Sci U S A,* 99(7)**,** pp 4233-8.

Wu, K.-D., Orme, L. M., Shaughnessy, J., Jacobson, J., Barlogie, B. & Moore, M. A. 2003. Telomerase and telomere length in multiple myeloma: correlations with disease heterogeneity, cytogenetic status, and overall survival. *Blood,* 101(12)**,** pp 4982-4989.

Wu, L., Gabriel, C. L., Parekh, V. V. & Van Kaer, L. 2009. Invariant natural killer T cells: innate-like T cells with potent immunomodulatory activities. *Tissue Antigens,* 73(6)**,** pp 535-45.

Wucherpfennig, K. W., Gagnon, E., Call, M. J., Huseby, E. S. & Call, M. E. 2010. Structural biology of the T-cell receptor: insights into receptor assembly, ligand recognition, and initiation of signaling. *Cold Spring Harb Perspect Biol,* 2(4)**,** pp a005140.

Xia, D., Chan, T. & Xiang, J. 2005. Dendritic cell/myeloma hybrid vaccine. *Multiple Myeloma: Methods and Protocols*, 225-234.

Xiao, J. & Chen, H.-S. 2004. Biological functions of melanoma-associated antigens. *World journal of gastroenterology,* 10(13)**,** pp 1849-1853.

Yasuda, T., Kamigaki, T., Kawasaki, K., Nakamura, T., Yamamoto, M., Kanemitsu, K., Takase, S., Kuroda, D., Kim, Y. & Ajiki, T. 2007. Superior anti-tumor protection and therapeutic efficacy of vaccination with allogeneic and semiallogeneic

dendritic cell/tumor cell fusion hybrids for murine colon adenocarcinoma. *Cancer Immunology, Immunotherapy,* 56(7)**,** pp 1025-1036.

Yin, L. K., Kosugi, M., Kufe, D. 2012. Inhibition of the MUC1-C oncoprotein induces multiple myeloma cell death by down-regulating TIGAR expression and depleting NADPH. *Blood,* 119(3)**,** pp 810-6.

Zarour, H., DeLeo, A., Finn, O.J. and Storkus, W.J. 2003. Categories of Tumor Antigens. *Cancer Medicine (BC Decker),* 6 Edition(chapter 12)**.**

Zhang, L., Gotz, M., Hofmann, S. & Greiner, J. 2012. Immunogenic targets for specific immunotherapy in multiple myeloma. *Clin Dev Immunol,* 2012(820394)**,** pp 1-6.

Zhang, X., Sun, S., Hwang, I., Tough, D. F. & Sprent, J. 1998. Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity,* 8(5)**,** pp 591-599.

Zhou, J., Weng, D., Zhou, F., Pan, K., Song, H., Wang, Q., Wang, H., Wang, H., Li, Y. & Huang, L. 2009. Patient-derived renal cell carcinoma cells fused with allogeneic dendritic cells elicit anti-tumor activity: in vitro results and clinical responses. *Cancer Immunology, Immunotherapy,* 58(10)**,** pp 1587-97.

Zhu, J. & Paul, W. E. 2008. CD4 T cells: fates, functions, and faults. *Blood,* 112(5)**,** pp 1557-1569.

Zou, W. 2006. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol,* 6(4)**,** pp 295-307.

Zuber, B., Levitsky, V., Jönsson, G., Paulie, S., Samarina, A., Grundström, S., Metkar, S., Norell, H., Callender, G. G. & Froelich, C. 2005a. Detection of human perforin by ELISpot and ELISA: ex vivo identification of virus-specific cells. *Journal of immunological methods,* 302(1)**,** pp 13-25.

Appendices

Appendix A:

A copy of the ethical approval for the present study from the Local Research Ethics Committee (Section 2.1.2).

University Hospitals of Leicester NHS
NHS Trust

DIRECTORATE OF RESEARCH & DEVELOPMENT

Director: **Professor D Rowbotham**
Assistant Director: **Dr David Hetmanski**
R&D Manager: **Carolyn Maloney**

Direct Dial: (0116) 258 8351
Fax No: (0116) 258 4226
20/06/2013

Research & Development Office
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

Dr Michael Browning
University of Leicester
Department of Immunology
Leicester Royal Infirmary
Leicester
University Road
LE1 5WW

Dear Dr Michael Browning

Ref: UHL 09708
Title: Immunologic studies in haematological malignancies
Project Status: Project Approved
End Date: 31/03/2014

Thank you for submitting documentation for Substantial amendment Number 1 Dated: 09th May 2013 for the above study.

I confirm that the amendment has the approval of the University Hospitals of Leicester NHS Trust R&D Department and may be implemented with immediate effect.

The documents received are as follows:

Description	Version
Participant Information Sheet: PIL and Consent	V3 Dated: 30.04.2013
Protocol	V2 Dated: 02.05.2013
Notice of Substantial Amendment (non-CTIMPs)	Amendment 1 Dated: 09.04.2013

Please be aware that any changes to these documents after approval may constitute an amendment. The process of approval for amendments MUST be followed. Failure to do so may invalidate the approval of the study at this trust.

Please ensure that all documentation and correspondence relating to this amendment are filed appropriately in the relevant site file.

Yours sincerely

Lisa Wann
R&D Team Leader

NHS
Health Research Authority

NRES Committee East Midlands - Northampton
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Tel: 0115 8839435
Fax: 0115 8839294

19 June 2013

Dr Michael Browning
Senior Clinical Lecturer in Immunology
University of Leicester
C/O Research Office
Leicester General Hospital, Gwendolen Road
Leicester
LE5 4PW

Dear Dr Browning

Study title:	**Immunologic studies in haematological malignancies**
REC reference:	**05/Q2502/26**
Protocol number:	**N/A**
Amendment number:	**Amendment 1**
Amendment date:	**09 May 2013**
IRAS project ID:	

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Participant Information Sheet: PIL and Consent	Version 3	30 April 2013
Protocol	Version 2	02 May 2013
Notice of Substantial Amendment (non-CTIMPs)	Amendment 1	09 May 2013

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

05/Q2502/26:	Please quote this number on all correspondence

Yours sincerely

Mr Ken Willis
Chair

E-mail: NRESCommittee.EastMidlands-Northampton@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *N/A. R&D contact not specified in database.*
Carolyn Maloney

Appendix B:

A copy of PIL and consent form, which each multiple myeloma patient read and signed, when they enrolled in this study, before withdrawal of their blood.

(Section 2.1.2):

Title of Study: Immunologic studies in haematological malignancies

Principal Investigator: Dr Michael Browning
Consultant Immunologist and Senior Clinical Researcher

You may contact: Dr Michael Browning on 0116 258 6702

Invitation paragraph

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

1. What is the purpose of the study?

The purpose of the study is to gain a better understanding of how the body's immune defence system recognises and responds to cancers of the white blood cells (multiple myeloma, lymphoma or leukaemia), and is aimed at identifying ways in which this immune response could be made stronger. The study involves a single donation of blood and / or bone marrow cells.

Most of the studies will involve examining how your lymphocytes (a type of white blood cells that fights infections and cancer cells) recognise and try to eliminate the cancer cells. However, some of the may involve genetic analysis of particular targets ("antigens") that allow the immune system to identify the tumour cells as abnormal, or analysis of host genes that may directly affect the host's ability to mount an immune response. These tests will help us to identify potential targets for treatment, or possibly groups of patients at increased risk of a particular disease. It is not intended to link the results of any genetic tests back to an individual, and these will not affect you or your family's insurance or employment prospects.

The samples taken will be stored in the Department of Infection, Immunity and Inflammation, University of Leicester until use. If there are any samples remaining at the end of the study, these will be disposed of in a safe and anonymous way.

2. Why have I been chosen?

You have been chosen because you have a type of white blood cell cancer that the research is interested in studying (myeloma). Up to 24 patients will be asked to take part in the study.

1. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

2. What will happen to me if I take part?

Taking part involves donation of blood and / or bone marrow sample. This will be done to coincide with a time when blood and / or bone marrow is being taken as part of your routine medical care. The size of blood donation we ask for is equivalent to up to 6 teaspoonfuls, and the amount of bone marrow (if applicable) to a maximum of 2 teaspoonfuls. You will not be required to make any additional visits to hospital or to your GP.

For patients donating bone marrow, this will be done under local anaesthetic by inserting a needle into your pelvic bone in order to remove a sample of bone marrow cells. You may experience some discomfort during this process, such as a feeling of pushing as the needle is inserted, or a sucking sensation as the marrow sample is taken up into the syringe. In some cases patients find this procedure quite painful, and bruising may occur as a result. In order to minimise the discomfort, we shall only ask for a sample of bone marrow for research if your doctor has indicated that a bone marrow sample has to be taken as part of your routine management. No patient shall be asked to give a sample of bone marrow purely for the purposes of the research.

3. What do I have to do?

You will have no further involvement than allowing the blood and / or bone marrow samples to be taken for the research. Your management will not be altered in any way by taking part.

4. Will I receive payment for the tissue that I donate for this research study?

You will not receive any payment for the tissue. The tissue is a gift - neither yourself nor your relatives will benefit from any inventions that result from the use of the tissue.

5. What are the possible disadvantages and risks of taking part?

The risks of taking part in the study are essentially those of the discomfort of giving a blood or bone marrow sample. To minimise this, the samples for research will be taken at the same time as you are having blood or bone marrow taken as part of your routine medical care.

6. What are the possible benefits of taking part?

There will be no direct benefit to you for taking part in the study. Its purpose is to try to improve care for future patients with the same illness as you, by studying your disease in more detail.

1. What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the disease that is being studied. If this happens, and could have an impact on your care, your research doctor will tell you about it and discuss its implications with you.

2. What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

3. Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

4. What will happen to the results of the research study?

When the study has been completed and all the data analysed, we would aim to publish the results in reputable medical journals, and through presentations at scientific meetings. This often takes several years to complete, although some of the data may be published or presented earlier than this. Copies of any published reports will be made available to participants on request to the Principal Investigator.

No participating patient will be identified in any report/publication.

5. Who is organising and funding the research?

The study is being funded from grants from the Leicester Immunology Research Fund and the Egyptian Educational Bureau.

None of the researchers is being paid over and above their normal salaries for conducting this research.

6. Who has reviewed the study?

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the Committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have

been given sufficient information on which to make an informed decision to take part or not. This study has been reviewed and approved by the Leicestershire Research Ethics Committee.

1. Contact for Further Information

Dr Michael Browning
Consultant Immunologist and Senior Clinical Researcher
Department of Immunology
Leicester Royal Infirmary
Leicester LE1 5WW

Tel 0116 258 6702
Fax 0116 258 6704

Thank you for reading this information leaflet.

You will be given a copy of the information sheet and a signed consent form to keep.

Patient name, address, DOB (or ID label)

Centre Number: _____
Study Number: _____
Patient Identification Number for this trial: _____

CONSENT FORM

Title of Project:
Immunologic studies in haematological malignancies

Name of Researcher / Principal Investigator: Dr Michael Browning

Please initial box

1. I confirm that I have read and understand the information sheet dated 30.4.13, Version 3 for the above study and have had the opportunity to ask questions. ☐
2. I understand that I may withdraw my consent to my tissue being used at any time without justifying my decision and without affecting my normal care and medical management. ☐
3. I agree to donate the tissue samples as detailed below and allow their use in medical research as described in the Patient Information Leaflet. ☐
4. I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue. ☐
5. I understand that tissue samples will not be used to undertake any genetic tests whose results may have adverse consequences on my or my families insurance or employment. ☐
6. **I agree / do not agree** to my tissue samples being used to undertake genetic research as described in the patient information leaflet
   (patient to delete as applicable) ☐
7. I understand that relevant sections of my medical notes and/or data may be looked at by responsible individuals from the study team, Research Ethics Committee, NHS Trust or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

1. The samples which I hereby consent to donate are:

Blood…… ☐

Bone marrow……..…… ☐

2. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Original for researcher/site file/CRF
copy for patient, copy for hospital notes

Appendix C:

A copy of the ethical approval for the present study from the University Research Ethics Committee approval respectively (Section 2.1.2).

University of Leicester Ethics Review Sign Off Document

University of Leicester

To: WAFAA SEDIK KHALAF

Subject: Ethical Application Ref: wsak1-012f

(Please quote this ref on all correspondence)

22/01/2014 08:59:32

Infection Immunity & Inflammation

Project Title: In vitro generation of cytotoxic T cells with potential for adoptive tumour immunotherapy

Thank you for submitting your application which has been considered.

This study has been given ethical approval, subject to any conditions quoted in the attached notes.

Any significant departure from the programme of research as outlined in the application for research ethics approval (such as changes in methodological approach, large delays in commencement of research, additional forms of data collection or major expansions in sample size) must be reported to your Departmental Research Ethics Officer.

Approval is given on the understanding that the University Research Ethics Code of Practice and other research ethics guidelines and protocols will be complied with

- http://www2.le.ac.uk/institution/committees/research-ethics/code-of-practice
- http://www.le.ac.uk/safety/

Page 1 of 2

University of Leicester Ethics Review Sign Off Document

The following is a record of correspondence notes from your application **wssk1-012f.** Please ensure that any proviso notes have been adhered to:-

Nov 28 2013 9:52AM I'm afraid you need a separate PIS and Consent form. There is a standard format for the consent form on the III website for taking blood just amend this and then send back to me through this system and then I should be able to approve this study.

--- END OF NOTES ---

Appendix D:

A copy of the consent form, which each normal healthy donor read and signed, before withdrawal of their blood (Section 2.1.2).

University of Leicester

Department of Infection, Immunity & Inflammation
Participant Consent Form

Title of Study: In vitro generation of cytotoxic T cells with potential for adoptive tumour immunotherapy

Principal Investigator: Wafaa Khalaf **e-mail/phone:** WSSK1@ le.ac.uk

This form should be read in conjunction with the Participant Information sheet

I agree to take part in the above study, by providing a blood sample, as described in Participant Information sheet *20- 1 – 014 Version 2*

I understand that if I have any reason to believe I am carrying a blood-borne infection such as Hepatitis B or C, or HIV, I should not volunteer to give blood.

I have read the Participant Information sheet on the above study and have had the opportunity to discuss the details with the investigator detailed below, and to ask any questions. The nature and purpose of the samples to be taken have been explained to me and I understand what will be required if I take part in the study.

Signature of Participant:_____Date _____

(Name in BLOCK LETTERS)_____

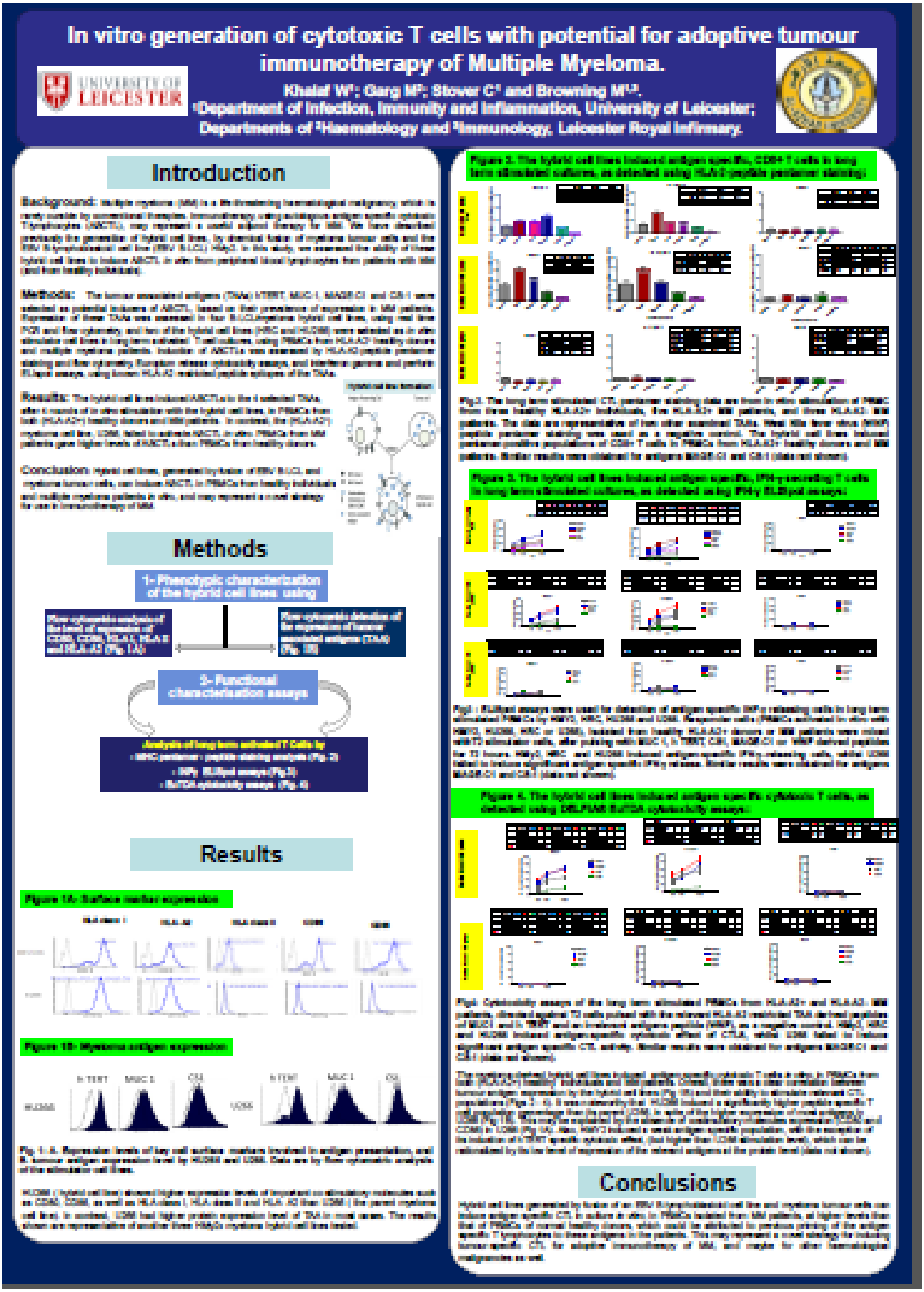
I confirm I have explained the nature of the Study, as detailed in the Participant / Donor Information sheet, in terms which in my judgement are suited to the understanding of the subject.

Signature of Investigator:_____Date_____

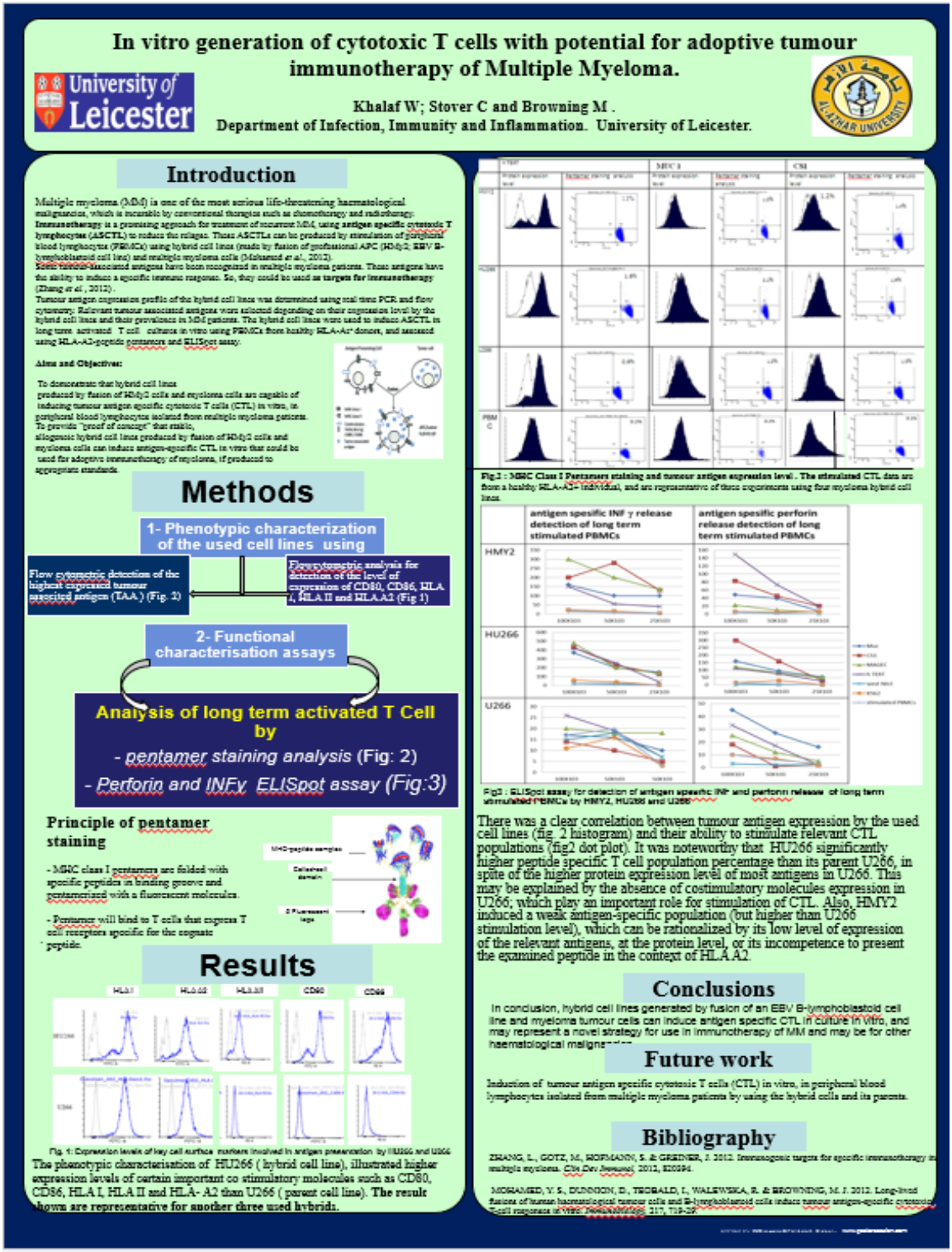
(Name in BLOCK LETTERS) _____

Appendics E: Posters presented during the time of the study:

Posters 1: presented in BSI /NVVI congress (December, 2016).



Posters 2: presented in Advanced Cell therapy symposium (March, 2016)



In vitro generation of cytotoxic T cells with potential for adoptive tumour immunotherapy of Multiple Myeloma.

Khalaf W; Stover C and Browning M .
Department of Infection, Immunity and Inflammation. University of Leicester.

University of Leicester

Introduction

Multiple myeloma (MM) is one of the most serious life-threatening haematological malignancies, which is incurable by conventional therapies such as chemotherapy and radiotherapy. **Immunotherapy** is a promising approach for treatment of recurrent MM, using **antigen specific cytotoxic T lymphocytes (ASCTL)** to reduce the relapse. These ASCTLs can be produced by stimulation of peripheral blood lymphocytes (PBMCs) using hybrid cell lines (made by fusion of professional APC (MM)(2, EBV B-lymphoblastoid cell line) and multiple myeloma cells (Mohamed *et al.*, 2012).
Cancer testicular-associated antigens have been recognized in multiple myeloma patients. These antigens have the ability to induce a specific immune response. So, they could be used as **targets for immunotherapy** (Zhang *et al.*, 2012).
Tumour antigen expression profile of the hybrid cell lines was determined using real time PCR and flow cytometry. Relevant tumour associated antigens were selected depending on their expression level by the hybrid cell lines and their prevalence in MM patients. The hybrid cell lines were used to induce ASCTL in long term activated T cell culture in vitro using PBMCs from healthy HLA-A2+ donors, and assessed using HLA-A2-peptide pentamers and ELISpot assay.

Aims and Objectives:

To demonstrate that hybrid cell lines produced by fusion of MMy2 cells and myeloma cells are capable of inducing tumour antigen specific cytotoxic T cells (CTL) in vitro, in peripheral blood lymphocytes isolated from multiple myeloma patients.
To provide "proof of concept" that stable, allogeneic hybrid cell lines produced by fusion of MMy2 cells and myeloma cells can induce antigen-specific CTL in vitro that could be used for adoptive immunotherapy of myeloma, if produced to appropriate standards.

Methods

1- Phenotypic characterization of the used cell lines using

- Flow cytometric detection of the highest expressed tumour associted antigen (TAA) (Fig. 2)
- Flowcytometric analysis for detection of the level of expression of CD80, CD86, HLA I, HLA II and HLA-A2 (Fig 1)

2- Functional characterisation assays

Analysis of long term activated T Cell by

- *pentamer staining analysis* (Fig: 2)
- *Perforin and INFγ ELISpot assay (Fig:3)*

Principle of pentamer staining

- MHC class I pentamers are folded with specific peptides in binding groove and pentamerized with a fluorescent molecules.
- Pentamer will bind to T cells that express T cell receptors specific for the cognate peptide.

Results

Fig. 1: Expression levels of key cell surface markers involved in antigen presentation by HU266 and U266

The phenotypic characterisation of HU266 (hybrid cell line), illustrated higher expression levels of certain important co stimulatory molecules such as CD80, CD86, HLA I, HLA II and HLA- A2 than U266 (parent cell line). **The result shown are representative for another three used hybrids.**

Fig.2 : MHC Class I Pentamers staining and tumour antigen expression level . The stimulated CTL data are from a healthy HLA-A2+ individual, and are representative of three experiences using four myeloma hybrid cell lines.

Fig3 : ELISpot assay for detection of antigen spesific INF and perforin release of long term stimulated PBMCs by HMY2, HU266 and U266

There was a clear correlation between tumour antigen expression by the used cell lines (fig. 2 histogram) and their ability to stimulate relevant CTL populations (fig2 dot plot). It was noteworthy that HU266 significantly higher peptide specific T cell population percentage than its parent U266, in spite of the higher protein expression level of most antigens in U266. This may be explained by the absence of costimulatory molecules expression in U266, which play an important role for stimulation of CTL. Also, HMY2 induced a weak antigen-specific population (but higher than U266 stimulation level), which can be rationalized by its low level of expression of the relevant antigens, at the protein level, or its incompetence to present the examined peptide in the context of HLA A2.

Conclusions

In conclusion, hybrid cell lines generated by fusion of an EBV B-lymphoblastoid cell line and myeloma tumour cells can induce antigen specific CTL in culture in vitro, and may represent a novel strategy for use in immunotherapy of MM and may be for other haematological malign[illegible]

Future work

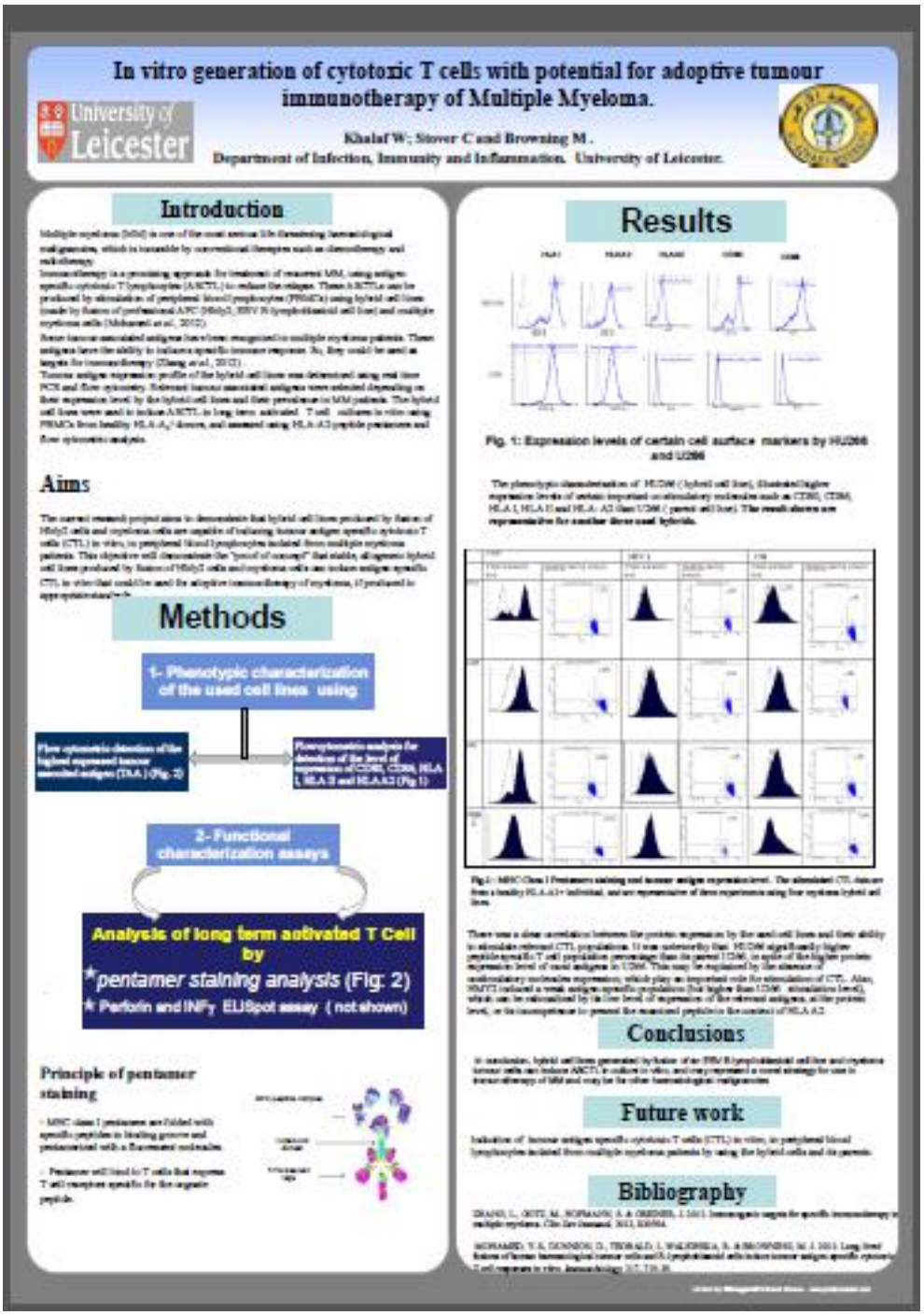
Induction of tumour antigen specific cytotoxic T cells (CTL) in vitro, in peripheral blood lymphocytes isolated from multiple myeloma patients by using the hybrid cells and its parents.

Bibliography

ZHANG, L., GOTZ, M., HOFMANN, S. & GREINER, J. 2012. Immunogenic targets for specific immunotherapy in multiple myeloma. *Clin Dev Immunol*, 2012, 820394.

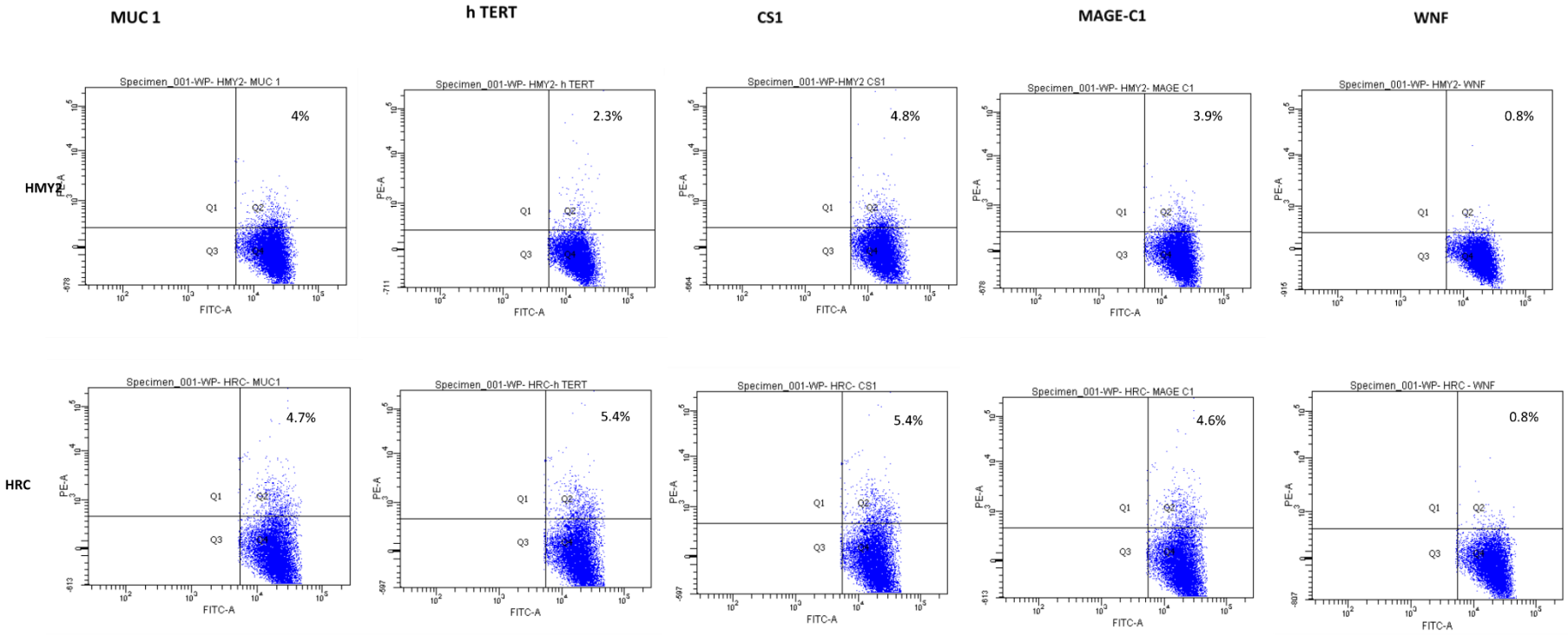
MOHAMED, Y. S., DUNDON, D., TRIBALD, I., WALEWSKA, R. & BROWNING, M. J. 2012. Long-lived fusions of human haematological tumour cells and B-lymphoblastoid cells induce tumour antigen-specific cytotoxic T-cell responses in vivo. *Immunology*, 317, 719-29.

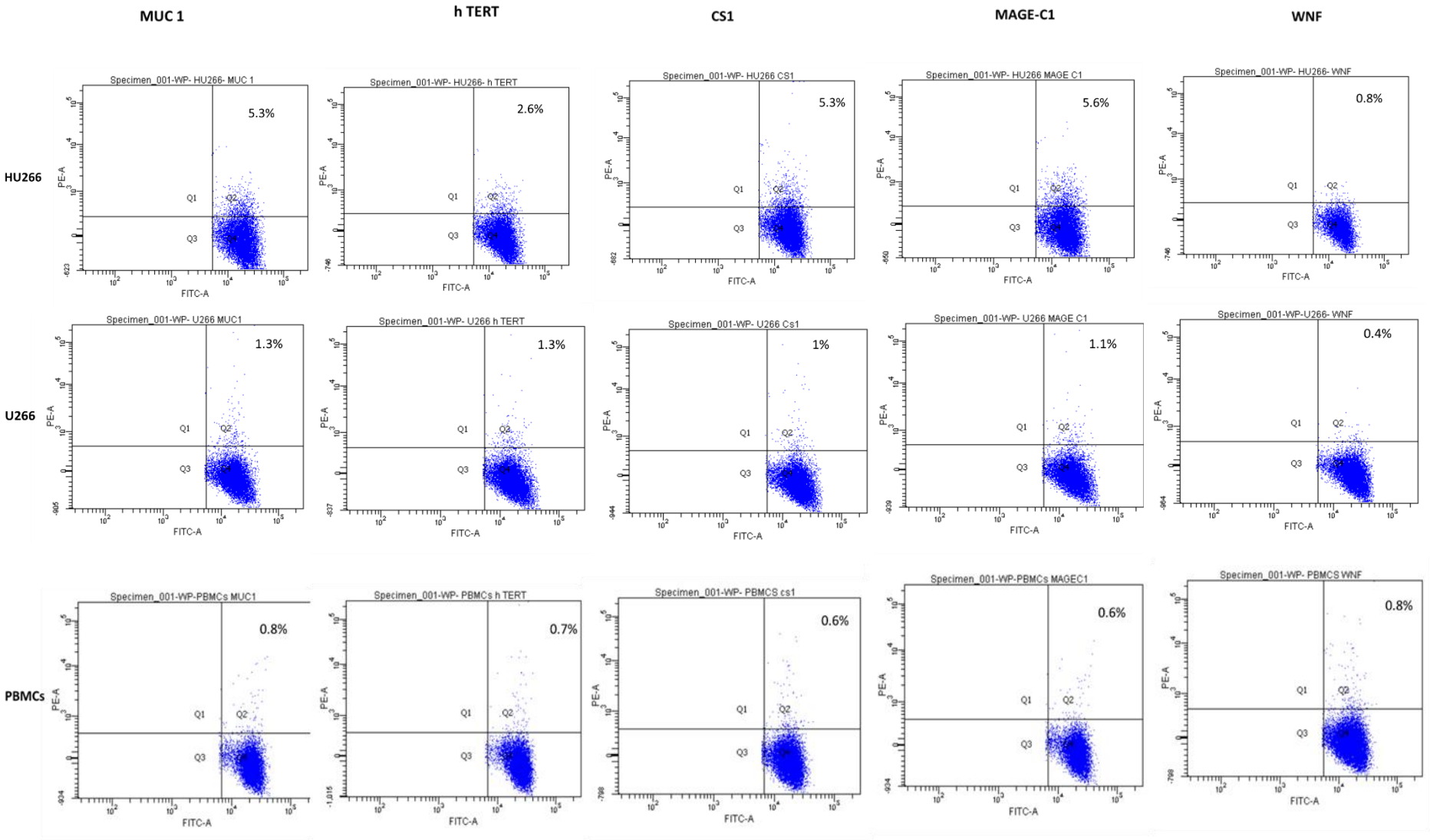
Poster 3: presented in M5 meeting (December, 2015):



Appendices F: Pentamer staining dot plots of long term stimulated PBMCs, isolated from MM patients:

1) HLA-A2^{+} patient 1:




MUC 1
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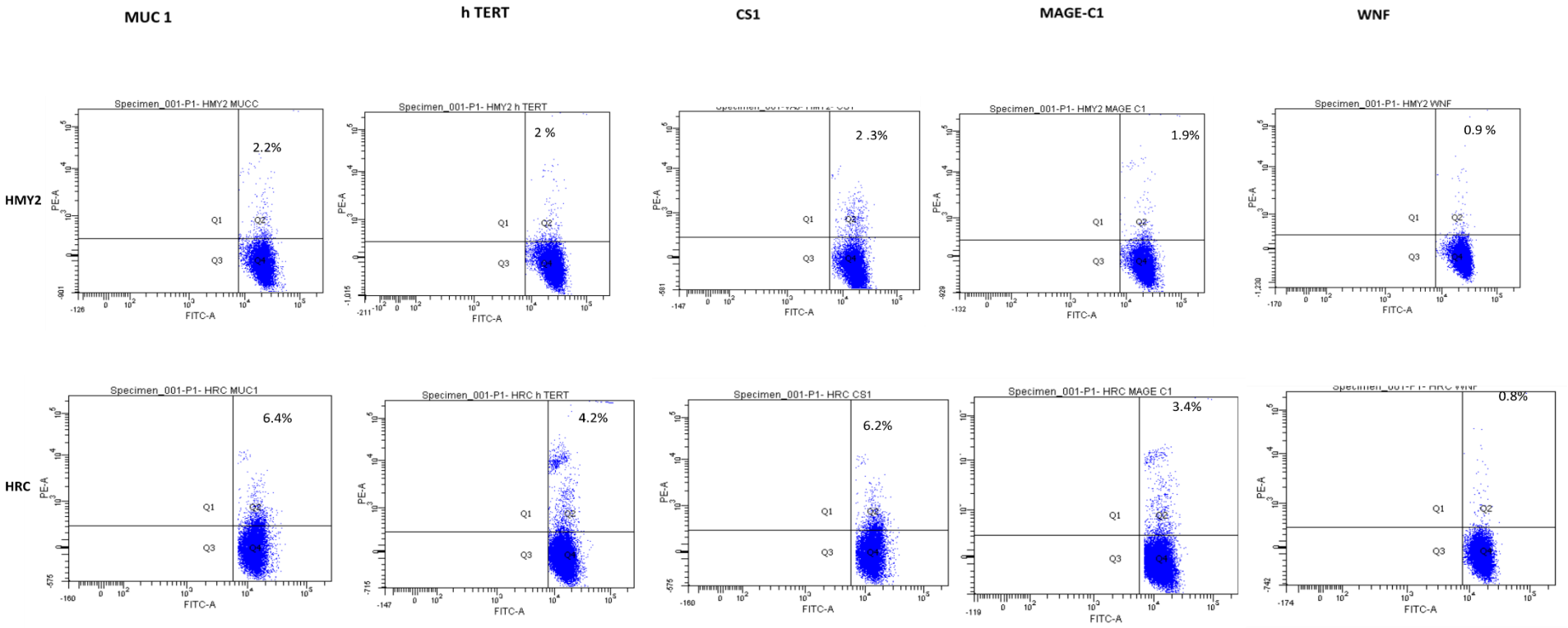
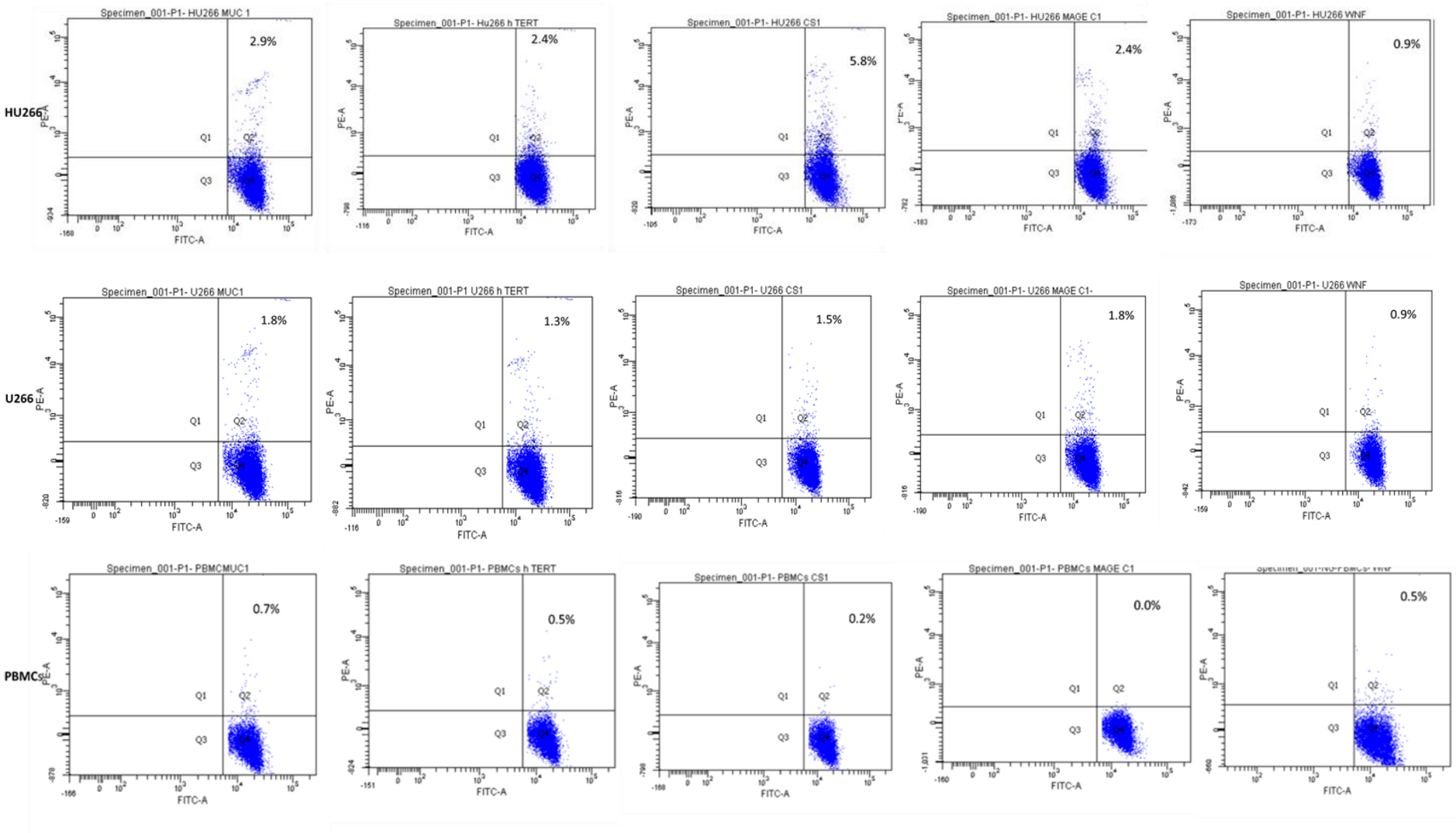
0.8%
PE-A
FITC-A
Q1
Q2
Q3
Q4

2) HLA-A2^{+} patient 2:

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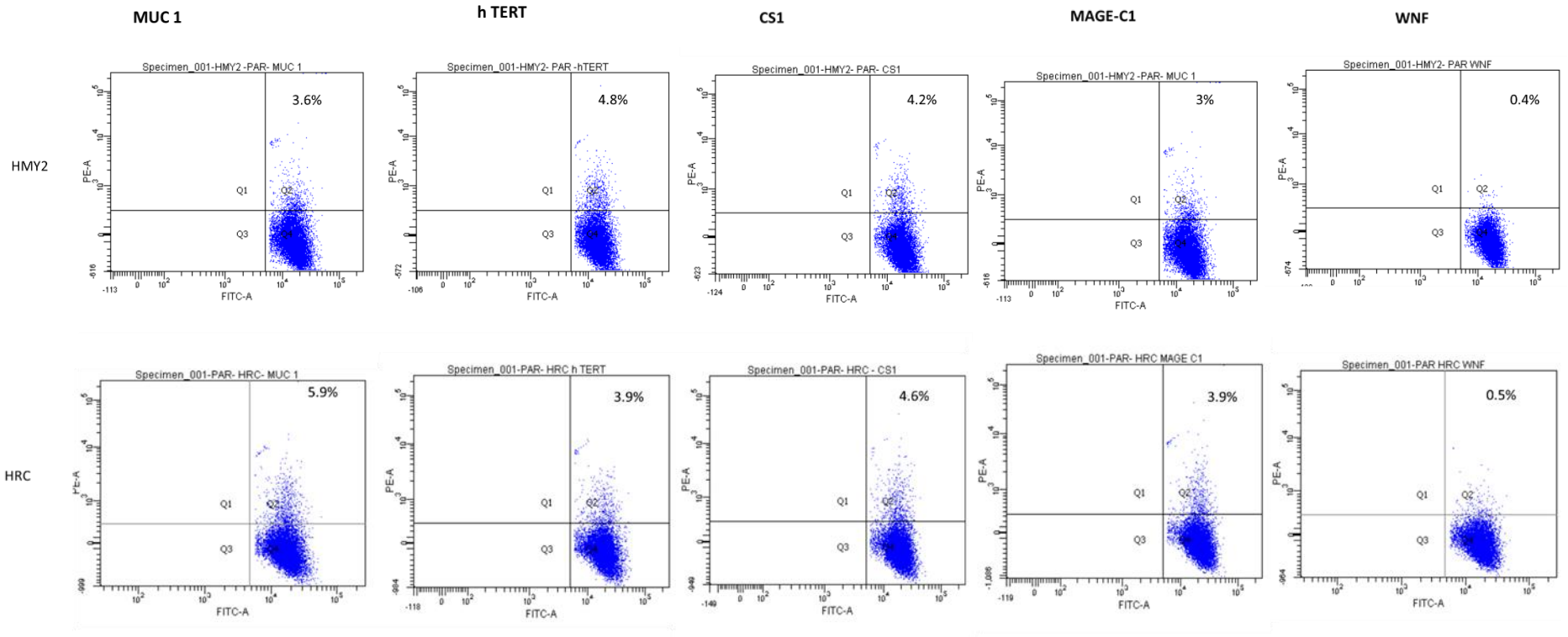
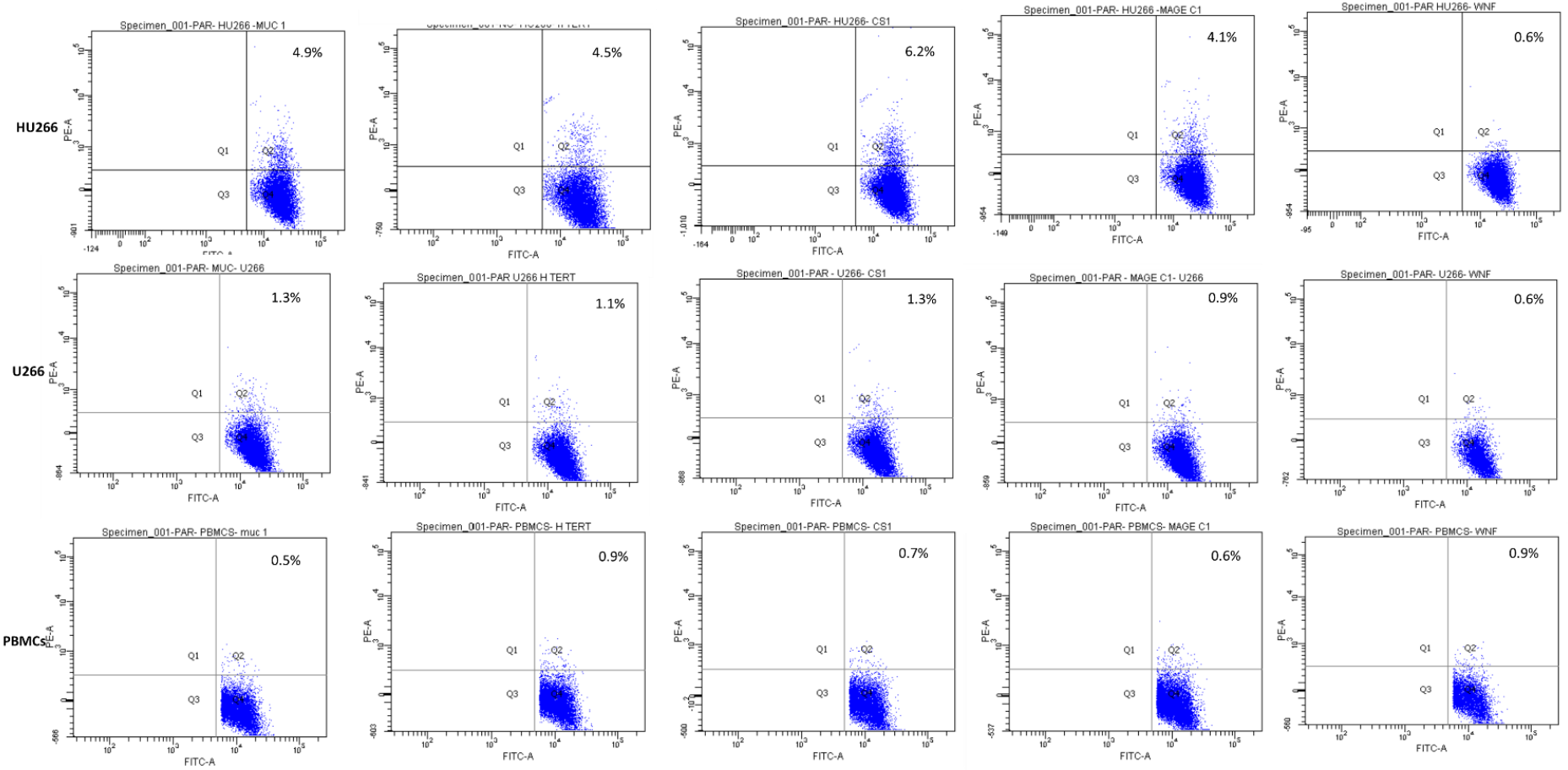
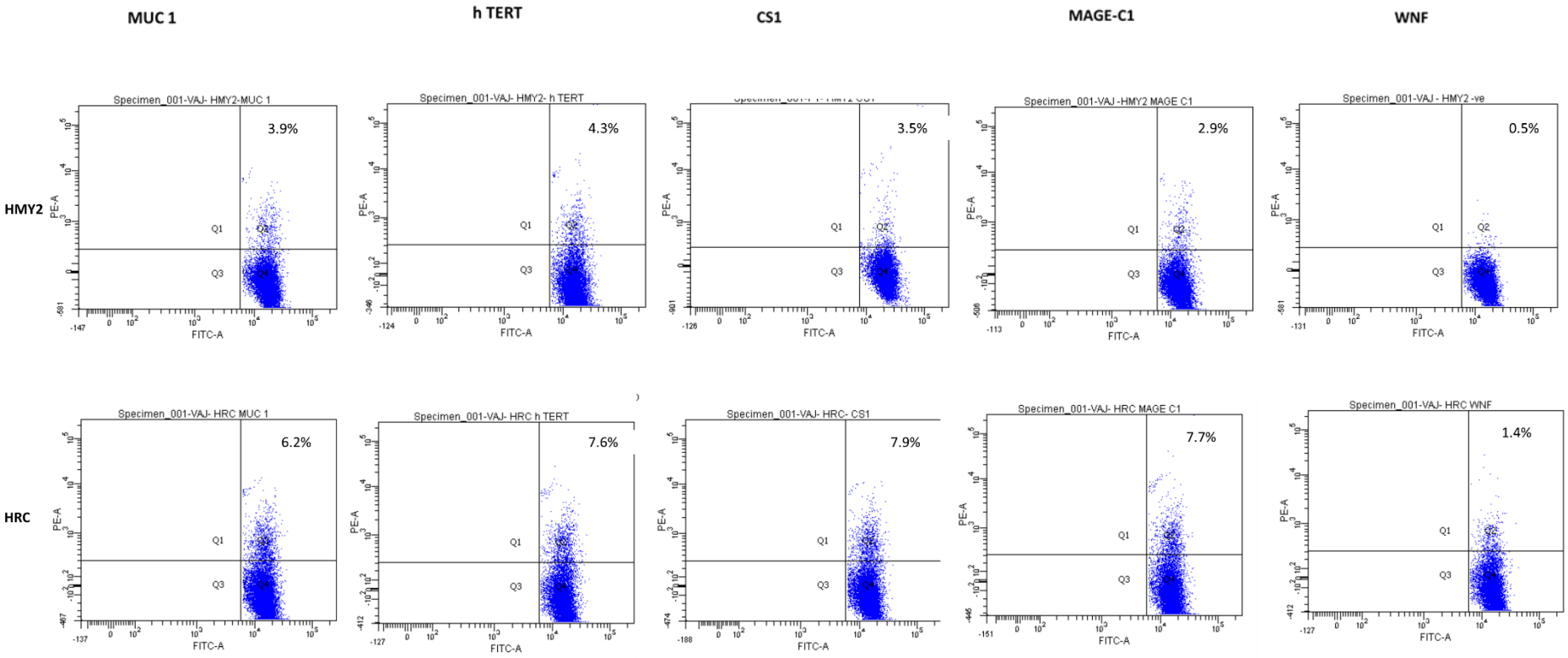
1.5%
Specimen_001-P1- U266 MAGE C1-
1.8%
Specimen_001-P1- U266 WNF
0.9%
PBMCs
Specimen_001-P1- PBMCMUC1
0.7%
Specimen_001-P1- PBMCs h TERT
0.5%
Specimen_001-P1- PBMCs CS1
0.2%
Specimen_001-P1- PBMCs MAGE C1
0.0%
0.5%
PE-A
FITC-A
Q1
Q2
Q3
Q4

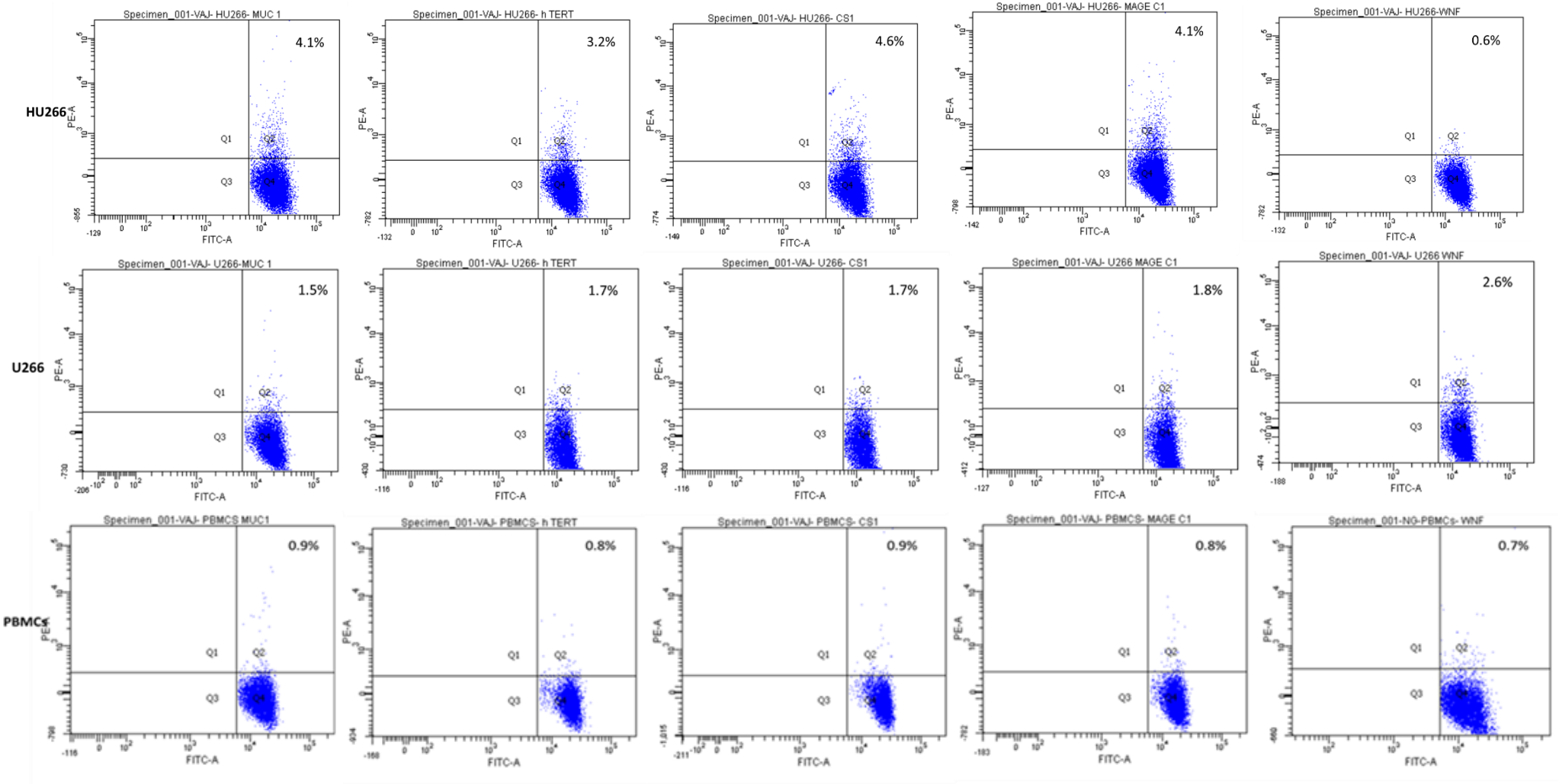
3) HLA-A2^{+} patient 3:



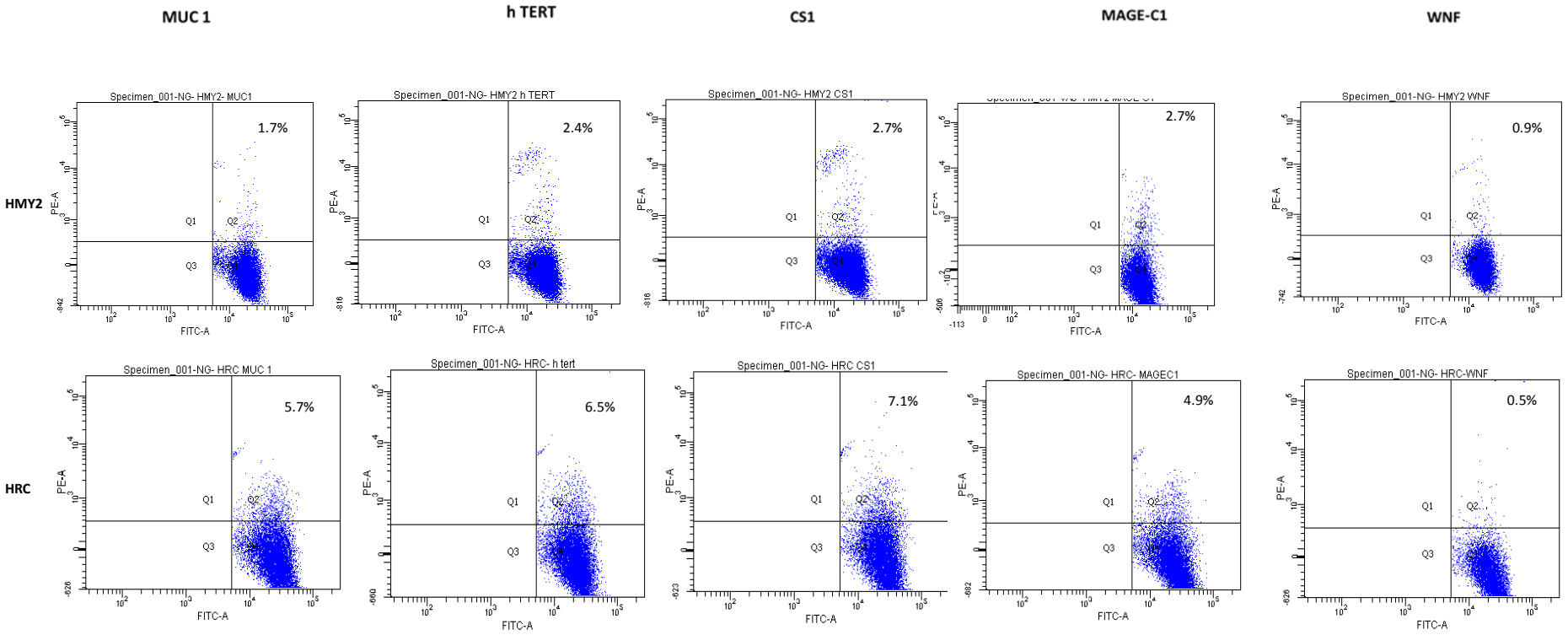

HU266
U266
PBMCs
Specimen_001-PAR- HU266 -MUC 1
4.9%
Specimen_001-PAR- HU266- CS1
6.2%
Specimen_001-PAR- HU266 -MAGE C1
4.1%
Specimen_001-PAR HU266- WNF
0.6%
Specimen_001-PAR- MUC- U266
1.3%
Specimen_001-PAR U266 H TERT
1.1%
Specimen_001-PAR - U266- CS1
1.3%
Specimen_001-PAR - MAGE C1- U266
0.9%
Specimen_001-PAR- U266- WNF
0.6%
Specimen_001-PAR- PBMCS- muc 1
0.5%
Specimen_001-PAR- PBMCS- H TERT
0.9%
Specimen_001-PAR- PBMCS- CS1
0.7%
Specimen_001-PAR- PBMCS- MAGE C1
0.6%
Specimen_001-PAR- PBMCS- WNF
0.9%
PE-A
FITC-A
Q1
Q2
Q3

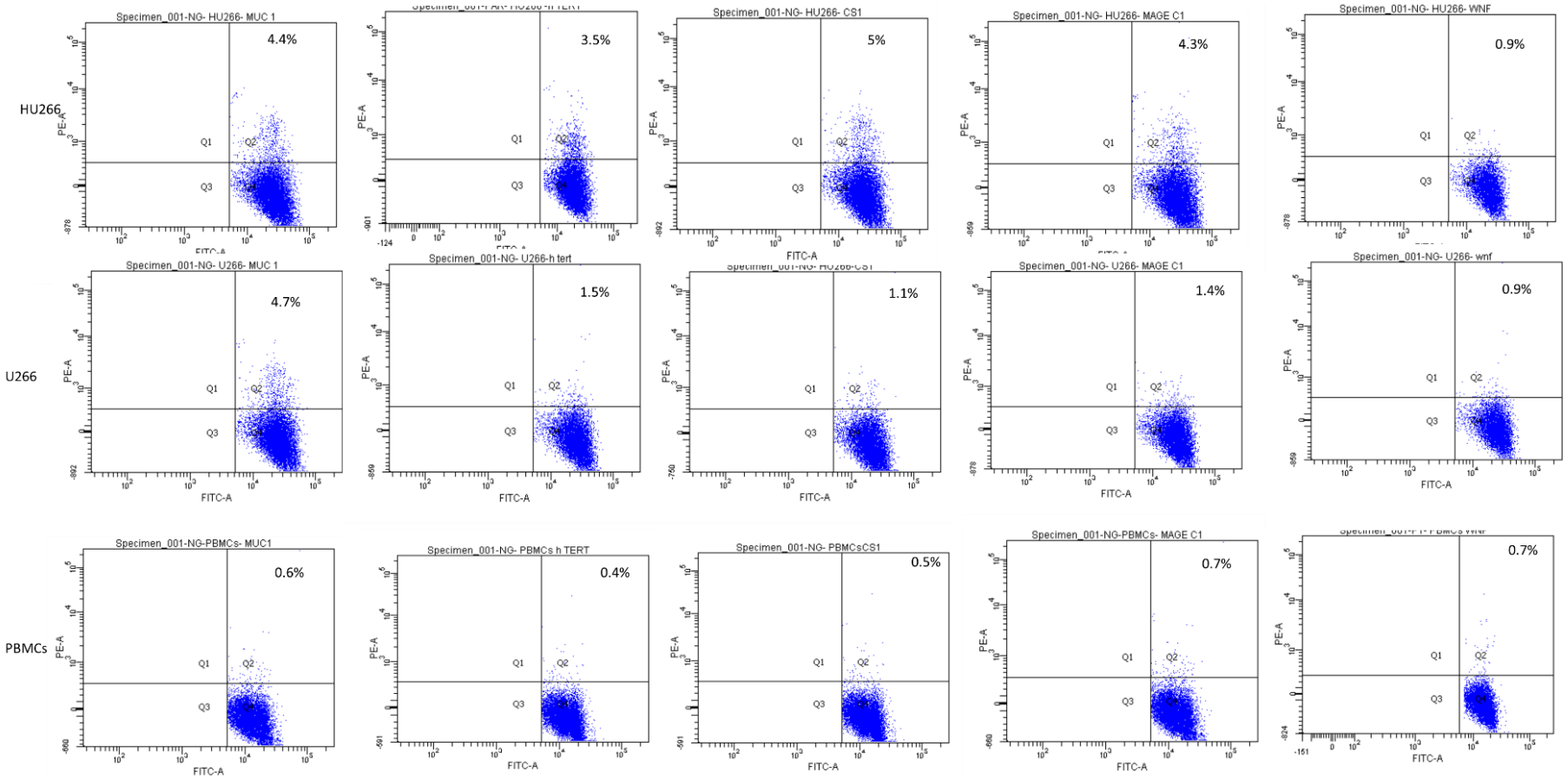
4) HLA-A2^{+} patient 4:



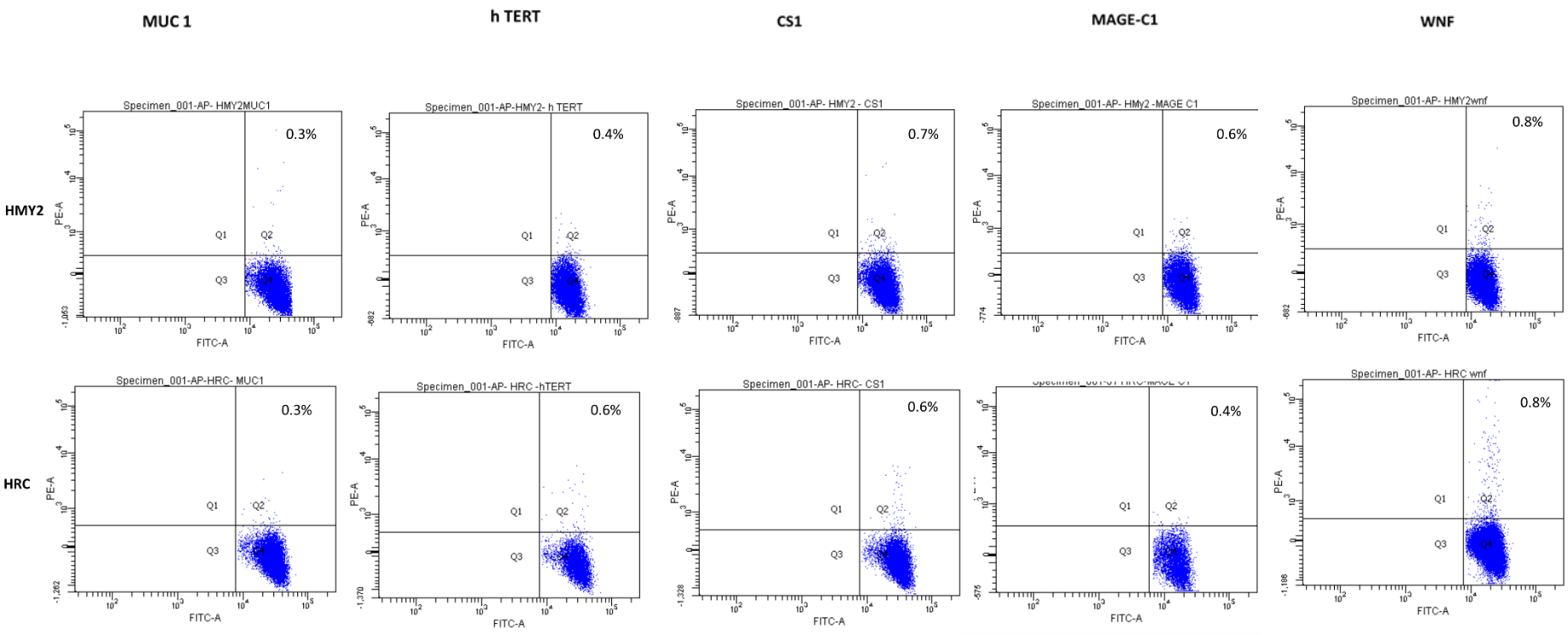

HU266
Specimen_001-VAJ- HU266- MUC 1
4.1%
Specimen_001-VAJ- HU266- h TERT
3.2%
Specimen_001-VAJ- HU266- CS1
4.6%
Specimen_001-VAJ- HU266- MAGE C1
4.1%
Specimen_001-VAJ- HU266-WNF
0.6%
U266
Specimen_001-VAJ- U266-MUC 1
1.5%
Specimen_001-VAJ- U266- h TERT
1.7%
Specimen_001-VAJ- U266- CS1
1.7%
Specimen_001-VAJ- U266 MAGE C1
1.8%
Specimen_001-VAJ- U266 WNF
2.6%
PBMCs
Specimen_001-VAJ- PBMCS MUC1
0.9%
Specimen_001-VAJ- PBMCS- h TERT
0.8%
Specimen_001-VAJ- PBMCS- CS1
0.9%
Specimen_001-VAJ- PBMCS- MAGE C1
0.8%
Specimen_001-NG-PBMCs- WNF
0.7%
PE-A
FITC-A
Q1
Q2
Q3
Q4

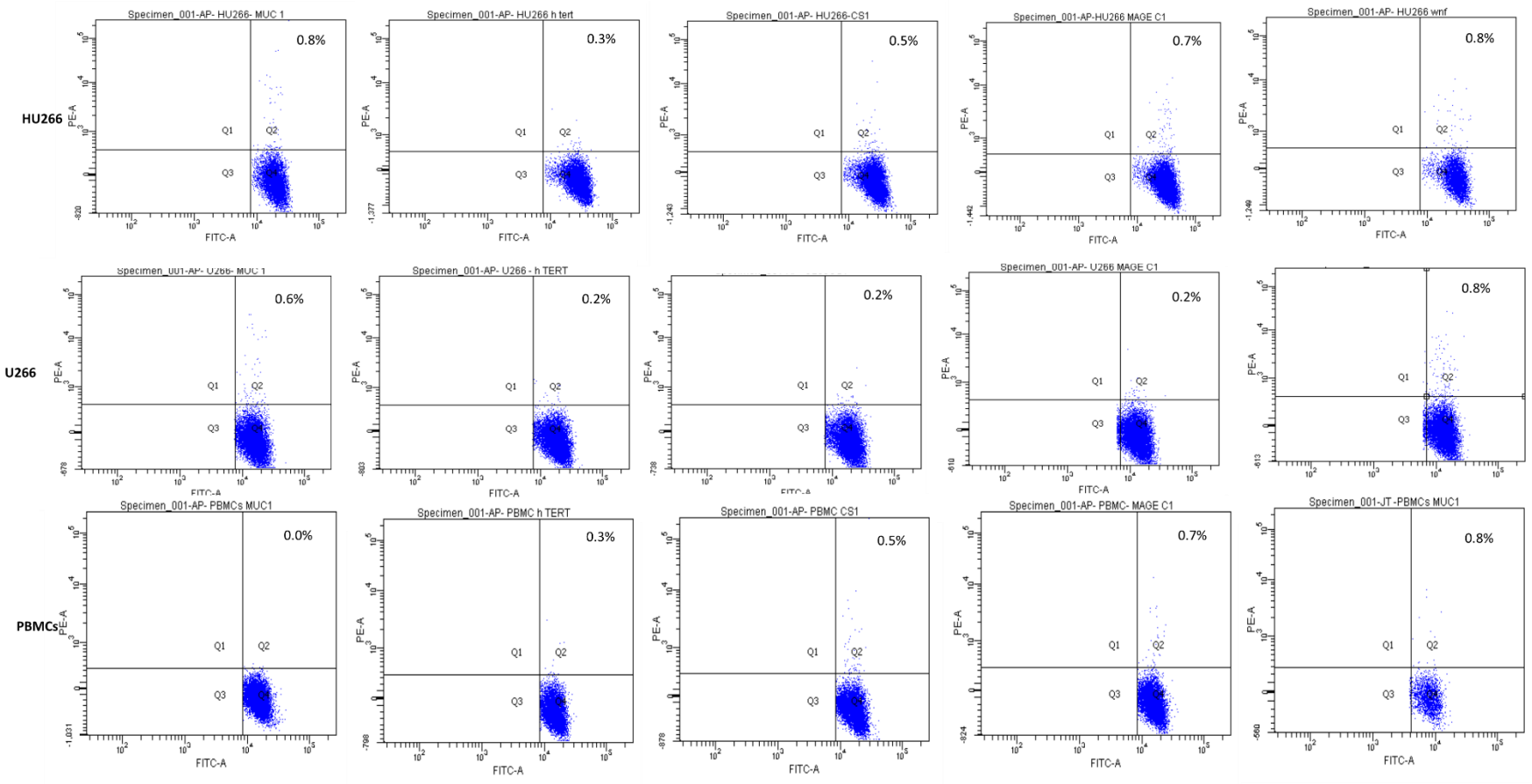
5) HLA-A2^{+} patient 5:



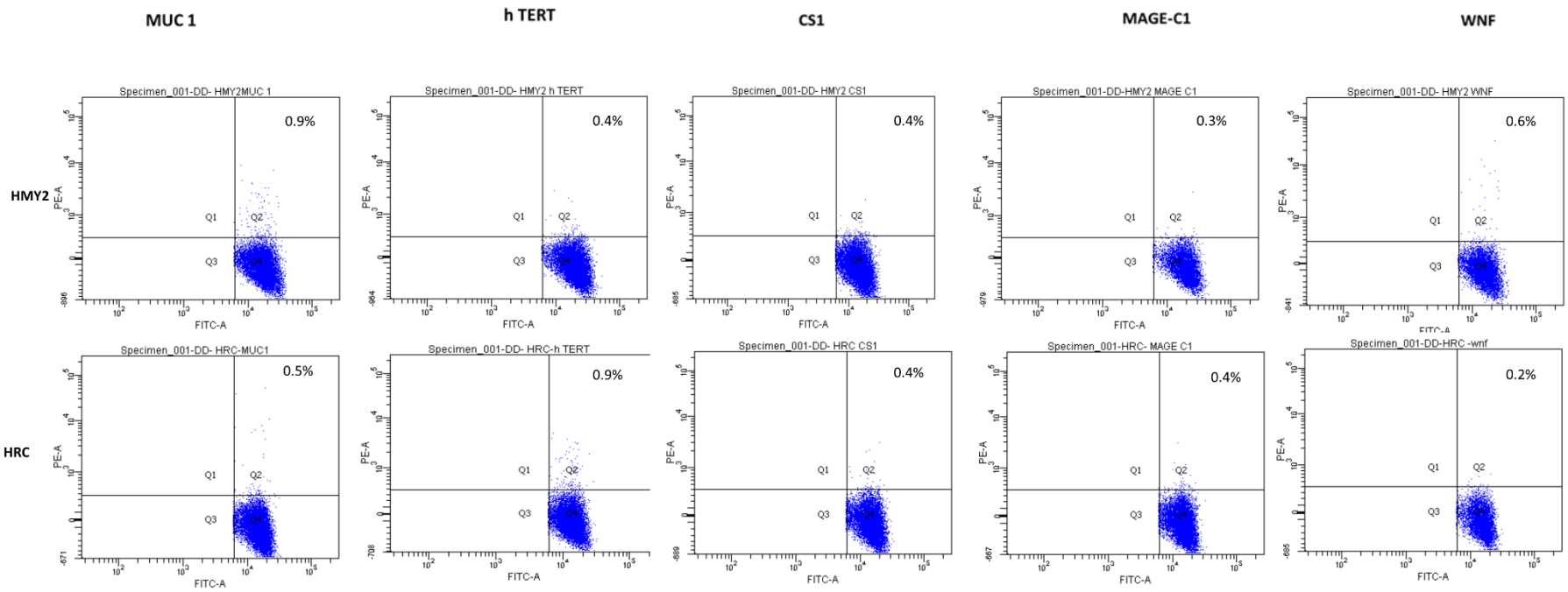

HU266
U266
PBMCs
Specimen_001-NG- HU266- MUC 1
4.4%
Specimen_001-NG- HU266- h TERT
3.5%
Specimen_001-NG- HU266- CS1
5%
Specimen_001-NG- HU266- MAGE C1
4.3%
Specimen_001-NG- HU266- WNF
0.9%
Specimen_001-NG- U266- MUC 1
4.7%
Specimen_001-NG- U266-h tert
1.5%
1.1%
Specimen_001-NG- U266- MAGE C1
1.4%
Specimen_001-NG- U266- wnf
0.9%
Specimen_001-NG-PBMCs- MUC1
0.6%
Specimen_001-NG- PBMCs h TERT
0.4%
Specimen_001-NG- PBMCsCS1
0.5%
Specimen_001-NG-PBMCs- MAGE C1
0.7%
0.7%
PE-A
FITC-A
Q1
Q2
Q3
Q4

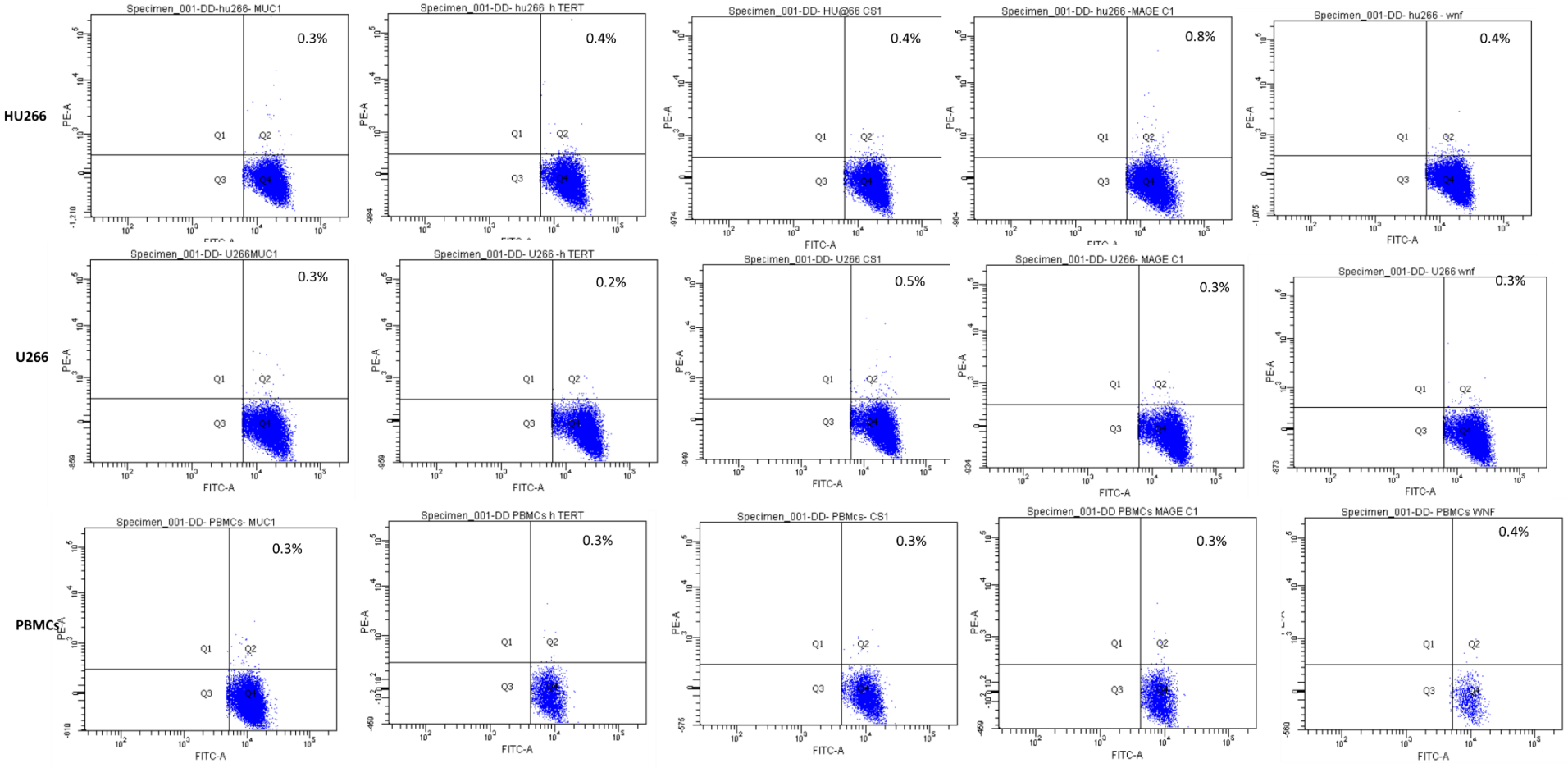
6) HLA-A2^{-} patient 1:



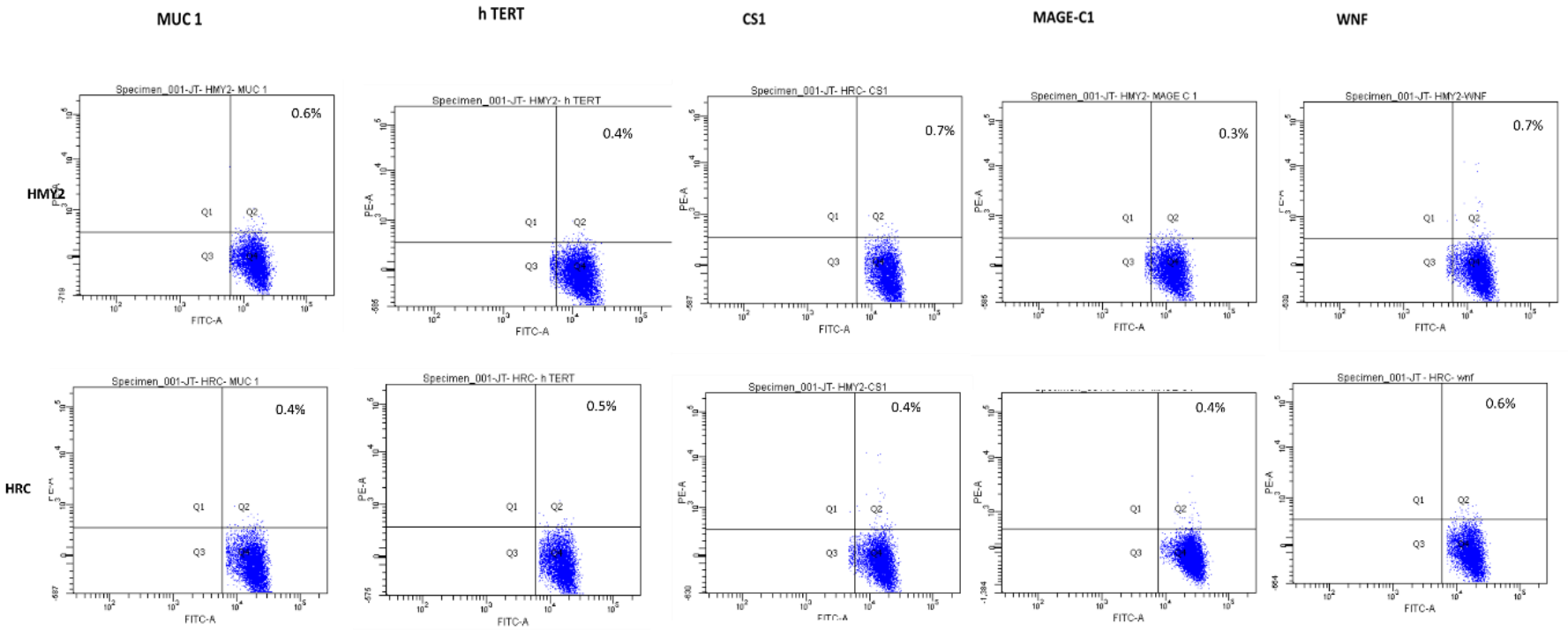

HU266
U266
PBMCs
Specimen_001-AP- HU266- MUC 1
0.8%
Specimen_001-AP- HU266 h tert
0.3%
Specimen_001-AP- HU266-CS1
0.5%
Specimen_001-AP-HU266 MAGE C1
0.7%
Specimen_001-AP- HU266 wnf
0.8%
Specimen_001-AP- U266- MUC 1
0.6%
Specimen_001-AP- U266 - h TERT
0.2%
0.2%
Specimen_001-AP- U266 MAGE C1
0.2%
0.8%
Specimen_001-AP- PBMCs MUC1
0.0%
Specimen_001-AP- PBMC h TERT
0.3%
Specimen_001-AP- PBMC CS1
0.5%
Specimen_001-AP- PBMC- MAGE C1
0.7%
Specimen_001-JT -PBMCs MUC1
0.8%
PE-A
FITC-A
Q1
Q2
Q3
Q4

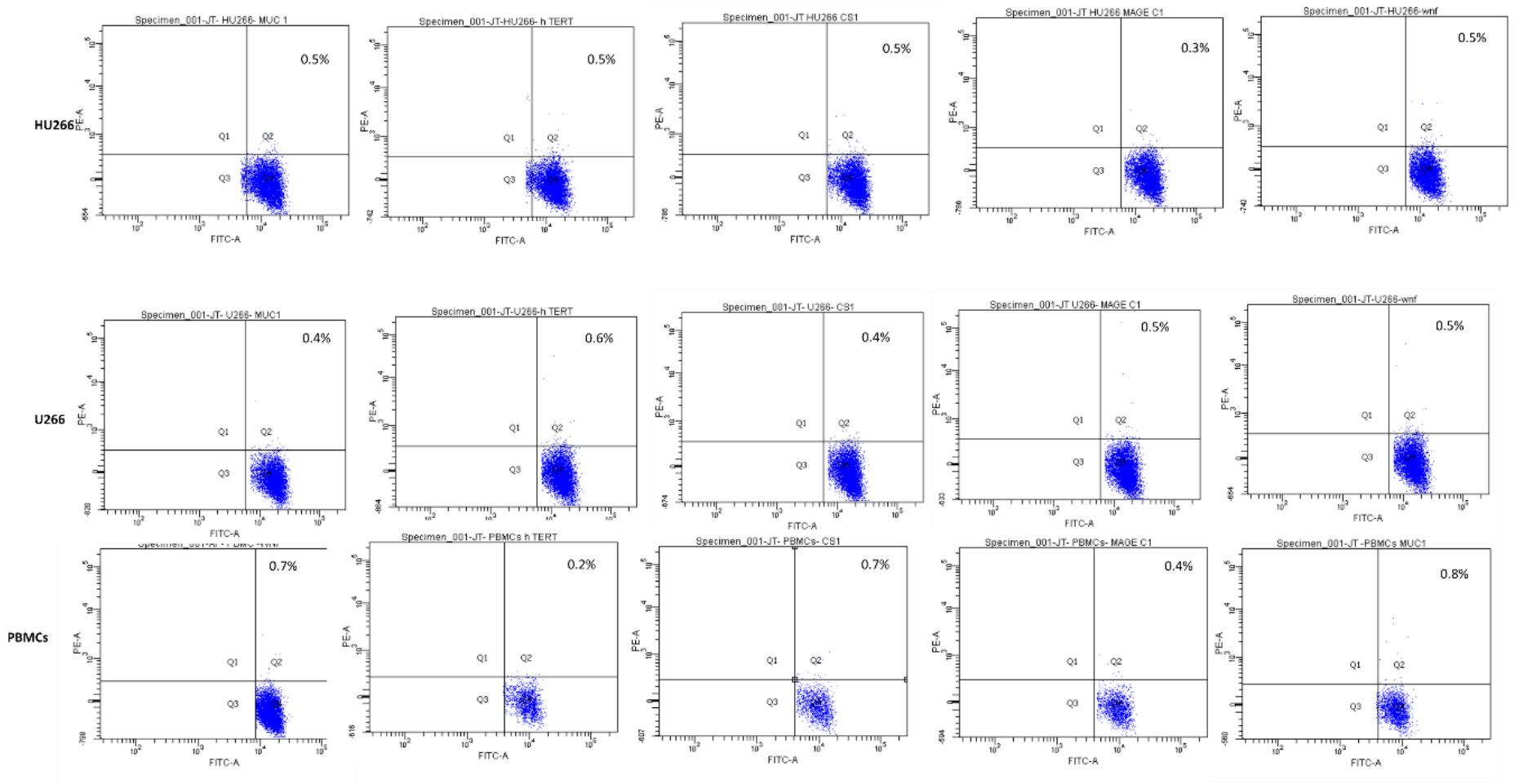
7) HLA-A2^{-} patient 2:




HU266
U266
PBMCs
Specimen_001-DD-hu266- MUC1
0.3%
Specimen_001-DD- hu266 h TERT
0.4%
Specimen_001-DD- HU@66 CS1
0.4%
Specimen_001-DD- hu266 -MAGE C1
0.8%
Specimen_001-DD- hu266 - wnf
0.4%
Specimen_001-DD- U266MUC1
0.3%
Specimen_001-DD- U266 -h TERT
0.2%
Specimen_001-DD- U266 CS1
0.5%
Specimen_001-DD- U266- MAGE C1
0.3%
Specimen_001-DD- U266 wnf
0.3%
Specimen_001-DD- PBMCs- MUC1
0.3%
Specimen_001-DD PBMCs h TERT
0.3%
Specimen_001-DD- PBMcs- CS1
0.3%
Specimen_001-DD PBMCs MAGE C1
0.3%
Specimen_001-DD- PBMCs WNF
0.4%
PE-A
FITC-A
Q1
Q2
Q3
Q4

8) HLA-A2^{-} patient 3:





Flow cytometric dot plots of HLA-A2-peptide pentamer staining of CD8+ T cells, isolated from MM patients (5 HLA A2+ and 3 HLA A2- patients), and stimulated by HMy2 cells, or by hybrid cells lines HU266 and HRC or myeloma cell line U266. The examined antigens are MUC1, h TERT, CS1 and MAGE-C1. WNF virus peptide pentamer was used as a negative control. The antigen specific CTL percentages (as a proportion of total CD8+ T cells in the culture) are shown in the upper right quadrant.