The use of antigen presenting cell/tumour cell hybrids for the *in vitro* induction of tumour-specific T cells

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Abstract

Malignant tumours are the second main cause of mortality worldwide, with haematological malignancies representing 10%. Current treatment strategies, such as chemotherapy, radiotherapy, and stem cell transplantation, are mostly effective but may induce serious side effects. In addition, tumours are developing resistance against most of these conventional therapies.

Immunotherapy is a promising investigational approach, especially hybrid cell vaccination, in which a professional APC is fused to a tumour cell, and the fusion product mostly combines the antigenicity of the tumour cell partner, processed and presented through the relevant APC-machinery, and associated with co-stimulatory molecules CD80, CD86, and CD40 expression, for proper induction of anti-tumour immune responses.

I investigated the phenotypic and functional characteristics of a group of previously-made hybrid cell lines, generated by *in vitro* fusion of a human APC (HMy2; EBV B-lymphoblastoid cell line) with haematological *ex vivo* or immortalized tumour cells.

On co-culture with allogeneic (normal donors) peripheral blood lymphocytes, hybrid cell lines induced elevated levels of T-cell proliferation compared with their relevant tumour cells, which was dependent on expression of co-stimulatory molecules CD80 and CD86, and MHC class I and class II antigens. The hybrid cell lines induced proliferation of naive, central memory, and effector memory populations of CD4$^+$, CD8$^+$ T-cells.

Moreover, the hybrid cells expressed a range of tumour associated antigens not expressed by HMy2 cells, or by normal PBMC. Depending on that, tumour-specific cytotoxic T lymphocytes were induced *in vitro* by stimulation of allogeneic or autologous PBMCs for multiple rounds with selected hybrid cell lines in the presence of rhIL-2. Tumour- and antigen-specificity of the activated T cells were assessed by IFN-γ releasing ELISpot, MHC class I (HLA-A2)-restricted tumour peptide-specific pentamer staining, and ^{51}Cr-release cytotoxicity assays.

Hybrid cell vaccines generated in this way may therefore represent a novel strategy for use in immunotherapy of haematological malignancies, and possibly in other forms of cancer as well.

Acknowledgements

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Abbreviations

ACI	Adoptive cellular immunotherapy
ACT	Adoptive cellular transfer
ADCC	Antibody-dependent cellular cytotoxicity
AFP	Alpha-feto protein
α-GalCer	Alpha-galactosylceramide
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloblastic leukaemia
APC	Antigen presenting cell
ASCT	Autologous stem cell transplantation
BCG	Bacillus Calmette-Guerin
BCIP/ NBT	5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium
B-CLL	B-cell chronic lymphocytic leukaemia
B-LCL	B-lymphoblastoid cell line
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
CLIP	Class II-associated invariant polypeptide
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CpG-ODN	Cytidine phosphate Guanosine-oligodeoxynucleotides
CPM	Count per minute
CR	Complete remission
CT	Cancer testis
CTLA-4	Cytotoxic T lymphocyte antigen-4
CTLs	Cytotoxic T lymphocytes
DAs	Differentiation antigens
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EBV	Epstein-Barr virus
EBNA	Epstein-Barr virus nuclear antigen
EDTA	Ethylene di-amine tetra-acetic acid
ELISpot	Enzyme Linked ImmunoSpot
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FB	Fixing buffer
FcR	Fc receptor
FCS	Fetal calf serum
FDA	US Food and drug administration
FITC	Fluorescein isothiocyanate

FSC	Forward scatter
GBM	Glioblastoma multiform
GM-CSF	Granulocyte macrophage -colony stimulating factor
GVHD	Graft versus host disease
GVL	Graft versus leukaemia
H x tumour cells	HMy2 hybrid of this tumour cells
HAT	Hypoxanthine aminopterin thymidine
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HSCT	Haematopoietic stem cell transplantation
IAPs	Inhibitory of apoptosis proteins
ICOS	Inducible co-stimulatory molecule
IDO	Indolamine-2,3-dioxygenase
IF	Immunofluorescence
IFN-γ	Interferon gamma
ILT-2	Ig-like transcript 2
ITAM	Immunoreceptor tyrosine-based activation motif
iTreg	Induced T regulatory cells
KIR	killer cell Ig-like receptor
KLH	Keyhole limpet hemocyanin
LMP	Latent membrane protein
MAC	Membrane attack complex
mAbs	Monoclonal antibodies
MAGE	Melanoma associated antigen
MCA	Methylcholanthrene
mCRP	Membrane-bound complement regulatory proteins
mDCs	Myeloid dendritic cells
MDSC	Myeloid derived suppressor cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mHC	Minor histocompatibility complex
MLR	Mixed lymphocyte reaction
ml	Millilitre
MM	Multiple myeloma
MUC-1	Mucin-1
MØ	Macrophage
NHL	Non Hodgkin lymphoma
NK	Natural killer
NOS	nitric oxide synthase
NPC	Nasopharyngeal carcinoma
NSCLC	Non small cell lung cancer
nTreg	Natural T regulatory cells
PAMPs	Pathogen associated molecular patterns

PBMC	Peripheral blood mononuclear cells
PCV	Pneumococcal conjugated-vaccine
pDCs	Plasmacytoid dendritic cells
PD-1	Programmed death-1 receptor
PE	Phycoerytherin
PEG	Polyethylene glycol
PG-E2	Prostaglandin-E2
PLC	Peptide loading complex
PRAME	Preferentially expressed antigen of melanoma
PTLD	Post transplant lymphoproliferative disease
qRT-PCR	Quantitative reverse transcription PCR
RCAS1	Receptor-binding cancer antigen expressed on SiSo cells
RCC	Renal cell carcinoma
RNA	Ribonucleic acid
Rpm	Round per minute
RPMI	Roswell park memorial institute
RT	Room temperature
SB	Sorting buffer
SCT	Stem cell transplantation
SCID	Severe combined immunodeficiency
SEREX	Serologic screening of recombinant DNA expression libraries
SGM	Supplemented growth medium
SR	Spontaneous remission
TAA	Tumour associated antigen
TAE	Tris acetate EDTA
Taq	*Thermus aquaticus*
TAM	Tumour associated macrophages
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
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T cells
TSA	Tumour specific antigens
VEGF	Vascular endothelial growth factor
WB	Washing buffer
WBCs	White blood cells
WT-1	Wilm's tumour-1
μl	micro litre

Chapter 1
General Introduction

1 Introduction

1.1 Tumour immunology, a historical perspective

The first assumption of the defensive and protective role of the human immune system against cancer was in the late nineteenth century by William Coley, and was known as Coley's phenomenon. Coley noticed tumour regression in some patients following accidental bacterial infection, which encouraged him to investigate the clinical importance of microbial components as an immune-enhancer in the treatment of cancer patients (reviewed in Bremers & Parmiani, 2000). As a result, modern exploitations of Coley's phenomenon suggested the existence of pre-treatment, effector anti-tumour immune mechanisms which were boosted by creating an inflammatory environment following administration of microbial or pro-inflammatory molecules (Old, 1992). Later, in 1909, Paul Ehrlich conceptualized the role of cell mediated immunity in tumour prevention and elimination, in addition to revealing the role of tumour associated antigens in the development of protective immune responses (reported in Bremers & Parmiani, 2000).

In the 1950's, Burnet and Thomas developed their concepts about the interrelation between the host immune system and transformed cells, which was known as the immunosurveillance theory (Burnet, 1957). In this route, the adaptive and innate immune effectors are patrolling the human body, looking for any transformed cells in their early stages and dealing with them before their overgrowth (Zitvogel *et al.*, 2006, Schreiber & Podack, 2009). There were some discrepancies between Burnet and Thomas in their explanation of the nature of immune recognition of tumour cells. While Burnet assumed the self/non-self discrimination mechanism, Thomas described the

graft rejection as a primary mechanism against tumour development (reported by Smyth *et al.*, 2001).

The development of inbred mouse strains enabled researchers to prove the antigenic nature of the tumours and their expression of tumour specific antigens, a phenomenon responsible for the induction of rejection of syngeneic tumour transplant in mice (Dunn *et al*., 2002). However, in the 1970s, Burnet and Thomas' concept of immunosurveillance was challenged when new data published by Stutman (Stutman, 1979), and supported by other reports (Qutzen *et al*., 1975, Koebel *et al.*, 2007) showed that there were no differences in spontaneous- or Methylcholanthrene (MCA)-induced tumour development between nude mice (CBA/ H mice, lacking the thymus gland) and syngeneic wild type mice (Koebel *et al.*, 2007, Teng *et al.*, 2008). However, subsequent studies showed the following; firstly, nude mice do not totally lack functional αβ T cells, and that all of interferon gamma (IFN-γ), perforin and other effector immune cells were playing essential roles in protection against tumour formation (Smyth *et al.*, 2001). Secondly, CBA/ H mice are characterised by expression of a highly active isoform of an enzyme (Aryl hydrocarbon hydroxylase enzyme system) which bio-transforms the MCA into a more active carcinogenic form, which makes the MCA-induced cellular transformation exceed the ability of immune system to protect from tumour development (Hiatt *et al.*, 1977). Thirdly, these experiments were carried out before the emergence of lymphocyte subpopulations such as Natural Killer (NK) cells and γδ T cells which do not need thymus maturation, and play an important role in the tumour immunosurveillance (Teng *et al.*, 2008). All of these explanations potentially disagree with Stutman's observation and support the tumour immunosurveillance hypothesis.

There was no great support for Burnet and Thomas theory until the mid 1990's, where more new data were published supporting the immunosurveillance hypothesis. In 1994, Kagi *et al*, reported the generation of perforin-1 knockout mice, which enabled scientists to confirm the role of perforin-1 and in turn its mediated cytotoxicity, in protection against certain chemically-induced tumours (Kagi *et al.*, 1994). In the same context, other reports from different authors confirmed the role of not only perforin-1, but also IFN-γ, STAT-1 and T cells in inhibition of the chemically induced tumour initiation and development (Shankaran *et al.*, 2001). Shankaran *et al*. also confirmed, for the first time, that tumours resected from immunocompetent animals were less immunogenic than those from immunodeficient ones; a phenomenon related to immune editing of tumour cells (will be addressed later in this Chapter). Furthermore, tumour infiltration with various immune effector cells, especially $CD8^+$ CTL, was regarded as a good indication of tumour prognosis and prolonged patient survival (Reiman *et al.*, 2007).

The further understanding of the tumour immunology, development, and escape mechanisms in immunocompetent individuals, and the phenotypic differences between tumours developed in immunocompetent and immunocompromised individuals led to modification and extension of the immunosurveillance concept to a wider term known as "immunoediting" (Shankaran *et al.*, 2001, Dunn *et al.*, 2002). The immunoediting process is defined as a dual host protective and tumour sculpting action of the host immune system. It was subdivided into three stages; elimination, equilibrium and escape, and so called collectively as "The three Es of cancer immunoediting" (Figure 1.1).
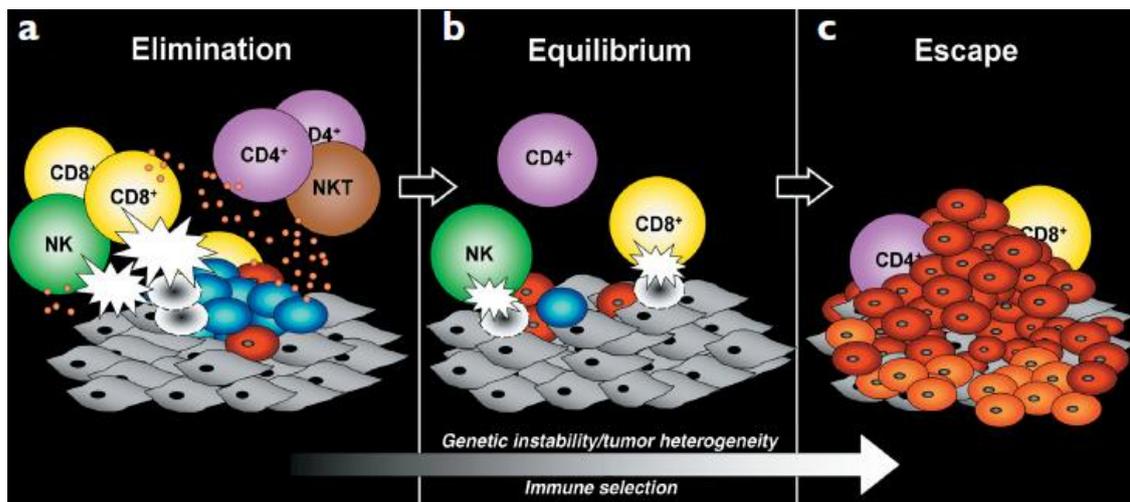
Figure 1.1: The three "Es" of cancer immunoediting. (a) Elimination: refers to immunosurveillance (recognition and elimination of the transformed cells by the immune effectors). (b) Equilibrium: represents a state of balanced interaction between resistant tumour cells and the immune system (tumour persists at a subclinical level without progression). (c) Escape: represents a process of overcoming the immune effectors by the "sculpted" tumour cells which led to tumour overgrowth. Blue rings: tumour cells; red rings: variant tumour; orange rings: highly variant tumour cells; small orange circles: cytokines; white flashes: lymphocyte-cytotoxicity; and group of different lymphocytes: as labelled (Figure from Dunn *et al*., 2002).

The first stage of immunoediting is elimination. This stage refers to the concept of immunosurveillance, in which the immune effector mechanisms, including innate, cellular and humoral responses are able to recognise and eliminate the newly emerged transformed cells. IFN-γ is one of the earliest anti-tumour cytokines released by different innate immune cells such as NKT cells; it induces direct effects on tumour cells by up-regulation of MHC molecules expression and even by induction of apoptosis through binding specific receptors on tumour cells, an effect which has been lost in IFN-γ knock out mouse models. The second stage is equilibrium, in which a continuous interaction is maintained between the transformed cells and the immune system over a period of time (called latency or dormancy) with no upper hand for either of the two parties. During this period, the tumour cells are being "edited" and new variants are developed, after which the balance is shifted toward the increased

resistance to immune recognition and attack, followed by tumour growth. The third stage is escape, which emerges as a result of down-regulation of MHC class I and/or immunodominant epitopes, induction of immunoregulatory mechanisms associated with T cell inhibition, with increased resistance to immune attack, and the balance of the equilibrium stage is shifted towards tumour overgrowth and metastasis to secondary tissues (Teng *et al.*, 2008, Stagg *et al.*, 2007). The diagram shows that escape is the phase in which the tumour can be clinically detectable in chemically induced tumour mouse models, but actually the situation is different in human, and the degree of lymphocyte infiltration and level of MHC/immunodominant–peptide expression play important role in choosing the best immunotherapeutic agent especially adoptive cell transfer. Further tumour escape mechanisms will be discussed in detail in a later Section (Section 1.4).

Regarding these aspects, many indications have been proposed relating cancer initiation and progression to integrity of the host immune system. These include: firstly, cancer spontaneous remission or regression (SR), which is defined as the spontaneous disappearance of tumours without any treatment or with clearly inadequate treatment, and SR has been estimated to be 1/140000 cases (Chang, 2000). Immune modulation was regarded as an important factor in cancer SR in addition to other possible factors e.g. cessation of oncogenic protein expression, repair of genetic damage, carcinogen withdrawal, hormonal or endocrine modifications, treatment of the underlying oncogenic infection, and the psychoneuro-religious participation (Chang, 2000). Secondly, immunocompromised individuals, such as human immunodeficiency virus (HIV) infected patients and persons on immunosuppressive medications, are regarded as high risk groups for development of microbial-induced cancers, especially Kaposi's sarcoma, non Hodgkin's lymphoma (NHL), Hodgkin's lymphoma; and lip, vulva and

squamous cell carcinomas (Clifford & Franceschi, 2007). In addition, they may moderately, but significantly, develop lung cancer, melanoma, and other cancers which are not associated with microbial infections (Bremers & Parmiani, 2000). Thirdly, in experimental animals, it has been shown by different research groups that mice were more resistant to their autologous, surgically removed tumours after being treated and cured. In addition, phenotypic investigations of *ex vivo* tumours taken from immunocompetent mice showed lower immunogenicity than tumours taken from immunodeficient mice (Schreiber & Podack, 2009, Koebel *et al.*, 2007). Moreover, immunodeficient mice developed more spontaneous and carcinogen-induced tumours than wild type mice (Zitvogel *et al.*, 2006). Fourthly, the clinical significance of some immunologic manipulations (high dose of interleukin-2 (IL-2); which is known as a lymphocyte growth factor) in the treatment of certain types of tumours (Rosenberg, 2001), raise the potential of tumour immunosurveillance. Metastatic melanoma, renal cell carcinoma, and NHL are examples of tumours which gave up to 20% response rate, ranging from partial to complete tumour regression, although often associated with a significant toxicity (Rosenberg *et al.*, 1998). Fifthly, the recent approval of the active immunotherapeutic agents; Sipuleucel (known generically as "Provenge"), and the T cell inhibitory-receptor, CTLA-4, antagonist mAb (Ipilimumab) in the treatment of certain tumour types, such as prostate and renal cancers, as well as melanoma, which is regarded as a breakthrough in the tumour immunotherapy (Carballido & Fishman, 2011, Peggs & Quenzada, 2010). Sixth, the successful use of allogeneic stem cell transplantation (ASCT) approach in the treatment of acute lymphoblastic leukaemia (ALL), acute myeloblastic leukaemia (AML), multiple myeloma (MM), and other haematological malignancies. Although several studies reported the development of graft versus leukaemia effects following ASCT (which were associated with a

minimum residual disease), it has been associated with a long term survival in half of the treated patients (Dreger *et al.*, 2010). Seventh, adoptive transfer of tumour-antigen specific CTL has been used *in vivo* in several studies (Section 1.5.2) and reported to achieve tumour regression and metastasis elimination (Ma *et al.*, 2010). Collectively, these data indicate a clear role of the anti-tumour immune responses in protection against tumour development, and suggest the potential of immunotherapy in the treatment of cancer.

1.2 Different components of tumour immunosurveillance

The immune system is generally divided into innate and adaptive immunity. The innate immune system is characterised by natural, non antigen-specific (but more or less can distinguish self from non-self), and fast acting responses. It includes several components, such as physicochemical barriers (e.g. skin, mucosa, ciliary movement of mucosal hairs, mucous secretions, tears, etc), blood proteins such as the complement system, cytotoxic cells (natural killer (NK) and NKT), phagocytic cells (e.g. monocytes, macrophages, neutrophils, and dendritic cells (DCs)), and a set of cytokines and chemokines which coordinate different immune cells to work co-operatively. The adaptive immune system is characterised by antigen specificity, memory, self and non-self discrimination, and ability to respond more rapidly and vigorously in the secondary exposure to the same antigens. Several molecules and cellular components of innate and adaptive immunity are involved in recognition and elimination of the transformed cells and summarised in Table 1.1.

Cytokines	Other molecules	Immune cells
Tumour necrosis factor (TNF-α)	mAbs	NK
Interferon-type I (IFN-α/β)	NKG2D receptor	NKT
IFN-type II (IFN-γ)	CD1d marker	γδ T cells
IL-12	MHC class I and class II	αβ T cells (CD8$^+$ and CD4$^+$)
IL-2	TRAIL/ Fas-L	Antigen presenting cells (APCs)
IL-7	Perforin/ granzymes	Phagocytes
IL-15	Complement proteins	

Table 1.1: Different cells, cytokines and other molecules involved in tumour immunosurveillance.

Moreover, it is now clear that many other factors are involved in tumour identification and elimination, depending on the tumour characteristics and the host immune system.

1.2.1 Innate tumour-immunosurveillance

This Section provides a detailed description of the main innate immune components involved in the immune response to tumours. Cellular components include NK, NKT, γδ T cells, APCs, neutrophils, and the other phagocytic cells. Non cellular components include cytokines, complement proteins, and other factors involved in protection and elimination of tumours. The role of APCs will be discussed in detail later (Section 1.2.3).

1.2.1.1 *Natural killer (NK) cells*

Natural killer (NK) cells are a subset of lymphocytes, and represent a unique component of innate immunity, with the ability to kill target cells without prior sensitization (hence the name "natural killer"). NK cells are also known as granular lymphocytes, and are phenotypically characterised by lack of CD3, expression of CD16, and variable levels of CD56 surface marker expression (Cooper *et al.*, 2001, Romagnani *et al.*, 2007). CD56 is a human cell adhesion molecule, and its main

function is to mediate NK adhesion to its target cells (Leibson, 1997). CD16 is a low affinity FcγRIII receptor, which recognises the Fc molecules of the antibodies (especially IgG isotype) to mediate target-cell cytotoxicity by NK cells (antibody dependant cellular cytotoxicity; ADCC) (Leibson, 1997, Suzuki *et al.*, 1991).

As a member of the innate immune system, NK cells express an invariant group of receptors. Unlike T or B lymphocytes, NK cells do not rearrange receptor-encoding genes in germ line, and therefore they are not able to recognise antigens presented in context of major histocompatibility complex (MHC) molecules (Hallett & Murphy, 2004). NK cells express different stimulatory and inhibitory receptors (Leibson, 1997). Inhibitory receptors include killer cell Ig-like receptors (KIR; their ligands are members of human leukocyte antigen-ABC molecules), CD94/NKG2A (its ligand is HLA-E molecule), and Ig-like transcript 2 (ILT-2) (Cooper *et al.*, 2001, Yu *et al.*, 2007). The stimulatory receptors include NKG2D (which recognises the MHC-class-I-related molecules; MIC-A and MIC-B, and UL-16 binding proteins), natural cytotoxicity receptors NKp30, NKp44, NKp46 and NKp80 (NCR; with unknown ligands), 2B4 (CD244; its ligand is CD48) (Cooper *et al.*, 2001, Yu *et al.*, 2007, Diefenbach & Raulet, 2002). Therefore, NK cells are one of the first line defence tools, which recognise and deal with the tumour cells by different mechanisms. One of these mechanisms is "the missing self recognition", by which NK cells can recognise tumour but not the normal cells, as many tumour cells lack, down regulate, or modify MHC class I molecule expression. Missing of major histocompatibility (MHC) class I molecule expression on the target cells results in loss of the NK inhibitory and dominance of the stimulatory signals. Another mechanism of NK cell activation is the cellular expression of specific ligands (such as MICA/B, Rae-1 and UL16-binding proteins) by the target cells, which

up-regulated under certain stressful conditions, such as viral infection and cellular transformation (Figure 1.2).
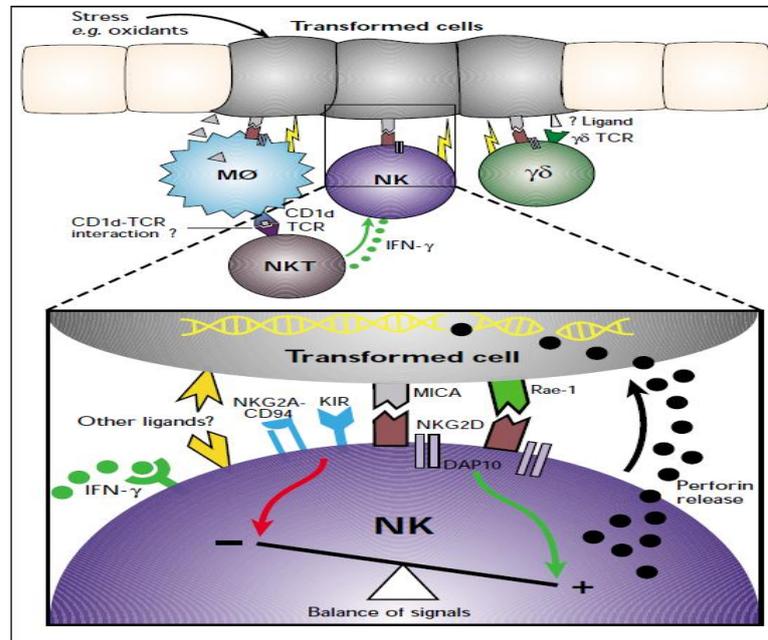
Figure 1.2: Recognition of tumour cells by innate immune cells, and the role of NK cell surface receptors. Stress-induced ligands (MICA and Rae-1) are recognised by NK stimulatory receptors (NKG2D-DAP10 complex). On the other hand, inhibitory signals are induced upon NK cells recognition of MHC class I alleles of tumour cells. In some cases, such as down regulation of MHC class I molecules (transformed or viral infected cells), activating signals dominate inhibitory signals, and this induces cytotoxicity granules and Th1-cytokine release by NK cells leading to target-cell killing. MØ= macrophage, NK= natural killer, NKT= natural killer T cells (Figure from *Smyth et al.* 2001).

NK cells can recognise these stress-induced ligands by their stimulatory receptor NKG2D which leads to NK activation. Activated NK cells have direct cytotoxic effects on target cells, in addition to other immune effector roles within the tumour environment. The direct cytotoxic effect of NK cells is mediated by attachment to the target cells using intercellular adhesion molecules (e.g. CD56), followed by cellular cytotoxicity using one of the following apoptosis mediators; Fas-L/Fas, TRAIL/TRAIL receptor, or by releasing cytotoxicity granules containing perforin, granzymes, and

granulysins (Caligiuri, 2008), Figure 1.2. Activated NK cells migrate to regional secondary lymphoid tissues and interact with different innate and adaptive immune cells by releasing Th1 cytokines (IFN-γ, IL-2, and TNF-α), leading to further activation of APCs (monocytes, macrophages, and DCs) which in turn up-regulate MHC molecules expression, and secretion of IL-12 and other cytokines. As a result, T cells are engaged by the activated APCs and release IL-2, which re-activates more NK cells to produce further IFN-γ and the pro-inflammatory cytokine TNF-α (Caligiuri, 2008, Tripp *et al.*, 1993). All of these indicate an important, instant role for NK cells as non-specific anti-tumour effector cells (Figure 1.3).
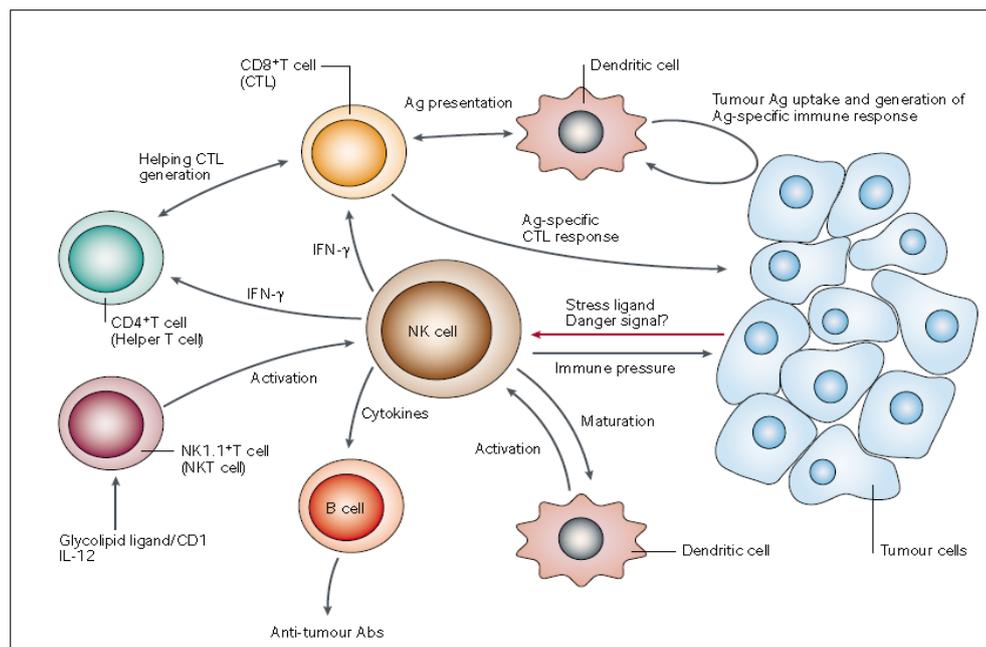
Figure 1.3: Role of NK cells in tumour immunosurveillance. Engagement of NK receptors by tumour ligands leads to net activating signals. In turn, NK cells interact directly and indirectly with the target cells, directly, by inducing apoptosis or cellular cytotoxicity, and indirectly by releasing several activating cytokines (e.g. IFN-γ) which influence the rest of innate (macrophages and DCs) and adaptive (CD4^{+}, CD8^{+}, and B) cells to induce positive immune pressure on the tumour cells (Figure from Smyth *et al.*, 2002).

1.2.1.2 NKT cells

Natural killer T cells are a group of lymphocytes, which have some characteristics of both T cells and NK cells. They have a functional TCR α chain and some NK receptors, such as NK1.1, NKG2D, CD94, and lyn 49 (Wu *et al.*, 2009). The recent description of NKT cells is as cells that recognise antigens expressed by non polymorphic MHC class I-like molecules (CD1d). The main antigens recognised by NKT cells are glycolipids, especially alfa-galactosylceramide (α-GalCer) and glycosylphosphatidyl inositols (Terabe & Berzofsky, 2007).

NKT cells are mainly CD8 and CD4 double negative, and rarely CD4 positive cells, although a significant proportion is CD8α$^+$ (Wu *et al.*, 2009). Activation of resting NKT cells requires two signals. Recognition of CD1d associated-antigens is regarded as the first signal for NKT cell activation, while the second signal is supplied by the surrounding APCs (such as DCs, macrophages or B cells) in the form of co-stimulation induced by CD80 and CD86 ligands of APCs to CD28 of NKT cells (Kawano *et al.*, 1998). Activated NKT cells can secret either protective or regulatory cytokines, such as IL-12 or IL-10 respectively. Hence, NKT cells can mediate either regulatory (Th2/ Treg) immune response, via release of immunoregulatory cytokine IL-4 and IL-10, or protective response through cell-mediated cytotoxicity and/or release of pro-inflammatory cytokines IFN-γ, IL-2, and IL-12, which stimulate further innate and adaptive immune responses (Figure 1.4) (Taniguchi *et al.*, 2010, Smyth *et al*., 2000). The specific anti-tumour protective role of NKT cells (through Th1 cytokine release and cytolytic activity) was proposed by Cui *et al*. as a response to endogenous IL-12 release by DCs, or engagement of α-GalCer in context of CD1d, or both together (Cui *et al.*, 1997). In 1998, Kawano *et al*. demonstrated that perforin release was the cytolytic mechanism by which NKT cell mediated target-cell apoptosis after its

activation with α-GalCer (Kawano *et al.*, 1998). Later, Smyth *et al.* suggested the

protective role of murine NKT cells as a spontaneous response toward chemically-

induced sarcomas without the need of IL-12 or α-GalCer (Smyth *et al.*, 2000)
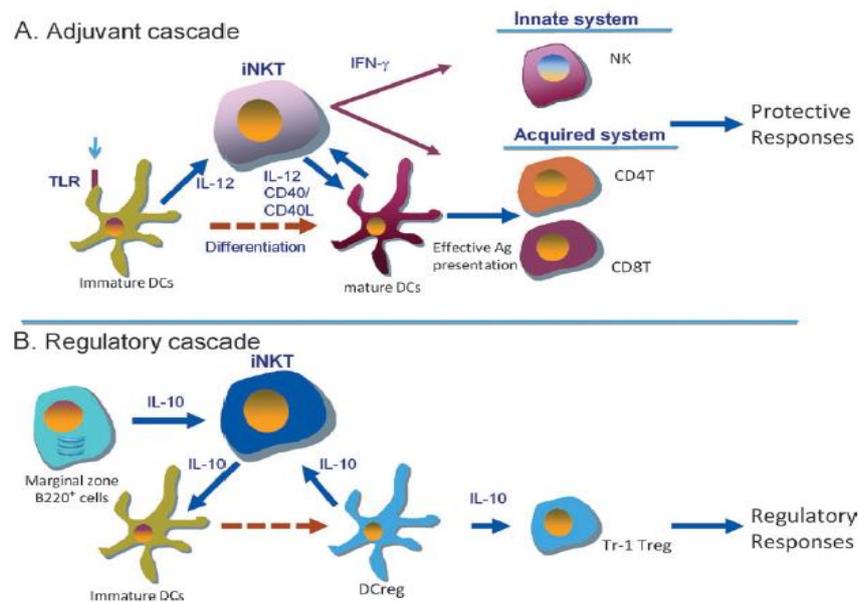
Figure 1.4: The role of NKT cells in mediating innate and adaptive immunity through both protective and regulatory responses. (A): interaction with IL-12-producing DCs induce NKT cell protective function by releasing IFN-γ, which in turn activates both adaptive and innate immune systems to protect against infective or transformed cells. (B): on the other hand, immunoregulatory signals (IL-10) induce the regulatory responses of NKT cells with subsequent induction of regulatory DCs and Treg cells (Figure taken from Taniguchi *et al.*, 2010).

1.2.1.3 Phagocytic cells

Macrophages, neutrophils, and other phagocytic cells share a complex relationship with

tumours; from one side, they have receptors for the Fc molecules of antibodies which

can make direct contact between phagocytic cells and antibody-linked tumour cells

(opsonisation) followed by tumour cell phagocytosis. On the other hand, macrophages

and neutrophils can be activated *in vivo* by bacterial products or Th1-derived cytokines

(e.g. IFN-γ). Activated macrophages are damaging for the surrounding tumours or even

self cells by releasing lysosomal enzymes and TNF-α. On the other hand, other reports showed production of reciprocal growth factors between macrophages and tumour cells which may indicate a sort of mutual beneficial relationship between them (Coico & Sunshine, 2009).

1.2.1.4 *Complement system and immunosurveillance*

The complement system, which comprises a group of over 30 serum proteins, is thought to play an anti-tumour role after its activation, through indirect pathway (ADCC) (Gelderman *et al.*, 2004) or directly through formation of cytolytic membrane attack complex (MAC), which consists of complement components C5b-9 (complement dependent cytotoxicity; CDC) (Gelderman *et al.*, 2004) (Figure 1.5). Several *in vitro* and *in vivo* studies reported the direct and indirect activation of the complement system by malignant cells through classical pathway activation (Fishelson *et al.*, 2003). However, no correlation was found between tumour progression and complement proteins levels in cancer patients. However, different reports showed normal or slightly elevated complement levels in patients with different haematologic malignancies (Southam & Siegel, 1966, Minh *et al.*, 1983). On the other hand, the normal cells protect themselves from complement-mediated attack by expression of membrane-bound complement regulatory proteins (mCRP) such as CD46, CD55, and CD59 that are often expressed at enhanced levels by tumour cells. In the case of CD55, it is expressed by tumour cells up to 100-fold more than normal cells, and its expression is up regulated in the presence of vascular endothelial growth factor (VEGF) (Morgan *et al.*, 2002). Complement activation is inhibited by mCRP through the induction of C3 and C5-convertases' decomposition, which in turn inhibits C3b formation and the downstream reactions which ends by MAC formation inhibition (reviewed in Capasso *et al.*, 2006). A recent report by Markiewski *et al.* (Markiewski *et al.*, 2008) showed

that, in some cases, complement activation enhanced the tumour growth and progression by formation of the anaphylatoxin and chemo-attractant C5a and C3a, which recruited myeloid derived suppressor cells (MDSC) to the tumour microenvironment, which in turn inhibited CD8^{+} CTL effector function. Recent reports suggested a role for complement in tumour development, invasion, and also in low susceptibility of tumour to apoptosis (Rutkowski *et al.*, 2010). Clinical investigations are now being undertaken to address the effect of complement activation on different types of tumours at various stages (Moghimi & Andresen, 2009).
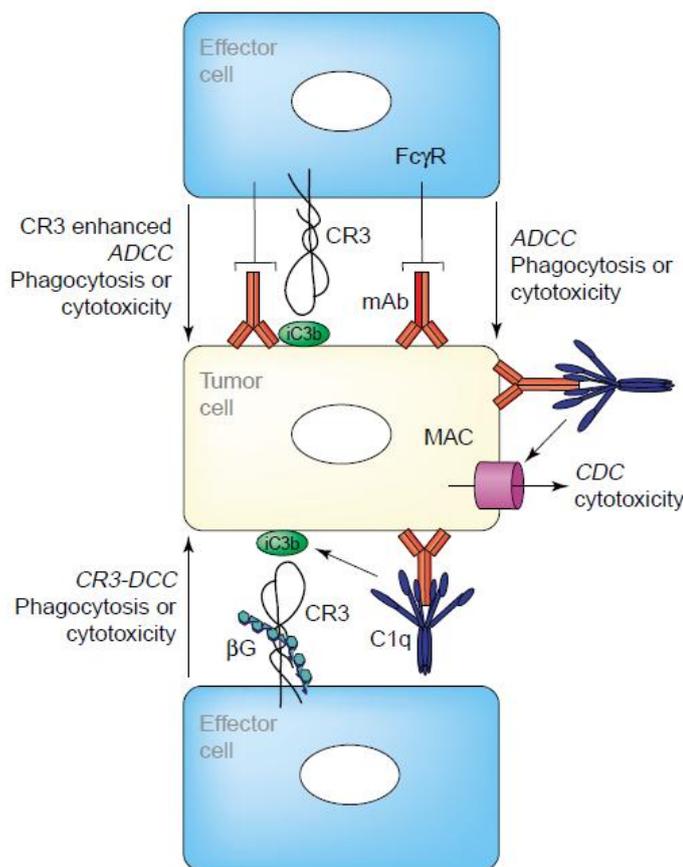
Figure 1.5: Effector mechanisms of complement system activation by tumour cells. In presence of mAb, activated C1q induces C3 deposition and activation with formation of membrane attack complex (MAC) followed by tumour cell lysis (complement dependent cytotoxicity), deposited C3b can bind to C3 receptor (CR3) and facilitate mAb-dependent cellular cytotoxicity or phagocytosis by effector cells (NK, granulocytes, or macrophages). Independently, C3b can bind the primed CR3 receptor and induce CR3-dependent cellular cytotoxicity (CR3-DCC). However, this is not the normal pathway of complement activation in tumour immunosurveillance (Figure from Gelderman *et al*., 2004).

1.2.2 Adaptive tumour-immunosurveillance

Adaptive (or acquired) immunity is the second arm of the immune system. It is characterised by specificity, memory, self/ non-self discrimination and exaggerated response on repeated exposure to the same antigens. The adaptive immune system can be classified into the cell-mediated immune response (T-cell dependent), and antibody-mediated or humoral (B-cell dependent) immune responses. T cells are the principal effector cells of tumour-immunosurveillance, in addition to the previously mentioned NK and NKT cells.

For activation of the adaptive immune system, tumour-associated antigens (TAAs) are taken up, processed, and presented by DCs (the main APC of the immune system). The DCs process and present the tumour-derived antigens in the context of MHC class II molecules, or via cross-presentation in the context of MHC class I molecules for recognition by T cells. Additionally, APCs need "danger" signals, such as inflammatory cytokines, Pathogen Associated Molecular Patterns (PAMP), or TLR agonists to mature and upregulate expression of costimulatory molecules. Upon maturation, APCs migrate to the lymph nodes and present TAAs (in the context of MHC class II molecules) to activate naïve $CD4^+$ T helper (Th) lymphocytes. Th cells recognize the MHC class II/peptide-epitope complex by their T cell receptor, but costimulation, via ligation of the B7 receptor family on the APC with surface receptors on the T cell, is required by the naive T cells for full activation. Upon inadequate stimulation by APCs, T cells become inactivated or tolerant towards tumour antigens and fail to elicit an effective antitumor response. On the other hand, full activation of the Th lymphocytes induces cytokine release, mainly IFN-γ that enhances $CD8^+$ cytotoxic T lymphocytes (CTL) proliferation and differentiation. Naive CTLs recognize their target antigens in the context of MHC class I molecules of DCs. Together with co-stimulation, induced by B7 molecules on

the DCs, induce CTL proliferation and effector/ memory differentiation. Activated

tumour-specific Th cells and CTLs home back to the tumour sites, where CTLs

recognize the cognate peptide presented in the context of MHC class I molecules on the

tumour cell surface, followed by induction of programmed cell death or apoptosis of

tumour cells. If cancer-immunoediting was effective in the elimination phase, the

developing tumour will be eradicated. Otherwise, the tumour is shaped by the immune

system, whereby less immunogenic variants will develop. In the equilibrium phase,

immune resistant tumour cells develop due to genetic instability and immune selection

pressure. Tumour cells surviving the equilibrium phase transfer to the escape phase and

gain the capacity to progress and evade the immune system. In the escape phase;

tumour growth proceeds unrestricted by the immune system, which ends by clinically

detectable disease. Mechanisms by which the tumour escapes the immune system will

be discussed in details in Section 1.4.

1.2.2.1   T cell response

T cells represent around 70-80% of the peripheral blood mononuclear cells (PBMCs)

and express mainly $\alpha\beta$ T cell receptor (TCR), with a subpopulation expressing $\gamma\delta$ TCR.

All T lymphocytes are $CD3^+$, and can be further divided according to their function and

phenotype into $CD4^+$ or $CD8^+$.

$CD8^+$ cytotoxic T lymphocytes (CTLs) recognise target cells, such as tumour or virally

infected cells, by binding peptide-epitopes presented on MHC class I, and mediate

cellular cytotoxicity by releasing cytolytic granules (perforin, granzymes and

granulysins), or through Fas-L/Fas interaction to induce target cell apoptosis

(Mantovani *et al.*, 2008). Effector $CD4^+$ T cells modify the function of other immune

cells like CTLs, DCs, NK and B cells directly by CD40L/CD40 and ICOSL/ICOS

engagement, or indirectly by release of cytokines. Functionally, naive CD4$^+$ Th0 cells

can be differentiated into Th1, Th2, Th17, or T regulatory (Treg) phenotypes (Martin-

Orozco *et al.*, 2009) according to the associated signals and the deriving cytokines; IL-

12, IL-4, IL-23/IL-6/IL-22/TGF-β, and IL-10/TGF-β polarise for Th1, Th2, Th17 and

iTreg respectively. Each Th phenotype is characterised by secretion of a set of

phenotype-specific cytokines to help perform its effector function. In general, Th1 cells

release IFN-γ, IL-2, and TNF-α to stimulate immune responses against intracellular

pathogens and tumour cells. Th2 cells release IL-4, IL-5, and IL-13 to trigger the

elimination of parasitic helminths, or induction of B-cell humoral responses. Th17 cells

respond to extracellular pathogens and they produce IL-17, IL-22, and IL-6, which were

reported to have a role in fighting infection at mucosal sites. Recently, an effector role

of Th17 was reported in activation of CTLs against cancer cells (Martin-Orozco *et al.*,

2009, Bonilla & Oettgen, 2010, Chaplin, 2010). Inducible Tregs (iTreg) are derived

from naive CD4$^+$ after inadequate activation by immature DCs in a tolerogenic

microenvironment (Figure 1.6). In addition, several factors also enhance the induction

of iTregs e.g. IL-10, TGF-β and others. Immunosuppressive function of Treg will be

discussed in details in Section 1.4.5.
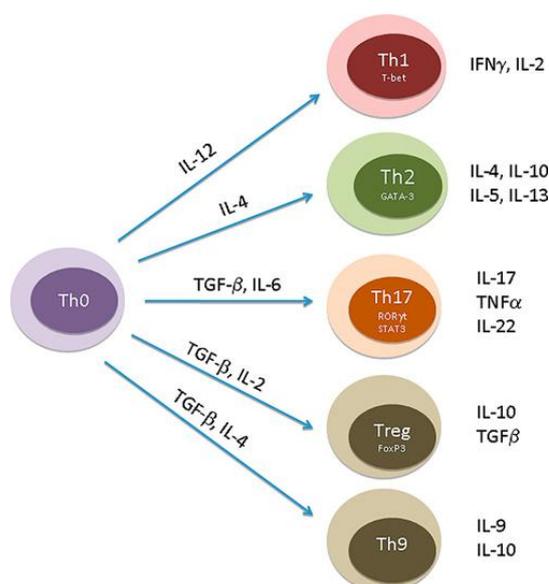
Figure 1.6: T helper cells subsets, naive Th0 cells are polarised to differentiate into Th1, Th2, Th17, Treg, or Th9 depending on the microenvironment polarisation cytokines IL-12, IL-4, IL-22/23/6, IL-10/TGF-β, or IL-4/TGF-β respectively (Figure from Bonilla & Oettgen, 2010)

In addition, a new subset of T helper cells has been reported recently, Th9, which differentiates after exposure of Th2 cells to IL-4/TGF-β, followed by release of IL-9 which has anti-helminths effector role and also acts as mast cell growth factor (Bonilla & Oettgen, 2010).

1.2.2.1.1 T cell receptor (TCR)

T cells are characterized by expression of highly variable, antigen-specific receptors (TCR) which have a clonal distribution i.e. each T cell clone expresses TCR with unique specificity. It has been estimated that each normal individual possesses a huge number of different TCRs within T cell repertoire. This repertoire was generated during embryonic development by somatic gene rearrangement of V(D)J gene segments. Each TCR is composed of two disulfide-linked polypeptide chains, α and β chain, (minor amount of T cells has γδ chains). Both chains comprise V (variable) and C (constant) regions. Vα and Vβ regions are folded together to form three hypervariable domains, CDR1, CDR2 and CDR3, which together form a monovalent antigen binding site which is different from Ig (immunoglobulin)-divalent antigen binding site of mAbs. The TCR genes are similar to immunoglobulin genes in their rearrangement process, except that Ig receptors perform somatic hypermutation to increase antigen binding. Some disorders may lead to failure of rearrangement in both Ig and TCR, and subsequent lack of both B and T cells repertoire, which causes lack of adaptive immunity and a condition called severe combined immunodeficiency (SCID), which is fatal by nature (Chlewicki *et al.*, 2005). Humanised SCID mice were used successfully to study role of B and T cells in *in vivo* response to viral infection and tumourigenesis (Roncarolo *et al.*, 1996). The TCR α and β chains are associated, non-covalently, with the CD3 complex [comprises 3 different polypeptides γ (gamma), δ (delta) and ε (epsilon)] and two identical ζ (zeta, CD247) chains (Figure 1.6). Both CD3 and ζ act as signal transducers

following antigen binding to α and β chains. The combination of TCR α and β chains plus CD3 and ζ is called the TCR complex. The CD3 molecule is invariant and is exclusively expressed on all T lymphocytes, so in addition of its role in activation-signal transduction through its tyrosine containing motive (immunoreceptor tyrosine-based activation motif; ITAM), CD3 also used as a marker to distinguish T cells from all other cell types (Figure 1.7).
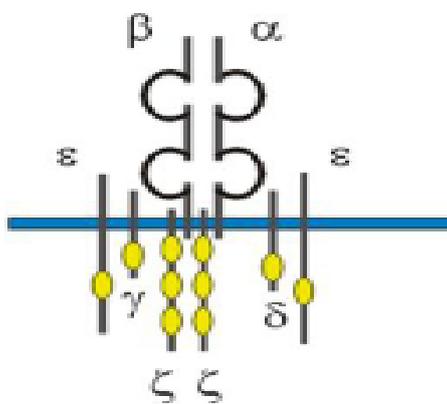
Figure 1.7: TCR complex; α and β antigen-recognising chains associated with signal transduction complex CD3 (γ, δ and ε chains) plus ζ. ITAMs are represented by the yellow oval shapes (Figure from www.wikipedia.org).

As mentioned earlier, mature T cells are classified into two main classes, according to their expression of CD4 or CD8 markers. CD4 and CD8 are trans-membrane molecules and regarded as members of the Ig super-family which do not bind the antigens, but enhance the antigen's ability to activate T cells by reducing the threshold for antigen response, through their intracellular attached protein kinases, by binding to the invariant portion of MHC class II or class I respectively. MHC class I molecules are expressed on all nucleated cells, while MHC class II molecules are expressed on professional APCs, such as DCs, macrophages and B cells. CD8^{+} T cells recognise antigens presented by MHC class I, and CD4^{+} T cells recognise antigens presented by MHC class II.

Although the majority of T cells have αβ TCR, there is a subset that has another type of chains in their TCR, which are γδ chains (which are different from γ and δ chains of CD3), and referred to as γδ T cells. As a general rule, γδ T cells lack CD4 and CD8 coreceptors, although some were found to express CD8, especially in the intestine. γδ T cells are found in high proportion in mucosal epithelium and regarded as part of the innate immune system in the first line of defence against invading pathogens, especially *Mycobacterium* species. The pattern of antigen recognition is different from αβ T cells, as γδ T cells respond to phospholipids, phosphoprotiens, and heat-shock proteins (proteins produced in heated or stressed cells) in association with non-polymorphic MHC class I molecules (CD1a, b or c). Upon activation, γδ T cells respond by cytokine release, especially IFN-γ, and by inducing cellular cytotoxicity of the target cells (Coico & Sunshine, 2009).

1.2.2.1.2 *Immunomodulatory T cell receptors*

Full activation of naive T cells (T cells which have not previously encountered antigen) needs at least two signals; the first is the interaction between TCR and antigenic-peptide presented in the context of MHC molecules, and the second signal is the interaction of the costimulatory ligands (B7 family or CD80 and CD86) of APCs with their receptors on naive T cells (CD28). This costimulation is critical for the activation of naive T cells to proliferate and perform their cytokine release, helper and cytolytic functions. In addition, T cells engage CD40 on APCs via its ligand, CD40L (CD154), which enhances costimulatory interaction by up regulating B7 expression, and also induces T cell-dependent B cell isotype switching. The activated T cells tend to return to their resting state, after performing their function and eliminating the target cells, through the inducible expression of regulatory receptor CD152 (known as cytotoxic T lymphocyte antigen-4; CTLA-4), which has the same CD28 ligands (CD80 and CD86) but of higher

binding affinity and which has an inhibitory effect on the activated T cells. A group of stimulatory or inhibitory receptors and their ligands are explained with their function in Table 1.2.

Receptor	Ligand	Effector function on T cells
CD28	CD80, CD86	↑ proliferation and IL-2 production
CTLA-4	CD80, CD86	↓ proliferation and TCR signalling
PD-1	PD-L1, PD-L2	↓ proliferation of non-functional T cells
CD27	CD70	↑ expansion and effector functions
4-1BB	4-1BBL	↑ generation, maintenance and enhance proliferation
OX40	OX40L	↑ survival and expansion
CD40L	CD40	↑ quality of other stimulatory/ regulatory signals
TRAIL receptors	TRAIL	↓ survival of helpless T cells
Lag3 and Tim3		↓ survival and expansion of T cells

Table 1.2 Summary of different costimulatory and coinhibitory T cell receptors, their ligands, and their suggested roles in T cell function (Table adapted from Abbas & Lichtman, 2008).

1.2.2.1.3 Role of MHC molecules in T cell mediated tumour-

immunosurveillance

Tumour cells express abnormal proteins or abnormal levels of endogenous proteins, which are known as tumour associated antigens (TAAs), and can be recognised and distinguished by T cells. To be recognised by CD8$^+$ CTL and CD4$^+$ T cells, TAAs have to be processed and presented as short peptides in the context of MHC class I or MHC class II molecules expressed on professional APCs respectively. *In vivo*, naive T cells

are primed in the regional lymph nodes through direct contact with mature, professional APCs (mainly DCs), by which TAAs were uptaken, processed, and presented via MHC class II and co-presented via MHC class I. Co-stimulation, by B7 engagement with CD28 on naive T cells, is the second mandatory signal supplied by DCs (in addition to MHC/peptide complex recognition by cognate TCR). T cell peptide recognition is MHC restricted, which means that MHC compatibility is required between the T cells and APCs. In humans, MHC molecules are encoded by a set of genes which are found on the short arm of chromosome six, and their products are represented by human leukocyte antigens (HLAs).

1.2.2.1.3.1 MHC class I structure and function

There is a group of independent genes encoding MHC class I molecules, and they produce highly polymorphic HLA-A, HLA-B, and HLA-C molecules. Recently, three more genes were identified producing less polymorphic antigens, HLA-E, HLA-F, and HLA-G (Coico & Sunshine, 2009). HLA class I molecules are constitutively expressed by almost every nucleated cells (there are some exceptions, such as some of nervous system and germline cells), and are commonly down regulated in viral infected and tumour cells (Sidney *et al.*, 2008, Algarra *et al.*, 2004). Each MHC class I molecule consists of a single chain composed of 3 domains (α1, α2 and α3), non-covalently linked to β2-microglobulin at the cell surface (Figure 1.8). The α1 and α2 domains are arranged in a special conformation to generate a peptide binding groove, known as the variable or polymorphic region, in which a 8-10 residue polypeptide is bound. The α3 domain represents the invariable region which binds to CD8 co-receptor of the CTL-TCR complex. The variability of the binding grooves allows for heterologous peptide presentation by the same MHC allele. This natural variability gives rise to presentation

of huge number of peptides to T cells and to diversity of the immune response (Kalish, 1995).
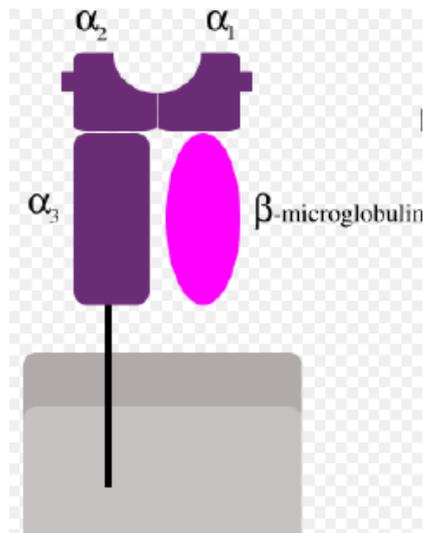
Figure 1.8: MHC class I schematic representation, showing the peptide binding groove formed of α1 and α2 Ig domains. α3 domain represents the invariable region which binds to the CD8 molecule of CD8$^+$ T cells. The three α domains together with β2-microglobulin form a stable complex with the presented peptide on the cell surface (Figure from www.wikipedia.org).

In addition to providing the endogenously presented or exogenously cross-presented peptides (mediated by professional APCs) to CD8$^+$ CTL, MHC class I molecules also provide the inhibitory signals to self or matched NK cells. Activated NK cells recognise MHC class I mismatched or missing targets (such as most tumour cells) and attack them through cellular cytotoxicity.

1.2.2.1.3.2   MHC class II structure and function

Like MHC class I, another group of independent genes on the same chromosome is responsible for production of HLA-DQ, HLA-DP, and HLA-DR molecules, which represent the three main MHC class II polymorphic antigen-presenting molecules. Professional APCs, including DCs, B cells, and macrophages, constitutively express MHC class II molecules, and their expression levels are elevated upon stimulation. Under certain inflammatory conditions, MHC class II molecules are inducibly expressed by non-professional APC (fibroblasts, epithelial cells, and keratinocytes), and activated T cells (Holling *et al.*, 2004, Costantino *et al.*, 2009). MHC class II molecules

are composed of one α and one β chain, each of two Ig domains (α1, α2, β1, and β2).
The two distal (α1 and β1) domains fold together to form the variable polymorphic
peptide binding groove, which is open at both ends and hence could bind to peptides
from 13 up to 30 residues (Kalish, 1995, Rudensky *et al.*, 1991). The invariable β2
domain is the binding site for CD4 co-receptor of TCR complex (Figure 1.9).
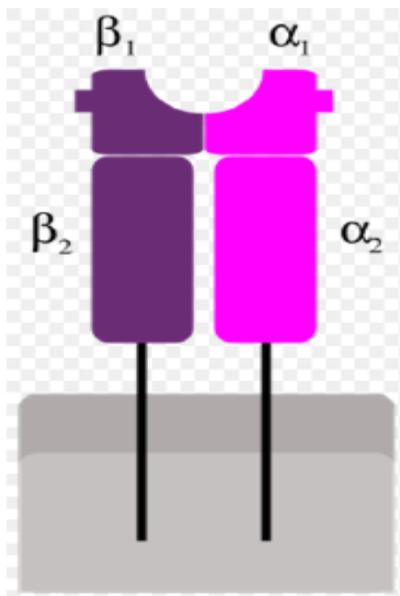
Figure 1.9: MHC class II molecule schematic representation, showing the peptide binding groove formed of β1 and α1 Ig domains. β2 domain represents the invariable region which binds to CD4 co-receptor of the CD4$^+$ TCR complex (Figure from www.wikipedia.org).

MHC class II molecules present the processed peptides derived from extracellular
proteins taken up by APCs through phagocytosis, endocytosis, or pinocytosis, and
expose them to be recognised by the TCR of CD4$^+$ T cells. *In vivo*, tumour-antigen
specific naive CD4$^+$ T cells are primed through APC (especially DCs), as most tumours
lack MHC class II molecule expression and are also unable to provide co-stimulation
mediated by B7 molecules.

1.2.3   Role of antigen presenting cells in tumour immunosurveillance

Antigen presenting cells are a group of innate immune cells which play an important
role in both adaptive immune responses (cellular and humoral), and in initiating anti-

tumour responses against tumour cells. Professional APC are characterised by constitutive expression of MHC class I, MHC class II, CD80, CD86, and CD40 molecules. In addition, they are able to uptake extracellular antigens (including tumour antigens), process, and present derived peptides in the context of MHC class II, and cross present them in the context of MHC class I molecules (Rock & Shen, 2005). Upon receiving danger signals (such as PAMPs, TLR agonists, pro-inflammatory cytokines, or heat shock protein), APCs get mature, migrate to regional lymph nodes where they meet and prime naive, antigen-specific $CD8^+$ and $CD4^+$ by direct contact, and indirectly stimulate proliferation of other innate immune cells (NK and NKT cells) by releasing of IL-12. In turn, the activated NK and NKT cells release IFN-γ and IL-2, leading to up regulation of both innate and adaptive immune responses.

1.2.3.1 Dendritic cells (DCs)

DCs are a heterogeneous family of professional APCs distributed in the circulation and several body tissues, especially portal sites. They are the most potent APCs, and are regarded as the principal activators of primary T cell responses (Davison, 2010). There are two main classes of DCs; myeloid and plasmacytoid. They differ in their surface marker expression panel, and effector function. Plasmacytoid dendritic cells (pDCs) are a major player in early innate responses against pathogens, by release of type I interferon (IFN-α,β). Phenotypically, pDCs are positive for HLA-DR and CD123 (IL-3 receptor α chain), while they lack lineage specific markers (CD3, CD19, CD56, CD11c, CD13 and CD33). It was suggested that they induce tolerance by polarizing toward production of Th2 or Treg cytokines such as IL-4 and IL-10 respectively, and by release of IDO (Cox *et al.*, 2005), however other studies reported a pro-inflammatory role of pDCs in viral infections by releasing of IFNα and IL-12 (Cella *et al.*, 1999).

On the other hand, conventional or myeloid DCs (mDCs) are the major player in activation and induction of T cell responses. Immature mDCs are found in different types of body tissues and express phagocytic receptors which take up protein antigens. Other external molecule internalization mechanisms include macropinocytosis and micropinocytosis. Immature mDCs are efficient in endocytosis and processing of protein antigens into short peptides. However, they lack the optimum levels of MHC and costimulatory molecules required for effective naive T cell priming. Therefore, engagement of TCR by DCs' MHC/peptide complex without appropriate co-stimulation leads to T cell un-responsiveness or tolerance. Maturation of mDCs can be mediated by several mechanisms, such as recognition of microbial substrates by their pattern recognition molecules (e.g. TLRs), engagement of their CD40 by CD40L, or by certain cytokines such as IFN-γ and IL-12. Mature DCs express high levels of MHC class II and costimulatory ligands which are required for priming both naive T and B cells, and are capable of providing both signals required for activation of naive T cells.

1.2.3.1.1 Endogenous antigen processing and presentation via MHC class I molecules

Proteasomes are cytosolic organelles that are responsible for processing of cytosolic self and foreign proteins. They contain proteolytic enzymes which digest antigens into small peptides before transporting them into the endoplasmic reticulum (ER), by the transporter associated with antigen processing (TAP) molecules. In the ER, TAP-containing peptides interact with MHC class I heterodimers lacking-peptides to form a large complex in association with tapasin (ER-oxidoreductase of 57KD; ERp57), protein disulfide isomerase (PDI) and calreticulin. This complex is called peptide loading complex (PLC). Once a MHC class I/peptide complex is formed, it is

transported by the Golgi apparatus to the cell surface for presentation of the peptide to

CD8$^+$ T cells (Figure 1.10).
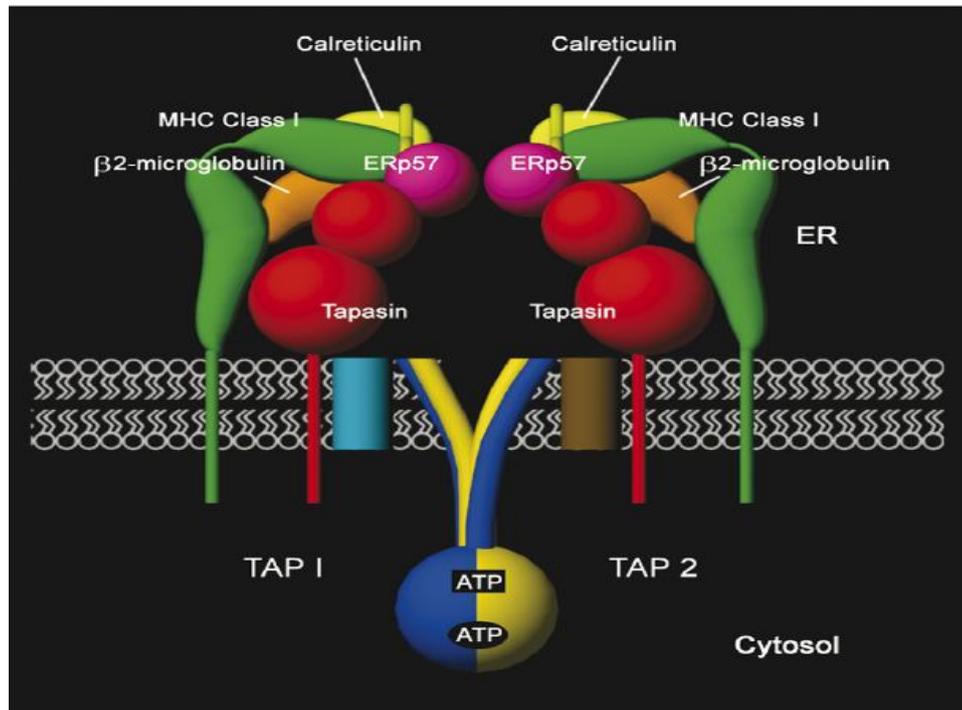
Figure 1.10: Peptide-loading complex (PLC) in the ER; MHC class I heavy chain (green) and β2-m (orange) in association with tapasin (red) and TAP1-TAP2 (blue and yellow respectively) complex. Many other factors are included in this complex, like calreticulin (light green), ERp57 (purple) and PDI (not shown) which together constitute PLC, (Figure from Raghavan *et al.*, 2008).

1.2.3.1.2 *Exogenous antigen processing and presentation via MHC class II*

molecules

MHC class II molecules present antigens derived from exogenous sources through

phagocytosis or endocytosis, which were processed by the acidic compartment with

association of lysosomes. Autophagy is another recent pathway by which cellular and

nuclear antigens can be processed and delivered to MHC class II molecules during

intracellular homeostasis (Crotzer & Blum, 2009). APCs, particularly DCs, B cells and

macrophages, internalise the exogenous antigen into an intracellular acid vesicle

(phagosome or endosome) followed by fusion with lysosomes. These acidic

endolysosomal vesicles recruit a set of proteolytic enzymes like proteases (known as

cathepsins) and peptidases to digest the associated antigens into short peptide fragments. MHC class II α and β chains are synthesised independently in ER and then assembled together, with association of CD74 (li), known as invariant chain, which acts as a trafficking chaperone to prevent binding of any peptides within the ER to this complex (Bryant & Ploegh, 2004). The MHC/li complex leaves the ER through the Golgi apparatus into the endolysosomal vesicle, where li is degraded by the proteolytic enzymes followed by attachment of CLIP (class II-associated invariant polypeptide) molecule to the free MHC II molecule. Fusion of CLIP/MHC class II complex into the acid vesicle enhances CLIP exchange with the processed peptides, with the help of HLA-DM molecule. Finally the MHC/peptide complex moves to the cell surface to expose and present bound antigen to CD4$^+$ T cells (Figure 1.11).
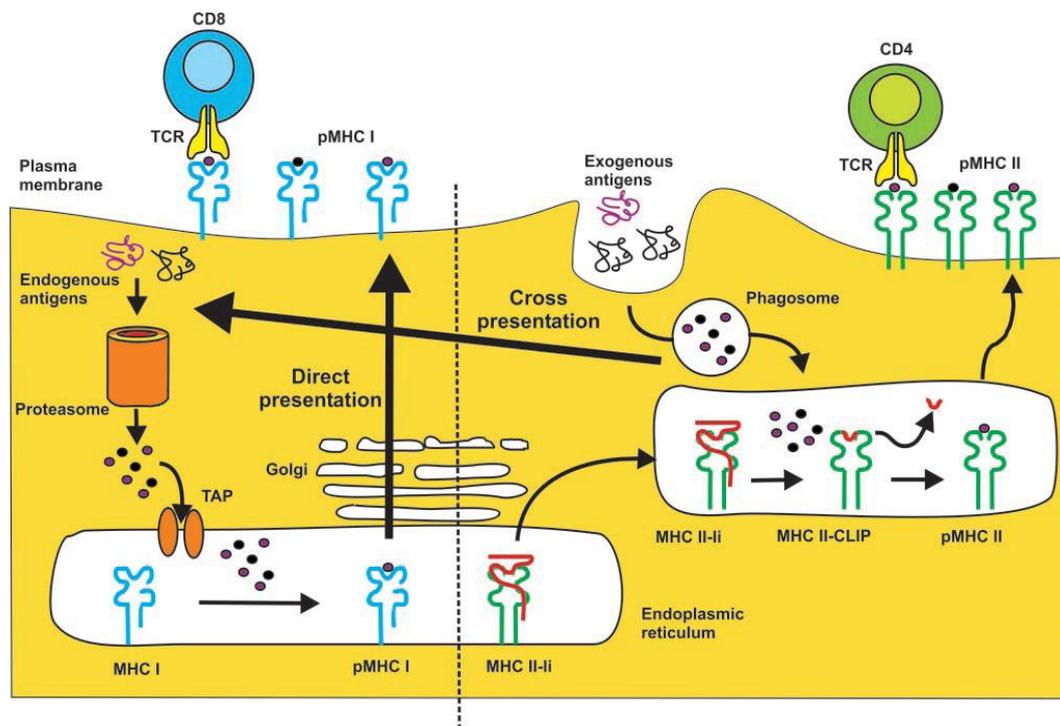
Figure 1.11: Antigen processing of endogenous and exogenous internalised antigens, followed by association with MHC class I or MHC class II molecules to be presented on the cell surface for recognition of CD8$^+$ or CD4$^+$ T cells. Figure also shows the ability of APCs to present exogenous antigen via MHC class I in a process called cross presentation (Figure from Friese & Fugger, 2005).

1.2.3.1.3 Cross presentation of exogenous antigens

Professional APCs, especially DCs, are characterised by their ability to present exogenous phagocytosed or endocytosed antigens in the context of MHC class I to induce CD8$^+$ cytotoxic T cell responses (cross priming of CTL), which plays an important role in induction of cytotoxic immune responses against infected and tumour cells (Crotzer & Blum, 2009) (Figure 1.11). In cross presentation, the exogenous internalised antigens can be processed through two different pathways. Firstly, antigens are transferred from phagosomes or endosomes into the cytosol, where they are fragmented by proteolytic enzymes and transported via TAP molecules into the ER to be processed by the normal MHC class I presentation pathway. In the second suggested pathway, antigens are digested by the endosomal proteolytic enzymes and bound to MHC class I molecules in the endosome itself. Depending on the type of antigen, one or

both of these pathways may be involved in cross presentation *in vivo* (Rock & Shen, 2005). Cross presentation is the only explanation of the *in vivo* pathway of tumour-specific naive $CD8^+$ CTL activation by DCs, especially in most solid and some haematologic malignancies, where tumour cells lack expression of co-stimulatory B7 molecules.

1.2.3.1.4 Autophagy and antigen presentation

Recently, a new route for presenting endogenous cytosolic and structural proteins into MHC has been identified, and called autophagy. In this route, and under certain nutritional and stressful conditions, parts of the cytoplasm are sequestered into double-membrane structures known as autophagosomes. This structure expands and engulfs parts of the cytosol, including entire organelles. The outer membrane of the autophagosome fuses with the lysosomal membrane forming an autolysosome, in which the contents are digested by action of the proteolytic enzymes. The hydrolysed antigens bind with either MHC class I or MHC class II molecules for presentation to and recognition by $CD8^+$ or $CD4^+$ T cell respectively (Crotzer & Blum, 2009).

Autophagy has been reported in recognition of viral, self, and tumour antigens in the context of MHC class II *in vitro* and *in vivo*, and its association in immune responses against pathogen, tumours and self antigens has been reviewed in several reports (Lunemann & Munz, 2009, Mizushima *et al.*, 2008).

1.2.3.2 Antigen specific B cells as initiators of T cell responses

B cells are often regarded as professional APCs. They are able to recognise their cognate-antigens through their surface antibodies. The recognised antigens are then internalised, processed and re-presented in the context of MHC class II to the cognate $CD4^+$ T cells. B cell/T cell interaction (which occurs in the lymph node follicles) is antigen and CD40-restricted. Following antigen recognition, $CD4^+$ Th2 cells induce B

cell maturation, and up regulate the expression of CD80/D86 co-stimulators by engaging CD40 through their CD40L (CD154). On the other hand, co-stimulatory molecules of the activated B cells can bind T cells' CD28 and induce further T cell proliferation and differentiation (Evans *et al.*, 2000). Therefore, the effector Th cells re-stimulate antigen-specific B cells resulting in proliferation, differentiation, Ig class switching and affinity maturation. The role of B cells in priming CD4^{+} T cells was investigated by different *in vivo* studies on transgenic mice lacking functional B cells. Rivera *et al*., reported that Th priming was highly dependent on the presence of B cells, in a study carried out on B cell deficient mice (muMT). Moreover, the authors showed the restoration of total T cell priming by B cell reconstitution of muMT mice (Rivera *et al.*, 2001). Another study carried out by Kleindienst and Brocker demonstrated the relative importance of DCs and B cells in the *in vivo* priming of CD4^{+} T cells in transgenic mice. They showed that DCs were sufficient to induce adequate proliferation, differentiation, and cytokine production of CD4^{+} T cells. However, the presence of B cells enhanced the response and number of activated T cells (Kleindienst & Brocker, 2005). On the other hand, an *ex vivo* study by Cassell *et al*. was carried to compare the stimulatory capacities of DCs and activated B cells. The authors observed equal proliferation efficiencies, with slightly less TCR signalling and great reduction in IL-2 releasing response in the case of T cells activated by B cells compared with DCs (Cassell & Schwartz, 1994). These studies showed the ability of B cells to induce CD4^{+} Th stimulation, albeit with a lower efficiency than DCs.

1.3 Tumour immunogenicity

As a result of transformation, tumour cells express either abnormal proteins or abnormal levels of normally expressed proteins. In both cases, tumour-antigens are usually recognised by patient's own immune systems as "foreign".

Tumour-specific antigens (TSAs) are those antigens that are expressed only by tumour but not by normal cells. They are usually highly immunogenic, and can induce strong antigen-specific cellular immune responses, due to lack of self tolerance. Oncogenic-viral antigens, and mutated tumour suppressor genes are examples of tumour-specific antigens. However, tumour-associated antigens (TAAs) are self-antigens which are expressed by certain specific tissues, in specific developmental stages, or within limited levels in normal state. They are mostly less immunogenic, as they represent self antigens and may be subject to self tolerance. Immunogenicity of tumour cells (represented by tumour-antigens) is an important factor for the *in vivo* induction of primary natural immune responses against them. It also represents a key factor for immunotherapeutic interventions in order to stimulate specific, highly selective, and safe therapeutic immune responses. Ideal tumour-antigens for use as immunotherapeutic agents are proteins that are tumour-specific, stably expressed by tumour cells, and essentially involved in growth and invasiveness of tumour cells, but are absent in normal tissues (Meklat *et al.*, 2007). In addition, the tumour environment plays an important role in tumour antigenicity, e.g. the availability of highly active APC (such as Langerhans cells in the skin, and its role melanoma immunogenicity) increases the likelihood of induction of a cellular immune response. The following Sections provide details of the known classes of tumour-antigens expressed by solid and/or haematological malignancies.

1.3.1 Cancer testis (CT) antigens

Cancer testis (CT) antigens are a group of antigens that normally expressed by certain normal immune-privileged tissues such as germline cells (testis and ovaries), and expressed in small amounts in other normal tissues (Chen *et al.*, 1997). The first CT antigens discovered in solid tumours were MAGE-A1 (van der Bruggen *et al.*, 1991),

BAGE, and GAGE (Boel *et al.*, 1995, De Backer *et al.*, 1999). Later and using bioinformatics and advanced antigen-screening approaches, new members of CT antigens were discovered. More than 100 CT antigen genes have been reported in last 2 decades, of which ~ 30 are members of multigene families on chromosome X (CT-X genes) (Caballero & Chen, 2009). A group of known CT families are listed in Table 1.3. Most CT antigens represent excellent targets for immunotherapy, as they are widely distributed in several types of solid and haematological malignancies, and are expressed by immune privileged tissues but not by normal somatic cells. However, testicular tissues are protected from immune attack by blood barriers and by lack of MHC class I expression (Westbrook *et al.*, 2004, Fiszer & Kurpisz, 1998). The tumourigenic roles of CT-antigens were demonstrated by Simpson *et al.*, and others, who reported their roles in tumour invasion, and metastasis (Simpson *et al.*, 2005).

Gene family	Disease and frequency of distribution	Antigen members
MAGE-A	MM (85-100%)	MAGE-A(1,2,3,..,12)
BAGE	MM (14%)	BAGE- (1,2,3,4,5)
GAGE-1	MM (41%)	GAGE- (1,2,...,8)
HAGE	CML (57%)	HAGE
NY-ESO	MM (60%)	NY-ESO-1
PRAME	MM (48%), AML (30%), ALL (17%), CML (34%), and CLL (28%)	PRAME
SP17	MM (26%)	SP17
SLLP1	AML (22%), CLL (27%), CML (29%), and MM (35%)	SLLP1

Table 1.3: CT-antigen families and their expression in haematological malignancies. MM: multiple myeloma, CML: chronic myeloid leukaemia, AML: acute myeloblastic leukaemia, ALL: acute lymphoblastic leukaemia, and CLL: chronic lymphocytic leukaemia (Table modified from Meklat *et al.*, 2007).

1.3.2   Over-expressed tumour antigens

The over-expressed tumour antigens are a group of antigens expressed by normal tissues in minute amounts, while over-expressed by a wide range of tumours. Their high levels of expression in different tumours indicate an important role in tumourigenesis. Mucin-1 (MUC-1) and HER-2/neu are prevalent over-expressed antigens in breast cancer, whilst Wilm's tumour-1 (WT-1) as well as MUC-1 is expressed by different types of haematological malignancies. However, human telomerase reverse transcriptase (h-TERT) and Survivin are expressed by both solid and haematological malignancies (Linley *et al.*, 2011), and ~ 85% of all tumours are positive for h-TERT. As these antigens are also expressed by normal somatic cells (albeit at low levels), the question is, does the induction of CTL against peptides derived from over-expressed antigens cause any autoimmune side effects?

The answer can be revealed from the following studies. Fisk *et al*. and Linehan *et al*. succeeded in separating HER-2/neu-specific T cell clones from TILs of ovarian carcinoma, and from metastasis effusion of breast cancer patients. It was shown that the over-expressed antigen HER-2/neu induced autologous, antigen-specific T cells without any signs of autoimmune tissue-damage (Fisk *et al.*, 1995, Linehan *et al.*, 1995). In another important study, the authors separated h-TERT peptide-specific CTL *in vivo* from TILs, and showed the *in vitro* ability of these TAA-specific clones to recognise cognate peptide on tumour cells in the presence of un-affected autologous haematopoietic cells expressing the same epitopes. This report showed the *in vivo* induction of "safe" h-TERT-specific CTLs which give a promise for using h-TERT (as an example of over-expressed antigens) in immunotherapy (Minev *et al.*, 2000). The safety of the antigen –specific T cells in the previous studies was an indicator of the high affinity but moderate avidity of the activated T cells (McKee *et al.,* 2005)

1.3.3 Differentiation antigens

Differentiation antigens (DAs) are a group of tissue specific antigens expressed by normal cells of particular lineages, from which the tumour arose. They are also expressed by several types of tumours, including melanoma, prostate, breast, and colon cancers (Novellino *et al.*, 2005). The fist discovery of DAs was after reporting the recognition of melanocytes by melanoma specific-CTLs. The expression of shared peptides (Tyrosinase, Melan-A, gp100, and TRP-1/2) between normal and malignant melanocytes was shown to be responsible for that recognition (Anichini *et al.*, 1993). Other DAs were observed in other tumour types, such as prostate (products of *PSA*, and *Kallikrein-A* genes), colon (product of *CEA)*, and breast cancers (products of *mammaglobin-A*, and *NY-BR-1)* (reviewed in Lucas & Coulie, 2008). Later, the spontaneous *in vivo* induction of CTLs against DAs was confirmed. However, mild autoimmune responses (such as vitiligo and anterior uveitis) were observed in some melanoma patients receiving immunotherapy against DAs (Yee *et al.*, 2000).

1.3.4 Tumour-specific antigens

Tumour-specific antigens (TSAs) are a group of tumour antigens that are derived from mutated somatic genes. More than 50 mutated antigenic peptides have been reported, in which 60% carried a single mutated amino acid, 20% were products of translocated genes, and the rest were products of frame shift or point mutation (reviewed in Lucas & Coulie, 2008). CDK-4, B-raf, K-ras, and N-ras are examples of TSAs. In immunotherapy, TSAs are regarded as ideal targets due to high tumour-specificity, relative high immunogenicity, and wideness of expression in different tumour types (reviewed in Parmiani *et al.*, 2007). TSAs have been used in un-identified autologous personal tumour vaccines. Examples of these vaccines are: tumour lysate pulsed DCs-vaccines (Chang *et al.*, 2002), GM-CSF transduced tumour cells (Zhou *et al.*, 2005),

and tumour-derived heat shock protein in combination with stimulating cytokines (Pilla *et al.*, 2006). In the context of good observations from the previous TSA-vaccine trials, further work is needed to individually identify and explore this group of antigens for better use as target antigens in immunotherapy.

1.3.5   Oncogenic viral antigens

Virally induced tumours are characterised by expression of invading viral-antigens. These antigens are essential for maintenance of transformational characteristic of the malignant cells, and are regarded as foreign and highly immunogenic targets for immunotherapy. E6 and E7 of human papilloma virus (HPV), latent membrane protein (LMP) and EBNA of Epstein-Bar virus (EBV), and core proteins of hepatitis B virus (HBV) are examples of viral antigens expressed by infected transformed cells (reviewed in Linley *et al.*, 2011). Viral antigens are regarded as ideal targets for tumour prophylaxis vaccines. In this context, HPV and HBV antigens have been used in prophylactic vaccination against cervical and hepatocellular carcinoma respectively (Harper, 2008).

1.4   Tumour immune escape mechanisms

Failure of the immune system, in many cases, to maintain protection against growing tumours was a result of different resistance mechanisms related to the tumour itself or the tumour environment, enabling the growing tumours to avoid and resist the immune effector mechanisms. The process of tumour escape plays a major role in mediating tumour progression and metastasis, and represents a major barrier to tumour immunotherapy. Major mechanisms, by which tumour cells escape innate and adaptive immune responses, will be discussed in this Section and are summarised in Figure 1.12.
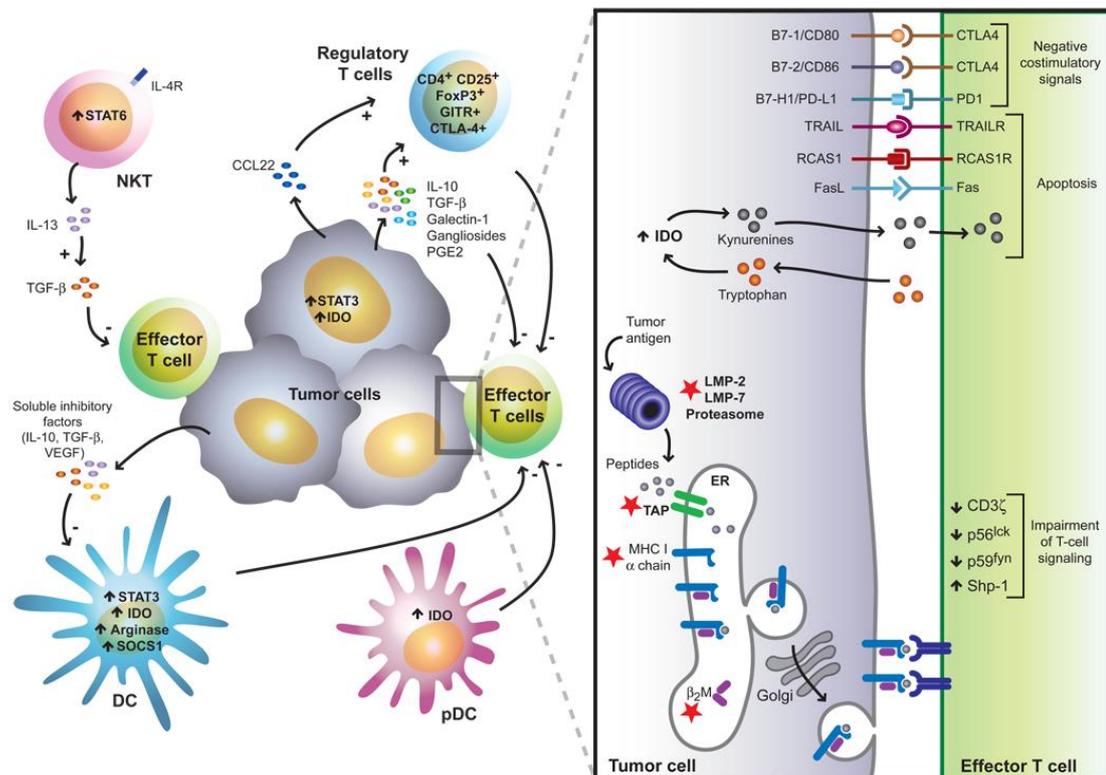
Figure 1.12: Immunosuppressive mechanisms employed by tumour cells and tumour microenvironment. These mechanisms include: impairment of antigen presentation (red star), induction of regulatory factors (IL-10, PG-E2, TGF-β, galectins, and gangliosides), providing inhibitory signals (CTLA-4/B7, PD-1/PDL1&2), activation of proapoptotic pathways (Fas-L, TRAIL, IDO, RCASI), and inhibition of DC maturation. In addition, different regulatory cell populations are involved in tumour immunosuppression; these include, natural Tregs (CD4$^+$, CD25$^+$, FoxP3$^+$), inducible Treg (iTreg), IL-13 producing NKT cells, myeloid derived suppressor cells (MDSCs), and pDCs, (Figure from Rabinovich *et al.*, 2007).

1.4.1 Modulation of MHC class I expression

In order to evade CTL recognition and elimination, tumour cells could selectively lose

HLA class I molecule expression by different mechanisms, such as deletion of β2-

microglobulin or loss of TAP-associated transportation (Garcia-Lora *et al.*, 2003).

Total MHC class I loss will protect the evading tumour cells from CTL attack, but will

make them easy targets for NK cytotoxicity, due to loss of the ligands of NK-inhibitory

receptors (missing self hypothesis) (Figure 1.13).
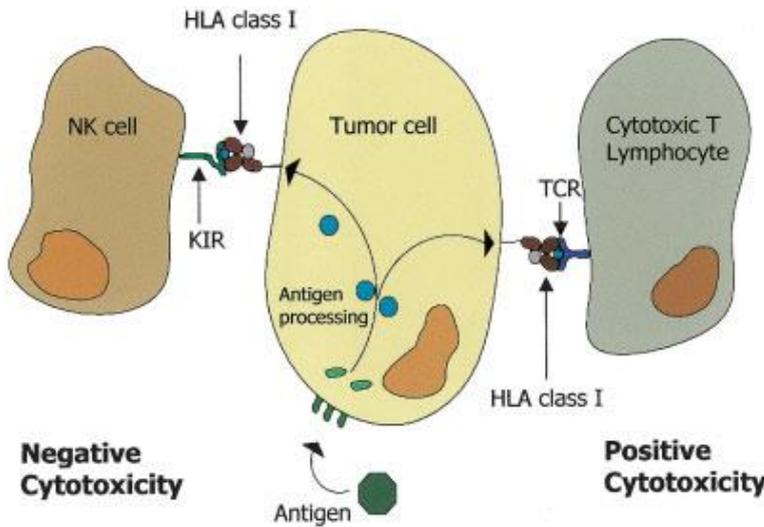
Figure 1.13: CTL and NK cells interaction with MHC class I molecules. CTLs are stimulated by recognition of peptides presented in the context of MHC class I molecules (right side). On the other hand MHC class I molecules provide inhibitory signals to NK-KIR (left side). Complete loss of MHC class I by tumour cells will make them escape CTLs while targeted by NK cytotoxicity (Figure from Garcia-Lora,Angel *et al*., 2003).

As a result, instead tumour cells may express the immune-modulatory HLA non classical-molecule (HLA-G/E) to avoid both the CTL and the NK-dependant cytotoxicity (Wilczynski & Duechler, 2010). HLA-G expression has been confirmed in different tumours and its expression was enhanced by IFN-γ, IL-10, hypoxia and inflammation of the tumour microenvironment (Wilczynski & Duechler, 2010, Lin *et al.*, 2007). In addition to its inhibition of NK cytotoxicity, HLA-G also has immunomodulatory effects on DCs, and its soluble form can induce Fas-L mediated T cell apoptosis (Urosevic & Dummer, 2008). HLA class I loss or down regulation is an immunological indicator of tumour invasion and progression. Frequency of this immunoediting is variable among different types of tumours (B cell lymphoma, colorectal, ovarian, breast, skin, lung and kidney cancers) (Reiman *et al.*, 2007)), and can reach up to 50% in breast cancer (Khong & Restifo, 2002).

1.4.2 Impairment of TAA processing and/or presentation

Several studies have shown that modulation of tumour antigen expression can allow tumours to escape the recognition and cytotoxicity induced by CTLs. This modulation may be either partial down-regulation, or complete loss of TAA expression. Zhou *et al.*

noticed down-regulation of haemaglutinin-antigen in murine renal tumour cells upon *in vivo* reconstitution with CD4^{+} T cells (Zhou *et al.*, 2004). Moreover, Knutson *et al.* observed the complete disappearance of HER-2/neu antigen from breast cancer cells when the tumour cells were injected into immunocompetent mice (Knutson *et al.*, 2006). The actual mechanism of tumour antigen loss is not clear; however, it may be attributed to the TAA-epitope immunodominance. Immunodominant tumour antigen-epitopes are regarded as central targets of immune system effectors, which enhance the tumour cell to down-regulate or completely lose these immunodominant antigens to avoid cellular immune attack (Dunn *et al*., 2002). In addition, complete TAA loss may also be attributed to lack of MHC class I molecules (Section 1.4.1), or impairment in antigen processing machinery of the tumour cells (reviewed in Rabinovich *et al.*, 2007). So, in tumour immunotherapy, efforts may in future be directed to targeting subdominant epitopes.

1.4.3   Secretion of immunosuppressive factors

Tumour and stroma cells release different biologically active agents which have suppressive effects on the immune system. One of these important agents is TGF-β; it is an immunosuppressive pleiotropic cytokine, which inhibits T cell proliferation, differentiation, and cytolytic function in response to self and non-harmful antigens (Li *et al.*, 2006). It also inhibits DCs maturation and migration, and induces Treg activation. The role of TGF-β in CTL-impaired cytolytic function was revealed by Thomas and Massague, who showed that TGF-β suppresses expression of most cytolytic gene products, such as perforin, granzyme A, granzyme B, Fas-L, and IFN-γ. In addition, antibody blockage of TGF-β restored the expression of these genes *in vivo* (Thomas & Massague, 2005). Moreover, inhibition of TGF-β signalling also blocked NKT cell mediated immunosuppression (Terabe *et al.*, 2000).

Furthermore, IL-10, prostaglandin E2 (PG-E2), sialomucins, vascular endothelial growth factor (VEGF), soluble intercellular adhesion molecules (ICAM-1), galectins, and adenosine are also biologically active immunosuppressive agents found in the tumour microenvironment. IL-10 impairs DCs function, and down regulates TAP-1 and TAP-2 synthesis, which reduces sensitivity of tumour cells to CTL. However, recent reports suggest an immunostimulatory and tumour rejection role of IL-10 in association with other cytokines (Lopez *et al.*, 2005). Adenosine and PG-E2 are potent inducers of IL-10 production, and inhibit IL-12 and DCs maturation. VEGF induces vascularisation of the growing tumours, and inhibits haematopoiesis and DCs maturation. ICAM-1 may shed from tumour cells and block binding of effector cells with their target, whilst galectins act by inducing effector CTLs apoptosis (reviewed in Rabinovich *et al.*, 2007).

1.4.4   Negative co-stimulatory pathways

Different immunological inhibitory checkpoints were investigated for their role in cellular effector functions. CTLA-4 is an important inhibitory receptor inducibly expressed by activated T cells, and its engagement with the cognate ligands (CD80/CD86) induces regulatory signals and T cell inhibition. Allison and co-workers reported the importance of CTLA-4 blockage on anti-tumour responses, including rejection of established tumours and protection from further exposure (Leach *et al.*, 1996). Recent clinical studies on checkpoint blockade, as an immunotherapeutic approach especially in treatment of melanoma, led to the new US, FDA approved anti-CTLA-4 mAb (Ipilimumab) in March 2011, (http://www.fda.Gov/AboutFDA/Centers Offichttp), in a combination regime for treatment of several types of tumours including metastatic melanoma (Peggs & Quezada, 2010).

Another checkpoint inhibitory receptor is programmed death receptor (PD-1). Engagement of PD-1 with its ligands (PD-L1 or PD-L2) results in inhibition of T cell activation and initiation of activated-CTL apoptosis (Dong *et al.*, 2002). Dong and his colleagues reported expression of PD-L1 by different tumour types but not by normal tissues (except myeloid lineage). Another target of PD-1 mediated negative regulation was revealed after the improved DC-mediated anti-tumour activity following PD-1/ PD-L1 inhibition *in vivo* (Curiel *et al.*, 2003). Therefore, PD-1/PD-L1 targeting therapy is a useful tool in tumour immunotherapy. On the other hand, positive co-stimulatory roles were reported for PD-L1 and PD-L2, which raise the possibility of the presence of alternative receptors of different function on T cells (reviewed in Rabinovich *et al.*, 2007).

1.4.5 Tumour immunoregulatory cells

Regulatory T cells (Tregs), myeloid derived suppressor cells (MDSCs), tumour associated macrophages (TAMs), and plasmacytoid dendritic cells (pDCs) are groups of tumour infiltrating immune cells and tumour stromal cells which could suppress the anti-tumour immune responses.

Tregs can be classified into two main subgroups, natural (nTregs) and inducible (iTregs). Both groups express the fork-head transcription factor 3 (Foxp3). nTreg are developed in the thymus and remain with unchanged properties. They mainly have the phenotype $CD4^+/CD25^+$, and a small fraction has a $CD8^+/CD25^+$ phenotype (Gross & Walden, 2008), lack IL-10 production but can induce DC tolerization. In contrast, iTregs are derived from naive $CD4^+$ after inadequate activation by immature DCs in a tolerogenic microenvironment. Several factors induce iTreg polarization of $CD4^+$ T cells, such as IL-10, TGF-β and others (Table 1.7) (Gross & Walden, 2008), IL-10 production is involved in inhibitory function. Both groups of Tregs suppress immune

responses and induce peripheral tolerance through direct (such as CTLA-4 dependent inhibition, or perforin/granzyme mediated effector CTLs killing) and indirect (through soluble factors such as TGF-β) mechanisms (Zou, 2006).

Tumour-stroma consists of different non-malignant cells and includes TAMs, pDCs, endothelial cells, fibroblasts, and immature MDSCs. Most of them suppress the tumour immune response by different mechanisms, especially TAMs and MDSCs, which act by production of arginase and nitric oxide synthase (NOS) which reduces the argenine concentration and generates peroxinitrites that inhibit tyrosine phosphorylation in the activated T cells leading to T cell apoptosis (Gross & Walden, 2008). TAMs release IL-6, IL-10, TGF-β and PG-E2 and can shift polarization of naive CD4$^+$ T cells into iTreg. In addition, IL-6 inhibits the Th1 anti-tumour response, and a high level of IL-6 was associated with reduced tumour-patient survival (Zhang & Adachi, 1999). pDCs produce indolamine-2,3-dioxygenase (IDO), which catabolises the circulating tryptophan, leading to tryptophan depletion, which in turn inversely influences the growth and activity of T cells (Bennaceur *et al.*, 2009).

1.5 Tumour immunotherapy

The current strategies of cancer treatment rely on surgery, radiotherapy, and chemotherapy.

A- Surgery: is an effective strategy, to remove the malignant solid tumour masses with a safety margin, but it is not effective in case of metastatic or haematological tumours.

B- Chemotherapy: selectively targets rapidly dividing cells. In which serious side effects arise because some normal host cells (like gastric mucosa, hair follicles, and haematopoietic stem cells) are rapidly dividing and so they are also targeted

by the chemotherapeutic agents, leading to significant side effects such as hair loss, nausea, peptic ulcer, and bone marrow suppression with frequent infections.

C- Radiotherapy: targets the tumour cells which have impaired DNA repair mechanisms, so tumour cells are more susceptible to damage caused by irradiation than normal cells. Several normal host cells are affected also from radiotherapy, especially blood and blood-forming progenitors, again leading to significant side effects.

The last two strategies are effective in eliminating malignant cells in many cases, but they lack the precise selectivity and specificity. These drawbacks result in serious side effects on normal cells, and also tumour recurrence is probable. In addition, in spite of the wide benefits and some cures offered by conventional therapies in many types of tumours, resistance mechanisms develop in some tumour cells. Hence, the need for additional approaches to control these residual and/ or resistant tumours is required.

Although naturally occurring immune responses are well documented, they are in most cases not able to prevent tumour growth. So, activation of the existing anti-tumour responses may provide an alternative approach. Tumour immunotherapy is an investigational strategy, which relies on the recruitment of different anti-tumour immune effectors, which are tumour-specific and tolerant for normal self tissues, to identify and eliminate developing tumour cells and their metastases, and also may provide long lasting prophylaxis against tumour recurrence. Tumour immunotherapy therefore provides a specific activation of the host immune system to eliminate malignant tumour cells without inducing damage to normal tissues. The ultimate goal of cancer immunotherapy is to sustain the first two phases of the cancer immunoediting process; tumour elimination, or at least maintaining long-lasting equilibrium with any

developing tumour cells. Targets of immunotherapy are tumour-associated antigens, which are antigens specifically expressed by the tumour cells. The first observation of immune responses toward tumour rejection was in the late nineteenth century by William Coley. This observation directed him to develop the first "cancer vaccine" to treat sarcoma patients. The trial gave rise a complete clinical response rate of 15% of cases (reviewed in Bergman, 2009). Recently, the prophylactic hepatitis B and papilloma virus vaccines have successfully contributed in prevention of hepatocellular and cervical carcinoma (Blumberg, 1997, Blumberg, 1997, Rogers *et al.*, 2008). In addition, the newly FDA-approved Sipuleucel and Ipilimumab, for use as immunotherapeutic agents in treatment of prostate cancer, melanoma and other tumour types respectively, and other agents which are under the clinical investigation are regarded as a result of the wide understanding of basic immune system/tumour interaction and tumour escape mechanisms (which represent attractive targets in promoting anti-tumour responses). Several manipulations were involved in immunotherapeutic approaches, such as use of Toll-like receptor agonists, cytokines, vaccines, and antibody-based immunotherapies (Baxevanis *et al.*, 2009). Collectively, the current strategies aim to synergise anti-tumour effectors and to inhibit regulatory mechanisms that maintain immune tolerance against tumours.

1.5.1 Monoclonal antibodies in cancer immunotherapy

Monoclonal antibodies (mAbs) provide passive and immediate immune responses, especially after recent modifications to avoid species-immunogenicity, and to enhance target-specificity and affinity of the used mAbs. In tumour therapy, mAbs can be used directly, or as conjugated to radioactive or cytotoxic agents to enhance the function and specificity of these agents. There are several suggested mechanisms of action by which mAbs perform their function; first, is the direct tumour targeting, such as inducing

tumour-apoptosis, blocking growth signals to inhibit proliferation, recruiting cytotoxic and phagocytic cells, and complement system activation for induction of ADCC and CDC. Second, is the *in vivo* regulation of immune effector pathways, such as blocking regulatory surface receptors (anti-CTLA-4, anti-PD-1), and induction of TNF-agonistic activity (anti-CD137), leading to potentiating of anti-tumour immune responses. A list of mAbs currently in use or under clinical investigations for treatment of cancer is presented in Table 1.4.

1.5.2 Adoptive cellular transfer

Adoptive cellular transfer (ACT) depends on *ex vivo* expansion of autologous or allogeneic tumour-specific T cells, and reintroducing these mature effector T cells into tumour bearing patients to eliminate the established tumour or inhibit its growth. ACT was first observed by Southam *et al.,* after the author's success in tumour inhibition in 50% of patients with advanced cancer by co-infusion of auto-leukocytes (Southam *et al.*, 1966), and the process was introduced clinically in late 1980s. This and similar reports suggested the natural development of a patient's own leukocytes which had inhibitory effects on growth and development of established tumours, and these leukocytes could be used efficiently in ACT. Moreover, recent observations indicate the improved prognosis of patients with different tumour types with the adoptive infusion of activated autologous tumour infiltrating lymphocytes (TILs) (reviewed in June, 2007a). ACT was dependent on the ability of specific CTL and $CD4^+$ Th cells to stabilise themselves, proliferate, and migrate to their target cells, especially when introduced in a tolerance-free tumour microenvironment (Baxevanis *et al.*, 2009). $CD8^+$ CTL are the main effector cells in ACT; however, $CD4^+$ are important for maintenance of CTLs activity (June, 2007b).

Antibody	Target	Indication	Mechanism of action
Ipilimumab	CTLA-4	Prostate, melanoma, breast	Immunomodulation
Cetuximab	EGFR	Head, neck, colon	Signalling, ADCC
Bevacizumab	VEGF	Breast, lung, colon	Signalling, anti-angiogenic
Trastuzumab (Herceptin®)	Her2/neu	Breast	Signalling, ADCC
Rituximab (Rituxan®)	CD20	B-NHL, CLL	Signalling, ADCC, CDC
90Y-ibriutumomab	CD20	B-NHL	Targeted radiation
Epratuzumab	CD22	B-NHL, CLL	Signalling, ADCC, CDC
Lumiliximab	CD23	CLL	Signalling, ADCC, CDC
SGN-30	CD30	Hodgkins lymphoma	Signalling, ADCC, CDC
Gemtuzumab (Mylotarg®)	CD33	AML	Conjugated with Cali-cheamicin
Alemtuzumab (Campath®)	CD52	B and T cell lymphoma, CLL, prolymphocytic leukemia, ALL	Signalling, ADCC, CDC
Milatuzumab	CD74	Myeloma, BNHL (under study)	Signalling
Abagovomab	Anti-idiotypic	Ovarian cancer	Anti-idiotypic CA-125 (Grisham *et al.*, 2011)
Galiximab	CD80	B-NHL	Signalling, ADCC, CDC (Under study)
Mapatumomab	TRAIL-R	Myeloma, lymphoma, solid tumours	Signalling, apoptosis, ADCC, CDC (Under study)
Tositumomab (Bexxar®)	CD20	NHL	[131]I conjugated

Table 1.4: Antibodies for tumour immunotherapy, EGFR: epidermal growth factor receptor, VEGF: vascular endothelial growth factor, ADCC: antibody dependent cellular cytotoxicity, B-NHL: B-non Hodgkin lymphoma, CLL: chronic lymphocytic leukaemia (Table adapted from Gelderman et al., 2004, Peggs & Quezada, 2010).

In ACT, autologous tumour-specific T cells are harvested from patient's PBMC, tumours, malignant effusions, and draining lymph nodes, and expanded *in vitro* before ACT. In order to improve the clinical outcomes of ACT, pre-infusion patient-conditioning improved the clinical outcomes, as observed in different reports.

In patients with metastatic melanoma, non-myeloablative lymphodepletion using chemotherapeutic agents before infusion of tumour-reactive autologous T cells achieved significant clinical responses in up to 50% of treated patients (Dudley *et al.*, 2005). There were several explanations for the synergistic effects of lymphodepletion before ACT. First, is the removal of the regulatory T cell reservoir from the tumour environment, which in turn improves proliferation and activation of CTL. Second, the space created for the infused T lymphocytes and the easier access to endogenous cytokines such as IL-2, IL-7, and IL-15 following lymphodepletion (Fry & Mackall, 2001, Lizee *et al.*, 2006). Furthermore, there were increasing evidences that *in vitro* primed T cells of central memory phenotype (T_{CM}) were more effective as long-term anti-tumour effectors *in vivo* than effector memory T cells (Gattinoni *et al.*, 2005, Klebanoff *et al.*, 2005). In this context, *in vitro* induction of tumour–specific CTL by stimulation of autologous PBMC with tumour antigen-pulsed DCs for multiple rounds in presence of IL-2, or IL-15 and IL-21, followed by non-specific anti-CD3/IL-12 expansion, produced tumour-specific lymphocytes of high T_{CM} phenotype. However, IL-15/IL-21 expansion protocol was characterised by a faster expansion and lower Treg content (Huarte *et al.*, 2009). Moreover, Mathias *et al*. used Melan-A-pulsed autologous DCs to induce HLA-A2-restricted Melan-A-specific CTLs, followed by non-specific expansion using anti-CD3 plus anti-CD28. Mathias showed that CTLs could be expanded for up to 600 fold without losing their specificity (Oelke *et al.*, 2000), which provided a method for *ex vivo* large scale specific CTL clone production.

On the other hand, allogeneic tumour-reactive T cells have been used successfully in clinical management of leukaemia, and other types of tumours. In a study carried out by Comoli *et al*. on EBV-induced nasopharyngeal carcinoma (NPC) patients, allogeneic HLA-matched PBMC were stimulated *in vitro* by EBV- LCL for several weekly rounds in the presence of IL-2. The activated T cells were investigated *in vitro* for EBV-specificity, and showed specific cytolytic activity against NPC tumours. *In vivo* infusion of allogeneic EBV-specific CTLs was well tolerated, and induced temporary disease stabilization. In addition, a marked increase in endogenous tumour infiltrating CD8$^+$ T cells and a long-term elevation of LMP-2-specific immunity were observed (Comoli *et al.*, 2004). Allogeneic adoptive T cell transfer has been used very successfully in the management of post-transplant lymphoproliferative diseases (PTLD). Several studies showed the clinical effectiveness of using EBV-specific CTL infusion in controlling PTLD tumour growth (Burns & Crawford, 2004, Sun *et al.*, 2002). In the same context, allogeneic CTLs specific for minor histocompatibility antigens (mHC) of selected haematologic malignancy recipients may provide graft-versus-leukaemia effects against malignant haematopoietic recipient cells. Unfortunately, some toxic graft versus host (GVHD) interactions were observed in a phase 1/II study of this model, which shows the need for further investigation to develop more specific T cell clones that can target only mHC expressed by leukemic cells but not by other normal tissues (Falkenburg, 2010). Generally speaking, the *in vitro* induction of tumour-antigen specific CTLs might serve as a potential therapy for different tumour types.

In an *in vivo* murine study showing the effect of *in vitro* induced allogeneic TA-specific and MHC-matched T cells on tumour rejection of murine metastatic lung cancer, Bartel *et al*. reported the ability of tumour-specific T cells to reject allogeneic 4-day induced

metastatic tumours by intravenous infusion to non-allo-sensitised host (Bartels *et al.*, 1996). The most prominent drawback of ACT was the development of antigen escape variants, however, this problem can be eliminated by either perfectly choosing the target antigen, or by using multi-epitopic CTL clones (Marincola *et al.*, 2000). It is worth to mention that in ACT we can benefit from the *ex vivo* reactivated T cells (exhausted but not the anergised T cells), reactivated exhausted cells can mediate effector anti-tumour function by IFN-γ release but not IL-2.

1.5.2.1 Bone marrow (BM) or stem cell transplantation

Bone marrow transplantation (BMT) is an important immunotherapeutic strategy for treatment of haematological malignancies. Syngeneic (e.g. from identical twin) and allogeneic (from unrelated donor) transplants were successfully used following myeloablative therapy, with a lower risk of relapse was shown by allogeneic transplantation. From previous observations, graft versus leukaemia (GVL) effect is thought to be the main mechanism in allogeneic BMT, which reduces risk of tumour relapse, although allogeneic BMT also carries the potential risk of GVHD caused by the allogeneic donor lymphocytes, which was the major cause of recipient tissue injuries (Ben-Bassat *et al.*, 2007).

1.5.2.2 Adoptive T cell transfer in association with tumour vaccines

Investigation of the effects of combination of ACT and therapeutic vaccines in management of haematological and solid tumours, showed improved results especially in lymphopenic patients (Parviz *et al.*, 2003, Teshima *et al.*, 2002). Clinical trials on myeloma patients reported promising results obtained by adoptive transfer of *ex vivo* primed T cells specific for the patient's myeloma-specific Ig idiotypes in an allogeneic stem cell transplantation setting (Neelapu *et al*., 2005). Moreover, other phase I clinical

trial was reported on autologous activated T cell transfer for haematological malignancy patients. It was carried out on relapsed NHL-patients, in which patients were given CD34$^+$ autologous stem cell transplantation before infusion of *ex vivo* expanded tumour-specific T cells. The infused cells induced a rapid reconstitution of the lymphocyte repertoire with high tumour-specific cytolytic activity as shown in *in vitro* IFN-γ release assays (Laport *et al.*, 2003). In addition to the previous promising results, Chang *et al*. performed a phase I clinical trial on a group of advanced melanoma and renal cell carcinoma (RCC) patients. Chang vaccinated the patients with autologous irradiated tumour cells plus BCG. Seven to ten days later, draining lymph nodes were harvested and vaccine-primed T cells were expanded *ex vivo* and re-infused into their relevant patients. Results showed a partial tumour remission in 1 of 11 melanoma patients, complete remissions in 2 of 12, and partial remissions in 2 of 12 of RCC patients (Chang *et al.*, 1997). To summarise; animal and human trials showed promising synergistic effect of ACT when conjugated with therapeutic vaccination, and to ovoid the tumour-escape, subdominant antigen-specific T cells were used.

1.5.3 Immunological adjuvants

Immunological adjuvants are biologically active agents of different natures which are able to activate innate immune effector cells (e.g. NK, NKT, DCs, and other APCs), and which act as danger signals through binding to specific cell surface receptors widely expressed by most innate immune cells. Activation of DCs, macrophages, or NK cells leads to their maturation, proliferation and cytokine release, which in turn induces an inflammatory environment followed by activation of adaptive immune effector cells. Adjuvants are mainly used in combination with TAAs in vaccine preparations to enhance the immunogenicity and to develop memory T and/or B cells (Singh & O'Hagan, 2002). Several adjuvants have been recognized, but only a few of these

adjuvants are approved for use in human (Singh & O'Hagan, 2002, Dougan & Dranoff, 2009) (Table 1.6).

Adjuvant	Members	Function
Toll like receptor agonists	CpG, Imiquimod, LPS	Induce maturation of APCs
Bacterial immunomodulatory components	BCG, diphtheria toxoids	Induce both adaptive and innate immune responses
Immunomodulatory cytokines	IL-2, IL-7, IL-12, GM-CSF, IFN-α, and IFN-γ	Induce both adaptive and innate immune responses
Others	Alum, Squalene, Saponin	Induce memory response to peptide vaccines

Table 1.6: Immunological adjuvants in use for tumour immunotherapy

1.5.4 Tumour vaccines

The continuous work for developing anti-tumour approaches of better specificity, safety for normal tissues, higher activity, and less tolerogenic effect is directed toward the anti-tumour potential of cellular immunity, the most specific anti-tumour system. Trials on human tumour therapy observed the potential of various vaccination strategies as active inducers of adaptive immunity toward effective control of developed and established tumours (Mocellin *et al.*, 2004), although less successful than in animal models.
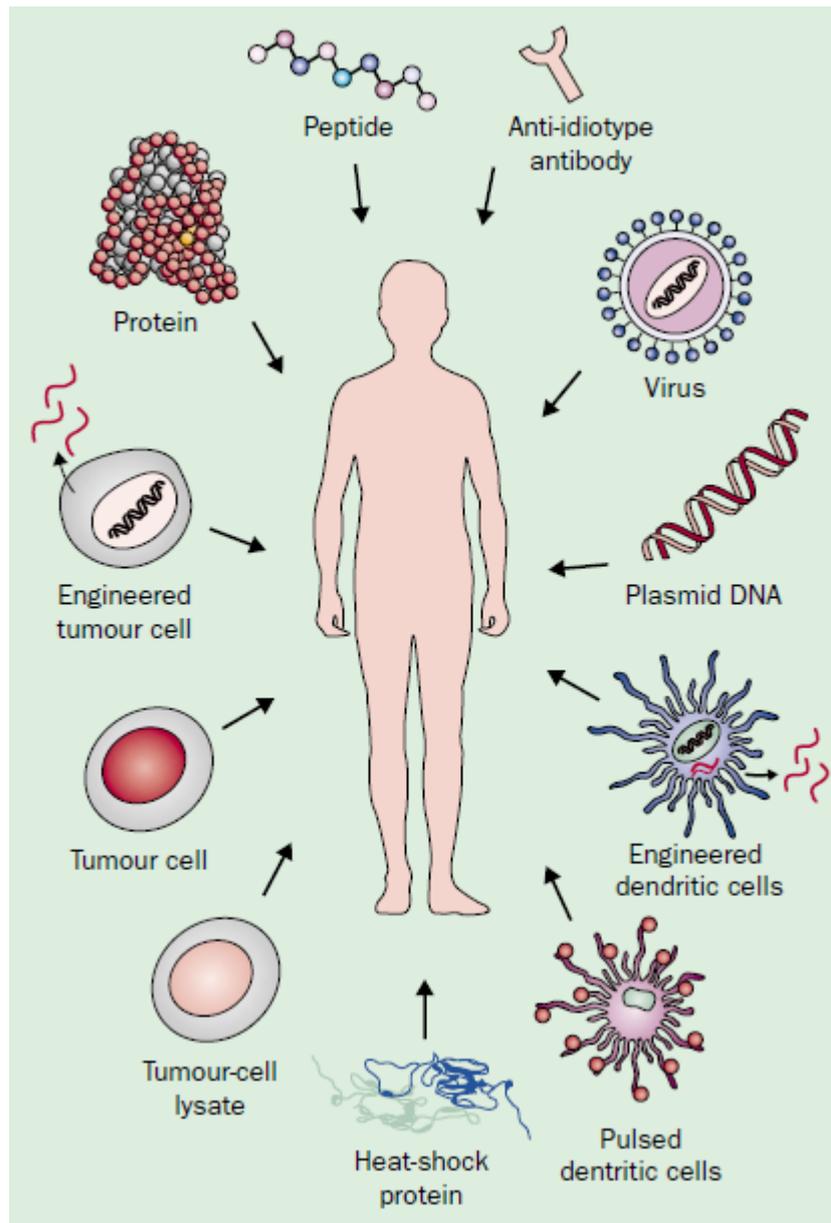
Figure 1.14: Tumour active immunotherapeutic strategies
(Figure taken from Mocelline *et al*., 2004).

The successful use of the anti-HPV and HBV peptide vaccines in prophylaxis against

development of cervical and hepatocellular carcinoma, and the recent approval of the

first therapeutic cancer vaccine (Provenge®; Sipuleucel), in addition to the anti-CTLA-4

mAb (Ipilimumab) by FDA for treatment of prostate cancer, melanoma, and other

possible types of tumours, offered a new promise in the old quotation of Ehrlich in 1897

"If it is possible to protect small laboratory animals in an easy and safe way against

infectious and highly aggressive neoplastic specimens, then it will be possible to do the same for human patients" (quoted in Bocchia *et al.*, 2000, Kantoff *et al.*, 2010, Emens, 2006).

In a previous Section, several advantages of immunotherapy over the current conventional treatment strategies were mentioned. Immunotherapeutic vaccines, in addition to these properties, are aimed at providing long lasting anti-tumour immune effectors which prevent the recurrence of any new transformed cells, or at least keep them under control (Vergati *et al.*, 2010). Although humoral immune mechanisms are induced, tumour vaccines are primarily aimed at activating the patient's anti-tumour T-cell mediated response (Dermime *et al.*, 2002). The antigens utilised in vaccine development should have maximum immunogenicity for T cell responses, play a principal role tumourigenesis, of wide range of expression by different tumour types, and have maximum tumour specificity (Mocellin *et al.*, 2004). Complete tumour regression and/or long lasting suppression of tumour growth has been reported in *in vivo* trials of cancer vaccines in animal models (Bijker *et al.*, 2007). On the other hand, less promising results were reported in the early clinical trials in human, and were presumably due to immune regulatory mechanisms of the tumour microenvironment (Baxevanis *et al.*, 2009). Experimental data suggest that vaccination would be clinically more successful in cases of low tumour burden, as in the case of residual tumours after conventional treatment, or tumours at early stage of development, or by co-administration of immunotherapeutic vaccines with an immunopotentiating agent (Baxevanis *et al.*, 2009, Bocchia *et al.*, 2000). One of the most recent phase III clinical trial was carried out using gp100 peptide vaccine in combination with IL-2 co-administration for advanced melanoma patients (schwartzentruber *et al*., 2011); clinical responses were achieved with long progression-free survival in 16% of cases compared

with just 6% in case of IL-2 alone. Different types of anti-tumour vaccines are illustrated in Table 1.7 and in Figure 1.14.

Vaccine category	Vaccine composition	Description
Antigen defined vaccines	TAA	Protein (e.g. CEA, P53), glycoprotein (e.g. MUC-1), or glycolipids (e.g. gangliosides)
	Peptides	HLA- class I or II restricted
	Recombinant DNA	Constructed to express TAA ± cytokines
	Recombinant virus	Engineered to express TAA ± cytokines
	Anti-idiotypic antibody	Mimicking the natural Ig expressed by malignant B and plasma cells
Polyvalent vaccines (No need for identification of special TAAs)	Whole tumour cell	Autologous or allogeneic tumour cells, ± engineered to secrete cytokines
	Tumour cell lysate	Mechanical, enzymatic, or viral lysate
	Shedding antigens	TAA or its derived peptides released *in vitro* from tumour cell lines
DCs based tumour vaccines (combine the tumour antigen expression and APC capacity in immune-stimulation)	Tumour-DC hybrids (by chemical or electrofusion)	New hybrid cells express tumour antigens, APC markers + HLA-class I and class II
	Peptide loaded DCs	HLA-class I or II restricted peptides
	Whole tumour cell loaded DCs	Autologous or allogeneic tumour cell lysate
	Tumour-mRNA loaded DCs	DCs express TAAs and present them on their HLAs
	Genetically engineered DCs	Engineered to secrete cytokines or to express known TAAs

Table 1.7: Cancer-vaccine strategies for tumour immunotherapy (Table adapted from Mocellin *et al*., 2004).

1.5.5 Dendritic cell-based vaccines

DCs, the most powerful APCs, are able to induce potent primary T cell immune responses due to their ability to process and present phagocytosed antigens in the context of HLA class I and class II molecules, plus the provision of the essential co-stimulatory signals (through CD80 and CD86 molecules) required for priming of the naive CD8$^+$ CTL and CD4$^+$ T helper lymphocytes, in addition to secretion of other immunostimulatory cytokines such as IL-12, IL-1, and IL-6 (Vergati *et al.*, 2010,

Rosenblatt *et al.*, 2005, Trefzer & Walden, 2003, Koido *et al.*, 2007, Orentas *et al.*, 2001).

1.5.5.1 Isolation and ex vivo maturation of DCs

DCs represent only 0.5-1% of peripheral blood cells. Recent advances have enabled obtaining larger numbers DCs. Fong *et al*. demonstrated that ligation of Flt-3 receptor of haematopoietic progenitors by its ligand (Flt-3L) resulted in great expansion of DCs *in vivo,* and the number obtained by leukapheresis was increased 60 fold compared with yields without Flt-3 ligation (Fong *et al.*, 2001b). In addition, DCs can be generated *ex vivo* from two main sources. First is from peripheral blood $CD14^+$-monocytes, second is from peripheral blood, bone marrow or cord blood $CD34^+$ precursor cells (Strunk *et al.*, 1996). Both methods produced types of DCs that were able to be loaded by different tumour antigens, but were unable to induce primary T cell responses (typical immature phenotype). Maturation of *ex vivo* produced DCs was achieved using different inflammatory stimuli, such as TNF-α, IL-1, LPS, or CD40 ligation (Sallusto & Lanzavecchia, 1994, Canque *et al.*, 1998). It has been reported that $CD34^+$ derived are more efficient than monocyte-derived DCs in the activation of CTL *in vitro* (reviewed in Markiewicz & Kast, 2004), although more studies are required to confirm these immunogenic differences.

As a result, DCs have been used as a principal partner in cancer vaccine development, and TAAs were loaded into *ex vivo* developed DCs in the form of synthetic tumour protein, HLA-restricted peptides, naked DNA or viral vectors encoding TAAs and/or cytokines, tumour antigen-expressing mRNA, whole tumour-cell lysate, or by fusion with autologous or allogeneic tumour cells/cell lines (Figure 1.15)

However, DC homing to regional lymph nodes is crucial for efficient induction of primary T cell responses. LFA-1, ICAM-1, CD62L, and CCR7 are essential for regional lymph node homing process of DCs (Warnock *et al.*, 1998). Preclinical studies showed that the efficiency of the immune response depends on the number of DCs getting access to lymph nodes. In humans, only 5% of intradermally injected DCs could reach regional lymph nodes (von Andrian & Mempel, 2003).

On the other hand, there was a debate about the ability of mature compared with immature DCs to migrate to regional lymph nodes and to induce a primary T cell immune response, as a result of chemokines, chemokine receptors, co-stimulatory, and adhesion molecules expression. Most studies showed that immature DCs may lead to T cell-tolerance due to lack of co-stimulation, in addition to their inability to migrate to the T cell zone in regional lymph nodes.

1.5.5.2 *DCs-based tumour immunotherapy trials*

The ideal vaccination protocol for DC-based vaccines has not yet been determined; however, different routes of administration were reported, such as subcutaneous, intradermal, intra-tumoural, intranodal, or intravenous administration. In addition, different doses and intervals have been suggested in each protocol (reviewed in Schultze *et al.*, 2004).

In the last two decades, hundreds of DC-based trials were performed. One of the early clinical outcomes of DC-based tumour immunotherapy was reported in 1996 by Hsu *et al*. (reviewed in Schultze *et al.*, 2004), who used antigen-pulsed DCs in lymphoma patients' trial. Later, Nestle *et al*. carried out a phase II trial on melanoma patients, where DCs loaded with Keyhole limpet hemocyanin (KLH) as a peptide antigen or as

tumour lysate, were used to vaccinate patients. The author reported long-lasting immune responses following vaccination (Nestle *et al.*, 1998).
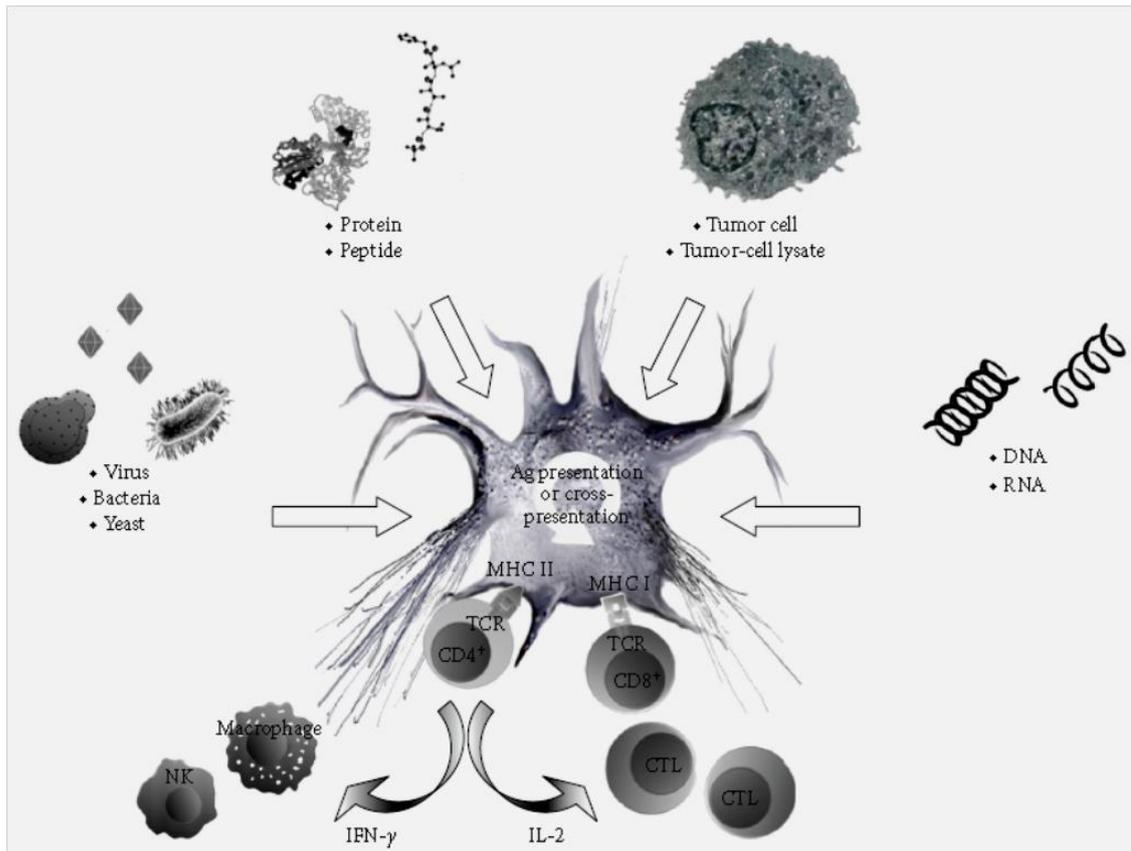
Figure 1.15: Different strategies used for loading DCs with tumour antigens, in the process of DC-cancer vaccines (Figure from Vergati *et al*., 2010).

Another trial was undertaken by Banchereau and co-workers, in which DCs were pulsed with KLH and HLA-A2-restricted peptides derived from Melan-A, Tyrosinase, gp100, or MAGE-A3 antigens. Results showed tumour regression in 7 of 17 cases, with induction of anti-melanoma specific immune responses (Banchereau *et al.*, 2001).

In another phase I/II trial by O'Rourke *et al*, DCs were loaded with autologous tumour lysate and used to vaccinate 12 melanoma patients biweekly for 6 consecutive weeks. Half of the patients showed clinical responses, with 3 complete remissions, 3 partial remissions, while the rest had a progressive disease (O'Rourke *et al.*, 2003). Similarly, Fong *et al*. vaccinated 21 prostate cancer patients with DCs loaded with murine prostatic acid phosphates (PAP); all patients showed immunogenic Th1 responses against the murine antigen, and human PAP-specific T cell were detected in 11 out of 21 patients, in addition to six patients had stable disease (Fong *et al.*, 2001a).

In another trial by Heiser *et al*., who loaded partially mature DCs with PSA-encoding mRNA and used them to vaccinate 13 prostate cancer patients. Induction of PSA-specific CD4$^+$ T cells were detected in all patients with significant reduction in serum level of PSA (Heiser *et al.*, 2002). These studies collectively demonstrate a promising benefit of using DC-based vaccines in treatment of different types of cancer.

1.5.5.3 DC-tumour fusion cellular vaccines

One promising method of developing DC-based vaccine is the cellular fusion of DCs and allogeneic (from unrelated) or autologous (from the same donor as DCs) tumour cells or cell lines, to generate hybrid cells carrying the antigenic properties (both known and un-identified antigens) of the parent tumour cell/or cell line, and the immunostimulatory and antigen presenting potential of the DCs (including HLA I , HLA class II, co-stimulatory CD40, CD80 and CD86 molecules expression) (Schultze *et al.*, 2004, Guo *et al.*, 1994, Schultze *et al.*, 1997, Trefzer & Walden, 2003, Lapointe *et al.*, 2003, von Bergwelt-Baildon *et al.*, 2002). Activated B lymphocytes also were introduced as fusion partner in hybrid cell vaccines due to expression of MHC class II in addition to T cell co-stimulatory and adhesion molecules. Fusion can be achieved chemically, using polyethylene glycol (PEG), virally, or physically by electrofusion (reviewed in Avigan, 2004) (Figure 1.16).
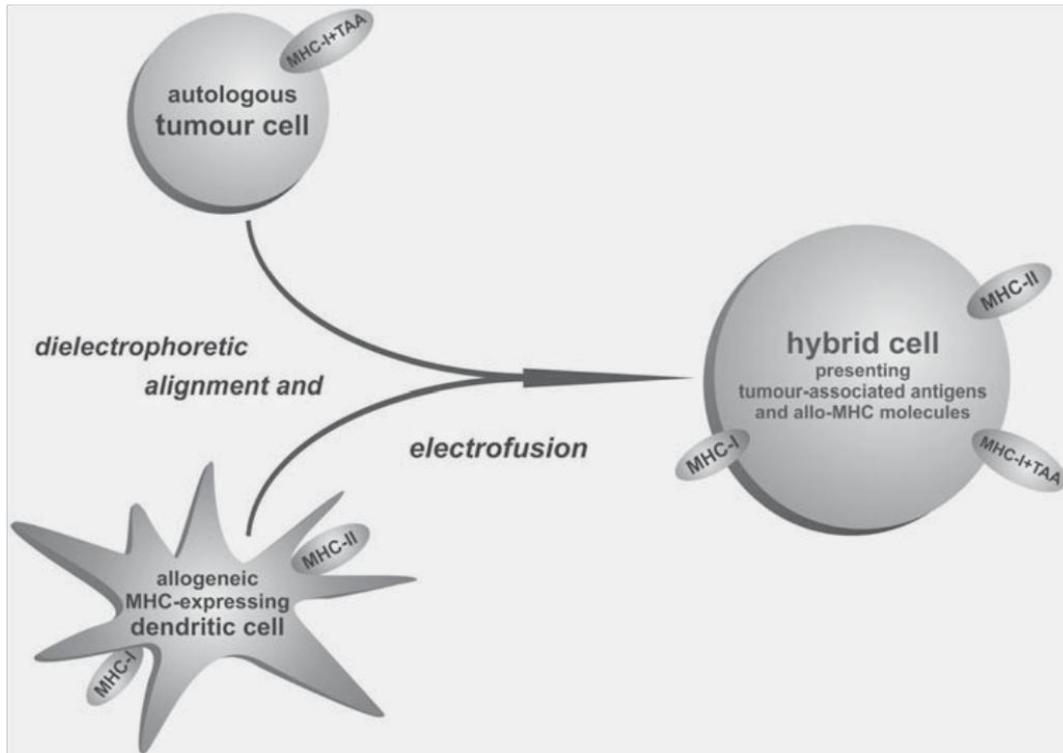
Figure 1.16: The hybrid cell formation. Patient's tumour or tumour cell line is fused to APC (allogeneic or autologous) to yield new fused cells carrying the parent tumour-cell antigens presented in the context of MHC-class I and APC-associated allogeneic or autologous MHC-class II, in addition to co-stimulatory and adhesion molecules (Figure from Trefzer & Walden, 2003).

Trefzer *et al*. (Trefzer & Walden, 2003, Trefzer *et al.*, 2000) described three requirements for hybrid cells to be successfully used in cancer immunotherapy, in which MHC class I and class II expressing cells are able to induce anti-tumour CD8^{+} and CD4^{+} T cell responses. Firstly, tumour-specific epitopes presented by MHC class I are needed for the specificity of the cytotoxic T cell immune response. Secondly, MHC class II restricted epitopes (which are more immunogenic in case of allogeneic MHC molecules) are required for induction of CD4^{+} Th cells. Thirdly, co-stimulatory molecules (CD80, CD86, CD27, and CD40) are required for activation of naive T cells. The APC/ tumour cell hybrids show the complex antigenicity of tumour cells and robust immunogenicity of allogeneic MHC class II/ co-stimulatory molecules of the APCs, thus fulfilling these requirements.

1.5.5.4 In vitro and in vivo DC-hybrid vaccination studies

Several *in vitro* and *in vivo* studies showed the ability of the APC-tumour cell hybrids to induce specific immune responses, and to recognise and eliminate autologous tumour cells of both animal and human origin. In an *in vitro* study undertaken by Goddard *et al*. (Goddard *et al.*, 2003) using hybrid cells made by electrofusion of autologous DCs and *ex vivo* B-CLL tumour cells, the generated hybrid cells (although with low fusion efficiency) were able to induce IFN-γ release and tumour specific CTL responses following activation of the autologous T lymphocytes. In another study (Gong *et al.*, 2000), autologous or allogeneic DCs were fused to ovarian carcinoma (OV CA) cells from two different patients. Both autologous and allogeneic hybrids were able to stimulate autologous T cells to induce tumour-specific CTL clones, which recognised and killed the parent tumour cells in MHC class I-restricted fashion, and this specific cytotoxicity was inhibited by pre-treatment of the target cells with anti-MHC class I mAb. In contrast, monocyte/tumour cell hybrids failed to induce a specific T cell immune response. Lespagnard *et al*. (Lespagnard *et al.*, 1998) investigated the *in vitro* and *in vivo* immunostimulatory effects of murine DC/P815 tumour cell hybrids. The fusion product was selected chemically using hypoxanthine aminopterin thymidine (HAT)-containing growth media. The selectively grown hybrid cells were used to induce murine purified-CD4$^+$ T cell proliferation and IL-2 releasing responses. Mature DC-tumour hybrids or mature DCs alone, but not P815 tumour cells or immature DC-tumour hybrids were able to induce primary T cell responses *in vitro*.

The role of CD80 and CD86 in induction of the primary T cell response was investigated by addition of neutralizing mAb specific for CD80 or CD86, and suppression of proliferation and IL-2 release was seen by inhibition of CD86 but not CD80 (Lespagnard *et al.*, 1998). Moreover, the *in vivo* immunization of animals, pre-

injected with a lethal dose of P815 tumour cell line, with 3-7 doses of DC-tumour hybrids resulted in long-term protection from lethal tumour growth in 55% of animals, in contrast with control group (0% survival), which was injected with immature hybrids or P815 tumour cells alone.

Some of the hybrid cell vaccination's drawbacks were the low fusion efficiency and the reduced purity of the hybrid cells, which affect the observed immunological and clinical results. Hereby, Li *et al*. (Li *et al.*, 2001) separated the true hybrid cells from their parent un-fused cells using two different fluorescent-dye staining of parental cells followed by fluorescent activated cell sorting of the fusion mixture to separate the true hybrid cells stained with both fluorescent colours. He succeeded in separation of the hybrid cells from a fusion of mouse DCs and melanoma tumour cells. *In vitro* results of IFN-γ release and tumour-specific cytotoxicity, and *in vivo* metastatic-lung tumour protection following vaccination, showed that vaccination with purified hybrids was more effective than with fusion mixture in inducing protective immunity and improved clinical responses (Li *et al.*, 2001).

Several published animal studies using hybrid cell vaccines confirmed the protective effect against tumour challenges, and in some cases repression of established metastatic disease (Rosenblatt *et al.*, 2005, Orentas *et al.*, 2001, Markiewicz & Kast, 2004, Guo *et al.*, 1994). Furthermore, the efficacy of DC-tumour hybrid vaccination in murine models was also investigated by Orentas *et al*. (Orentas *et al.*, 2001), who showed that electrofusion product of DC-neuroblastoma cell line was able to induce T-cell immune responses *in vitro* and upon animal vaccination, a significant murine protection from tumour challenge was achieved.

Allogeneic APCs may provide an additional immunogenicity to the fusion hybrids, due to stimulation of allogeneic MHC class II-specific CD4$^+$ Th cell responses. This suggestion was supported by reports of human clinical studies (Trefzer & Walden, 2003) showing the adjuvant effect of MHC class II alloantigens, which provide help for induction and maintenance of CTL. Recently, Yasuda *et al.* (Yasuda *et al.*, 2007) showed the superiority of allogeneic and semi-allogeneic DCs over syngeneic DCs (when fused with autologous tumour cells) in providing protection against tumour challenges in murine colon adenocarcinoma models. The protection was proven to be associated with tumour-antigen specific CTL and NK cellular activity, and an increased Th1/Th2 cytokine profile ratio.

1.5.5.5 Human preclinical and clinical trials on DC-hybrid vaccination

Pre-clinical studies on DC-based hybrid vaccines showed their feasibility and anti-tumour efficacy in different tumour types (Section 1.5.5.4). Based on these features in murine and preclinical studies, several phase I/II clinical trials on human using this approach have been described, with reports of clinical and/or immunological responses (Rosenblatt *et al.*, 2005, Trefzer *et al.*, 2000, Koido *et al.*, 2005, Matsumoto *et al.*, 2006, Wei *et al.*, 2006, Kim *et al.*, 2007, Yin *et al.*, 2008, Koido *et al.*, 2010a, Kalinski & Okada, 2010, Tanaka *et al.*, 2002). DC-tumour fusion vaccine immunotherapy was used in patients with melanoma, where allogeneic DCs were fused to patient-derived tumour cells, and the fusion product was used to vaccinate 17 melanoma patients. The vaccine induced robust anti-melanoma immune responses; however the immunological response was not correlated with the observed clinical responses. The vaccine was shown to be safe, and 1 of 17 patients had complete remissions, 1 of 17 a mixed response, and 6 of 17 had stable diseases (Trefzer *et al.*, 2003). Similar results on melanoma patients were reported by other groups using allogeneic DCs fused to

autologous tumours (Krause *et al.*, 2002, Barbuto *et al.*, 2004). In 2001, Kikuchi *et al.* published a phase I clinical trial using autologous DC-glioblastoma cell fusion to vaccinate 8 glioblastoma patients. Fusion cells were irradiated and injected intradermally, near to cervical lymph node, every three weeks between 3 and 7 injections. Clinical results showed no serious side effects and two patients (25%) had partial responses following vaccination (Kikuchi *et al.*, 2001). In similar trials by the same group, vaccination with autologous fusion cells was administered with IL-12 to patients with malignant glioma, melanoma, breast, gastric, colon and ovarian cancers. Three of 12 patients with malignant glioma achieved partial responses and 1 of 12 a minor response (Kikuchi *et al.*, 2004). The clinical responses in the other tumour types were minor (Homma *et al.*, 2005). In a separate study on 23 patients with metastatic renal and breast cancers, DC-fusion vaccines showed immunological and clinical responses. Two breast cancer patients showed complete regression of some tumour masses, and one breast cancer and five renal carcinoma patients showed stable diseases (Avigan *et al.*, 2004). In a subsequent trial by the same group, allogeneic DCs were fused to autologous RCC cells. Vaccination of 21 patients using fusion product showed clinical responses in 10 patients, with partial clinical responses in two, and stable diseases in 8 patients (Avigan *et al.*, 2007).

Recently, Zhang *et al*. (Zhang *et al.*, 2007) reported results of hybrid cell vaccination of metastatic breast cancer patients. Zhang and co-workers showed the feasibility and efficacy of fusing the tumour cell line (MCF-7; breast cancer cell line), instead of *ex vivo* tumour cells, with autologous patient-derived DCs to generate semi-autologous hybrid-cell vaccines. The use of a tumour cell line instead of *ex vivo* tumour cells bypasses the shortage of *ex vivo* tumour cells, especially in patients where the primary tumour has been resected. Also the tumour cell lines can be easily grown and

investigated/manipulated before fusion, without risk of contamination or loss. Vaccination with DCs/ MCF-7 fusion induced proliferation of autologous T cells, with induction of IFN-γ releasing and specific CTL responses. The CTL recognized and killed the autologous patient's tumour cells in antigen and HLA class I-restricted manner (Zhang *et al.*, 2007).

Similar results were observed by Zhou *et al*. (Zhou *et al.*, 2009) in metastatic renal cell carcinoma (RCC) patients, in a study of allogeneic APC fused to autologous *ex vivo* patient-derived tumour cells. Vaccination with the fusion cells induced T cell immune responses *in vitro* and anti-tumour clinical responses *in vivo*. *In vitro* co-culture of autologous T cells with hybrid cells induced T cell proliferation with enhanced IFN-γ versus IL-4 cytokine release in 5 out of 10 patients, and induction of tumour antigen-specific CTLs which were able to recognise and kill autologous tumour cells. Clinical responses were obtained in 7 out of 10 patients, with one partial response as regression of lung metastasis, and six patients with disease stabilization for over 18 months. More recently, chemical fusion of autologous DCs with patients' bone marrow-derived myeloma cells was successfully carried out by Rosenblatt *et al*. (Rosenblatt *et al.*, 2011). Patient vaccination with fusion cells showed high tolerability without evidence of dose limiting toxicity, and in 11 out of 15 evaluated patients, vaccination with hybrid cells induced T cell proliferation and clonal expansion of CTLs and CD4^{+} helper cells specific for autologous myeloma tumour cells. Humoral immune responses were also demonstrated by SEREX analysis in the majority of cases, and most of the patients showed disease stabilization for up to 41 months. A summary of some clinical trials and their results are shown in Table 1.8. These studies showed improved immune responses to vaccination with DC-tumour cell fusion, although with limited clinical outcomes. In

order to improve the clinical benefits of hybrid cell cancer vaccines, several measures should be taken against immunosuppressive nature of tumour microenvironment.

1.5.6 B cell-based fusion vaccines

Activated B cells represent an alternative source of professional antigen-presenting cells, which are characterised by the ability to process and present antigens in the context of MHC class II and MHC class I molecules for recognition and activation of CD4^{+} T helper and CD8^{+} CTL respectively, in addition to expressing of co-stimulatory CD80, CD86, and CD40 ligands, and intercellular adhesion molecules, which are important molecules required for full activation of primary cellular immune responses (Schultze *et al.*, 1997, Trefzer & Walden, 2003). Although DCs are the most potent inducer of primary T cell response, B cells are characterized by relative abundance compared with DCs in normal PBMC (5-10%), and they can be easily isolated, expanded, and matured through simple laboratory processes, such as CD40 and TLR ligation using CD40L and TLR-agonists respectively. In addition to activation and maturation, B lymphoblastoid cell lines can be obtained by EBV transformation of normal B cells to produce cell lines with immortal growth *in vitro*. Moreover, activated B cells can induce primary T cell immune responses *in vitro* and *in vivo*. (Schultze *et al.*, 2004, Lapointe *et al.*, 2003, von Bergwelt-Baildon *et al.*, 2002). Although DCs may be superior in priming and activation of naive T cells, the labour intensive techniques of their *ex vivo* maturation, low number in PBMC, and inability to be grown *ex vivo* for prolonged periods limit their use (reviewed in Ahmadi *et al.*, 2008).

Tumour	Tumour/DC fusion			Patient number	Clinical response	Authors
	Tumour cells	DCs	Co-administered agents			
Melanoma (disseminated, late stage)	Auto	Allo		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)
	Auto	Allo		13	8 (SD) 3 (SD) 2 (N)	(Barbuto *et al.*, 2004)
	Auto	Auto	rh IL-12	4	4 (PD)	(Homma *et al.*, 2005)
Malignant Glioma	Auto	Auto		8	2 (PR) 1 (SD) 5 (PD)	(Homma *et al.*, 2005)
	Auto	Auto	rh IL-12	12	3 (PR) 2 (MR) 4 (SD) 3 (PD)	(Kikuchi *et al.*, 2004)
Renal cell carcinoma (metastatic)	Auto	Allo		22	14 (SD) 2 (PD) 3 (OR) 3 (N)	(Barbuto *et al.*, 2004)
	Auto	Auto		13	5 (SD) 8 (PD)	(Avigan *et al.*, 2004)
Breast Cancer (metastatic)	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)
Gastric/ Colorectal cancers	Auto	Auto	rh IL-12	3	1 (SD) 2 (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)

Table 1.8: Summary of some clinical trials of DC-tumour fusion vaccines. CR: complete response, PR: partial response, MR: mixed response, SD: stable disease, PD: progressive disease, OR: objective response, N: not evaluated. (Table adapted from Koido *et al.*, 2010b).

Guo *et al*. (Guo *et al.*, 1994) were the first to demonstrate the potential of activated B cells fused to hepatocellular carcinoma cells to induce effective, tumour-specific CD4^{+} and CD8^{+} T cell responses *in vivo*, and to protect laboratory animals against subsequent challenge of tumour cells. A phase I/II clinical trial of activated B cell-tumour hybrid vaccine was reported by Moviglia, G.A. (Moviglia, 1996), where activated-B cells were fused with tumour cells from 21 patients with different solid and haematological malignancies. Complete remission was seen in 4 breast cancer and one prostate cancer cases; partial remission in 3 breast cancer, and 2 haematological tumour cases; minor regression in 2 breast, one prostate, one nasopharyngeal and 3 colon cancer cases; stable disease in 2 colon cancer and one osteochondro-sarcoma cases; and progressive disease in one bladder cancer patient (Moviglia, 1996).

In 2000, Trefzer *et al*. described a new hybrid cell vaccine generated by fusion of allogeneic activated B cells with autologous *ex vivo* melanoma tumour cells. Of 16 patients with advanced metastatic melanoma given the vaccine, one underwent complete remission, one partial remission, and 5 patients showed stable diseases maintained for more than 24 months by booster doses of the vaccine (Trefzer *et al.*, 2000). Recently, Moviglia *et al*. (Moviglia *et al.*, 2008) reported a preliminary study on immunotherapy of glioblastoma multiform (GBM), a malignant disease which is characterized by progressive tumour mass and severe immunosuppression. The study was carried out on 12 post-surgical-removal patients, in which e*x vivo* tumours were fused with autologous activated B cells using PEG, which produced tumour-B cell hybrids (TBH) with a fusion efficiency of 14%. Results of patients vaccination showed complete remission in more than 75% of patients, and the rest of cases (25%) were ranging from partial regression to unresponsiveness (Moviglia *et al.*, 2008). Minor side effects such as hypotension, gastrointestinal upset and flu-like symptoms with

hyperpyrexia were reported in some cases. Moviglia concluded that hybrid cell vaccine composed of autologous activated B cells and autologous tumour cells induced potent immune and clinical responses that were able to overcome the persistence and immune suppression induced by tumour cells of glioblastoma multiform.

As an alternative to *in vitro* activated and normal B cells, EBV B-LCL represent *in vitro* transformed B cell lines that show all features of activated B-cells, such as expression of HLA-class I and class II, co-stimulatory ligands (such as CD80, CD86 and CD40), and adhesion molecules (e.g. ICAM-1) which are necessary for priming naive T cells, in addition to the immortalised growth in tissue culture. EBV B-LCL has an extra advantage by expression of some EBV-specific gene products (e.g. EBV-LMP 1 and 2, and EBNAs), which may provide an adjuvant effect (through activation of existing EBV-specific memory helper T cells) and may potentiate the anti-tumour immune responses. In addition, the EBV-transformed B-LCL, HMy2, cell line combines these features and also is chemically selectable, due to its resistance to ouabain and sensitivity to HAT enriched media (Edwards *et al.*, 1982, Kerr *et al.*, 1992). HMy2 cells have been used as an APC partner, fused with a range of haematological and solid tumour cell/ cell lines, in a group of studies done in my supervisor's lab, which led to the work done in this project.

Dunnion *et al*. reported the fusion of HMy2 with different lymphoid and melanoma tumour cell lines, using PEG, MW 1500, followed by double chemical selection of the HMy2 x tumour fusion hybrids, using growth medium supplemented with HAT and ouabain, to exclude homogenous fusions, un-fused HMy2 and tumour cells. The resulting hybrid cell lines grew continuously in tissue culture; most of them showed high expression levels of the parent-APC surface markers, and expression of several known immunogenic TAAs, and were able to induce strong T cell proliferation in

mixed lymphocyte reaction *in vitro*, which was shown to be CD80, CD86 and CD40 dependent. In addition, Dunnion *et al*. reported that co-culturing the hybrid cells with allogeneic T cells induced production of CTL lines, which were able to recognise the relevant parent tumour cell lines, and destroy them in ^{51}Cr release cytotoxicity assays. Dunnion *et al*. carried out similar experiments on both parent tumour cell lines and their HMy2-hybrids, and showed markedly enhanced immunogenicity and T cell induction ability of the hybrid cell lines compared with their respective parent tumour cells in these assays (Dunnion *et al.*, 1999).

Extending these results, Cywinski *et al*. (Cywinski *et al.*, 2006) showed major differences in the immunogenicity for T cell responses *in vitro* between hybrid cell lines formed by fusion of HMy2 cells with haematological as opposed to non-haematological tumour cells. Fusion of HMy2 with non-haematological tumours showed enhanced T cell stimulation compared with the unfused parent tumour cells, but significantly lower than that of haematological tumour-derived hybrids.

The first report that showed fusion of EBV B-LCL with *ex vivo* tumour cells was in 2007 by Walewska *et al*. (Walewska *et al.*, 2007). Walewska carried out fusion of HMy2 with 5 different *ex vivo* multiple myeloma tumour cells, to produce 5 stable hybrid cell lines that grew continuously in tissue culture and survived successive freezing-re-thawing cycles. The hybrid nature of the fused cell lines was confirmed by microsatellite genetic analysis, and all hybrid cell lines showed consistent expression of HLA class I and class II antigens, and CD80 and CD86 co-stimulatory molecules. A high level of T cell proliferation (allogeneic and autologous) was induced by the hybrid cell lines, in contrast with their parent tumour cells, and this ability was shown to be CD80 and CD86 dependent, and was also dependent on both of HLA class I and class II expression. Moreover, stimulation of autologous T cells using relevant hybrid cell lines

for several rounds induced activated T cells with significant increased IFN-γ production upon sensitization with the relevant parent tumour cells. However, none of these studies with hybrid cell lines produced by fusion of HMy2 and tumour cells addressed the induction of tumour antigen-specific T cell responses *in vitro*.

HMy2-tumour cell hybrids therefore offer an alternative model to DC-derived hybrids, with higher stability, continuous tissue culture growth, and double chemical selectivity to obtain highly pure hybrid cell line product.

1.6   Aims of the study

My focus in this study was to extend the *in vitro* characterization of a panel of previously-generated LCL-tumour hybrids in relation to their potential as agents in cancer immunotherapy. This involved the investigation and evaluation of the ability of the hybrid cell lines generated by chemical fusion of HMy2 (EBV B-LCL) with a group of haematological and lymphoid tumour cells/cell lines, to effectively stimulate proliferation and functional differentiation of naive and/or memory T cells, and in particular, their ability to induce tumour and tumour antigen-specific cytotoxic T cell responses *in vitro*.

These objectives were achieved through

1- Phenotypic characterization of hybrid cell lines

2- Qualitative and semi-quantitative evaluation of a group of TAA-expression by hybrid and parent HMy2 cell lines

3- Functional characterization of hybrid cell lines, and investigation of their ability to stimulate allogeneic and autologous T lymphocyte proliferation, and to produce anti-tumour IFN-γ release responses *in vitro*.

4- Induction of anti-tumour CTL lines, able to recognise and kill autologous parent tumour cells *in vitro.*

5- *In vitro* induction of HLA-A2-restricted, TAA-specific T cell lines using HLA-A2-positive PBMC from either healthy allogeneic donors or tumour-bearing patients.

These results, and further animal model investigations shall provide important preclinical information on the potential of this hybrid cell line model as candidate cancer vaccines and/or as *in vitro* inducers of TAA-specific CTL cell clones for adoptive tumour immunotherapy.

Chapter 2
Materials and Methods

2 Materials and Methods

2.1 Primary cells and cell lines

2.1.1 Established cell lines

A summary of the cell lines used in this study is shown in Table 2.1. HMy2 is the APC fusion partner cell line, and was used in all experiments as a control positive, KG-1 and U266 are AML and MM tumour cell lines respectively, which were investigated in comparison with their hybrid cell lines (HxKG-1 and HxU266 respectively) to show the difference in phenotypic and functional characteristics of tumour cells before and after fusion to HMy2. K562 is a chronic myeloid leukaemia (CML) cell line, and due to lack of MHC class I molecule expression, it was used as an NK target in several immune assays. T2 cell line is a T and B cell hybridoma cell line characterised by its TAP-deficiency and ability to bind exogenously pulsed peptide to its HLA-A2 molecules. Accordingly T2 cells were used as targets in ^{51}Cr release assays after pulsing with the appropriate peptide, followed by radioactive isotope labelling. The hybrid cell lines used in this study are mentioned later in Section 2.1.3

Cell line	Description	Lineage
HMy2	EBV- transformed B-LCL	B-cells
KG-1	Acute myeloblastic leukaemia	Myeloid cells
K562	Chronic myeloid leukaemia	Myeloid cells
U266	Multiple myeloma	Plasma cells
T2	Hybridoma	T cell x B cell

Table 2.1: Tumour and APC cell lines used in different protocols in this study

2.1.2   Primary cells

These include peripheral blood mononuclear cells (PBMC) from normal donors and from tumour bearing patients, in addition to *ex vivo* primary tumour cells.

2.1.2.1   Peripheral blood mononuclear cells

Normal donors' blood was collected (after getting Local Research Ethics-Committee approval, and subject to informed consent by the volunteer donors) followed by PBMC separation. Patients' PBMC were taken from frozen stocks stored in liquid nitrogen.

2.1.2.2 Separation of blood mononuclear cells

PBMC were isolated from heparinised whole venous blood (collected by venepuncture) and separated by density gradient over lymphocyte separation medium, Lymphoprep solution (Axis-Shield Diagnostic, Norway). Blood was diluted with an equal volume of Roswell park memorial institute (RPMI-1640) medium, and 20-25ml of diluted blood was layered gradually over 15ml of Lymphoprep. Tubes were then centrifuged at 400 xg, at room temperature (RT), low acceleration and zero deceleration (brake off), for 25 minutes. PBMC were collected from the buffy interface between the RPMI-medium and the Lymphoprep layer, washed twice with fresh RPMI-medium, centrifuged at 1200 rpm for 10 minutes and resuspended in supplemented growth media (SGM; RPMI-1640 supplemented with L-glutamine (2mM), streptomycin (100µg/ml), penicillin (100U/ml) (all from Sigma, UK), and 10% Foetal Calf Serum (FCS) (Harlan laboratory, UK). Cells were counted using fast counting chamber slides (ISL, UK), 20µl of trypan blue stained cells (10µl of the dye + 10µl of cell culture) was introduced into the application area and counted under inverted microscope using 20x magnification. The cell concentration was calculated by the following equation:

Cell number/ ml= (Total counts x 10^4 / Number of 4x4 grids counted) x Dilution factor.

At this stage, PBMC were ready for either immediate use or freezing for later use.

2.1.2.3 Separation of the primary tumour cells using magnetic microbeads

Primary tumour cells were separated from peripheral blood or bone marrow mononuclear cells (PBMCs or BMMCs) of the tumour bearing patients by means of magnetic microbeads cell sorting (MACS), and stored in liquid nitrogen till further use in different assays.

2.1.2.3.1 Principle of MACS sorting

Magnetic cell sorting is a standard method for group-specific cell separation. It depends on the use of monoclonal antibody (mAb)-linked magnetic microbeads, which can be chosen according to lineage-specific CD markers for the cells to be separated e.g. CD19 for B cell separation. MACS (Miltenyi Biotec, UK) microbeads are super paramagnetic particles of approximately 50nm diameter, and composed of completely biodegradable material, so the separated, labelled cell fraction can be used directly in the subsequent experiments with no structural, functional or activity changes (*MACS user manual, Miltenyi Biotec, UK*). The separation process was carried out in three steps, according to the manufacturer's instruction. Firstly, cell labelling, in which patient's PBMCs or bone marrow mononuclear cells were labelled with the magnetic bead-conjugated mAb directed against the target tumour cells' lineage-specific marker. Secondly, cell separation, in which the labelled cells were loaded to the pre-activated separation column (LS, Miltenyi Biotec, UK) fixed in a magnetic field of MACS separator (MidiMACS®; Miltenyi Biotec, UK). The magnetically labelled cells were retained on the column and the unlabelled fraction run through. Finally, the column was removed from the magnetic field and the target cells were eluted by using the provided plunger.

Using this method, the PBMC/ BMMC were separated into two fractions, the labelled tumour cell fraction, and the rest of the patient's PBMC including T lymphocytes as the untouched fraction (Figure 2.1).
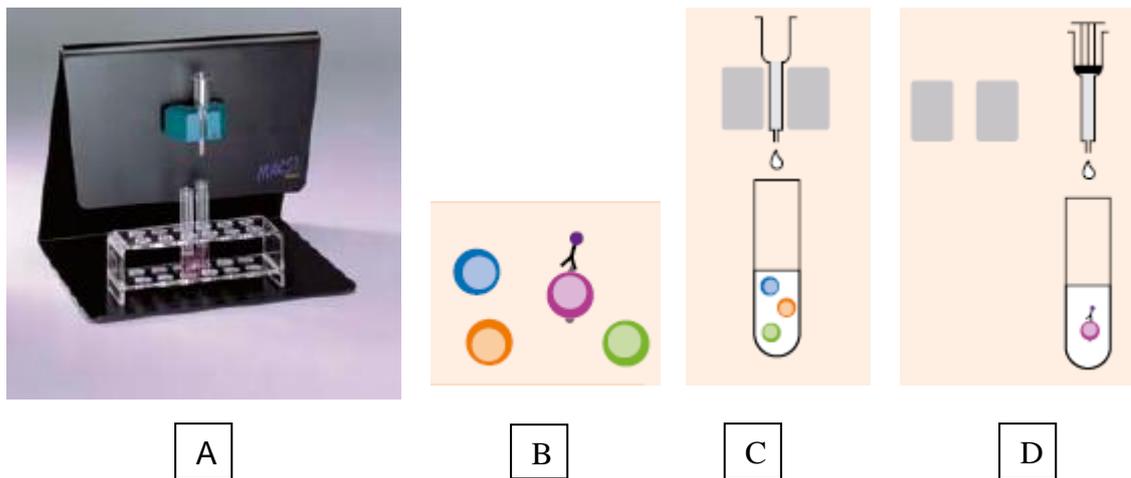
Figure 2.1: MACS magnetic cell sorting. A; a separation column connected to a magnet on the MACS separator, B; Target cell (violet) labeled with a specific mAb-magnetic microbeads, C; Unlabeled cell fraction is collected, D; Labelled cell fraction is eluted. (Modified from; MACS separation protocole, Miltenyi Biotec, UK)

2.1.2.3.2 PBMC labelling

Patients' PBMC were collected from liquid nitrogen store, thawed, counted and suspended in 80µl of pre-cooled, degassed and sterile filtered sorting buffer (SB), 0.5% bovine serum albumin (BSA; Sigma, UK) in PBS, per 10^7 total cells, and 20µl of specific monoclonal antibody-conjugated microbeads was added per $1x10^7$ total cells. A list of the magnetic labelled monoclonal antibodies used is shown in Table 2.2 (all from Miltenyi Biotec, UK). The cell suspension was mixed well and incubated at 4°C for 15 minutes. After incubation, cells were washed using 2ml SB per $1x10^7$ cells and centrifuged at 1200 rpm for 10 minutes. The supernatant was pipetted out completely and the cells were resuspended in 500µl SB for up to $1x10^8$ cells and the cell suspension was kept on ice until cell sorting.

Tumour cell type	mAb-magnetic microbeads
Myeloid leukaemia	CD33 labelled magnetic microbeads
B- lymphocytic leukaemia	CD19 labelled magnetic microbeads
Mantle cell lymphoma	CD19 labelled magnetic microbeads
Multiple myeloma	CD138 labelled magnetic microbeads

Table 2.2: mAb-conjugated magnetic microbeads used in patients' tumour cell sorting

2.1.2.3.3 *Magnetic separation*

The LS column (Miltenyi Biotec, Germany) was placed in the magnetic field of a MACS separator (MidiMACS®, Miltenyi Biotec, Germany) and 3ml of SB were pipetted in and allowed to run through the column to prime it. Labelled cold cells were applied to the column, the unlabelled fraction (pass through fraction) was collected, and the column was washed three times with 3ml of SB, and all the flow through was collected and pooled. The column was removed from the magnetic field and placed onto a suitable collection tube, and 5ml of sorting buffer was pipetted onto the column and all content was flushed out by applying the provided plunger; both fractions were washed in SB prior to use.

2.1.2.3.4 *Flow cytometric analysis of the separated fractions*

Purity of the separated fractions was determined by flow cytometric analysis. Cells were labelled with the appropriate monoclonal antibodies (listed in Table 2.3) and analysed for purity by immunofluorescence staining followed by flow cytometric analysis (Section 2.3.1).

Analysed cell lineage	Specific labelled mAb	Source	
T lymphocytes	CD3-FITC	Beckman UK	Coulter,
B-cell tumour	CD20-FITC	Beckman UK	Coulter,
Myeloid tumour	CD13-PE	Beckman UK	Coulter,
Multiple myeloma tumour	CD38-FITC	Beckman UK	Coulter,

Table 2.3: mAb used in IF staining of the separated cells

Purity of the positively sorted population exceeded 90%, and the T cell content of the untouched fraction was variable, depending on the immune status of the patient.

2.1.3 Hybrid and parent tumour cells/cell lines

The origin and type of the used hybrid cell lines are shown in Table 2.4. HMy2 was fused to *ex vivo* tumour cells or cell lines from six different haematological malignancy types, including acute myeloblastic leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), follicular cell lymphoma (FCL), mantle cell lymphoma (MCL), and multiple myeloma (MM).

2.1.3.1 EBV B- LCL x Tumour cell hybrid production

The hybrid cell lines used in this study had been generated prior to the beginning of this project. The generation protocol has been described previously (Dunnion *et al.*, 1999, Cywinski *et al.*, 2006) and is summarized briefly hereafter.

Hybrid cell line	Description	Tumour cells	Type of the tumour partner
HMy2 x KG-1	Acute myeloblastic leukaemia hybrid	KG-1	Established tumour cell line
HMy2 x DO050	Acute myeloblastic leukaemia hybrid	DO050	*Ex vivo* tumour
HMy2 x VP024	Acute myeloblastic leukaemia hybrid	VP024	*Ex vivo* tumour
HMy2 x PG052	Acute lymphoblastic leukaemia hybrid	PG052	*Ex vivo* tumour
HMy2 x AW072	Chronic lymphocytic leukaemia hybrid	AW072	*Ex vivo* tumour
HMy2 x LC069	Chronic lymphocytic leukaemia hybrid	LC069	*Ex vivo* tumour
HMy2 x JR004	Chronic lymphocytic leukaemia hybrid	JR004	*Ex vivo* tumour
HMy2 x SG059	Chronic lymphocytic leukaemia hybrid	SG059	*Ex vivo* tumour
HMy2 x RS048	Follicular cell lymphoma hybrid	RS048	*Ex vivo* tumour
HMy2 x TH044	Mantle cell lymphoma hybrid	TH044	*Ex vivo* tumour
HMy2 x FC029	Mantle cell lymphoma hybrid	FC029	*Ex vivo* tumour
HMy2 x U266	Multiple myeloma hybrid	U266	Established tumour cell line
HMy2 x CM053	Multiple myeloma hybrid	CM053	*Ex vivo* tumour
HMy2 x KS037	Multiple myeloma hybrid	KS037	*Ex vivo* tumour
HMy2 x IC038	Multiple myeloma hybrid	IC038	*Ex vivo* tumour
HMy2 x RC056	Multiple myeloma hybrid	RC056	*Ex vivo* tumour

Table 2.4: Description of the hybrid and relevant parent tumour cells/ cell lines used in this study.

2.1.3.2 Cell fusion procedure

The antigen presenting cell fusion partner (Epstein-Barr virus transformed B-lymphoblastoid cell line; EBV B-LCL; HMy2) is characterised by double chemical growth selection (sensitive to Hypoxanthine, Aminopterin and Thymidine (HAT) and resistant to ouabain), which allows for selection of hybrid cell growth *in vitro*. The

tumour partners were a group of different haematologic *ex vivo* tumour cells and cell lines, as described in Table 2.4. The tumour cells were characterized by their ouabain sensitivity and HAT resistance, whilst HMy2 cells are ouabain resistant and HAT sensitive. Equal numbers of tumour cells and HMy2 cells were mixed together in presence of PEG/DMSO for 1 minute before SGM was added drop wise to the cells. After 24 hours, HAT and ouabain were added to select for heterokaryons' growth (Walweska *et al.*, 2007).

2.2   Cell culturing and freezing conditions

Cell lines' growth was maintained in SGM in 25 or 75cm3 low attachment tissue culture flasks (Nunc, UK), and incubated in a humid atmosphere at 37°C and 5% CO_2. The cell growth was maintained by cell splitting 1:4 every 2-3 days with fresh media replacement. High cell number was obtained by growing the cells in 75 cm3 flask, and fresh SGM was added up to 50ml to maintain cell growth to logarithmic phase.

Prior to freezing, cells were mixed well, stained with Trypan blue solution and counted using stained cell exclusion method to assess the cell viability. Around $0.5 - 2 \times 10^7$ viable cells were harvested, centrifuged at 1480 rpm for 5 minutes in case of cell lines, or at 1200 rpm for 10 minutes in case of primary cells. Cell pellets were loosened by gentle flicking, placed on ice for 10 minutes, and 1ml of cold freezing medium [FM; 10% dimethyl sulfoxide (DMSO; Sigma, UK) in FCS], was added to the cells gradually, mixed gently, and pipetted into pre-chilled, labelled 1.5ml cryovials (Sarstedt, UK). Cryovials were placed directly in a pre-cooled Mr. Frosty (Nalgene, USA) to allow the gradual drop in temperature by -1°C per minute, transferred directly into a -80°C freezer, and after 24 hours, cryovials were transferred into cryobox in a liquid nitrogen storing vessel (-196°C). To re-culture cells from frozen, cryovials were

collected on dry ice from the liquid nitrogen vessel, defrosted in a water bath at 37°C for 1-2 minutes, and the cells immediately washed by addition of 10ml pre-warmed SGM drop wise with swirling. Cells were pelleted, and pellets were resuspended in 5-10ml SGM, transferred into a 25 cm^3 low attachment tissue culture flask, and incubated at 37°C, 5% CO2, in a humid incubator.

2.3 Phenotypic characterization of HMy2, parent tumours, and hybrid cell lines

2.3.1 Surface marker and HLA expression profile

Immunophenotyping of HMy2, hybrid cells and parent tumour cell lines was carried out using immunofluorescent (IF) staining and flow cytometry to investigate the surface markers and human leukocyte antigen expression. Direct surface IF staining of the viable cells was carried out using the labelled mouse anti-human monoclonal antibodies (mAbs) listed in Table 2.5.

2.3.1.1 Immunofluorescent staining

Cells were harvested, washed thoroughly in cold PBS, counted and dispensed as 5×10^5 cells (or as required) in a minimum amount of supernatant per tube. 10µl of the appropriate mAb was added (except for HLA-A2, where 2 µl were used per test), the tubes were mixed and incubated at 4°C in the dark for 20 minutes. After incubation, cells were washed twice by addition of 2ml of cold washing buffer (WB; 1% BSA in sterile PBS) and pelleted. After the final washing, cell pellets were loosened by gentle flicking and fixed by addition of 0.5ml cold fixing buffer (FB; 2% paraformaldehyde (Sigma, UK) in sterile PBS) and tubes were stored in the dark at 4°C until flow cytometric analysis.

mAb	Isotype	Labell.	Final conc.	Source
Isotype control	IgG1	FITC	0.5µg/ml	Beckman Coulter, UK
Isotype control	IgG1	PE	0.5µg/ml	Beckman Coulter, UK
Isotype control	IgG1	PerCP	0.5µg/ml	R&D Systems, UK
Isotype control	IgG1	PC5	0.5µg/ml	Beckman Coulter, UK
Isotype control	IgG$_{2b}$	APC	0.5µg/ml	BD Biosciences, UK
HLA-ABC	IgG1	FITC	0.5µg/ml	Beckman Coulter, UK
HLA-A2	IgG$_{2a}$	FITC	0.5µg/ml	BD Biosciences, UK
HLA-DP, DQ, DR	IgG1	FITC	0.5µg/ml	Dako Cytomation, UK
MHC class I (W6/32)	IgG$_{2a}$	Plain	0.75µg/ml	Tissue culture supernatant
MHC class II (L243)	IgG$_{2a}$	Plain	0.75µg/ml	Tissue culture supernatant
CD3	IgG1	PE /FITC/PC5	0.5µg/ml	Beckman Coulter, UK
CD4	IgG1	PE /FITC/PC5	0.5µg/ml	Beckman Coulter, UK
CD8	IgG1	PE /FITC/PC5	0.5µg/ml	Beckman Coulter, UK
CD19	IgG1	FITC	0.5µg/ml	Beckman Coulter, UK
CD40	IgG1	PE	0.5µg/ml	Beckman Coulter, UK
CD45RA	IgG$_{2b}$	APC	0.5µg/ml	BD Biosciences, UK
CD80	IgG1	FITC	0.5µg/ml	Beckman Coulter, UK
CD86	IgG1	PE	0.5µg/ml	Beckman Coulter, UK
CCR7	IgG1	PerCP	0.5µg/ml	R&D Systems, MN, USA

Table 2.5: mAbs used in flow cytometric analysis and in mixed lymphocyte reaction, optimisation was carried out as a part of previous projects in the lab find the ideal mAb final concentration.

2.3.1.2 Flow cytometric analysis

Flow cytometry of fluorescently labelled cells were carried out using a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, UK), and data were analysed using CellQuest Pro software (Becton Dickinson, UK). Fluorescently labelled isotype control antibodies were used as negative controls, and at least 1×10^4 (unless

stated) events were acquired per sample, and gates were set as required on living or specific cell populations on the forward-side scatter dot plot. Voltage of each channel was adjusted so that isotype control mAb-stained cells produced a linear fluorescence intensity of value around 10.

2.3.2 Tumour associated antigen expression by RT-PCR

2.3.2.1 Total RNA extraction

Total RNA was extracted from about $1x10^7$ cells using RNeasy Minikit (Qiagen, UK), according to the manufacturer's instructions. Cells were harvested in RNase free Falcon tubes, centrifuged for 5 minutes at 1480 rpm, washed in PBS, the supernatant was completely discarded, and cells were loosened by flicking the tube gently before addition of 600µl of RLT buffer followed by vortexing to completely lyse cells (N.B: β-Mercaptoethanol was added to RLT buffer in a ratio 1:100; this mixture was stable for a month at room temperature). The cell mixture was homogenized by passing it through a 23 gauge needle for 5-10 times, and then an equal volume of 70% ethanol was added and mixed by pipetting. 700µl of the mixture was transferred to the RNeasy mini column, centrifuged at 10^4 rpm, at RT for 15 seconds, flow-through was discarded, and last step was repeated if required. Columns were washed by addition of 700µl of RW1 buffer followed by centrifugation at 10^4 rpm for 15 seconds, and flow-through was discarded. 500µl of RPE buffer was added, followed by centrifugation at 10^4 rpm for 15 seconds, flow-through was discarded, and a new 2ml collecting tube was applied to the column. A further 500µl of RPE buffer was added with centrifugation at 10^4 rpm for 2 minutes. The column was transferred to a 1.5ml Eppendorf tube and 40µl RNase free water was added to the middle of the column on the silica membrane lining. The column was left standing on the bench for 5 minutes and centrifuged at 10^4 rpm for 1 minute. The last step was repeated using the same RNA solution to get a more

concentrated RNA preparation, which was subjected to quantification and purity estimation, and stored at -20°C until used.

2.3.2.2 Calculation of RNA concentration

RNA concentration was assessed by measuring the absorbance at 260 nm (A_{260}) using a spectrophotometer (Pharmacia Biotech, UK) in a 70µl sized RNase free cuvette (Brand, UK). RNA was firstly diluted with RNase free water, then the reading was taken against blank (RNase free water). An absorbance of 1 unit at 260 nm corresponds to a solution of 40µg of RNA per ml. The total RNA concentration was calculated using the following equation:

{Total RNA concentration (µg/ml) = 40 x A_{260} x Dilution Factor}

{Total amount of RNA in µg = Concentration x Volume in millilitres}.

2.3.2.3 Estimation of RNA purity

Purity of the extracted RNA was estimated through measuring the absorbance ration at 260nm/280nm ($A_{260/280}$). A ratio of 1.9 or above indicated an acceptable purity of the RNA preparation.

2.3.2.4 Genomic DNA degradation

Contaminating genomic DNA was removed from the total RNA extract using RNase-free, DNase I (Qiagen, UK), according to the manufacturer's instructions. In a microcentrifuge tube, 5 - 10µg of RNA was mixed with 2µl 10x DNase buffer, 2µl DNase I, and the total volume was made up to 20µl using RNase-free sterile distilled water. The mixture was heated at 37°C for 30 minutes, and 2µl of 1mM EDTA solution was added before incubation at 65°C for 5 minutes to de-activate the remaining DNase I enzyme.

2.3.2.5 *First strand complementary DNA (cDNA) synthesis*

RNA was reverse transcribed to first strand complementary DNA (cDNA) using cDNA Superscript[TM] First-Strand Synthesis kit (Invitrogen, UK), according to the manufacturer's instructions. 5µg of total RNA was mixed with 1µl of Oligo deoxythymidine $(dT)_{12-18}$ (0.5µg/µl) as a primer and 1µl of deoxynucleotide tri-phosphate mix (dNTP mix) (10mM each) in a PCR tube on ice and tubes were incubated at 65°C for 5 minutes. The tubes were then chilled on ice for 1 minute. A reaction mixture containing 4µl of 5 x reverse transcriptase buffers, 2µl of 0.1M Dithiothreitol (DTT) and 1µl RNaseOut[TM] (40U/µl) was prepared, mixed, and 7µl of this mixture was added to each tube and incubated at 42°C for 2 minutes. Next step was to add 1µl of 200U/µl reverse transcriptase enzymes to each reaction, and incubate at 42°C for 50 minutes, followed by reaction termination at 70°C for 15 minutes. Finally, 1µl of RNase-H (2U/µl) was added to each tube, followed by incubation for 20 minutes at 37°C, to degrade the remaining RNA.

2.3.2.6 *Reverse transcription- polymerase chain reaction (RT-PCR)*

RT-PCR was carried using cDNA template to investigate the expression of the tumour associated antigens of interest. In 0.2ml PCR tubes on ice, 1µl of each cDNA template was mixed with 5µl of 5x well mixed Hot-start green PCR buffer, 1µl of 10mM dNTP, 2µl of 25mM well mixed $MgCl_2$, 1µl of 10µM solution of forward and reverse primers, 0.2µl of Hot-start Taq-polymerase 5U/µl, all from (Promega, USA), and PCR grade water to a final volume of 25µl. The tubes were mixed, briefly spun and transferred to the Thermo cycler (T1 Thermo cycler, Biometra, Germany). Each PCR reaction was subjected to running conditions of an initial denaturation step at 94°C for 2 minutes, 35 cycles of polymerization and a final elongation step at 72°C for 10 minutes. Each cycle was composed of denaturation step at 94°C for 60 seconds followed by annealing at the

appropriate primer-set specific temperature for 60 seconds, and elongation at 72°C for

another 60 seconds. The primer sequences, amplicon sizes, and annealing temperature

are summarized in Table 2.6.

Antigen	Primers sequences	Prod. size	Anneal temp.	Reference
Survivin	5´-CACCGCATCTCTACATTCAA-3' 5'-CACTTTCTTCGCAGTTTCCT-3'	275 bp	59°C	Schmidt *et al.*, 2003
WT-1	5'-ATGAGGATCCCATGGGCCAGCA-3' 5'CCTGGGACACTGAACGGTCCCCGA-3'	790 bp	69°C	Greiner *et al.*, 2004
NY-ESO-1	5'-GGCTGAATGGATGCTGCAGA-3' 5'-CTGGAGACAGGAGCTGATGGA-3'	251 bp	57°C	Sugita Y. *et al.*, 2004
PRAME	5'-CTGTACTCATTTCCAGAGCCAGA-3' 5'TATTGAGAGGGTTTCCAAGGGGTT-3'	561 bp	67°C	Van Baren *et al.*, 1999
MAGE-A1	5-'CGGCCGAAGGAACCTGACCCAG-3' 5'-GCTGGAACCCTCACTGGGTTGCC-3	421 bp	68°C	Van Baren *et al.*, 1999
MAGE-A3	5'-TGGAGGACCAGAGGCCCCC-3' 5'-GGACGATTATCAGGAGGCCTGC-3'	725 bp	69°C	Van Baren *et al.*, 1999
MUC-1	5'-CGTCGTGGACATTGATGGTACC-3' 5'-GGTACCTCCTCTCACCTCCTCCAA-3'	287 bp	59°C	Brossart *et al.*, 2001
h-TERT	5'-GCTGTTTGCGGGGATTCGGC-3' 5'-CCACGCAGCCATACTCAGGGAC-3'	137 bp	61°C	NCBI, id = JSID_1_684 35_130.14.2 2.21_9000
β-Actin	5'-GCTCGTCGTCGACAACGGCTC-3' 5-'CAAACATGATCTGGGTCATCTTC TC-3'	353 bp	60°C	Invitrogen

Table 2.6: primer sequences, product size and annealing temperature used in RT-PCR
for detection of the investigated TAA expression.

Following RT-PCR, 12µl of the end product was subjected to agarose gel

electrophoresis using 2% agarose gel (Sigma, UK) in the presence of 0.5µg/ml

Ethidium bromide (Sigma, UK) in Tris acetate EDTA (TAE) buffer. After loading RT-

PCR end products and 6µl of 100 bp size ladder (New England Biolabs, UK) the

agarose gel was immersed in an electrophoresis tank containing 1X TAE buffer and

electrophoresis was carried out at a voltage of 90V for 60 - 75 minutes. The PCR bands were visualized using a UV transilluminator (UVP, USA).

2.3.3 Semi-quantitative estimation of tumour antigen expression levels

2.3.3.1 Principle

Real time PCR was used for the relative estimation of TAA gene expression levels of different hybrid and parent tumour cell lines, using Roche Light Cycler® system 2 (Roche Diagnostic, Mannheim, Germany), according to the manufacturer's instructions. SYBR green master mix (SYBR® Green JumpStart™ Taq ReadyMix™ for Quantitative PCR, Capillary Formulation, Sigma, UK) was used for cDNA amplification, which contains hot start Taq polymerase enzyme, dNTP mix, polymerase buffer and fluorescent dye (SYBR green I).

SYBR green I is a green fluorescent dye, which is able to bind to the minor grooves of the double helix of DNA molecules. The unbound dye has little fluorescence; however, the fluorescence is greatly enhanced after double-stranded DNA binding, enabling the Light Cycler to evaluate the amount of the double stranded DNA produced in each cycle. The amplification reaction mixture was prepared as follow; SYBR green mix 7.5µl, forward primer (5 µM) 1.5µl, reverse primer (5 µM) 1.5µl, PCR-grade water 3.5µl and cDNA template 1µl (equivalent to 0.25µg RNA).

All reagents were premixed together and added to the cDNA template in the 20µl Light Cycler capillaries (Roche Diagnostic, Germany), and capillaries were fixed with the provided stoppers. The capillaries were then transferred to the Cycler aluminium adaptor, centrifuged at 3000 rpm, 4˚C for 60 seconds, transferred to Cycler carousel and placed in known order in the Light Cycler rotor. Four x tenfold serial dilutions of the control positive template were included for standard curve preparation.

Cycling conditions and primer sequences for each antigen are summarised in Table 2.7. The run started by initial activation of the hot start Taq polymerase at 95˚C for 30 seconds, followed by 45 to 55 amplification cycles (denaturation at 95˚C for one second, annealing at the given temperature for 10 seconds and elongation was performed at 72°C for 45 seconds). At the end of each elongation cycle, there was a single data acquisition step to quantify the amount of amplified DNA per cycle.

On completion of the run, a melting curve was set up by gradual increase in the temperature from 65˚C up to 95˚C, with continuous data acquisition to investigate the specificity and purity of the end product. A single pure end product should result in a melting curve of one clear peak, Figure 2.2.

Antigen	Primers sequences	Prod. size	Ann. Temp	Reference
Survivin	5´-CACCGCATCTCTACATTCAA-3' 5'-CACTTTCTTCGCAGTTTCCT-3'	275	59°C	Schmidt *et al.*, 2003
WT-1	5'-TTCCCCAACCACTCATTCAA-3' 5'-GGCGTCCTCAGCAGCAAAGC-3'	150	60°C	Yukiko N. *et al.*, 2004
NY-ESO-1	5'-GGCTGAATGGATGCTGCAGA-3' 5'-CTGGAGACAGGAGCTGATGGA-3'	251	57°C	Sugita Y. *et al.*, 2004
PRAME	5'-CTGTACTCATTTCCAGAGCCAGA-3' 5'TATTGAGAGGGTTTCCAAGGGGTT-3'	561	67°C	Van Baren *et al.*, 1999
MAGE-A1	5-'CGGCCGAAGGAACCTGACCCAG-3' 5'-GCTGGAACCCTCACTGGGTTGCC-3	421	68°C	Van Baren *et al.*, 1999
MAGE-A3	5'-TGGAGGACCAGAGGCCCCC-3' 5'-GGACGATTATCAGGAGGCCTGC-3'	725	69°C	Van Baren *et al.*, 1999
MUC-1	5'-CGTCGTGGACATTGATGGTACC-3' 5'-GGTACCTCCTCTCACCTCCTCCAA-3'	287	59°C	Brossart *et al.,* 2001
h-TERT	5'-GCTGTTTGCGGGGATTCGGC-3' 5'-CCACGCAGCCATACTCAGGGAC-3'	137	61°C	NCBI, id = JSID_1_6 8435_130. 14.22.21_ 9000
β-Actin	5'-GCTCGTCGTCGACAACGGCTC-3' 5-'CAAACATGATCTGGGTCATCTTC TC-3'	353	60°C	Invitrogen

Table 2.7: Primer sequences, amplicon size, and annealing temprature of the investigated TAAs by qRT-PCR

2.3.3.2 Data analysis and gene quantification

2.3.3.2.1 Purity of the end products

Purity and specificity of the end product was demonstrated via the melting curve.

Production of a single peak is an indicator of a single specific product, see Figure 2.2

File: F:\LightCycler3\Users\ysa2\Data\2009-10\B-Actin..ABT   Program: melt   Run By: Home
Run Date: Jul 16, 2009 16:18   Print Date:

Digital Filter: Enabled   Calculation Method: Polynomial

Color Compensation: Off
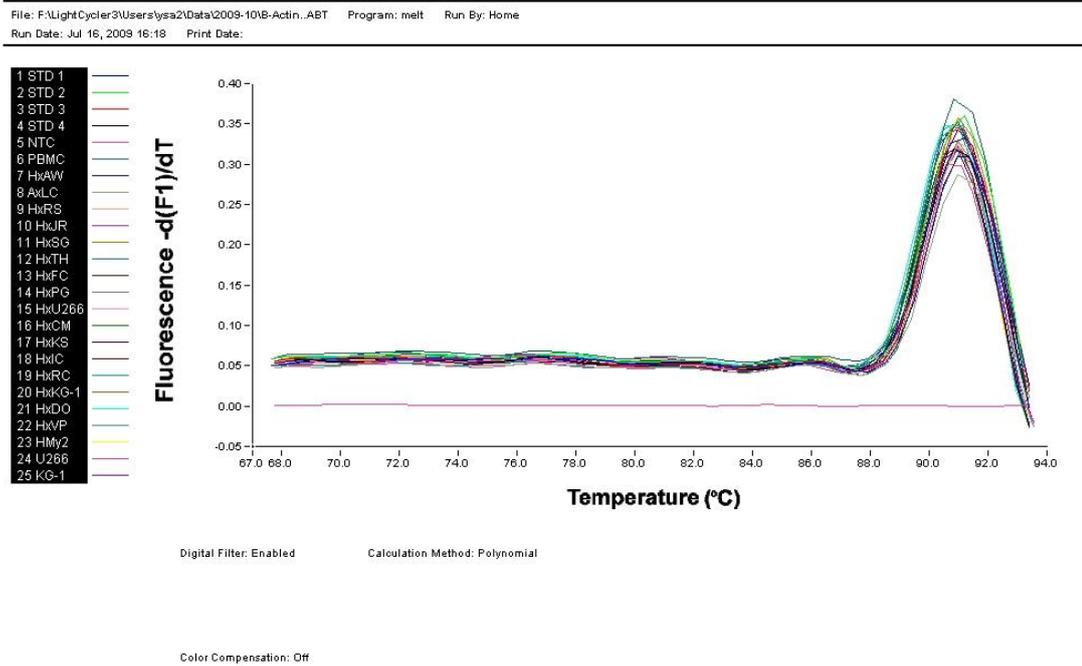
Figure 2.2: Representative melting curve; showing a single clear peak with a baseline for the control sample, indicating a pure monoproduct

2.3.3.2.2   *Quantification of the antigen expression*

The analysis of TAAs expression in different cell lines was calculated automatically by the machine-provided software, using the standard curve (Figure 2.3), which is the plotting of C_T (Threshold cycle, the cycle number at which there was a detectable increase in the SYBR green fluorescence, and usually measured in the range 15 – 25 cycles (Figure 2.3), versus log concentration of four (tenfold) dilutions of the antigen positive standard template (1, 0.1, 0.01, and 0.001). The standard curve was made automatically by the Light Cycler software at the end of each run.

Results were analyzed by using the "fit point" protocol in the provided Roche Applied Science software (LDCA). Relative mRNA expression levels of each antigen were

estimated from the calculated mRNA concentration, and normalized to the level of beta actin (housekeeping gene) expression in the same cDNA sample.

2.3.3.2.3 Data analysis

Data were presented as mean ±SEM of triplicate runs, using GraphPad Prism 5.5 Software (San Diego, CA, USA).
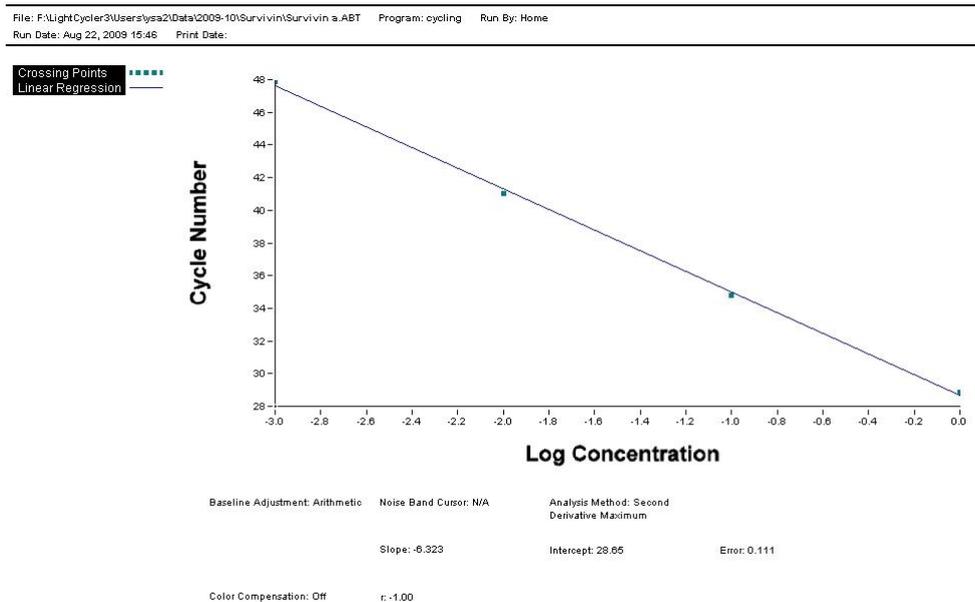
Figure 2.3: Representative standard curve; showing straight line representing the linear relationship between log concentrations of amplified cDNA and the cycle numbers.

2.4 *In Vitro* Immunostimulatory assays using the hybrid and parent tumour cell lines

2.4.1 Mixed lymphocyte reaction (MLR)

The ability of the hybrid cell lines, compared with their parent tumour cells (where available) to induce allogeneic lymphoproliferative responses was assessed in MLR by incorporation of radioactive thymidine (methyl ^{3}H-thymidine) into the replicating

cellular DNA within the reaction mixture, using normal healthy donors' PBMCs as responder cells.

2.4.1.1 Preparation of the stimulator cell lines

HMy2, hybrid and parent tumour cell lines were used in separate proliferation assays as immune-stimulators for allogeneic PBMC. The stimulator cells were first treated with 0.5ml (50µg/ ml) Mitomycin-C (Sigma, UK; optimization of final concentration was carried out, data not shown) for 30 minutes at 37°C in 5% CO2 and in a humid atmosphere, to inhibit cell division (Mitomycin-C inhibits the cellular division by induction of DNA cross linking). After incubation, the cells were washed twice in RPMI-1640 medium, counted, and resuspended in SGM in the following concentrations; $1x10^6$, $3x10^5$, and $1x10^5$ per ml. 100µl of each concentration was plated in triplicate in 96-U bottomed well plates.

2.4.1.2 Preparation of the responder cells

PBMC from healthy allogeneic donors were separated, as mentioned in Section 2.1.2.2.1, counted and resuspended in SGM as $1x10^6$ per ml. 100µl per well was mixed with Mitomycin-C -treated stimulator cells in three different ratios 1:1, 1: 0.3 and 1:0.1 respectively, in triplicate wells. Unstimulated PBMC and stimulator cells alone were plated in triplicate as background controls in a final volume 200µl.

2.4.1.3 Incubation, harvesting, and counting of MLR

The plates were incubated at 37°C, 5% CO_2 in a humid incubator for 5 days. At the end of the fifth day, and to estimate T cell proliferation by measuring DNA synthesis, cells were pulsed with 1µCi ^{3}H-thymidine (Amersham, UK) per well. The plates were re-incubated for a further 18 hours under the same conditions, then the cells were harvested with a Tomtec automated cell harvester onto a glass fibre filter mat (Wallac,

Turku, Finland), and filter mats were left to dry. The dry mat was sealed in a plastic envelope (Perkin Elmer, UK), and 5ml of scintillation liquid cocktail (Beta plate scint; Perkin Elmer, UK) was added, distributed equally on the mat and the extra liquid was removed. Incorporated radioactivity was counted (as counts per minute, cpm) in a plate scintillation counter (1450 Microbeta plus liquid scintillation counter, Wallac, Finland).

2.4.1.4 Statistical analysis for MLR assays

Results were represented as mean ± SEM of triplicate wells and analysed using paired Student t test, GraphPad Prism 5.5 software (San Diego, CA, USA)

2.4.2 MLR using HLA-class I and/or class II blockers

$CD8^+$ and $CD4^+$ T cells can recognise only peptides presented in the context of HLA class I and HLA class II of the antigen presenting cells respectively. In order to investigate the role of HLA class I and class II in the immune responses directed against the stimulator cells in MLR, the assays were performed as mentioned in Section 2.4.1, except that blocking monoclonal antibodies directed against HLA class I (W6/32) and HLA class II (L243) were added to the stimulator cells and incubated for 45 minutes at 37°C in 5% CO2 before addition of the responder cells, as mentioned before (Walweska *et al*., 2007). The blocking mAbs were used as purified tissue culture supernatant from hybridoma cells in culture, and were used at a final concentration of 0.75µg/ ml. A tissue culture supernatant isotype control IgG was added to the control wells at the same concentration, as a negative control.

2.4.3 MLR in the presence of cytotoxic T lymphocyte antigen-4 fusion protein (CTLA-4 Ig)

CTLA-4 Ig fusion protein is a soluble ligand of the co-stimulatory molecules, CD80 & CD86. By addition of this protein to the stimulator cell lines, it will block the effect

induced by CD80 and CD86 ligation to CD28, and in turn their role in induction of T cell proliferation and differentiation can be investigated.

MLR was set as mentioned before (Section 2.4.1), except the Mitomycin-C treated-stimulator cells were incubated with CTLA-4 Ig at a final concentration of 0.5μg/ ml for 45 minutes at 37°C in 5% CO_2, as mentioned before (Walweska *et al*., 2007), before mixing with the responder cells. The same concentration of isotype matched IgG was added to the control wells, as a negative control.

2.4.4 Phenotyping of the proliferating lymphocytes in MLR

In order to investigate the phenotype of responding T cells, MLR was performed using CFSE-labelled PBMC. The responder T cells then were analysed using flow cytometry before and after incubation with the stimulator cells.

2.4.4.1 Carboxyfluorescein diacetate succinimidyl ester (CFSE)

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a tracking dye used for cell labelling and tracking, and has been suggested for phenotyping the dividing T cells in MLR. The diacetate ester is colourless and has the ability to passively diffuse into the cells and react via its ester groups with the intracellular amines to form dye-protein adducts (which are fluorescent, with excitation and emission peaks of 492 an 517 nm respectively) which are well retained by the cells. Upon cell division, the amount of the label is divided equally between the new daughter cells; hence intensity of the dye can be used as an indicator of the cellular proliferation level.

2.4.4.2 Dye preparation

According to the manufacturer's instruction, 5mM stock solution of CFSE reagent was prepared immediately before use by dissolving the components of one vial, 50μg lyophilized CFDA-SE powder, in 18μl of DMSO (provided in the kit; CellTrace™

CFSE Cell Proliferation Kit, Invitrogen, Paisley, UK). Any remaining reagent was stored in the dark at -20°C.

2.4.4.3 PBMC labelling

Cells were separated as mentioned before (Section 2.1.2.2), and were counted and centrifuged at 1200 rpm for 10 minutes. A pre-warmed sterile solution of 0.1% BSA in PBS was used to resuspend the cells to a final concentration of $1x10^6$ cells/ ml. According to the required final concentration of CFSE, a defined volume of stock solution (5mM) was added to the cell suspension. Pilot experiments showed that 5μM final concentration of CFSE was adequate and non-toxic for PBMC labelling. Consequently, 1μl of CFSE stock solution was added to each 1 ml of cell suspension, and incubated at 37°C in 5% CO_2 for 10 minutes. The excess dye was quenched from the cells by addition of 5 volumes of ice cold RPMI-1640 media, then tubes were incubated on ice for 5 minutes, and cells were pelleted by centrifugation at 1200 rpm for 10 minutes and washed twice with fresh RPMI media. The cells were ready to be used for setting up the MLR or for staining and flow cytometric analysis of CD marker phenotyping.

2.4.4.4 Setting up CFSE labelled-PBMC stimulation reaction

In a 96-well U-bottomed plate, $2x10^5$ (in 100μl SGM) of CFSE-stained PBMC were plated in triplicate and mixed with $6x 10^4$ (in 100μl SGM) of Mitomycin-C treated stimulator cells (HMy2, HxU266, HxKG-1, U266, or KG-1). Phytohaemaglutinin (PHA) was used (50μg/ml final concentration) as a control positive stimulator, and the unstimulated CFSE-stained PBMC as negative controls. The plates were incubated at 37°C and 5% CO_2 for 6 days prior to harvesting for CD marker/ CFSE flow cytometric analysis.

2.4.4.5 Characterization of the proliferating cell populations

Cells were harvested after 6 days, washed with cold PBS and the following mAbs were used for surface IF staining; CD3-PE, CD4-PE or CD8-PE; IgG1-FITC and IgG1-PE (Beckman Coulter, UK); CD45RA-APC, CCR7-PerCP, IgG1-PerCP and IgG2a-APC (R&D systems, UK). 10µl of mAb was added to the cells suspended in a minimum amount of PBS, shaken well and incubated in the dark at 4°C for 20 minutes. Cells were washed twice in WB and fixed as described in Section 2.3.1.1. In order to evaluate the proliferating populations, stained cells were analysed for T cell subpopulations; naive, effector and memory associated markers, in addition to CFSE intensity. Cell acquisition was carried out using a FACSCalibur flow cytometry system and data analysed by CellQuest Pro software (Becton-Dickinson Ltd, UK). Multiple gating was performed on CD3, CD4 or CD8 populations, and then on the naive, central memory and effector memory populations depending on quadrant gating of CCR7 *versus* CD45RA dot plot.

2.5   Enzyme Linked ImmunoSpot (ELISpot) Assays

ELISpot assay is a technique which allows quantification of the functional, responding lymphocytes at a single cell level, by detection of Th1 cytokine release under a given stimulatory conditions. IFN-γ is one of the Th1 cytokines; it is produced mainly by activated T cells, NK cells, and other immune cells, and regulates immune responses by its stimulatory effect on APCs, T cells and B cells. Production of IFN-γ by CD4$^+$ and CD8$^+$ lymphocytes is a hallmark of a Th1 phenotype response, so high levels of IFN-γ production can be used as a marker of immune response directed against intracellular infections or tumour cells.

ELISpot assays were carried out in two different protocols. First, was a short-term assay (48 hours), which investigated the ability of the stimulator cells (Hybrid, HMy2, or

tumour cells or cell lines) to stimulate IFN-γ releasing responses of the responding (mainly memory) T cells. Second, was a long-term (3-4 weeks stimulation plus the experiment duration), which is characterized by more prolonged stimulation of T cells with selected hybrid cell lines to induce and expand primary as well as memory T cell immune responses against the stimulator hybrid cell line's epitopes, and these activated T cells were used as responder cells in IFN-γ releasing ELISpot assays against different stimulator cells or cell lines.

2.5.1   Short-term ELISpot using allogeneic normal donor PBMC

The test was performed using human IFN-γ ELISpot kit (Mabtech AB, Sweden), and according to the manufacturer's instructions. The 96 well Multiscreen PDVF filter plates (Millipore, USA) were activated by addition of 50μl/ well of 70% ethanol, and plates were incubated at RT for 2 minutes. Plates were washed 5 times with 200μl/well sterile distilled water, and wells were coated with the capture antibody (anti-IFN-γ mAb clone 1-D1K 15μg/ml in sterile PBS) by addition of 100μl/ well. The plates were incubated overnight at 4°C before the excess antibody was removed. The plates were washed 5 times by addition of 200μl/ well of sterile PBS, blotted dry, and 200μl/ well of SGM was added to block the non-specific binding sites, and plates were left at RT for not less than 30 minutes before addition of the cell mixture. The responder cells (allogeneic normal PBMC) were washed with RPMI medium, counted, resuspended to the desired concentration in SGM and seeded as 100μl/ well in triplicate wells.

The stimulator cells were harvested, washed in RPMI medium and treated with Mitomycin-C as described in Section 2.4.1.1. Cells were resuspended in SGM at the required concentration and 100μl/ well were mixed with the responder cells in 96-well plates. The ratio of responder to stimulator cells was adjusted to 3:1 in a total volume of 200μl per well. Plates were incubated at 37°C in 5% CO_2 in a humid atmosphere for 48

hours. During the incubation period, T and other cells were induced by the stimulator cells to release IFN-γ and other cytokines. The produced IFN-γ was captured by the coating anti-IFN-γ mAb attached to the plate surface. After incubation, the cell mixture was removed and the plates were washed 5 times with sterile PBS 200 μl/well and blotted dry. A biotinylated anti-IFN-γ mAb (clone 7-B6-1; Mabtech, Sweden), was diluted to 1μg/ ml in sterile PBS containing 0.5% FCS, and 100μl/ well was added, followed by incubation for 2 hours at RT. After incubation, the excess antibody was removed, the plates were washed as in the last step, and Streptavidin conjugated alkaline phosphatase (Streptavidin-ALP; Mabtech, Sweden) was diluted 1:1000 in PBS containing 0.5% FCS and 100μl/ well was added, followed by one hour incubation at RT. The unbound enzyme was removed, plates were washed as described before, and 100μl/ well of filtered substrate solution, 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP/ NBT; Mabtech, Sweden), was added, and plates were incubated at RT for 20-40 minutes, or until distinct purple spots appeared in the positive wells. The reaction was terminated by extensive plate-washing under tap water. The plates were left to dry, and spots were counted under a dissection microscope using x40 magnification lens (Figure 2.4). The number of spots per 10^6 of responder cells was calculated using the following equation:

$$= \frac{1 \times 10e6}{\text{number of responder cells per well}} \times \text{number of emerged spots}$$

2.5.2 Short-term ELISpot assay using autologous patients' PBMC

ELISpot assays were set as mentioned in Section 2.5.1, except that the responder cells were the patient tumour-depleted PBMC (including T cells), which were stimulated using the patient autologous tumour cells, semi autologous hybrid cells, HMy2, and a group of cross matched allogeneic tumour and hybrid cell lines.

Anti-gamma interferon mAb immobilized on the ELISpot plate-well surface

PBMCs and stimulator cells added to the coated wells

Plate was incubated for 48 hours

Activated T cells secrete IFN γ, which is captured by specific coating anti-IFN-γ mAb

Cells were removed by washing

Incubation with biotin-conjugated capture antibody

Excess antibody was removed by washing

Incubation with streptavidin conjugated alkaline phosphatase

Excess was removed by washing, followed by addition of substrate solution BCIP/NBT

Purple spots were emerged, reaction stopped by washing, and plate was dried.

Spots were counted

Anti-IFN γ capture mAb
PBMCs
Stimulator cells
IFN γ

Biotin conjugated IFN γ mAb
Streptavidin conjugated ALP
BCIP/NBT

Figure 2.4: An illustrated overview of steps of IFN γ ELISpot assay (adapted from *Proimmune, UK, ELISpot manual*)

2.5.3   Long-term ELISpot assays

2.5.3.1 Induction of long-term activated T cell cultures

In order to investigate the ability of the hybrid cell lines to induce functional, cytokine releasing, and cytotoxic T cell responses *in vitro,* long term T cell lines were established by repeated stimulation of autologous and allogeneic T cells using selected HMy2, hybrid or parent tumour cell lines.

Responding T cells were generated by co-culturing the tumour-depleted patients' PBMC or allogeneic donors' PBMC with Mitomycin-C treated stimulator cell line, for several consecutive rounds (each of 7 days). In the first round at day 0, up to 30×10^6 PBMC, from either semi-autologous patient or normal healthy allogeneic donors, were mixed with Mitomycin-C treated stimulator cells in 2:1 ratio in 6 well plates as 2-2.5×10^6 responder cells per ml, and in a total volume of 2 ml/ well SGM. Fresh media was added on day 3. In the second round, cells from the first round were harvested, counted and re-stimulated with a new batch of Mitomycin-C treated stimulator cells in a ratio of 3:1, in a total volume of 2 ml/ well of SGM in presence of 50U/ ml rhIL-2 (R&D system, UK) in 6-well plates. Fresh media was added on days 9 and 11. In the third round, on day 14, responder cells were harvested, counted and re-stimulated with the a new batch of Mitomycin-C treated stimulator cells in a ratio of 3:1 in a total volume of 2ml/ well SGM in the presence of 300U/ml rhIL-2 in 6-well plates. Fresh media was added on days 16 and 18. On day 21, cells were harvested as before, and according to the number of the resulting T cells, cells were subjected to further activation rounds, or kept for use in subsequent assays. Further activation rounds were carried out for selected groups of the activated autologous T cell lines for up to 8 rounds, under the same co-culturing conditions, except that IL-2 concentration was increased up to 1000U/ml, and IL-7 (BD Bioscience, UK) was added at a final

concentration of 1ng/ ml of SGM. Parent tumour cells failed to stimulate T cell lines for more than 10 days under the same culturing conditions. The activated allogeneic and autologous cell cultures were used as responder cells in setting of IFN-γ releasing ELISpot assays, and in ^{51}Cr release cytotoxicity assays as well.

2.5.3.2 ELISpot assay using activated cell cultures

The procedure of ELISpot assays was as described in Section 2.5.1, except using the long-term activated T cells as responder cells. Stimulator cells were the same hybrid cell lines used for activation of the responder T cells, relevant tumour cells of, HMy2, and K562 cells as NK target.

2.5.4   Statistical analysis for ELISpot assays

Two healthy allogeneic donors were used whenever possible in short- and long-term ELISpot assays, and each experiment was performed in triplicate wells. Results were presented as number of spots per 1x10^6 responder cells in form of mean ± SEM. Data were analysed using paired Student t test and P value ≤ 0.05 represented a significant difference.

2.6   Induction of tumour antigen-specific CTLs

The hybrid cell lines' ability to induce tumour and/or tumour antigen-specific T cell lines was investigated by long-term activation of allogeneic normal donors- or tumour bearing patients-derived, HLA-A2$^+$, PBMC for several consecutive rounds *in vitro*. The T-cell cultures were re-stimulated with the same hybrid cell line at weekly intervals, in the presence of exogenous rhIL-2, as described in Section 2.5.3.1, for 4-6 weeks using a selected group of hybrid cell lines, and HMy2 cells, depending on their expression of TAAs (Table 2.8). The activated cell cultures were investigated for induction of TAA-

specific T cell clones using pentamer staining (Section 2.7) and cytotoxicity assays (Section 2.8).

Stimulator cell line	Responder PBMC source	Type of the response
HMy2	Healthy donors	Allogeneic
HxU266	Healthy donors	Allogeneic
HxCM053	Healthy donors	Allogeneic
HxRC056	Healthy donors	Allogeneic
HxKG-1	Healthy donors	Allogeneic
HxVP024	Healthy donors/ Patient (VP024)	Allogeneic/ Semi-autologous
HxPG052	Healthy donors	Allogeneic
HxTH044	Healthy donors	Allogeneic
HxDO050	Patient (DO050)	Semi-autologous

Table 2.8: Summary of stimulator cell lines and responder PBMCs used for induction of antigen specific T cell lines

2.7 HLA-A2-peptide pentamer staining

Pro5® pentamers (Proimmune, UK) comprise five MHC-peptide complexes assembled and connected through a coiled-coil domain, and each Pro5® pentamer also comprises up to five fluorescent tags for bright and efficient labelling. Due to their configuration, all five MHC-peptide complexes in the pentamer are available for binding to complementary TCRs (Figure 2.5).
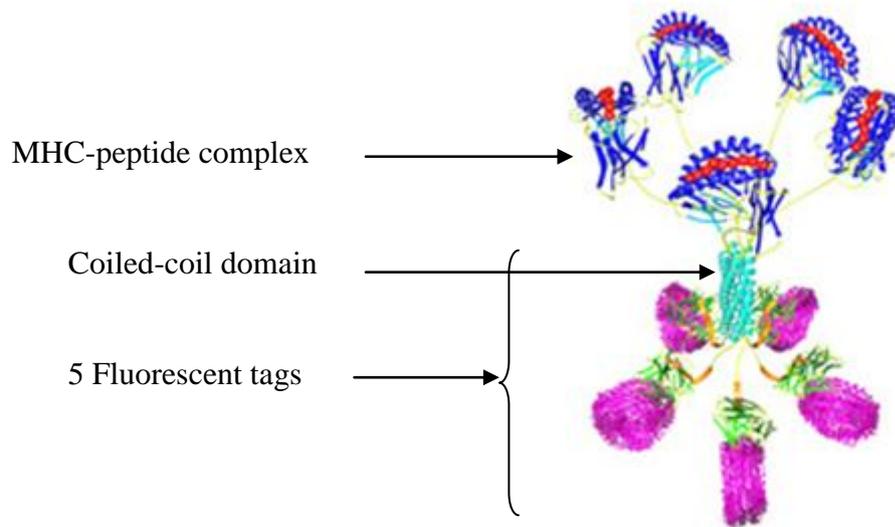
Figure 2.5: Labelled Pro5 MHC Pentamer structure; it consists of a coiled-coil domain connected from one side with five MHC/peptide complexes and from the other side with five fluorescent tags. Figure from (Proimmune pentamer manual).

2.7.1 Pentamer staining and cell analysis

Detection and evaluation of tumour antigen-specific CTLs were performed using R-PE-labelled Pro5 MHC class I pentamers (Proimmune Limited, UK), and FITC-labelled anti-CD8, clone LT8, IF staining followed by flow cytometric analysis of the fixed cells. Five different HLA-A2-restricted tumour-peptide pentamers of the candidate TAAs were selected to investigate the corresponding CTLs clones produced in the long-term activation of HLA-A2^{+} T cells (Table 2.9).

Tumour antigen	HLA-A2-restricted peptide	Peptide sequence	Labelling
Survivin	Survivin (96-104)	LMLGEFLKL	R-PE
MAGE-A1	MAGE-A1 (278-286)	KVLEYVIKV	R-PE
PRAME	PRAME (300-309)	ALYVDSLFFL	R-PE
NY-ESO-1	NY-ESO-1 (157-165)	SLLMWITQV	R-PE
WT-1	WT-1 (235-243)	CMTWNQMNL	R-PE

Table 2.9: list of the HLA-A2-peptide pentamers, showing relevant TAA, peptide sequence, and labelling (Proimmune, UK), which were used for detection of antigen-specific T cell clones.

According to the manufacturer's instructions for staining, T cells were harvested, washed with PBS, and counted and dispensed as $2x10^6$ cells in the last drop of supernatant in FACS tubes. In the first use of the pentamer, vials were spun at 14000x g for 3 minutes at 4°C to remove any protein aggregates, which may cause non-specific binding. 10µl of labelled pentamer (Proimmune Limited, UK) was mixed with the cells, followed by 10 minutes incubation at room temperature in the dark. After incubation, cells were washed once with 2ml cold washing buffer (0.5 % BSA in PBS), pelleted by centrifugation at 1200 rpm for 10 minutes, the supernatant was removed and 2µl of FITC-labelled anti-CD8 mAb, clone LT8 (Proimmune Limited, UK) was added. Cells were mixed and incubated at 4°C, shielded from light for 20 minutes. Cells were then washed twice with the same washing buffer, spun as before, loosened by flicking and either fixed using 200µl of cold fixing buffer (2% Paraformaldehyde in PBS) or left unfixed. Tubes were stored in the dark at 4°C prior to flow cytometric analysis. Cells were acquired on a FACSCalibur flow cytometer and analysed using CellQuest Pro software (Becton Deckinson, UK). At least 500,000 total events were acquired per sample, with appropriate gating on living lymphocytes. In the FACS analysis dot plot, X and Y axes showed CD8-FITC and pentamer-PE respectively. The positive

population for both CD8 and pentamer was reported in the upper right quadrant, and its percentage, derived from the total live lymphocyte population, was calculated from the quadrant statistics. Normal unstimulated PBMC from the same donor were stained with the same pentamers and anti-CD8 mAb under the same conditions, and used as negative controls.

2.7.2   Statistical analysis of pentamer-reactivity versus TAA-expression

Data were analysed for statistical significance of differences by Fisher's exact test, using GraphPad Prism 5.5 software (GraphPad Software, San Diego, CA, USA). A value of $p < 0.05$ was taken as signifying a statistically significant difference.

2.8   Radio-active chromium (^{51}Cr) release cytotoxicity assays

The radioactive chromium release assay was used to investigate the specific cytolytic activity of long-term activated cell cultures. In this assay the effector cells were mixed in 3 different concentrations with a known number of radioactive chromium-labelled target cells in 96 well U-bottom plates and incubated for 4 hours. During the incubation time, CTLs (if present) attacked their targets in a TCR-dependent fashion and responded by releasing cytolytic molecules (e.g. perforin, granzymes and granulysins) which penetrated the target cell membrane and induced apoptosis and cell lysis, with subsequent ^{51}Cr release into the medium. The supernatant was collected and counted for radioactivity counting.

2.8.1   Target cells labelling with [^{51}Cr]-sodium chromate

Target cells were harvested, washed with RPMI media, counted and pelleted. In 2ml screw-caped Eppendorf tubes, $2x10^6$ target cells were suspended in 200ul SGM containing 100μCi or 3.7 MBq, of [^{51}Cr]-sodium chromate (PerkinElmer, UK), and incubated with half-open screw cap at 37°C for an hour. The cells were washed three

times with RPMI media, counted, and seeded in 96-well round bottom plates as 50µl per well, at a concentration 1x 10^5 cell/ ml, in triplicate wells.

2.8.2 Setting up the reaction

Effector cells (activated T lymphocytes) were counted and resuspended in SGM at the required concentration for the assay. Three effector to target cell ratios were used; 3:1 (1.5x10^5 cell/ ml), 10:1 (5x10^5 cell/ ml); 30:1 (1.5x10^6 cell/ ml), or 100:1 (5x10^6 cells/ml) (depending on the number of the available effector cells). Effector cells were added to the target cells (100µl per well) to give a final volume of 150µl per well. The plates were incubated for 4 hours at 37C, 5% CO2, and in a humid atmosphere. Two types of controls were set, in which target cells were seeded in the same number (5x10^3/ well) in a total volume of 150µl; first was the spontaneous release-, and second was the maximum release-control. Target cells were set in maximum as in spontaneous release control wells, except with addition of 20µl of 5% Triton-X (Fisher, UK), to lyse all cells, in a total volume of 150µl SGM. At the end of incubation time, plates were centrifuged to pellet the cells/ debris, and 50µl of supernatant was collected in 6ml Pico polystyrene vials, followed by addition of 3ml liquid scintillation counting cocktail (both from PerkinElmer, USA). Vials were counted in a liquid scintillation counter (2000CA/LL TRI-CARB, Liquid Scintillation Analyzer, Packard, Netherland), and the percentage of specific lysis was calculated as follow:

$$= \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

2.8.3 Tumour antigen-specific cytotoxicity assays

In order to investigate the tumour antigen specificity of the long-term stimulated T cell lines, a group of HLA-A2-restricted tumour antigens, which was highly expressed by

the hybrid cell lines, was selected to check the availability of its cognate-specific CTLs cell line, using peptide-pulsed T2 cells as targets.

2.8.3.1 T2 cell line

T2 cell line is a TAP deficient, HLA-A2$^+$ lymphoblastoid hybrid cell line. It is characterized by its inability to present endogenous peptides in the context of MHC class I molecules. T2 cells were loaded with the exogenous peptide of interest on to their HLA-A2 molecules, to be used as targets for the relevant CTLs.

2.8.3.2 HLA-A2-restricted tumour antigenic-peptide

A selected group of HLA-A2-restricted peptides of tumour antigens of interest, in addition to HLA-A2-restricted irrelevant peptide (*Plasmodium falciparum* CSP antigen 334-342) (all peptides purchased from Proimmune, Oxford, UK) were selected for pulsing of T2 cells (Table 2.10).

Solubility of the used peptide was variable according to the peptides' nature (see Table 2.10). All the vial content was dissolved as described in the manufacturer's instructions and dispensed into small volumes and kept frozen at -80°C prior to use.

Tumour antigen	Peptide sequence	Peptide solubility
NY-ESO-1 (157-165)	SLLMWITQV	1mg was dissolved in 100µl DMSO, and volume topped up to 1ml using nano-pure water
Survivin (96-104)	LMLGEFLKL	1mg was dissolved in 100µl DMSO, and volume topped up to 1ml using nano-pure water
MAGE-A1 (278-286)	KVLEYVIKV	1mg was dissolved in 200µl of 10% glacial acetic acid, volume was topped up to 1ml using nano-pure water
WT-1 (235-243)	CMTWNQMNL	1mg was dissolved in 100µl DMSO, and volume topped up to 1ml using nano-pure water
PRAME (300-309)	ALYVDSLFFL	1mg was dissolved in 100µl of ammonium hydroxide and nano-pure water was added up to 1ml
Plasmodium falciparum CSP (334-342)	YLNKIQNSL	1mg was dissolved in 200µl of 10% glacial acetic acid, and volume was topped up to 1ml using nano-pure water

Table 2.10: Summary of sequence and solubility of TAA-derived and control peptides used for pulsing T2 cells to be used as targets in ^{51}Cr release cytotoxicity assays

2.8.3.3 T2 cell-peptide pulsing

T2 cells were harvested in their log phase of growth, spun at 1480 rpm for 5 minutes, washed in serum free SGM and counted. Cells were suspended in serum free SGM at 3×10^6/ ml. Lyophilised peptides were dissolved as described in Table 2.10, and 2 ml of the cell suspension was pulsed with 100µg (50µg/ ml) of the soluble peptide of interest, and 6µg (3µg/ml) β2-microglobulin (Sigma, UK) in 6-well plates. Control T2 cells were pulsed with 100 µg (50µg/ml) of the control peptide (Table 2.10) plus 3µg/ ml β2-microglobulin (control 1), or with 3µg/ml β2-microglobulin alone (negative control 2). Plates were mixed and incubated for 4 hours at 37°C and 5% CO2 in a humid atmosphere. After incubation, cells were harvested, washed twice in serum free SGM

and labelled with ^{51}Cr as mentioned in Section 2.10.1, for use as targets in cytotoxicity assays.

2.8.4 Statistical analysis for cytotoxicity assays

Data were analysed for statistical significance of differences by ANOVA or Fisher's exact tests as indicated, using GraphPad Prism 5.5 software (GraphPad Software, San Diego, CA, USA). A value of $p < 0.05$ was taken as signifying a statistically significant difference.

Chapter 3

Phenotypic characterization of hybrids and their parent tumour cells/cell lines

3 Phenotypic characterization of hybrids and their parent tumour cells/cell lines

3.1 Introduction

This Chapter discusses the phenotypic and antigenic characterization of a panel of hybrid cell lines, made prior to this study, by fusion of EBV B-LCL (HMy2) and a group of haematological tumour cells or cell lines. It describes the phenotypic characterization of cell surface markers and HLA class I and class II expression by the hybrid, HMy2, and parent tumour cells/cell lines where available, and the qualitative and semi-quantitative estimation of expression of TAAs known to be expressed by haematological and other tumour types.

3.1.1 Hybrid cell generation

Various techniques have been used for hybrid cell production, including virus mediated cell fusion (White *et al.*, 1981), chemical fusion e.g. fusion using PEG (Davidson *et al.*, 1976), or physical fusion e.g. electrofusion (Trevor *et al.*, 2004). The hybrid cell lines used in this study were generated by fusion process using PEG, followed by chemical selection of the true hybrids' growth, as described previously (Dunnion *et al.*, 1999; Cywinski *et al.*, 2006; Walewska *et al.*, 2007).

3.1.1.1 Chemical fusion using polyethylene glycol (PEG)

Fusion using PEG offers several advantages over the other fusion methods. It is inexpensive, relatively quick and simple to use, and can be used to fuse different types of cells or cell lines. This method was used successfully in the fusion of EBV B-LCL with different haematological and solid tumour cell lines (Dunnion *et al.*, 1999, Cywinski *et al.*, 2006, Walewska *et al.*, 2007, Kerr *et al.*, 1992), including the hybrid cell lines used in this project. The fusion mechanism involves cell aggregations, which

is followed by disturbance of the phospholipid membrane leading to formation of pre-fusion sites. Expansion of the pre-fusion sites following the sudden dilution of PEG, allows for complete membrane fusion of adjacent cells (Lentz & Lee, 1999). Different factors, such as PEG concentration, pH, serum content, cell exposure time, cell size and origin, and ratio of parent cells all may affect the fusion efficiency. On the other hand, cytotoxicity of PEG for certain mammalian cell lines and low fusion rate are limiting factors for this method (Davidson *et al.*, 1976, Lentz & Lee, 1999). Estimated fusion efficiency has been reported to be at the highest level in mammalian cells at PEG 50%, at 37°C, and for up to 60 seconds followed by gradual dilution with growth medium (Davidson *et al.*, 1976), conditions which were followed during production of hybrids of this project.

3.1.1.2 HMy2 x tumour cell hybrid production and selection

Parent HMy2 and tumour cells were fused using PEG/DMSO of molecular weight 1500 (Sigma-Aldrich, UK), diluted to 50% (w/v) using serum free medium and at 37°C. The fusion procedure has been described before (Dunnion *et al.*, 1999, Cywinski *et al.*, 2006, Kerr *et al.*, 1992). Briefly, the APC parent cell line (EBV B-LCL; HMy2) and a panel of haematologic and lymphoid *ex vivo* tumour cells/or cell lines (Table 2.4), were prepared for the fusion process. A mixture of $1x10^7$ cells of each parent cell/ cell line was washed in serum free RPMI 1640 medium and was pelleted. One ml of pre-warmed PEG 50% solution was added over one minute to the cell pellet with continuous swirling, the mixture was diluted using 10 ml of pre-warmed serum-free medium over 10 minutes, and the cells were pelleted and resuspended in SGM, and flasks were incubated for 24 hours before culturing in the double chemical selective media.

As the hybridization between two different cell types is a random process, there were three possibilities for the fusion end products.

1- Un-fused cells, which may be HMy2 or parent tumour

2- Heterokaryons (hybrid of HMy2/tumour cell), which are the aim of the fusion

3- Homokaryons (hybrid of homo cells), either HMy2/HMy2 or tumour cell/tumour cell hybrids

In order to select the heterokaryons growth, and to eliminate un-fused cells and homokaryons, the cell mixture was transferred after 24 hours into selective SGM containing 2% HAT and an optimum concentration of ouabain to allow the growth of heterokaryons and to inhibit both of homokaryons and un-fused parent cells. HMy2 cell line has a dominant ouabain resistance and a recessive sensitivity for 2% HAT-containing growth medium (Edwards *et al.*, 1982), while the parent tumour cells are able to grow in HAT but are sensitive to ouabain. Preliminary viability tests were performed in order to determine the optimum concentration of HAT and ouabain required to select the growth of hybrid cells and inhibit the growth of un-fused parent cells.

The hybrid cell lines used in this study had all been generated prior to my PhD project by D. Dunnion, I. Teobald, and R. Walewska.

3.1.2 Phenotyping of EBV B-LCL x tumour cell hybrids

3.1.2.1 *Functional importance of co-stimulatory receptors, and HLA class I and class II antigen expression*

Co-stimulatory molecules (CD80, CD86, and CD40) are the main inducers of the second signal required, in association with antigen-HLA/ TCR engagement, for induction of primary T cell immune responses. Other ligand/ receptor pairs are involved in maintenance of effective $CD8^+$ and $CD4^+$ immune responses, and include ICOS/ICOSL, CD40/CD40L, 4-IBB/4IBBL, CD27/CD70, and CD30/CD30L (Croft,

2003). CD8^{+} and CD4^{+} T cells recognise antigens presented in the context of HLA class I and class II respectively. Although CD8^{+} T cells represent the main cellular effectors in tumour cell elimination by direct cellular cytotoxicity, CD4^{+} T cells have an essential role in providing direct cell to cell, and cytokine-mediated help for both cellular (via Th1 cells) and humoral (via Th2 cells) immune responses. So, in addition to co-stimulatory molecules, the expression of both of HLA class I and class II is of crucial importance for optimum cellular immune stimulation.

3.1.2.2 Role of HLA-A2 in tumour immunotherapy

HLA-A2 is an allelic subtype of HLA class I. It is characterised by widespread distribution in multiple ethnic groups. HLA-A2 plays an important role in restriction of CTL to tumour antigens. Its role in tumour immunosurveillance was revealed from the recurrent loss or down regulation as a result of tumour escape of T cell recognition (Komlos *et al.*, 1995). Moreover, the ability of HLA-A2 to present endogenously and exogenously processed antigens to CTL was reported *in vitro* (Harao *et al.*, 2008), and in transgenic animals studies (Kaplan *et al.*, 2005). Furthermore, immunogenic peptides derived from several TAAs have been shown to be presented in its context.

3.1.2.3 Tumour associated antigens (TAAs)

TAAs are proteins expressed by tumour cells that can be recognised by the immune system as "foreign". It has been documented that T lymphocytes can specifically recognise and eliminate tumour cells, which is the principle of antigen specific tumour immunotherapy. Most tumour cells process the relevant tumour antigens, and present them in the context of an appropriate HLA class I complex, which in turn can be recognised by tumour-specific CTL followed by tumour cell lysis, in the presence of Th1 helper T cells. The discovery of MAGE antigen expression by melanoma cells, in 1991, increased the promise of using TAA as targets in immunotherapy. As the TAA

role in tumourigenesis and their pattern of expression in different tumours are variable, they showed variable success as immunotherapeutic targets. The ideal TAA candidates should be of crucial importance in tumour growth and progression, containing highly immunogenic epitopes presented by a range of different HLA types, and distributed in a wide range of tumour types (Linley *et al.*, 2011).

3.1.3 EBV B-LCL/tumour cell hybrids, the importance of phenotypic characterization

Previous studies have reported the potential of fusion of EBV B-LCL with lymphoid and non-lymphoid human cell lines (Kerr *et al.*, 1992, Contreras-Brodin *et al.*, 1991). Moreover, several studies showed that lymphoid derived tumour x EBV B-LCL hybrids consistently retained EBV-gene expression, and displayed typical cell surface markers and molecules associated with activated B cell phenotypes (Kerr *et al.*, 1992, Cywinski *et al.*, 2006).

In a study carried out by Dunnion *et al*. (Dunnion *et al.*, 1999), HMy2 cells were fused with a group of haematological tumour cell lines, including promyelocytic (HL-60), T cell-derived (CEM), and erythroleukaemia (K562) cell lines. The produced hybrid cells were stable and grew continuously as suspension cells in culture. The hybrid cell lines had phenotypic characteristics of professional APCs, such as constitutive expression of HLA class I and class II molecules, T cell co-stimulatory ligand molecules (CD80, CD86, and CD40), and further adhesion and accessory molecules, such as ICAM-I. In contrast, fusion of HMy2 with the melanoma cell line, 518.A2, produced a hybrid cell line with typical parent tumour cell phenotype, which grew in adherent cell culture and lacked HLA class II and co-stimulatory molecules expression. Extending these studies, Cywinski *et al* (Cywinski *et al.*, 2006) showed that hybrids made with EBV B-LCL and tumours of haematopoietic origin grew in suspension in tissue culture, with stable

phenotype characterised by expression of HLA class I and class II, co-stimulatory and adhesion molecules. However, hybrids of EBV B-LCL x solid tumour cell lines, such as melanoma and cervical tumours, grew as adherent cells and lacked the expression (like their parent tumours) of HLA class II and T cell co-stimulatory molecules.

From Dunnion and Cywinski's studies, it can be concluded that fusion of HMy2 with haematologic or solid tumour cells produced hybrid cell lines with the ability to grow continuously and to express relevant tumour antigens. However, HLA class II and T cell co-stimulatory molecule expression were retained only by hybrids of haematopoietic origin. In a separate study, Walewska et al. (Walewska et al., 2007) generated a group of myeloma hybrid cell lines by fusion of HMy2 with malignant plasma cells from five multiple myeloma patients. The hybrid cell lines grew stably in tissue culture, but only 4/5 hybrids were successfully recovered after liquid nitrogen freezing-thawing. The heterogenic hybrid nature of the surviving four hybrid cell lines was confirmed by cytogenetic and microsatellite finger print analysis, in comparison with HMy2 and parent tumour cells. These studies highlighted the importance of characterising the hybrid cells *in vitro* as a first step in their evaluation as potential tumour immunotherapeutic agents.

In this Chapter, I shall show the phenotype and antigen expression profile of the hybrid cell lines used in this study, HMy2, and the available parent tumour cells, which can be summarised in the following points:

1- The cell surface CD marker expression, including CD80, CD86, and CD40
2- HLA expression, including HLA class I, class II and the allelic type HLA-A2

3- TAA expression profile, including MAGE-A1, NY-ESO-1, WT-1, PRAME, Survivin, MAGE-A3, MUC-1, and h-TERT, in addition to beta actin (housekeeping gene), using RT-PCR.

4- Semi-quantitative expression levels of these TAAs, using qRT-PCR

3.2 Results

3.2.1 Stability and growth maintenance of hybrid cell lines

Fourteen hybrid cell lines were studied as described in Section 3.1.1, generated by fusion of HMy2 as APC partner with *ex vivo* tumour cells of different haematological malignancy including acute myeloblastic leukaemia (two hybrids), acute lymphoblastic leukaemia (one hybrid), chronic lymphocytic leukaemia (four hybrids), follicular cell lymphoma (one hybrid), mantle cell lymphoma (two hybrids), and multiple myeloma (four hybrids) (Table 2.4). In addition, other two hybrid cell lines were generated by fusion of HMy2 with established tumour cell lines, KG-1 (acute myeloblastic leukaemia) (Koeffler & Golde, 1978), and U266 (multiple myeloma) (Nilsson *et al.*, 1970) (Table 2.1). Four of these hybrids (HxRC056, HxCM053, HxKS037, and HxIC038) have been investigated previously by the group, but not for induction of antigen-specific T cells; in addition, karyotyping was used to prove their hybrid nature (Walweska *et al.*, 2007). The remaining 12 hybrid cells are novel and have not been investigated before.

All of the hybrid cell lines grew continuously in tissue culture as suspension cells in the form of single cells or small clumps. They required passage in fresh media by 1:4 every 2-3 days, and have been frozen in liquid nitrogen (suspended in FM) for long periods, with successful growth re-establishment under tissue culture conditions (data not shown).

3.2.2 Immunofluorescence staining and flow cytometry phenotyping

Immunophenotyping of cell surface marker and HLA expression by the hybrid cell lines, HMy2, and parent tumours (where available) was carried out by direct IF staining using specific fluorescently-labelled mouse anti-human mAbs followed by flow cytometric analysis using a FACSCalibur flow cytometer (Becton Dickinson, UK) . Non specific isotype matched mAbs were used as negative controls. Data were analysed using CellQuest Pro software (Becton Dickinson, UK). All IF staining and flow cytometric analysis of CD marker and HLA expression was carried out at least two times for each hybrid cell line, with a time interval of two months or more, with consistent results.

HMy2 cells expressed high levels of CD80, CD86, CD40, HLA class I and HLA class II molecules, typical of an EBV B-LCL phenotype (Edwards *et al.*, 1982). HMy2 also expressed high levels of HLA-A2 (Figure 3.1). This professional APC-phenotype makes HMy2 an ideal fusion partner for generation of self-replicating, CD8^{+} and CD4^{+}-reactive, and multi-epitopic cellular hybrid vaccines.

A group of hybrid cell lines was analysed against their parent tumour cells for expression of CD80, CD86, CD40, HLA class I, HLA class II and HLA-A2, and data are shown in Figure 3.1. The hybrid cell lines of the study were all shown to express co-stimulatory molecules (CD80, CD86, and CD40), HLA class I and HLA class II, and HLA-A2. The expression of these markers by the hybrid cell lines was consistent with HMy2 expression, but showed some variability in levels of expression and was independent of parent tumour cells' expression of these molecules (Table 3.1 and Figure 3.1), HMy2 appears in black histograms, parent tumour and hybrid cells appear in red and blue histograms.

Due to a shortage of patients' *ex vivo* tumour cells, it was not possible to investigate the expression of HLA and co-stimulatory molecules of all the parent tumour cells. Table 3.1 shows the mean fluorescence intensity (ΔMFI) of the surface markers analysed for each of the hybrid cell lines and for HMy2 cells.

The multiple myeloma hybrid cell line, HxU266, compared with its relevant parent tumour cells, showed up regulated levels of CD80, CD86, CD40, HLA class I, and HLA class II. Although lower than the parent HMy2 cell line, HxU266 levels of CD marker and HLA expression were elevated when compared with its parent tumour cell line, U266, which showed very low expression levels of all co-stimulatory markers and HLA class II, and relatively high level of HLA class I, especially HLA-A2 allele (Figure 3.1). The phenotype of U266 is consistent with its cell lineage as mature plasma cells. The other MM hybrid cell lines, HxCM053, HxRC056, HxIC038, and HxKS037 showed moderate levels of CD80, high-moderate levels of CD86, and high level of CD40 expression. In addition, MM hybrid cell lines demonstrated moderate to high levels of HLA class I, including HLA-A2 allele, while HLA class II was expressed, but at lower levels than by parent HMy2 cell line.

CLL hybrid cell lines, HxAW072, HxJR004, HxLC069, and HxSG059, shared low to moderate expression level of CD80 (compared with HMy2), however these levels were up regulated when compared with the relevant parent tumour cells, such as AW072 and JR004, both of which lacked expression of CD80 (Figure 3.1). However, CD86 expression by CLL hybrid cell lines showed moderate levels in HxAW072, HxJR004, and HxLC069, while high CD86 expression level was seen in HxSG059 hybrid cell line. In contrast, all of the CLL hybrid cell lines (and their relevant parent tumours where available) showed moderate to high expression levels of CD40, except HxLC069 hybrid cell line. Moreover, CLL hybrid cell lines showed moderate to high expression

levels of HLA class II (except HxLC069, which showed relatively low level). HLA class II also was expressed by relevant parent B-CLL tumour cells (AW072 and JR004). The expression of CD86, CD40, and HLA class II, as characteristic molecules for APC phenotype, by parent CLL tumour cells was expected, as they represent mature B cell lineage. High levels of HLA class I, and its allele HLA-A2, were seen in all CLL hybrid cell lines (and their available parent tumour cells), (Figure 3.1).

AML hybrid cell lines, HxKG-1, HxDO050, and HxVP024, showed high expression levels of co-stimulatory markers (CD80, CD86 and CD40), HLA class I, HLA class II and HLA-A2 molecules. HxKG-1 hybrid cell line showed high levels of expression of all these molecules, whilst, parent KG-1 cells lacked expression of CD80, CD40, and HLA-A2, and only weakly expressed CD86 (Figure 3.1).

In the case of MCL hybrids, HxTH044 and HxFC029, co-stimulatory molecules CD80 and CD86, in addition to HLA class II, and HLA-A2 antigens were relatively highly expressed compared with relevant parent tumour cells, whilst CD40 and HLA class I were expressed at similar levels in the parent tumour cells and hybrid cell lines (Figure 3.1). FCL hybrid, HxRS048, showed moderate to high levels of expression of CD86, CD40, HLA class I, HLA class II, and HLA-A2, but moderate level of CD80 expression. ALL hybrid cell line, HxPG052, showed relatively high expression of CD86, CD40, HLA class I and class II, but only moderate expression of CD80 and HLA-A2. Parent ALL tumour cells, PG052, were not available for comparison with the relevant hybrid cell line.

This phenotypic characterization data of the hybrid cell lines were consistent with previous work done on hybrid cell lines made by fusion of HMy2 with different haematological tumour cells and cell lines (Dunnion *et al.*, 1999, Walewska *et al.*, 2007,

_____Phenotypic characterization of the hybrids and their parent tumour cells

Cywinski *et al.*, 2006), where the hybrids were shown to express CD80, CD86 and both

HLA class I and class II, even when the parent tumour cells lacked expression of one or

more of these molecules.
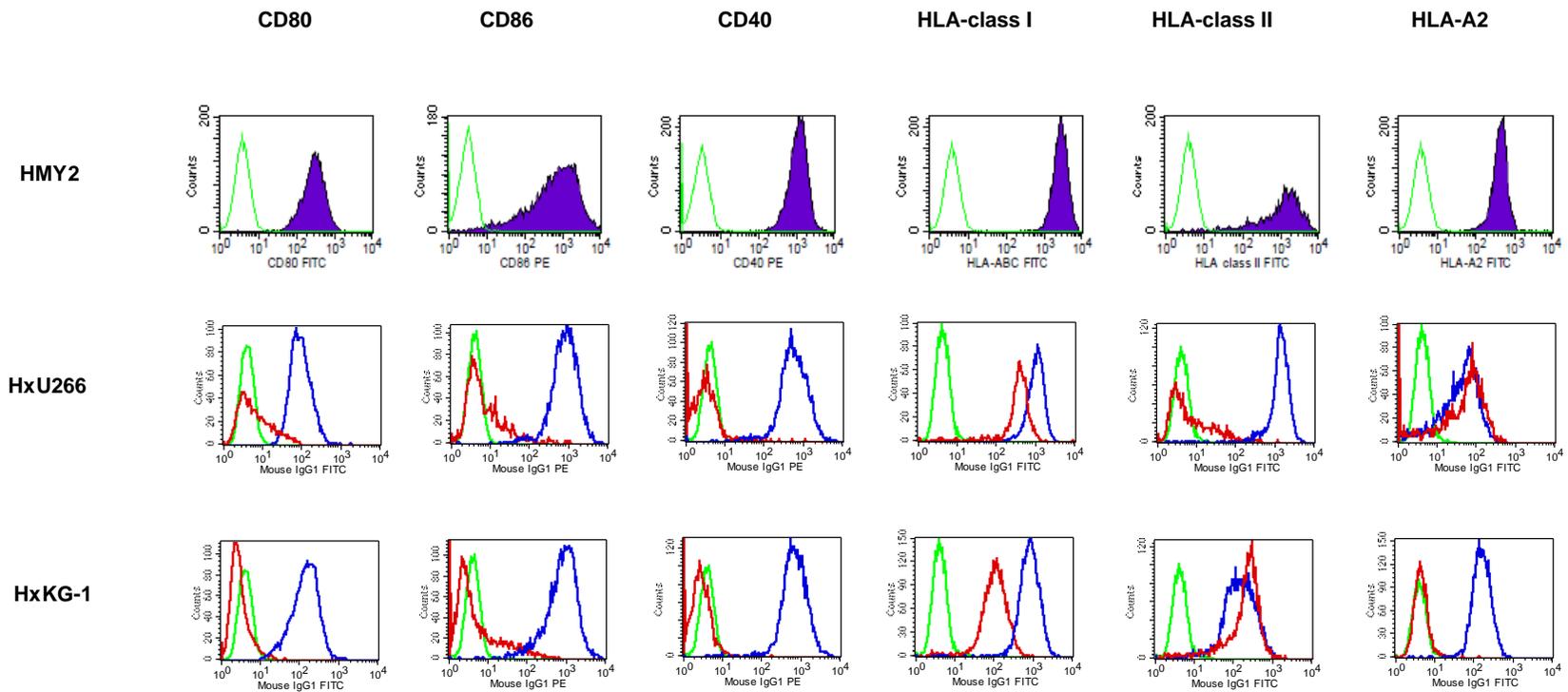
Figure is continued overleaf
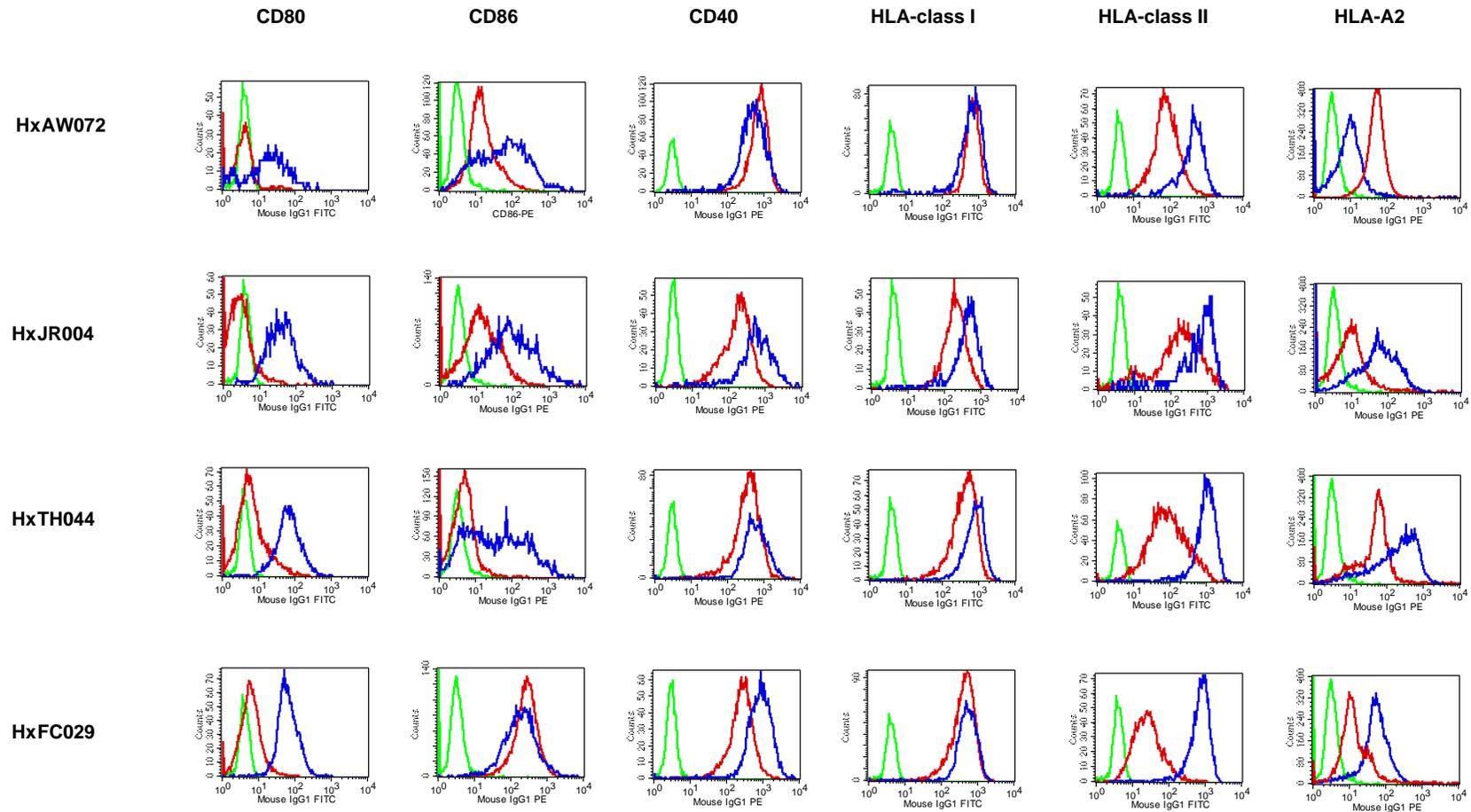
Figure 3.1: Flow cytometric histogram plots of HMy2, hybrids cell lines and parent tumours of U266, KG-1, AW072, JR004, TH044 and FC029; the plots represent level of expression of CD80, CD86, CD40, HLA class I, class II and HLA-A2. Green histograms represent isotype mAb stained cells, red and blue histograms represent parent tumour, and hybrid respectively, HMy2 represented by closed blue   histograms.   .

Tumour cell type	Cell line	CD80	CD86	CD40	HLA I	HLA II	HLA-A2
	HMy2	298	991	1108	2571	1537	448
MM	HxU266	100.25	862.45	613.23	914.88	1509.66	416.98
	HxCM053	32.06	130.9	554.04	244.99	100.3	117.65
	HxIC038	47.22	68.9	787.72	555.53	176.58	164.09
	HxKS037	42.48	92.81	1029.04	709.87	188.09	164.46
	HxRC056	15.31	359.15	326.78	346.48	278.3	258.07
CLL	HxAW072	64.69	86.92	458.29	551.66	202.66	234.73
	HxJR-004	25.93	100.24	484.82	320.92	502.77	78.15
	HxLC069	41.32	63.17	101.93	684.42	49.48	695.81
	HxSG059	34.71	310.33	717.21	338.55	490.41	175.69
MCL	HxTH044	62.63	92.09	589.19	715.62	780.54	388.46
	HxFC029	64.31	232.07	889.4	533.29	627.1	58.24
AML	HxKG-1	143.52	827.38	719.75	766.81	1050.5	140.95
	HxVP024	123.4	961.38	906.74	759.23	708.94	121.99
	HxDO050	92.16	630.92	751.47	765.68	330.79	304.33
ALL	HxPG052	66.22	605.21	673.52	626.7	574.59	86.1
FCL	HxRS048	45.04	279.42	548.4	447.92	487	138.19

Table 3.1: Summary of ΔMFI of CD80, CD86, CD40, HLA class I, class II and HLA-A2 expression by HMy2 and hybrid cell lines. Values represent (MFI of tested marker expression - MFI of matched isotype control).

3.2.3 Tumour antigen expression profile of HMy2 and hybrid cell lines using

RT-PCR

To evaluate the expression of a range of tumour associated antigens by HMy2 and the

hybrid cell lines, reverse transcription-PCR was performed on total RNA extracted from

the cells, and reverse transcribed into single stranded complementary DNA (cDNA) (Section 2.5.2). The cDNA synthesised from each cell line was used as a template in thermal cycler reactions to multiply the transcribed genes as mentioned in Section 2.5.2.6. I did not study antigen expression in parent tumour cells, as there were insufficient tumour cells available for most of the hybrid cell lines.

Complementary DNA from normal PBMC was used as a negative control in the PCR, and cDNA from known positive tumour cell lines as a positive control for each antigen. Eight different primer-pairs representing eight members of TAAs known to be expressed by haematological and/or solid tumours were used. All of the candidates TAA have been identified as potential targets for tumour specific CTL responses. Primer pairs specific for MAGE-A1, NY-ESO-1, WT-1, PRAME, Survivin, MAGE-A3, MUC-1 and h-TERT were used for PCR runs, under appropriate conditions for each primer set as described in Table 2.6. RT-PCR experiments were carried out at least two times for each cell line with time interval of 2 months or more using new RNA extract, with consistent results.

Results of RT-PCR are shown in Figure 3.2, HMy2 and all of the hybrid cell lines expressed Survivin, MAGE-A3, MUC-1, and h-TERT. HMy2 lacked expression of MAGE-A1, WT-1 and PRAME antigens, while it weakly expressed NY-ESO-1. High level of MAGE-A1 was expressed by HxAW072, HxTH044, HxPG052, HxU266, HxCM053, HxRC056, and HxKG-1 hybrid cells. In addition, HxJR004, HxSG059, HxFC029, HxIC038, HxDO050, and HxVP024 hybrid cells weakly expressed MAGE-A1, whilst HxLC069, HxRS048, and HxKS037 hybrid cells did not express it at all. NY-ESO-1 was expressed by HxAW072, HxTH044, HxPG052, HxU266, and

HxCM053 cell lines, and weakly expressed by HxKG-1, HxDO050, and HxVP024 hybrid cell lines. HxLC069, HxJR004, HxSG059, HxRS048, HxFC029, HxKS037, HxIC038, and HxRC056 hybrids were negative for NY-ESO-1 expression. WT-1 antigen was expressed only by HxKG-1 and HxVP024 cell lines, and was not expressed in all other hybrids. PRAME antigen was strongly expressed in HxPG052, HxU266, HxRC056, HxKG-1, HxDO050, and HxVP024 hybrid cell lines, and less strongly expressed in HxJR004, HxSG059, HxAW072, and HxTH044 hybrids. In contrast, PRAME was not expressed by HxLC069, HxRS048, HxFC029, HxCM053, HxKS037, and HxIC038.   Normal PBMC lacked expression for all the TAAs, but was positive for beta actin (Figure 3.2).
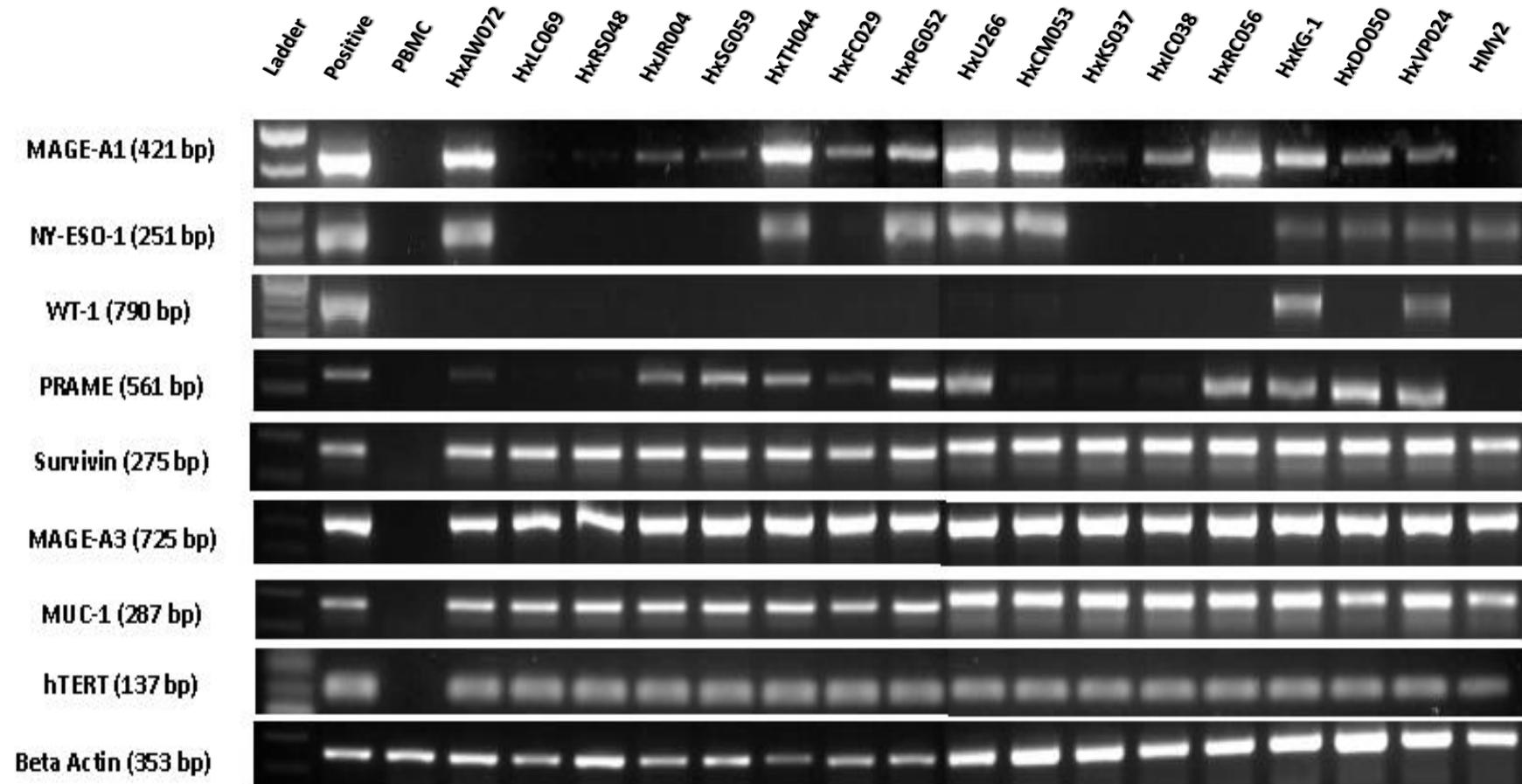
Figure 3.2: RT-PCR-product bands representing TAAs and housekeeping gene (β-actin) expression by HMy2 and all hybrid cell lines. First lane: is for 100 bp ladder. Each subsequent lane represents a cell line, as indicated by the legends. Tumour antigen and relevant amplicon sizes are indicated also in the legends. Positive control for each antigen was an appropriate positive cell line, and negative control was the normal PBMC   .

3.2.4 Semi-quantitative estimation of TAA expression

After detection of TAA-expression using RT-PCR, I wanted to evaluate the level of TAA- mRNA in each cell line using a qRT-PCR. The importance of this experiment was to confirm the RT-PCR results, and to identify the hybrid cell members with higher expression levels of certain TAA of interest, to be used later in the induction of tumour-antigen specific T cell responses. Also, quantitative evaluation of these TAAs-mRNA levels in normal PBMCs (as normal somatic cells) may indicate a limitation of using certain candidate TAA in immunotherapy to avoid development of autoimmune diseases.

Relative mRNA expression of certain tumour antigens of interest was evaluated in normal PBMCs, HMy2, and all hybrid cell lines using Light Cycler PCR machine (Roche Diagnostic, Mannheim, Germany) in the presence of SYBR Green-containing PCR master mix, and using specific primer sets for the antigens of interest (Table 2.7). The qRT-PCR running conditions were adjusted individually, according to the melting temperature of the primer pairs and to the reagents-manufacturer's instructions (Table 2.7). Each experiment was repeated three times under fixed settings and running conditions, and relative mRNA concentrations from each run were normalized against the mean values of triplicate PCR runs for beta actin expression levels. Results were plotted for each cell line as mean of triplicate ± SEM, and data were analysed using GraphPad Prism 5.5 (CA, USA).

qRT-PCR data for HMy2 and the hybrid cell lines are illustrated in Figure 3.3, and for easy interpretation of RT-PCR and qRT-PCR data, summary Table was made to show

positive, negative, and weak expression of different TAAs by RT-PCR, compared to qRT-PCR for each hybrid cell line (Table 3.2).

Relative mRNA expression values for NY-ESO-1 antigen showed highest level of expression by HxU266, followed by HxAW072, HxCM053, HxPG052, and HxTH044 respectively. This order of expression was consistent with the RT-PCR bands shown in Figure 3.2. HMy2, HxRC056, HxKG-1, HxDO050, and HxVP024 cells expressed NY-ESO-1, but in lower levels, which were also consistent with data in Figure 3.2, except for HxRC056 and HxKS037, which showed weak expression by qRT-PCR but were negative by RT-PCR. In contrast, HxLC069, HxJR004, HxSG059, HxRS048, HxFC029, and HxIC038 hybrid cell lines showed no NY-ESO-1, as revealed by semi-quantitative PCR (Figure 3.3A and Table 3.2); cut off values of an arbitrary unit were set for positive NY-ESO-1 expression as $>2.5 \times 10^{-3}$, and for negative expression as $<1 \times 10^{-3}$. Data of semi-quantitative NY-ESO-1 expression by hybrid cells and HMy2 cell line were consistent with that of conventional RT-PCR, except for HxRC056 and HxKS037 cell lines.

MAGE-A1 was highly expressed by HxRC056, HxU266, HxCM053, HxAW072, HxKG-1, HxPG052, and HxTH044. Whilst, HxFC029, HxIC038, HxDO050, and HxVP024 hybrid cell lines showed weak expression. HMy2, HxLC069, HxRS048, HxJR004, and HxSG059 cell lines were negative for MAGE-A1 expression, (Figure 3.3 B, and Table 3.2). The cut off values for MAGE-A1 positive and negative expression were set as $>0.5 \times 10^{-2}$, and $<0.2 \times 10^{-2}$ respectively. All cell lines showed consistent results with RT-PCR bands, except for HxJR004, and HxSG059 cell lines.

HxKG-1 hybrid cell line expressed the highest amount of WT-1 mRNA followed by HxVP024 (although in low level) (Figure 3C and Table 3.2). The rest of the hybrid cell lines, and HMy2 were negative or showed negligible values of WT-1 mRNA. The cut off values for WT-1 positive and negative expression were set as $>1\times10^{-1}$ and $<0.7\times10^{-1}$ arbitrary unit. By these criteria, all cell lines showed consistent results with that from RT-PCR.

PRAME antigen expression showed cut off values of $>25\times10^{-1}$ and $<18\times10^{-1}$ for positive and negative expression respectively. Accordingly, HxJR004, HxSG059, HxTH044, HxPG052, HxU266, HxRC056, HxDO050, and HxVP024 cell lines were positive. HxAW072 and HxKG-1 cells weakly expressed PRAME. However, HMy2, HxLC069, HxRS048, HxFC029, HxCM053, HxKS037, and HxIC038 cell lines were negative. All semi-quantitative PCR results of PRAME expression were consistent with RT-PCR results.

MUC-1, MAGE-A3, h-TERT, and Survivin antigens semi-quantitative expression results were consistent with RT-PCR, and all hybrid and HMy2 cell lines were positive for these TAAs (Figure 3.3, and Table 3.2). Normal PBMCs were almost zero in all the investigated TAAs expression, a result which is consistent with RT-PCR bands.

Overall, thirteen out of 16 hybrid cell lines expressed at least one TAA that was not expressed by HMy2 cells, whilst nine of the hybrid cell lines expressed at least two TAAs that were not expressed by HMy2 cells (such as MAGE-A1, WT-1, and PRAME). On the other hand, three hybrid cell lines, HxLC069, HxRS048, and HxKS037, did not express any TAAs that were not also expressed by HMy2 cells. Accordingly, differential TAA expression by hybrid versus HMy2 cell lines can be

considered as an indicator of the hybrid nature of these cell lines. In certain cell lines (thirteen out of sixteen), expression of at least one TAA that was not expressed by HMy2 is a confirmation of the hybrid nature, and that the antigen has been derived from the parent tumour cell rather than from HMy2. These results, in addition to double chemical selection of the generated hybrid cell lines were regarded as evidence of the heterokaryotic nature of these hybrid cell lines, however further karyotyping of the other three hybrids is essential to confirm their hybrid nature.

NY-ESO-1

A

MAGE-A1

B

WT-1

PRAME

MUC-1

MAGE-A3

hTERT

G

Survivin
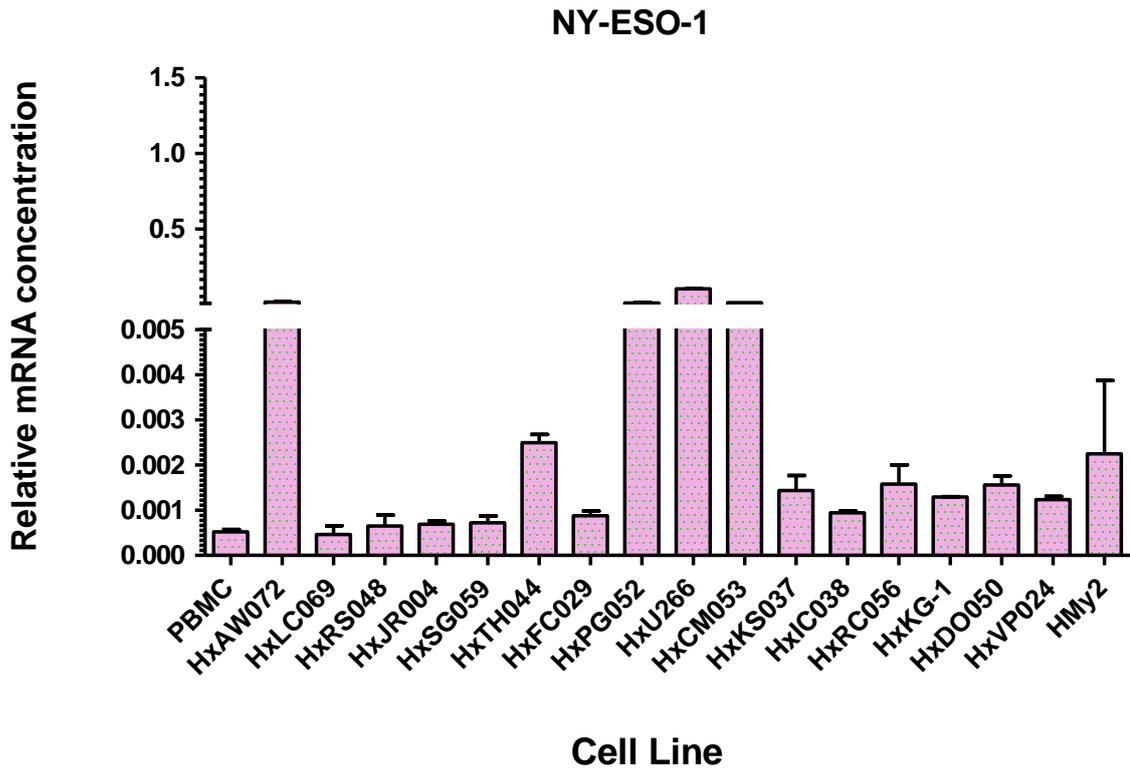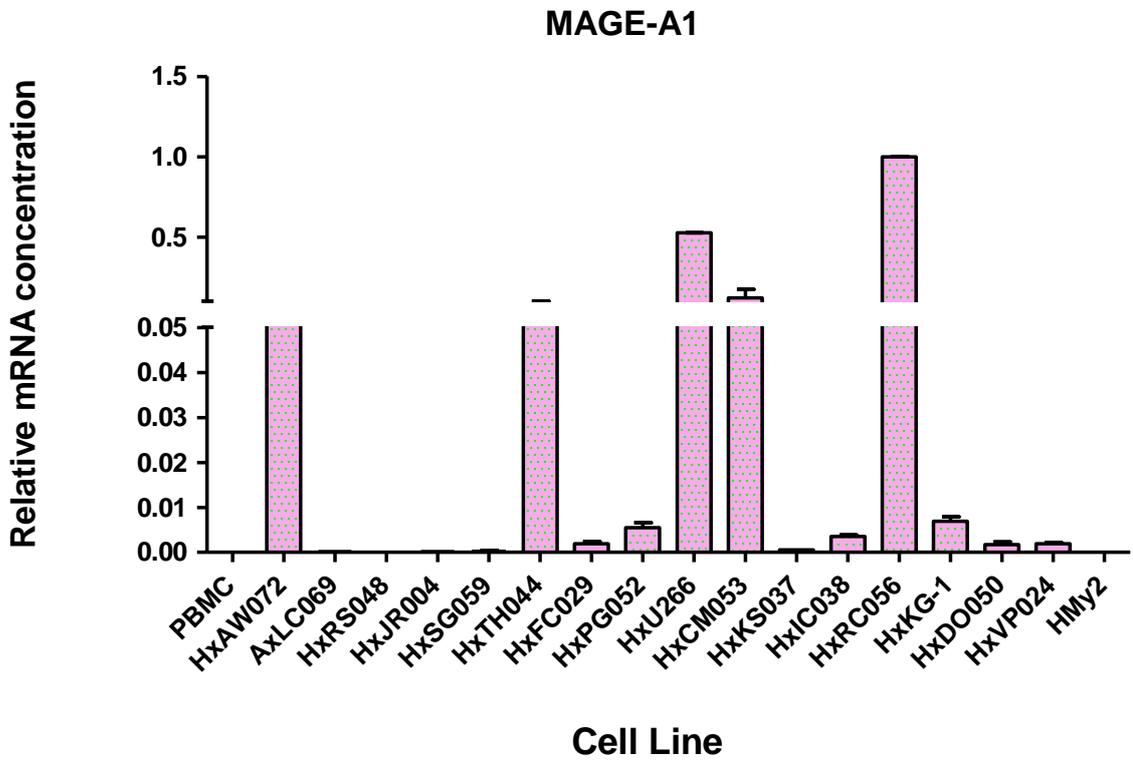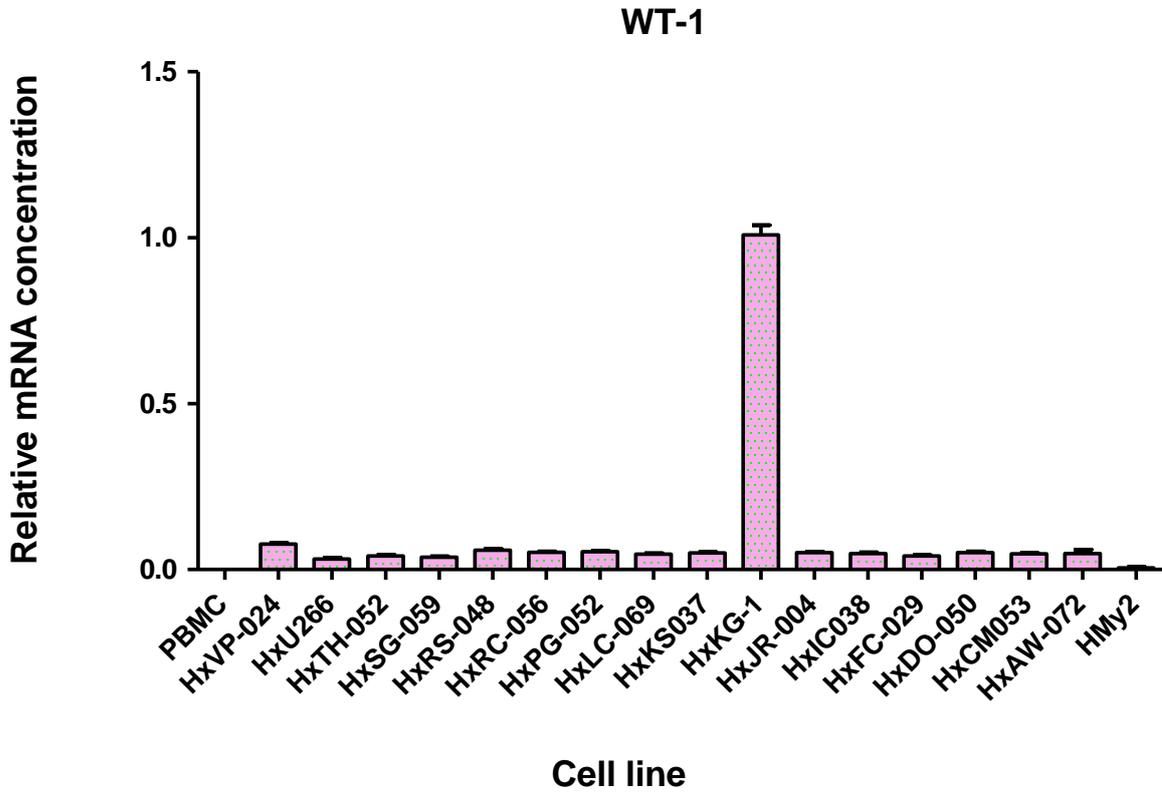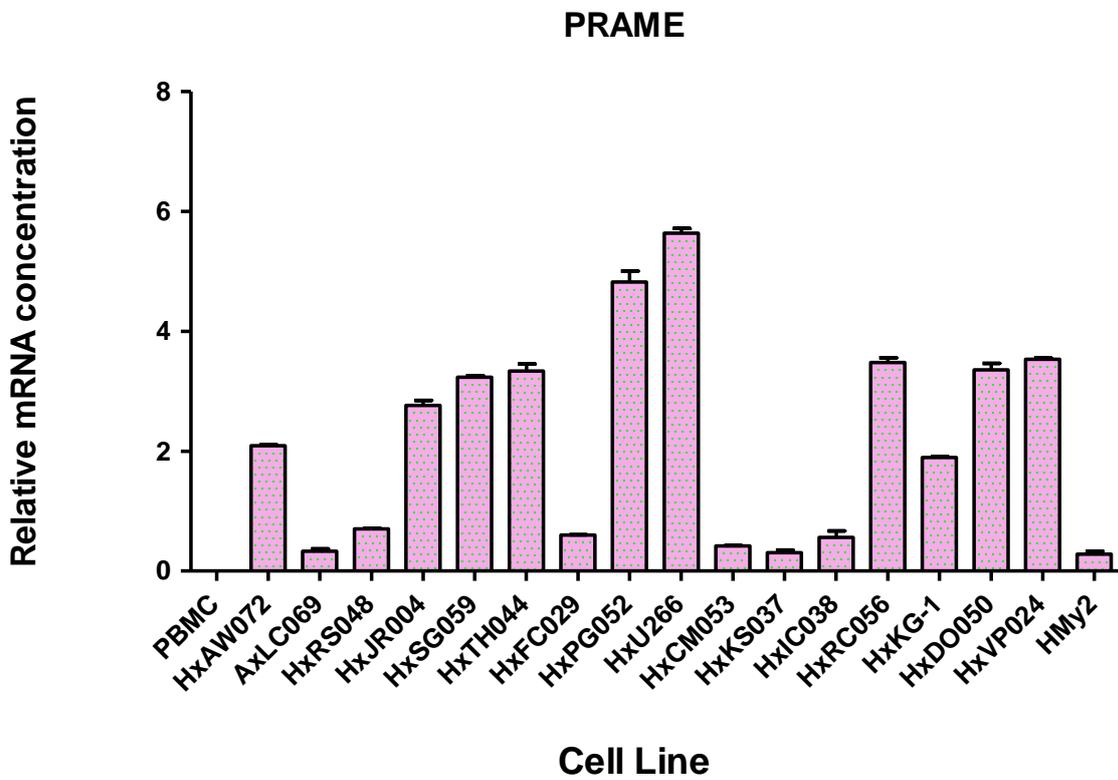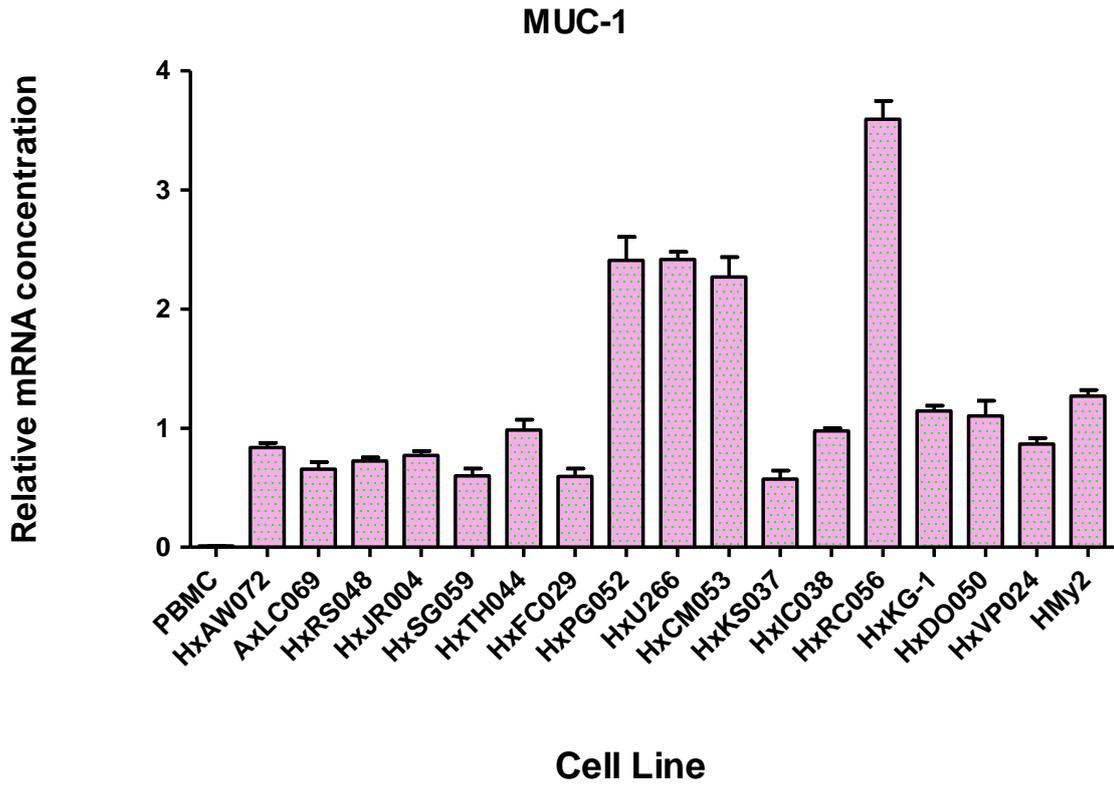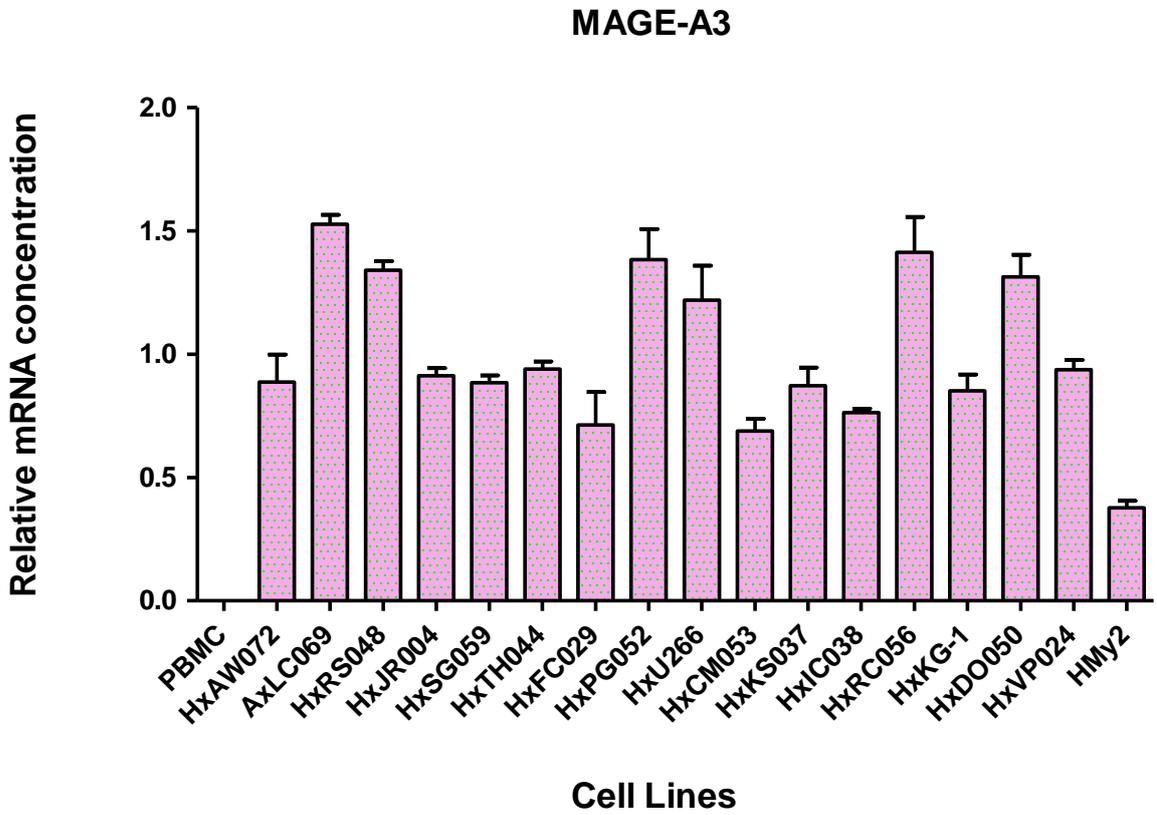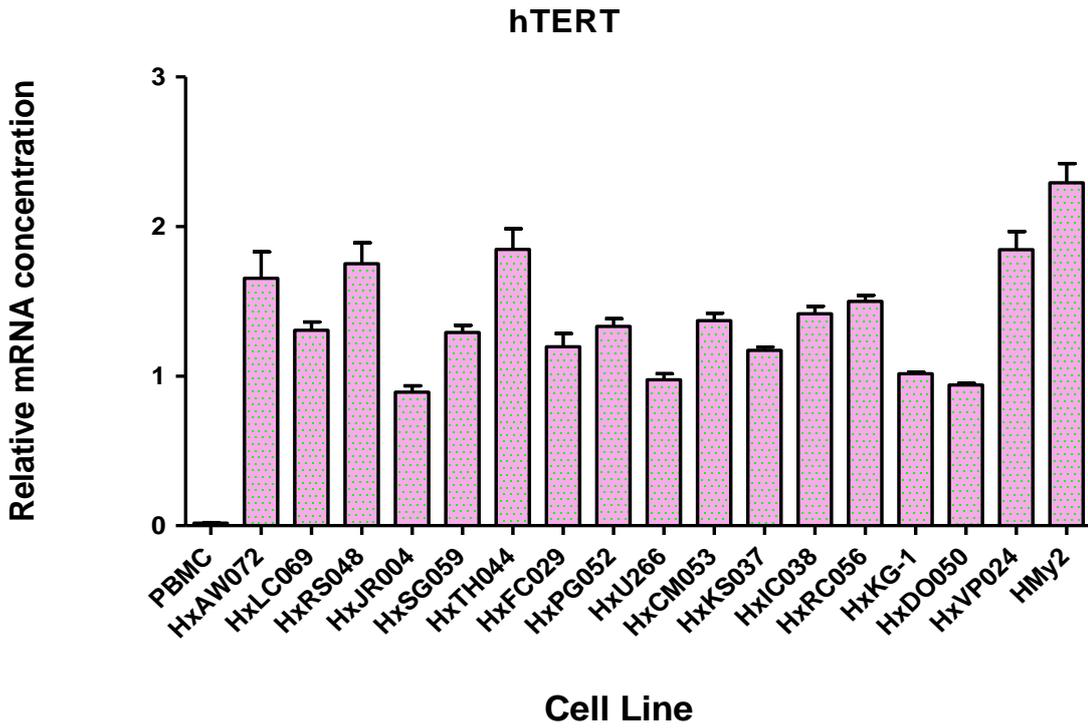
H
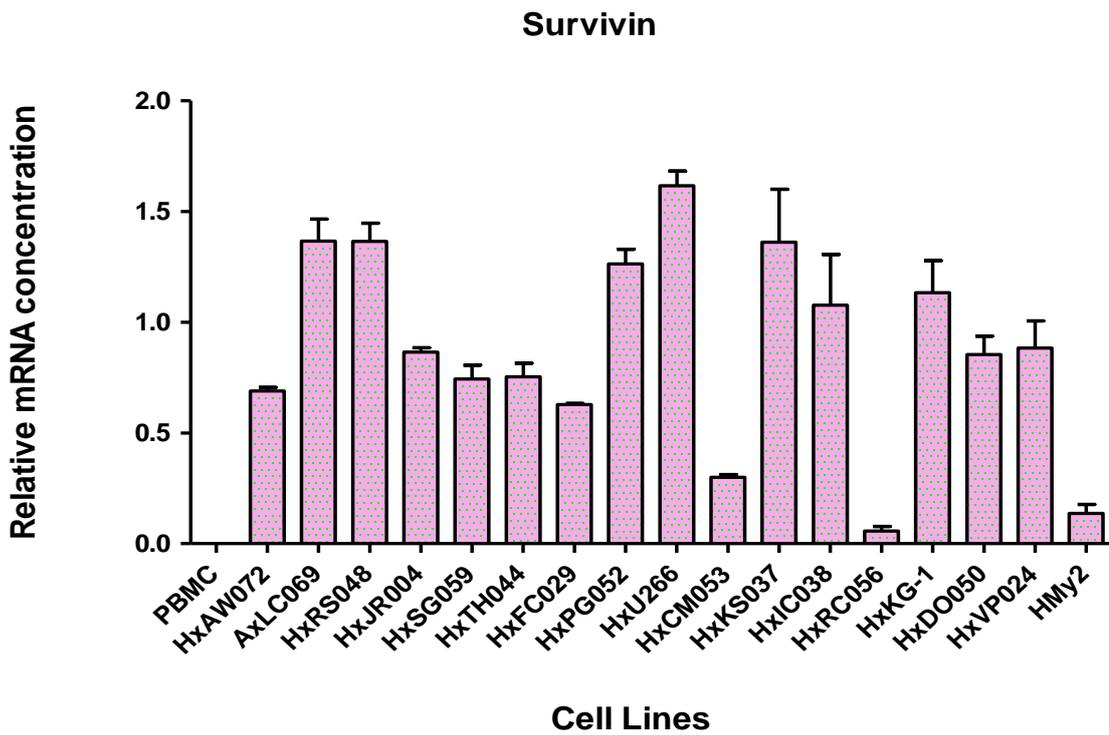
Figure 3.3: Relative tumour associated antigen mRNA transcriptional levels of normal PBMCs, HMy2, and hybrid cell lines. Charts A, B, C, D, E, F, G, and H represent eight TAAs of study as indicated by the upward-legend. In each chart, the individual column represents mean value of triplicate runs ± SEM.

Cell line	NY-ESO-1 x 10^3		MAGE-A1 x 10^2		WT-1 x 10		PRAME x 10		MUC-1 x 10		MAGE-A3 x 10		h-TERT x 10		Survivin x 10	
PBMC	0.53	(-)	0.00	(-)	0.00	(-)	0.00	(-)	0.10	(-)	0.01	(-)	0.17	(-)	0.00	(-)
HMy2	2.25	(-/+)	0.006	(-)	0.05	(-)	2.82	(-)	12.70	(+)	3.77	(+)	22.92	(+)	1.37	(+)
HxAW072	18.27	(+)	8.307	(+)	0.49	(-)	20.90	(-/+)	8.39	(+)	8.87	(+)	16.54	(+)	6.90	(+)
HxLC069	0.50	(-)	0.017	(-)	0.46	(-)	3.33	(-)	6.55	(+)	15.27	(+)	13.07	(+)	13.66	(+)
HxRS048	0.70	(-)	0.005	(-)	0.58	(-)	7.03	(-)	7.25	(+)	13.41	(+)	17.52	(+)	13.66	(+)
HxJR004	0.72	(-)	0.019	(-/+)	0.50	(-)	27.62	(+)	7.71	(+)	9.13	(+)	8.92	(+)	8.65	(+)
HxSG059	0.73	(-)	0.025	(-/+)	0.37	(-)	32.36	(+)	6.00	(+)	8.85	(+)	12.91	(+)	7.44	(+)
HxTH044	2.50	(+)	7.036	(+)	0.41	(-)	33.37	(+)	9.84	(+)	9.39	(+)	18.48	(+)	7.54	(+)
HxFC029	0.91	(-)	0.191	(-/+)	0.41	(-)	6.02	(-)	5.95	(+)	7.13	(+)	11.97	(+)	6.28	(+)
HxPG052	13.87	(+)	0.551	(+)	0.53	(-)	48.26	(+)	24.10	(+)	13.83	(+)	13.32	(+)	12.63	(+)
HxU266	105.00	(+)	52.57	(+)	0.32	(-)	56.37	(+)	24.17	(+)	12.20	(+)	9.77	(+)	16.17	(+)
HxCM053	15.40	(+)	12.144	(+)	0.47	(-)	4.20	(-)	22.70	(+)	6.88	(+)	13.72	(+)	3.00	(+)
HxKS037	1.1	(-)	0.054	(-)	0.50	(-)	3.09	(-)	5.73	(+)	8.72	(+)	11.73	(+)	13.62	(+)
HxIC038	0.94	(-)	0.353	(-/+)	0.49	(-)	5.64	(-)	9.77	(+)	7.63	(+)	14.16	(+)	10.77	(+)
HxRC056	1.6	(-)	99.920	(+)	0.52	(-)	34.81	(+)	35.93	(+)	14.13	(+)	15.01	(+)	0.57	(+)
HxKG-1	1.29	(-/+)	0.691	(+)	10.1	(+)	18.98	(-/+)	11.43	(+)	8.51	(+)	10.16	(+)	11.34	(+)
HxDO050	1.56	(-/+)	0.174	(-/+)	0.51	(-)	33.59	(+)	11.01	(+)	13.13	(+)	9.41	(+)	8.54	(+)
HxVP024	1.24	(-/+)	0.194	(-/+)	0.77	(-/+)	35.34	(+)	8.67	(+)	9.38	(+)	18.46	(+)	8.83	(+)

Table 3.2: Summary of qRT-PCR values (as mean of triplicates; values are multiplied by the indicated factors) of relative levels of TAAs expression by normal PBMCs, HMy2, and hybrid cell lines (left panels), and the corresponding RT-PCR results (right panels).

3.3 Discussion

Tumour immunotherapy represents a promising strategy for cancer treatment, with more specific, long acting, and less toxic anti-tumour effects than conventional therapies. Professional APCs are characterised by enhanced ability to stimulate primary anti-tumour cellular immune responses through their ability to capture, process, and present TAAs in the context of MHC class I and class II molecules, in addition to provision of co-stimulation required to induce naive CD4$^+$ and CD8$^+$ T cells. *Ex vivo* TAA-loaded APC were regarded as a good inducer of anti-tumour immune responses. A strategy which showed a great ability to induce effective anti-tumour immune responses in clinical trials (reviewed in Yasuda *et al.*, 2007, Koido *et al.*, 2009). A major drawback of this strategy is the limited number of known tumour peptide epitopes available in the context of many HLA specificities, and the potential evasion of immunological targeting by tumours through down-regulation of their immunodominant antigens. In addition, the processing and presentation of TAA by APCs are most effective for endogenous, but not for exogenously loaded antigens (Koido *et al.*, 2010a). Accordingly, different strategies were proposed for the effective loading of APC with TAAs. Hybrid cell vaccination is an approach of active cancer immunotherapy, as the APC x tumour cell fusion offers a number of advantages over other strategies, including processing and presentation of multiple known and un-identified tumour-associated antigens (derived from tumour partner) in the context of HLA class I and class II, which allows antigen processing and presentation to both CD8$^+$ and CD4$^+$ T cells. In addition, the expression of HLA, accessory and T cell co-stimulatory molecules (including CD80, CD86, and CD40) allows for induction of effective primary cellular immune responses. In animal studies, APC/tumour hybrids have been shown to stimulate anti-tumour immune responses capable of mediating tumour rejection (Guo *et al.*, 1994, Yasuda *et*

al., 2007, Koido *et al.*, 2005, Kim *et al.*, 2007, Suzuki *et al.*, 2005). The potential of the hybrid cells as immunotherapeutic agents is dependent on the APC-derived phenotype and the effective processing and presentation of tumour associated antigens in the context of MHC class I and class II, in a way which effectively induces CD8$^+$ and CD4$^+$ T cells immune responses respectively (Stuhler *et al.*, 1998). The possibility of chromosomal losses and transcription factors' modifications during hybridization emphasises the importance of careful phenotypic and functional characterization of the hybrid cells before their use in immunotherapy (Tripputi *et al.*, 1988).

In this Chapter, I have shown that fusion of EBV B-LCL (HMy2) with a panel of *ex vivo* or immortalised haematological tumour cells/ cell lines using PEG/DMSO, produced phenotypically stable hybrid cell lines. The previously generated hybrids grew and survived in selective growth medium containing two inhibitory chemicals, HAT and ouabain, a step which encouraged the survival of the true heterokaryons but neither homokaryons nor unfused cells. The hybrid cell lines' growth was maintained, after the selection process, as suspension cells in tissue culture for several weeks to months with stable phenotypic and antigenic properties, and the cell lines survived cycles of freezing in liquid nitrogen and subsequent thawing and re-establishment in tissue culture.

Investigation of co-stimulatory CD molecules and HLA expression showed high levels of CD80, CD86, CD40, HLA class I, and HLA class II expression by HMy2 cells, consistent with their EBV B-LCL phenotype and their potential role as professional APCs (Edwards *et al.*, 1982). Similarly, the hybrid cell lines showed high expression levels of HLA class I and class II, and the co-stimulatory ligands CD80, CD86, and CD40, compared with the relevant parent tumour cells. The role of each of these

molecules in the induction of primary T cell responses was documented in several studies (Grujic *et al.*, 2010, Chan *et al.*, 2005, Schendel *et al.*, 2000).

T cells can only recognise the processed and presented short peptide antigens in the context of HLA class I, or class II for recognition by CD8^{+} or CD4^{+} T cells respectively. This recognition is regarded as the first signal for T cell activation. Another signal is required, in addition to antigen recognition, for full activation of naive T cells, which can be provided by engagement of the co-stimulatory ligands of APC (CD80 and CD86) with the cognate cell receptors on T cells (CD28). Moreover, CD40 expression by APC plays an important role in immune responses, and CD40 interaction with CD40L gives rise to dual activation of both APC and responding T cells. This may explain the inability of most tumour cells to induce effective cellular immune responses *in vivo*; however, the immunostimulatory ability of tumour cells has increased dramatically after transfection of these cells with co-stimulatory ligand gene (Chan *et al.*, 2005). In this study, the predominant LCL phenotype of the investigated hybrid cell lines would suggest high immunogenic properties, together with the roles of the co-stimulatory ligands and HLA expression in induction of cellular immune responses, which will be investigated in the next Chapter.

The difference between immune stimulatory potential of LCL-like and non LCL-like HMy2-derived hybrids was reported by Dunnion *et al.* She observed the association of high expression levels of CD80, CD86, CD40, HLA class I, and class II of hybrid cells with the *in vitro* enhanced immunostimulatory capacity in mixed lymphocyte reaction (Dunnion *et al.*, 1999).

Moreover, HLA-A2 was expressed by HMy2 and all of the hybrid cells of the study, and its expression was independent of relevant parent tumour cells' expression. The

importance of investigating HLA-A2 expression comes from its ability to present many HLA class I-restricted tumour associated antigenic peptides for recognition by TCR of CD8^{+} T cells (Sette & Sidney, 1999), and its wide constitutive expression by most ethnic groups (Huo *et al.*, 2006) As a consequence, tumour antigens presented by HLA-A2 antigens are good candidates for *in vitro* use in induction of restricted CD8^{+} T cell responses using these hybrid cells.

To conclude the phenotypic characterization, all the hybrid cell lines expressed co-stimulatory ligands (CD80, CD86, and CD40) in addition to HLA class I and class II molecules, more than relevant tumour cells, which would enhance the immunostimulatory capacities of hybrid cells. The efficiency of the hybrid cell lines as immunostimulatory agents is not only dependent on expression of HLAs and co-stimulatory molecules, but also on the expression of relevant associated antigens, especially the tumour-derived antigens, and efficient presentation of these antigens in the context of appropriate HLA for recognition of specific T cells.

Expression of a group of known TAAs by HMy2 and hybrid cell lines was investigated using RT-PCR and semi-quantitative RT-PCR (qRT-PCR). MAGE-A1, NY-ESO-1, WT-1, PRAME, Survivin, MAGE-A3, MUC-1and h-TERT antigens were chosen for investigation, as represent potential targets for tumour-specific CTL responses. NY-ESO-1, MAGE-A1, MAGE-A3, and PRAME are members of CT antigens, a group of highly immunogenic and widely expressed tumour antigens. CT antigens include several subgroups such as, GAGE, CAGE, BAGE, MAGE, NY-SAR-35, OY-TES-1 and others; these and other antigens are listed in Section 1.3.1 (Van der Bruggen *et al.*, 1991, Boel *et al.*, 1995, Novellino *et al.*, 2005, Parmiani *et al.*, 2007, Caballero & Chen, 2009). MAGE-A1 was the first identified CT gene, in a melanoma cell line, and

reported as one of twelve related homologous members of MAGE-A family. All are located at the long arm of chromosome X. CT antigens are widely expressed by a range of different tumour types such as, melanoma, lung, head and neck, and bladder carcinoma. Their expression in normal tissues is very low, and restricted to germ line tissues, such as testis and ovaries, so they are considered as an ideal target for tumour immunotherapy. In addition, germline tissues are regarded as immune privileged tissues, due to lack of HLA class I expression and the presence of blood-tissue barriers. Therefore, the immunotherapeutic use of CT is largely not associated with autoimmune side effects (Meklat *et al.*, 2007). In haematological malignancies, CT antigen expression was reported in several different studies. One of these studies showed the expression of MAGE-A family antigens (especially MAGE-A1 and MAGE-A3) in around 50% of human T and B cell leukaemias, but was absent in myeloid leukaemia (not seen in 23 cases) (Shichijo *et al.*, 1995). In another study by Chambost *et al.* (Chambost *et al.*, 2001), MAGE-A family members were detected in a large panel of investigated leukaemia cells, including AML, ALL, and B-CLL. A recent study by Martinez *et al.*, carried out on 115 leukaemia patients, showed that 14 of 34 (41%) AML, 23 of 76 (30%) B-ALL and one CML cases were positive for at least one of the three genes of study (MAGE-A1, MAGE-A3, or MAGE-B2) (Martinez *et al.*, 2007). PRAME (preferentially expressed antigen in melanoma) is expressed in high levels in germ tissues, while in low levels by most normal tissues (Wadelin *et al.*, 2010). Although expressed at low levels in normal haematopoietic progenitor cells, PRAME is widely expressed in most haematological malignancies such as AML (45%), ALL (up to 42%), CLL (27%), myeloma (up to 52%), and CML (36%), as well as in some solid tumours such as skin, breast, lung, and neck cancers (Wadelin *et al.*, 2010). PRAME was used as a target antigen in tumour immunotherapy due to its aberrant expression in

normal tissues and wide expression in a variety of tumour cell types (Wadelin *et al.*, 2010). NY-ESO-1 was expressed by different types of tumours, such as ovarian, lung, and prostate cancers. In addition, high levels of NY-ESO-1 were expressed by melanoma, bladder cancer (reviewed in Caballero & Chen, 2009) and by MM tumours (Meklat *et al.*, 2007).

Over-expressed tumour antigens, represent a group of proteins that may be expressed in small amounts in normal tissues, but are over-expressed by certain transformed cells. WT-1, Survivin, MUC-1, and h-TERT are members of this group. WT-1, or Wilms' tumour gene, is a zinc finger transcription factor involved in cellular proliferation and differentiation, and was described originally as tumour suppressor gene. WT-1 is normally expressed by embryonic kidney cells, and has been described to have an oncogenic function in different malignancies including leukaemia (Cesaro *et al.*, 2010), especially in AML and ALL. WT-1 was also reported to be over-expressed in several types of solid tumours such as, gastrointestinal tumours, renal and breast cancers (Hou *et al.*, 2010, Kang *et al.*, 2010).

Survivin is a member of the inhibitor of apoptosis protein (IAPs) family, and is over-expressed in most solid and haematologic tumours, but not in normal tissues (Schmidt *et al.*, 2003). Moreover, several studies have shown the expression of Survivin in almost all leukaemia blasts, especially in AML and ALL (Schmidt *et al.*, 2003, Carter & Andreeff, 2008). Survivin is an important factor in tumourigenesis, inhibition of apoptosis, and regulation of cell division, which, together with its wide expression in different types of tumours and the poor prognosis associated with its up regulation, make it an ideal target for tumour immunotherapy (Carter & Andreeff, 2008).

The epithelial trans-membrane type I glycoprotein (Mucin-1; MUC-1) antigen is over-expressed in different epithelial tumours, such as pancreatic, ovarian and breast cancers. Its role in the tumour immune evasion and tumour metastasis promotion, through loss of tumour contact to extracellular matrix and adjacent cells, was documented (O'Connor *et al.*, 2004). MUC-1 expression was also detected in B-lymphoma, multiple myeloma and myeloid leukaemia cells (Brossart *et al.*, 2001).

Human telomerase reverse transcriptase (h-TERT) is an important universal TAA expressed by more than 85% of tumours, but not expressed by normal tissues except in germline, lymphocyte and some stem cells (Wenandy *et al.*, 2008). The vital role of h-TERT in tumourigenesis and its expression pattern, in addition to the ability to induce humoral and cellular-specific immune responses, confirm its importance in tumour immunotherapy (Huo *et al.*, 2006).

In my study, qRT-PCR results showed that MAGE-A1 mRNA was absent in HMy2, but was expressed in 3 of 3 in AML, 4 of 5 in MM, 2 of 3 in B-cell lymphoma, 3 of 4 in B-CLL, and 1 of 1 in ALL hybrid cell lines according to the indicated cut off values. These results were consistent with the conventional RT-PCR data, except for two B-CLL hybrid cell lines, where expression levels were underestimated in the semi-quantitative compared with the conventional RT-PCR. In addition, the qRT-PCR showed more precise comparison of expression levels between the positive cell lines. MM hybrids, showed the highest level of MAGE-A1 expression. On the other hand, lack of MAGE-A1 expression was confirmed in 3 hybrid cell lines in both conventional and semi-quantitative RT-PCR.

NY-ESO-1 was weakly expressed by HMy2, and was expressed in 3 of 3 in AML, 4 of 5 in MM, 1 of 3 in B-cell lymphoma, and 1 of 4 in B-CLL, and 1 of 1 in ALL according

to the indicated cut off values of qRT-PCR. These results were consistent with the conventional RT-PCR, except in 2 MM hybrids, where the qRT-PCR overestimated the expression levels in both cell lines. The top four cell lines expressing NY-ESO-1 revealed by qRT-PCR were of MM, B-CLL, and ALL, whilst was not detected in 5 other hybrid cell line from different lineages, as revealed in both semi-quantitative and conventional RT-PCR. This suggests that weak antigen expression may have been missed by RT-PCR, and that qRT-PCR method was more sensitive for TAA detection. Consistent results were obtained in WT-1 antigen expression, which was expressed by 2 AML hybrid cell lines, but not expressed, by any of the other investigated cell lines.

Similarly, PRAME expression was consistent in both semi-quantitative and conventional RT-PCR. HMy2 was negative; while 3 of 3 AML, 2 of 5 MM, 1 of 3 B-cell lymphoma, 3 of 4 B-CLL, and 1 of 1 ALL hybrid cell lines expressed PRAME according to the indicated cut off values of qRT-PCR. The rest of the hybrid cell lines were negative for PRAME expression consistently in semi-quantitative and conventional RT-PCR. Positive expression levels were seen in HMy2 and all hybrid cell lines for MUC-1, h-TERT, MAGE-A3, and Survivin, with consistent results in both semi-quantitative and conventional RT-PCR. Over expressed TAAs were variably detected in the cell lines of the study. Survivin, MUC-1, and h-TERT were expressed by HMy2 and the entire investigated hybrid cell lines, with slightly variable levels as indicated by qRT-PCR. The unexpected up-regulation of MAGE-A3 in HMy2 might be a result of activation after the EBV transformation. Results of TAA expression were consistent in both types of PCR, with more precise values in qRT-PCR. The degree of consistency was in 140 out of 144 occasions, with percentage exceeding 97%. The

remaining 3% inconsistency may be attributed to the higher sensitivity of qRT-PCR technique, or to the different primer pair sets.

To conclude the TAA expression data; normal PBMCs showed no expression at all for the candidate tumour antigens (except minor detectable amount, far less than cut off values of negative expression, of NY-ESO-1, MUC-1, and h-TERT), while HMy2 and all hybrid cell lines expressed Survivin, MAGE-A3, MUC-1 and h-TERT. HMy2 lacked expression of MAGE-A1, WT-1 and PRAME, and weakly expressed NY-ESO-1 antigen. MAGE-A1 was strongly expressed by seven of the hybrid cell lines, and was not expressed by three other hybrid cell lines. WT-1 was only expressed by two hybrids. NY-ESO-1 was strongly expressed by five of the hybrid cell lines and not expressed by six other hybrid cell lines. Finally, PRAME was expressed by eight of the hybrid cell lines, weakly by two, and not expressed by six hybrid cell lines.

The majority of the hybrid cell lines, except for three (HxKS037, HxRS048 and LC069), were positive for expression of at least one of the TAAs that were not expressed by HMy2. The TAA expression profile was confirmed by semi-quantitative RT-PCR to show precise levels of expression of these antigens by different hybrid cell lines. By this precise technique, lack of expression of MAGE-A1, WT-1 and PRAME by HMy2, and the absence, or minimal expression of all these antigens by normal PBMC, were confirmed.

Moreover, the ability of the fused cells to survive and multiply in selective media containing both ouabain and 2% HAT was the first proof of the hybrid nature of the fused cells. Moreover, data from cell surface marker and HLA phenotyping of the hybrid cells, when compared with their parent tumour cells, in addition to TAAs profiling, were the second proof for the heterokaryotic nature of the fused cell lines.

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Finally, data in this Chapter showed that the hybrid cell lines retained the APC phenotype of HMy2 cells, by retaining co-stimulatory molecules, HLA class I and HLA class II expression, and the expression of a group of TAAs derived from parent tumour cells. Although important, the observed HLA, co-stimulatory ligands and TAA expression does not in themselves demonstrate the enhanced immunogenicity and ability of hybrid cells to induce T cell responses. Therefore, functional characterization of the hybrid cell lines to investigate their ability to stimulate T cell responses *in vitro* was the second step to be addressed in this study. Moreover, the variability of TAA expression levels by different hybrid cells gave the chance to select a group of them to be used as stimulators of allogeneic and semi-allogeneic PBMCs for induction of TAA-specific CTL clones. This is described in detail in Chapters 4 and 5.

Chapter 4

Functional characterization of hybrids and their parent tumour cells/cell lines

4 Functional characterization of hybrids and their parent tumour cells/cell lines

4.1 Introduction

In the absence of a direct animal model for *in vivo* study of EBV B-LCL, *in vitro* immunostimulatory assays were performed to evaluate the hybrid cell lines' immunostimulatory capacity (compared with the respective parent tumour cells), and their ability to process and present endogenous and tumour-specific antigens in the context of HLA class I and class II for T cell recognition. T cell immunostimulatory assays included proliferation, cellular differentiation, IFN-γ release, and the induction of specific T cell clones able to recognise and eliminate target cells expressing the cognate antigens. T cell proliferation, differentiation, IFN-γ release responses, and induction of allogeneic- (which may be accompanied by antigen-specific) CTLs will be discussed in this Chapter, while the induction of tumour antigen-specific cytotoxic T lymphocyte responses will be addressed in Chapter 5.

4.1.1 T cells and anti-tumour immunity

The aim of tumour immunotherapy is to enhance antitumour immunity by induction of effector humoral and/ or cellular responses. Most tumours express different types of antigens which can be recognised as foreign by the host immune system. However, in most cases the immune system fails to induce effective anti-tumour responses, which leads to tumour out growth and progression. The induction of *in vivo* primary T cell immune responses is mediated by professional APCs, which take up the tumour antigens (in the form of necrotic or apoptotic tumour cells), and process and present their peptides in the context of HLA class I and class II. APCs also provide the second

signal required for naive T cell priming, in addition to presentation of TAAs in the context of HLA for recognition by the cognate TCR of antigen-specific T cells. The main co-stimulatory signal required for naive T cell stimulation is the engagement of T cell receptor CD28 by APC-co-stimulatory ligands, CD80 and CD86, (other co-stimulatory ligands were mentioned in Section 1.2.2.1.2).

The lack of co-stimulatory ligand expression in most types of tumours, in addition to the inhibitory effect of tumour-microenvironment, induces anergic or tolerant T cells upon recognition of tumour antigens.

4.1.2 Effector T cell responses

The T cell subclasses are primed, as mentioned before, by recognition of cognate antigen in the context of the appropriate HLA molecules, and by receiving the required co-stimulation. $CD8^+$ and $CD4^+$ T cells recognise peptides associated with HLA class I and class II molecules respectively. Activated $CD8^+$ T cells perform their effector function directly by recognizing the target tumour cells followed by inducing target cell apoptosis (by release of cytolytic molecules; perforin, granzyme, and granulysin, or through Fas/Fas-L binding), and indirectly by releasing Th1 cytokines (TNF-α, IL-2 and IFN-γ). On the other hand, activated $CD4^+$ T cells mediate their helper function according to the polarization pattern of the tumour microenvironment. There are different phenotypic patterns of helper T cells, which include Th1, Th2, Th17, and inducible regulatory T cells (iTreg). Th1 cells produce type I cytokines like IFN-γ, TNF-α, and IL-2 which have anti-tumour effector functions by induction of NK, $CD8^+$ and APCs. Th1 $CD4^+$ T cells also have a direct stimulatory effect on APCs by CD40L/CD40 engagement (Banchereau & Steinman, 1998). Th2 cells produce type II cytokines such as IL-4, IL-13 and IL-5, which have a role in induction of mature

humoral response against tumour antigens, in addition to other roles. Th17 cells have a role in tissue inflammation, autoimmunity and in protection against infection at mucosal sites, mediated by IL-17, IL-22 and TNF. Inducible Treg are provoked by TGF-β and have an inhibitory effect on other T cell types (Koido *et al.*, 2010b). Th1 T cells are thought to be the major helper T cell population involved in promoting anti-tumour immunity, whilst Tregs inhibit induction of anti-tumour immunity. In addition, some groups reported the induction of MHC class II-restricted CD4^{+} killer cells in animal models and in human, especially during viral infection. Moreover, *in vitro* cytotoxicity studies reported that CD4+ killer cells mediate cytotoxicity function through TNF-α, FasL, or perforin-dependent killing (Jellison *et al*, 2005).

4.1.3   Anti-tumour role of IFN-γ

IFN-γ is a member of a protein family which is characterized by its role in protection against intracellular infection, and malignant transformation. According to the functional and structural characteristics, this group of cytokines was subdivided into two types; type I interferon, which are induced in response to viral infection and includes IFN-α and IFN-β, and Type II interferon, which includes IFN-γ released by activated CTL, Th1 helper cells, NK, NKT, and other cell types (reviewed in Ikeda *et al.*, 2002). IFN-γ mediates anti-tumour effects by activation of APCs which indirectly activate CD8^{+} T cells. It also sensitises tumour cells to CTL attack by up regulation of HLA class I molecule expression, and antigen processing by APCs. NK and NKT cells also release IFN-γ upon stimulation, in turn IFN-γ induces more APCs to release IL-12 which has an inducible effect on NK and NKT cells, and so on (Caligiuri, 2008, Taniguchi *et al.*, 2010). So, IFN-γ release is an indication of anti-tumour cellular immune responses, and its detection is regarded as an ideal assay to investigate cellular

immune response. In this study, I used IFN-γ ELISpot assays to detect IFN-γ secreting cells in response to antigenic stimulation.

4.1.4 Principle of allogeneic T cell responses

Allorecognition is a phenomenon of interaction of T cells of a defined donor, of specific HLA profile, with cells from a different and un-related donor (with mismatched HLA type), which are then recognised as foreign or non-self antigens. Allogeneic T cell responses are amongst the main mechanisms of tissue transplant rejection.

4.1.4.1 Mechanisms of allopeptide recognition by T cells

The main targets of an allogeneic response are the mismatched HLA molecules of the allogeneic stimulator cells. Responder T cells can recognise mismatched HLAs as foreign antigens through three pathways (Bharat & Mohanakumar, 2007): direct, indirect, and semi-direct alloantigen recognition. In the direct pathway of alloantigen recognition, CD8$^+$ and CD4$^+$ alloreactive T cells directly recognise alloantigens of HLA class I and class II respectively on the surface of allogeneic APC. Several studies reported the prolonged persistence of allograft in cases where they were depleted (by using appropriate mAb) from donor APCs prior to transfer (reviewed in Bharat & Mohanakumar, 2007). The direct pathway allorecognition is largely responsible for the acute phase allograft rejection.

Secondly, in the indirect pathway of allorecognition, T cells recognise alloantigens that have been taken up, processed, and presented in the context of HLA of self (recipient) APC. Different reports supported the role of indirect allorecognition in delayed allograft rejection. Auchincloss *et al*. (Auchincloss *et al.*, 1993) reported the ability of MHC class I-deficient mice to reject skin grafts from MHC class II deficient donor mice suggesting the uptake, processing, and presentation of alloantigens in the context of

MHC class II of recipient APC. This mechanism could play an important role in chronic allograft rejection. Thirdly, the semi-direct pathway depends on the transfer of intact MHC molecules between cells. By this model, self DCs were reported to accept MHC class I and II from other allogeneic-cells' exosomes, and could prime self $CD8^+$ and $CD4^+$ T cells using these accepted allogeneic complexes (Tsang *et al.*, 2003).

4.1.5 Immunogenicity of hybrid cell lines versus relevant-parent tumour cells

Several mechanisms have been proposed to explain the failure of tumour infiltrating lymphocytes in tumour rejection in both animal and human studies (Wilczynski & Duechler, 2010, Gross & Walden, 2008). Some of these explanations are related to low immunogenicity of most tumour cells, due to tolerance inherited to some self-derived TAAs, down regulation of HLA and/ or TAA expression, tumour induced suppressive mechanisms, and lack of co-stimulation.

In order to overcome the low immunogenicity of tumour cells, it was proposed that their fusion with professional APC (e.g. EBV B-LCL) would produce hybrid cells of enhanced immunostimulatory capacities that expressed, processed, and presented tumour associated antigens in the context of HLA class I and HLA class II, together with providing essential co-stimulatory signals and adhesion molecules required for successful initiation and maintenance of effective anti-tumour, cellular immune responses of both $CD8^+$ and $CD4^+$ T cell nature. This proposal was based on results obtained from similar works done on several animal tumour models and clinical studies. Guo *et al.* (Guo *et al.*, 1994) using a hepatocellular carcinoma rat model, reported that fusion of tumour cells with activated B cells produced hybrid cells with high capability to induce protective immunity against established tumours. Similarly, but in humans,

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Moviglia (Moviglia, 1996) reported promising results of phase I and II trials on 21 different tumour-patients using hybrid cells of activated B cells fused to the patient's tumour cells. Further reports showed the effectiveness of hybrid cells made by fusion of APC x tumour cells as immunotherapeutic agents in *in vitro* studies (Goddard *et al.*, 2003, Gong *et al.*, 2000, Li *et al.*, 2001), animal models (Rosenblatt *et al.*, 2005, Orentas *et al.*, 2001, Markiewicz & Kast, 2004), and clinical trials (Trefzer & Walden, 2003, Trefzer *et al.*, 2000, Tanaka *et al.*, 2002, Koido *et al.*, 2005). Furthermore, several *in vitro* studies of LCL x tumour cell hybrids have been reported in a set of papers published by my supervisor's group. Dunnion *et al.* (Dunnion *et al.*, 1999) investigated a group of hybrids made by fusion of HMy2 x solid or haematological tumour cell lines. Both solid and haematologic tumours produced chemically selectable hybrids which grew continuously in tissue culture. However, she reported the phenotypic similarity between haematologic-derived hybrids, but not the solid tumour-derived hybrids, and the HMy2 partner regarding expression of HLA, co-stimulatory, and adhesion molecules. In addition, hybrid cells showed higher allogeneic T cell immunostimulatory and lymphoproliferative capacity than their respective parent tumours, and these responses included $CD8^+$, $CD4^+$, naive, and memory T cells. These functional properties were inhibited by blocking co-stimulatory molecules (CD80 and CD86), and by blocking HLA class I and HLA class II molecules. Similar results were reported on other solid and haematologic tumour x HMy2 hybrid cell lines *in vitro* (Cywinski *et al.*, 2006). The majority of the hybrid cell lines used in my study have not been reported previously. Therefore, in this Chapter, I investigated the ability of the hybrid cell lines to induce T cell proliferative, IFN-γ releasing, and allogeneic cytolytic responses, by *in vitro* stimulation of normal donors' PBMC in mixed lymphocyte reaction (MLR), IFN-γ ELISpot, and ^{51}Cr release cytotoxicity assays. In MLR, the roles

of MHC class I and class II, and co-stimulatory molecules (CD80 and CD86) were also investigated after their blockade using mAbs. In IFN-γ ELISpot assays, T cell response to stimulation with hybrid cell lines, compared with their parent tumour cells (in both allogeneic and autologous PBMC), was investigated for short-term and long-term stimulation. Finally, the specificity of the induced allogeneic CTLs was investigated in cytotoxicity assays using different cell targets.

4.2 Results

4.2.1 Allogeneic lymphoproliferative immune response

In order to investigate the effect of their fusion with EBV B-LCL on their immunogenicity, hybrid cell lines and their relevant parent tumour cells were evaluated for their abilities to induce allogeneic T cell immune responses *in vitro*. Hybrid cell lines, their relevant tumour cells, or HMy2 cell lines were co-cultured with PBMCs (in MLR) from normal, healthy, allogeneic donors for the appropriate time under tissue culture conditions as mentioned in Section 2.4.1. Each experiment was carried out using PBMC from at least 2 different donors, with consistent results. Representative data are shown, and hybrid cell lines used in each assay are listed in the relevant Figure legends.

4.2.1.1 *Stimulator cell-dose versus PBMCs-response relationship*

As a preliminary experiment, and to investigate the response to different responder/ stimulator ratios, three different concentrations of stimulator cells ($1x10^5$, $3x10^4$, or $1x10^4$) were plated in 96-U bottom well plates with $1x10^5$ of responder cells per well (Figure 4.1A). The data showed, in most cases, a proportional increase in T cell proliferation in response to increased number of stimulator cells.

4.2.1.2 Proliferative PBMC responses to HMy2, hybrid cell lines, and their parent tumour cells

HMy2, hybrid cell lines, and respective parent tumour cells were used as stimulators for allogeneic normal PBMC in MLR, in a ratio of 1:3 respectively, and the levels of T cell proliferation induced are shown in Figure 4.1B. HMy2 cell line was used as a positive control stimulator, whereas unstimulated PBMCs were used as a background control. All of the hybrid cell lines stimulated allogeneic T cell proliferative responses to a significantly higher degree than their respective parent tumour cells in MLR; albeit, some hybrids showed lower proliferation than HMy2, which may be explained by the release of immunosuppressive cytokines or expression of inhibitory co-stimulatory receptors.

In contrast with hybrid cell lines responses, respective parent tumours induced poor or minimal allogeneic proliferative responses, in spite of being derived from individuals unrelated to the responder PBMC (Figure 4.1 B). This might be due to lack of co-stimulation, having lower allogeneic MHC molecules than relevant hybrid cells.
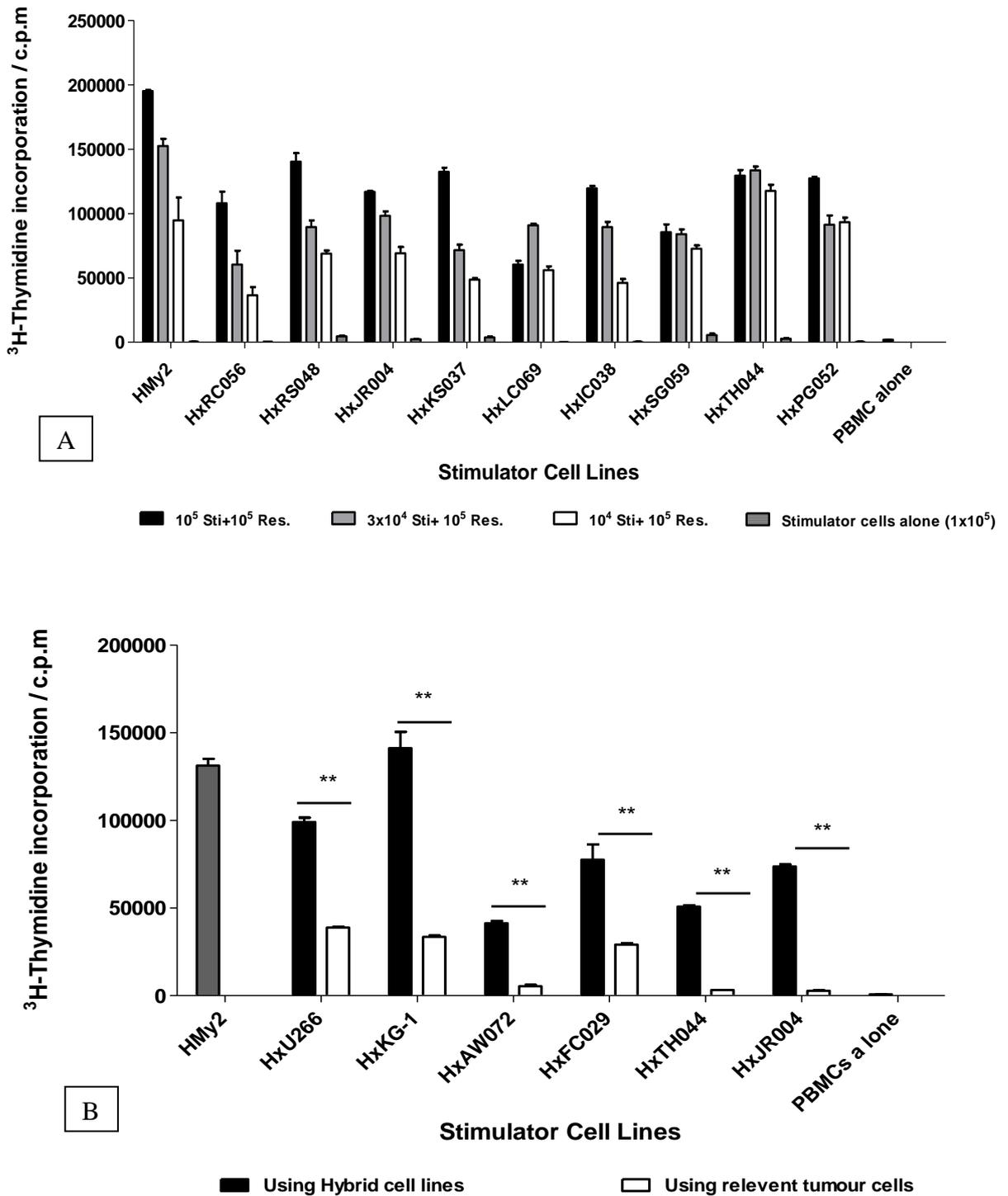
Figure 4.1: Mixed lymphocyte reactions. Chart (A): shows dose-response relationship, as three different concentrations of stimulator cells mixed with 1×10^5 of allogeneic PBMCs. Chart (B): shows lymphoproliferative responses to a group of hybrid cell lines, compared with their respective parent tumour cells. HMy2 was used as a positive allogeneic stimulator control, and unstimulated PBMCs as a background control. Only six tumour cells and hybrid pairs were studied, due to the shortage in *ex vivo* tumour cells. Results represent mean ± SEM of triplicate wells

4.2.1.3 *Effect of anti-HLA class I and class II antibodies on PBMC responses to HMy2 and hybrid cell lines*

Monoclonal antibodies W6/32 and L243 were used to block the function of HLA class I and HLA class II (DR) respectively, in order to investigate their roles in the allogeneic lymphoproliferative responses to hybrid cell lines. The antibodies were used as purified culture supernatant, and an isotype matched control antibody was used in control wells. The antibodies W6/32 and L243 were incubated with the stimulator cells before addition of responder cells, to show the blocking effect to HLA class I, HLA class II (DR), or both together on the hybrid cell-induced proliferative responses of allogeneic PBMCs. The proliferative responses were not affected by addition of isotype control IgG1, but were partially inhibited by addition of W6/32 or L243 mAbs. The inhibition was greater in the case of W6/32 than L243, and addition of both mAbs together blocked the proliferation to near background control level (unstimulated PBMC) (Figure 4.2). These results indicate the involvement of both HLA class I and class II (DR) molecules in the allogeneic responses to the hybrid cell lines of the allo-specific T cells, and (indirectly) that both CD8^{+} and CD4^{+} T cells are involved in the responses in MLR.
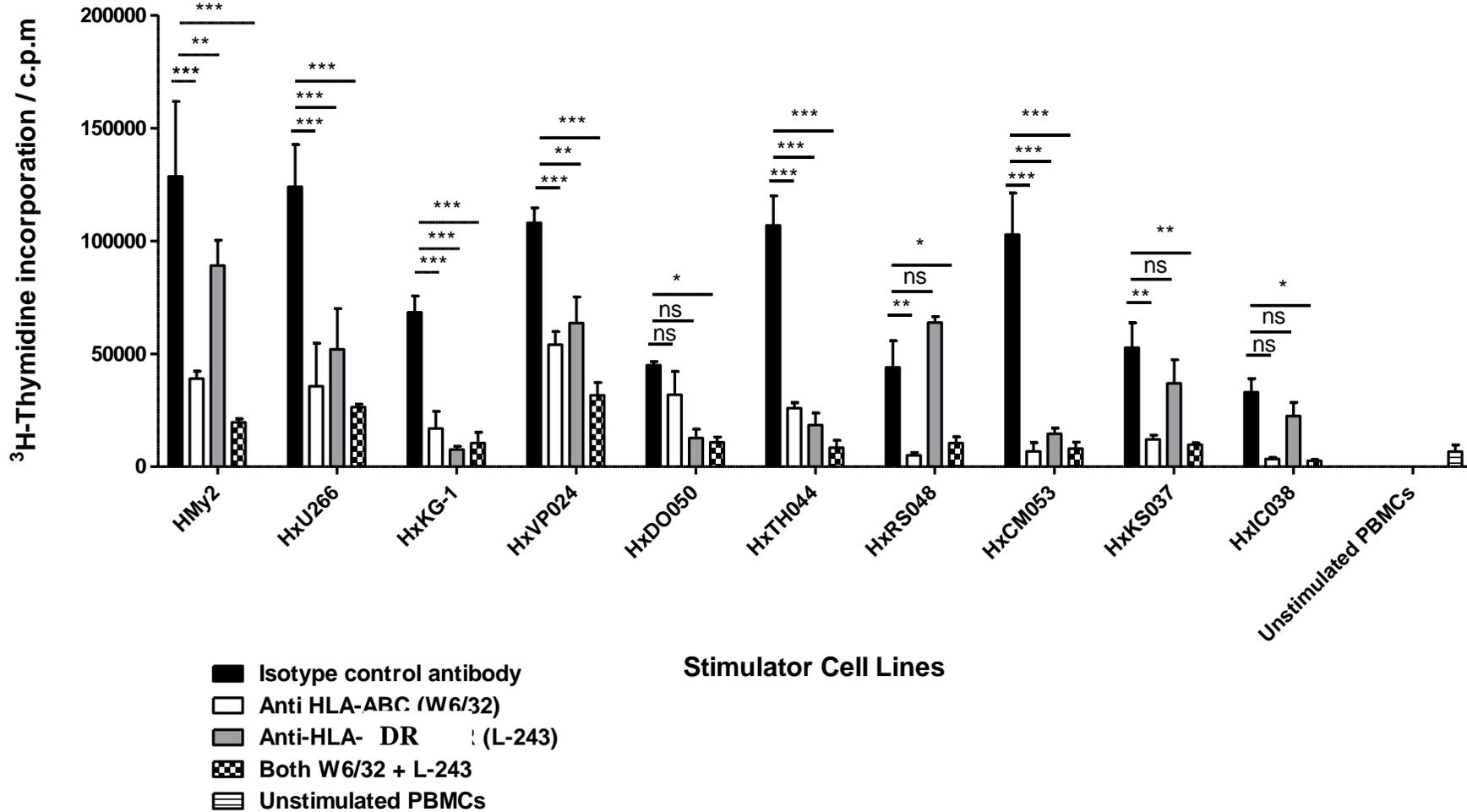
Figure 4.2: Mixed lymphocyte reaction in the presence of anti-HLA class I mAb (W6/32), anti-HLA class II (DR) mAb (L243), or both together. A non-specific IgG1 mAb was used as an isotype control at the same final concentration (0.75µg/ ml). Results represented as mean ± SEM of triplicate wells,

4.2.1.4 Effect of cytotoxic T lymphocyte antigen-4 immunoglobulin (CTLA-4 Ig) fusion protein on proliferative response to HMy2 and hybrid cell lines

CD80 and CD86 are important co-stimulatory ligands expressed on the surface of professional APC. CD80 and CD86 were also expressed by HMy2 and by all of the hybrid cell lines under investigation (Section 3.2.2). In order to investigate the role mediated by interaction of CD80 or CD86 of the hybrid cell lines with cognate receptors on T cells (CD28) in the enhanced, allogeneic lymphoproliferative responses, modified *in vitro* MLR assays were carried out in the presence of CTLA-4 Ig fusion protein (inhibitor of CD80 and CD86). An isotype control mAb was used as a non-specific control. Stimulator cells were incubated with CTLA-4 Ig or IgG-1 before addition of responder cells (for more details see Section 2.4.3).

Significant inhibition of the allogeneic proliferative responses was seen after addition of CTLA-4 Ig fusion protein, in contrast to IgG1 isotype control, in all cases. These results showed the important role mediated by co-stimulatory ligands (CD80 and CD86) in the enhanced T cell stimulatory capacity of hybrid cell lines (Figure 4.3). Moreover, another approach which can be used to investigate the importance of CD80/CD86 expression by hybrid cells to induce primary T cell response, is to knock down the expression of these molecules (siRNA) by the hybrid cells.   .
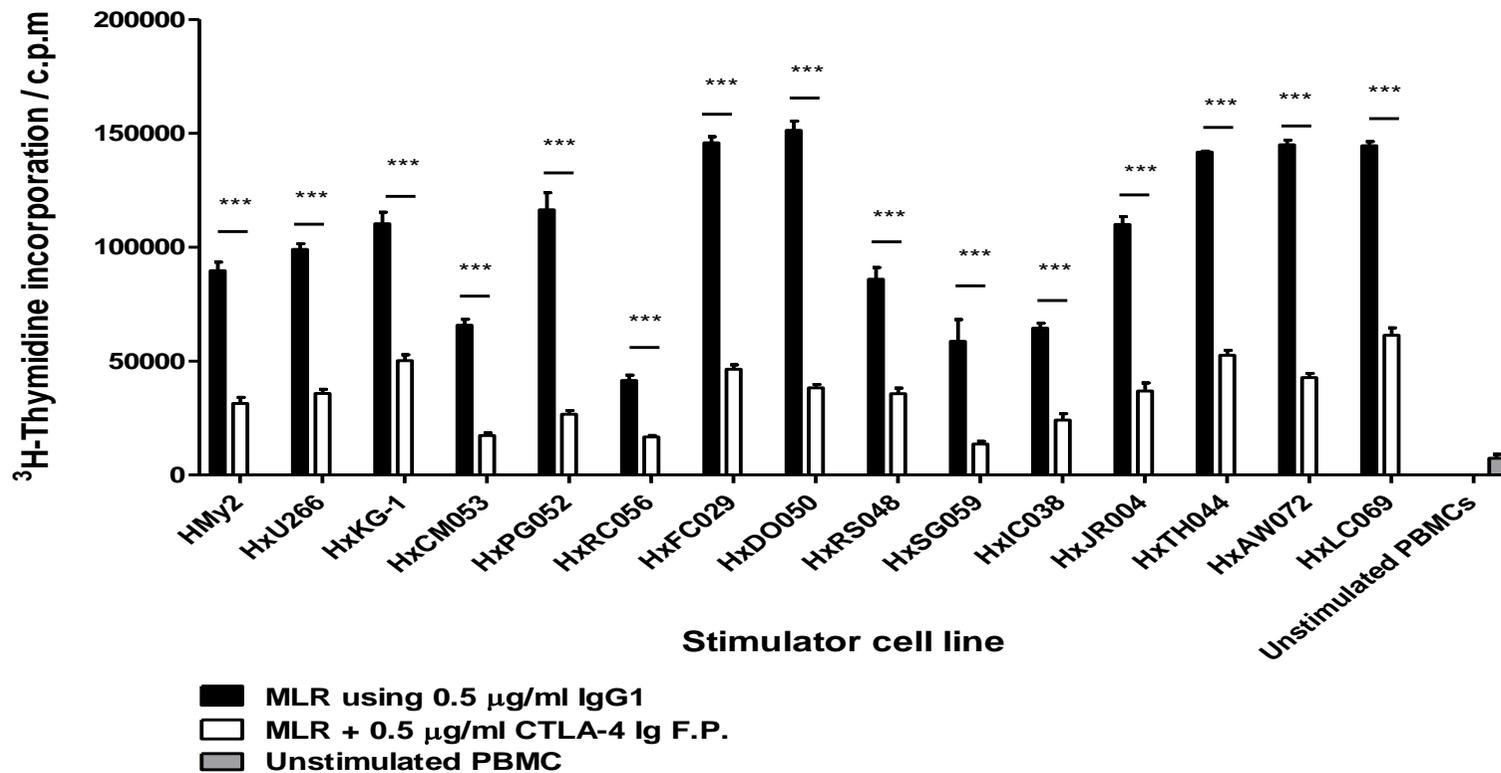
Figure 4.3: Mixed lymphocyte reaction, showing the CTLA-4 Ig fusion protein blocking effect on CD80 and CD86 induced co-stimulation. A non-specific IgG1 mAb was used as an isotype control. Stimulator cells were incubated with CTLA-4 Ig or IgG1 (at a final concentration of 0.5µg/ ml) before addition of responder PBMC. Columns represent mean ± SEM of triplicate wells.

4.2.1.5 Phenotypic investigation of the responding T lymphocytes

The previous results showed that the immune response of allogeneic PBMC to stimulation with hybrid and HMy2 cell lines was dependent on HLA class I and class II expression by the stimulator cells, in addition to co-stimulation mediated by CD80/ CD86 ligands. HLA class I and class II were involved in antigen presentation and recognition of CD8$^+$ and CD4$^+$ T cells respectively. Co-stimulatory ligands (CD80 and CD86) provided the second signal required for optimum T cell priming.

The major and minor mismatches in histocompatibility antigens between stimulator cells and the allogeneic PBMC are regarded as a strong inducer of proliferation and differentiation of the responder allo-specific CD8$^+$ and CD4$^+$ T cells. The induction of cellular immune responses in MLR in this context does not indicate a specific anti-tumour response to the stimulator cells. However, it does reflect the profound immunogenicity of the hybrid cell lines (compared with parent tumour cells) for stimulation of T cell responses more generically.

The intracellular staining of T cells with 5, 6-carboxyfluorescein diacetate succinimedyl ester (CFSE) was used in combination with flow cytometry for tracking responder T cell populations in MLR. HMy2, HxU266, U266, HxKG-1and KG-1 stimulator cell lines were used in MLR to stimulate CFSE-stained allogeneic PBMC in order to investigate the phenotype of T cells involved in the immune response to hybrid cell lines versus their parent tumour cells. Phytohaemagglutinin (the strong T cell mitogen) was used as a control positive stimulator, and unstimulated PBMC at zero and 6 day time points as background controls.

4.2.1.5.1 T cell-subsets' proliferation in response to different stimulators

Prior to and at the end of MLR, responder cells were harvested, stained with anti-human CD3, CD4, or CD8 mAb, and analysed by flow cytometry to evaluate the different subsets of responder T cells, according to the cellular CFSE-intensity shift, which is directly proportional to the degree of T cell proliferation. To analyse flow cytometric data, a gate was applied on the forward scatter (FSC) x side scatter (SSC) dot plot to exclude dead cells and debris, and another gate on the dot plot representing positive populations of CD3, CD4 or CD8 (Figure 4.4). Histogram plots then were made for each T cell subtype derived from the individual gates, to evaluate the proportion of proliferating cells based on CFSE content (Figure 4.5). A summary of the proportions of proliferating T cells (CD3$^+$, CD4$^+$, and CD8$^+$ populations) is shown in Figure 4.6.
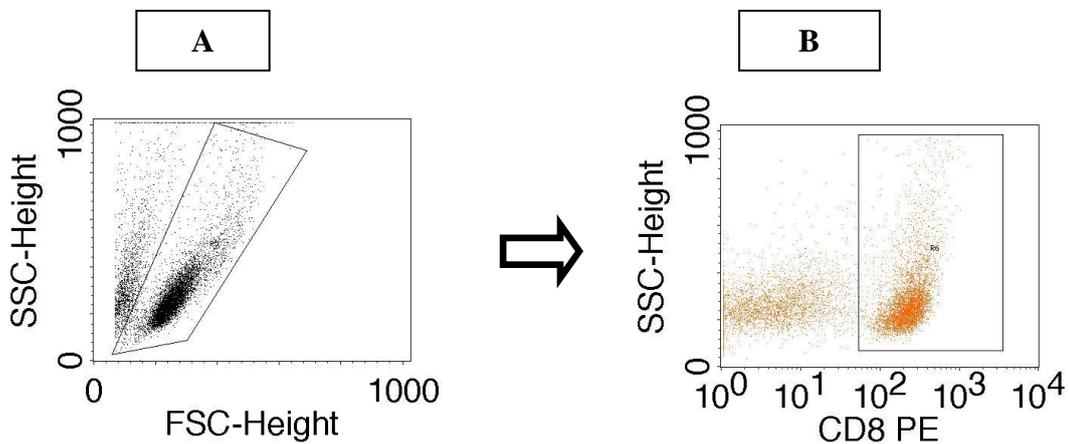
Figure 4.4: Gating process in flow cytometry data analysis. Dot plot (A) represents FSC x SSC gating on living PBMCs, and dot plot (B) represents staining of the live cells with CD8 mAb and a gate was made on CD8$^+$ T cell population to allow for analysis of CFSE content by the CD8$^+$ T cells.

In Figure 4.5, unstimulated PBMC showed a very low level of proliferation by the end of the incubation time. On the other hand, PHA stimulated cells showed successive rounds of proliferation, and more than five new generations of proliferating cells were observed, especially in total CD3$^+$ and CD4$^+$ populations. HMy2 also induced a high level of proliferation, but with less distinct peaks, and the highest proliferation level was seen in the CD8$^+$ population.

In order to compare the proliferative responses to HxU266 and HxKG-1 hybrid versus their parent tumour cells, U266 and KG-1 respectively, proliferating lymphocyte were compared with non-proliferating peaks following 6 days of incubation with stimulators. CD8$^+$ populations gave the highest proportion of responding cells to stimulation with HxU266 and HxKG-1, compared with their parent tumours, which induced much lower levels of proliferation. Furthermore, CD3$^+$ and CD4$^+$ populations also showed high degrees of proliferation in response to HxU266 and HxKG-1 hybrid cells, and much lower levels in response to stimulation with U266 and KG-1 parent tumour cells. .
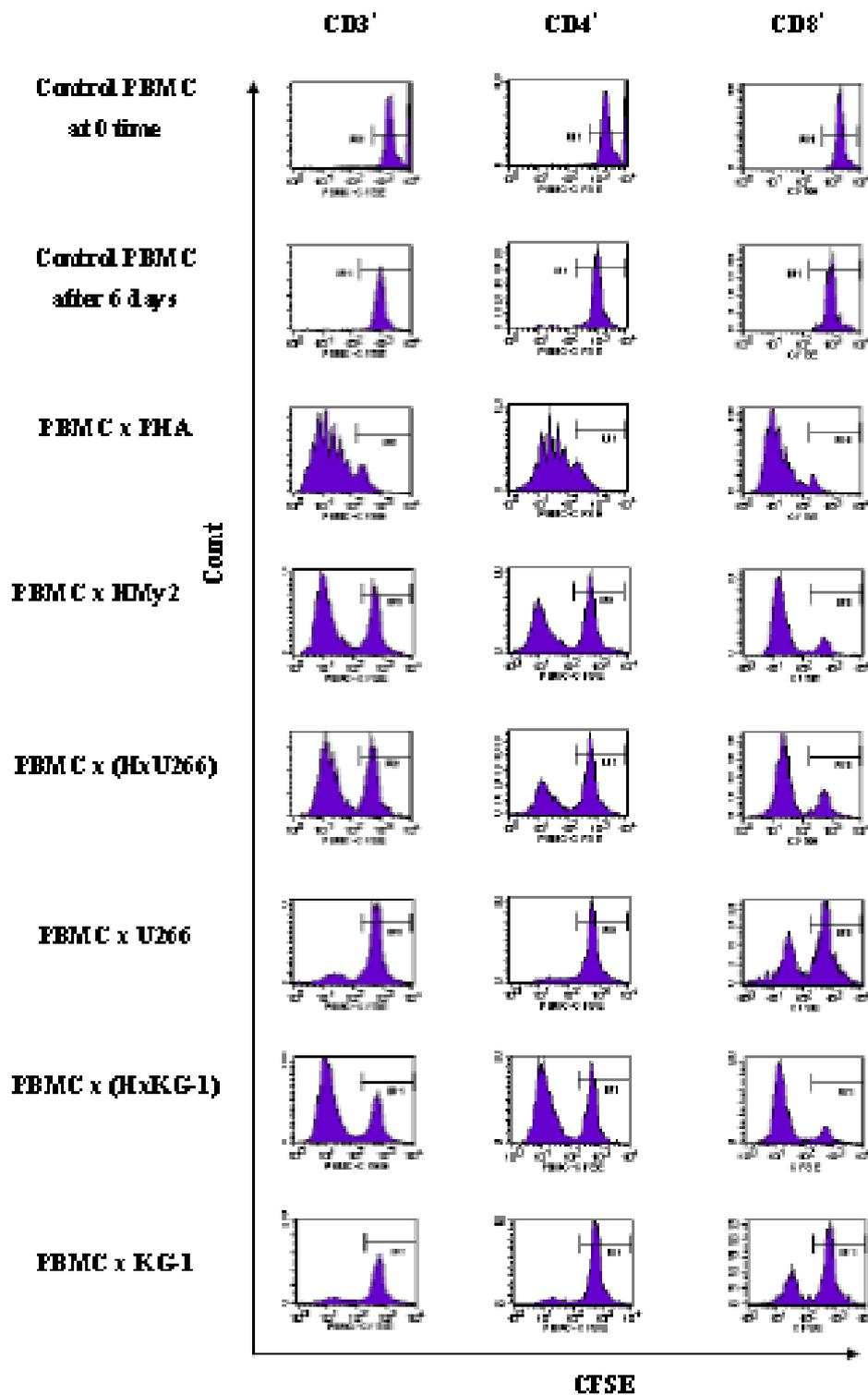
Figure 4.5: Flow cytometric histogram analysis of proliferating T cell subtypes in mixed lymphocyte reactions. Stimulatory cell lines are listed in the left hand legend. PHA-stimulated cells were used as the positive control, and unstimulated PBMC before and after incubation were used as background controls to set marker gates based on CFSE staining, to evaluate the % of proliferating populations under different stimulatory conditions.

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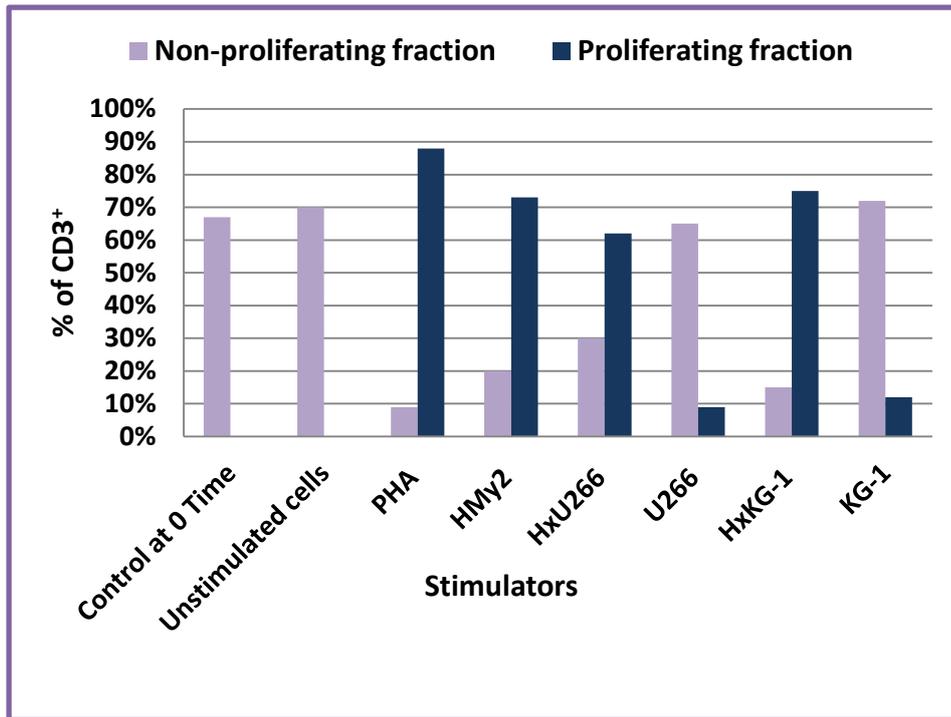
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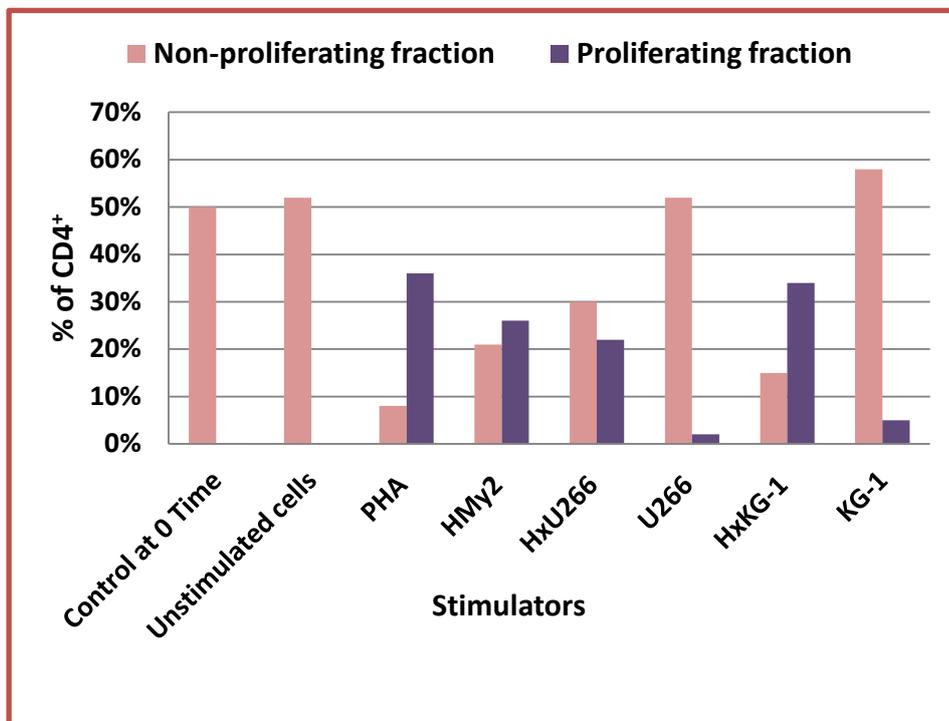
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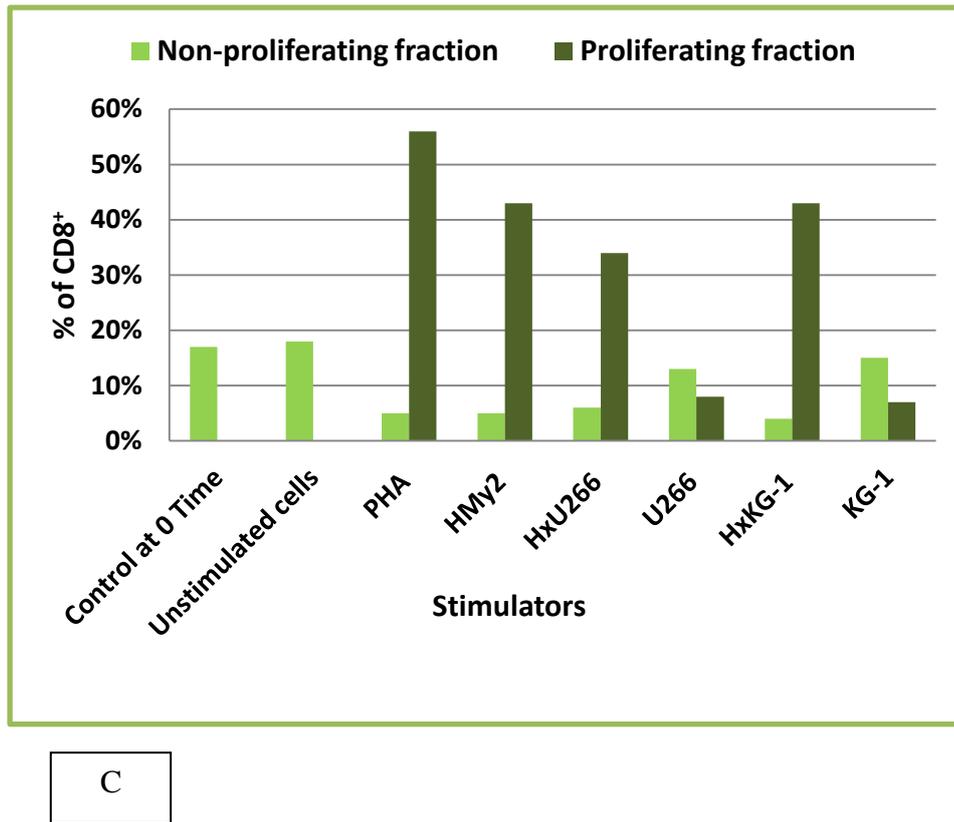
C

Figure 4.6: Chart representation of percentage of proliferating and non-proliferating T cell populations in response to stimulation with PHA, HMy2, a pair of hybrids and their parent tumour cell lines. Unstimulated PBMCs were used as control at 0 and 6 days time points. Chart (A): represent CD3$^+$, chart (B): CD4$^+$, and chart (C): CD8$^+$ T cells, all are presented as percentage value of the total live PBMC.

Figure 4.6 shows the percentage of the proliferating populations for each T cell subtype. Different populations are presented as % of the total live PBMCs, to show the change in normal ratios of CD8$^+$ and CD4$^+$ T cells before and after stimulation with the hybrid cell lines. There were no noticeable changes in the control PBMC before and after the incubation period, in which around 70% of living PBMC stained positive for CD3 mAb, 50% for CD4$^+$, and ~20% for CD8$^+$. PHA (the strong T cell mitogen) stimulated over than 80% of T cells to proliferate, mostly CD8$^+$ subset (50%) and to a lesser extent CD4$^+$ (35%). A similar result was observed with HMy2, where a higher proportion of the proliferating cells were CD8$^+$ (45%) than CD4$^+$ (25%). HxU266 gave rise CD3$^+$ T

cells proliferation 6 fold higher than its parent tumour cell line (U266), with over 60% of total T cells responding to HxU266, compared with 10% in case of U266. The $CD8^+$ population responded to HxU266 stimulation to a greater degree than $CD4^+$ population (35% compared with 25% respectively) although both $CD4^+$ and $CD8^+$ T cells responded more strongly to HxU266 than to the parent U266 cell line. In the same context, HxKG-1 hybrid showed over 7 fold more stimulatory effect than parent tumour KG-1 (70% and 10% respectively) for total $CD3^+$ T cells, and responding fractions were slightly higher in $CD8^+$ than $CD4^+$ populations (40% and 30% respectively). To conclude, CFSE staining was used to identify the proportions and phenotypes of responding T cells in MLR, in response to two hybrid cell lines and their relevant tumour cells. Data obtained showed that the hybrid cell lines induced elevated proliferative T cell responses of all T cell subtypes, but mainly $CD8^+$ T cells, compared with their parent tumour cells. Data of CFSE-labelling showed consistent results with the radioactive thymidine incorporation in MLR, in terms of the enhanced stimulatory capacities of hybrid cell lines compared with their respective parent tumour cells.

4.2.1.5.2 *Proliferation index in MLR*

Further flow cytometric analysis of the CFSE-stained, proliferating T cells in MLR was achieved by measuring the percentage of proliferating cells in each replicative generation from total living PBMC. Using PHA-stimulated cells as a positive control, six different marker gates were made on successive generations of the proliferating cells, in addition to the parent population, in each of $CD3^+$, $CD4^+$ and $CD8^+$ T cells. The proportion of cells in each gate was measured as percentage of the relevant subpopulation.

First, a gate was applied on living lymphocytes, excluding dead cells and debris, then a second gate was applied to the individual T cell subsets ($CD3^+$, $CD4^+$ or $CD8^+$),

followed by a histogram plot showing the generations of proliferating cells induced by each individual stimulator. Figure 4.7 shows an example of the gating process used for T cell subsets, followed by gating on each generation on CFSE histogram plot. Each generation of the proliferating cells was represented by an individual peak and measured as a percentage of the relevant T cell subpopulation (i.e. total proliferating generations plus non-proliferating cells equal to 100% for each T cell subtype). Different generations are presented in a column chart as a proliferation index (PI) for each T cell subset for all stimulators, and for unstimulated cells (Figure 4.8). PI of PHA, HMy2, and hybrid cell lines showed 6 distinct T cell generations plus non-proliferating cells, which indicates a mean generation time of 24 hours. The results also showed the induction of dividing T cells which were able to proliferate continuously in semi-logarithmic fashion in cycles 1-4 and declined back in cycle 5 and 6. Parent tumour cells could not induce clear proliferation cycles; albeit, lower levels of proliferation were seen in response to U266 and KG-1stimulation (Figure 4.8).
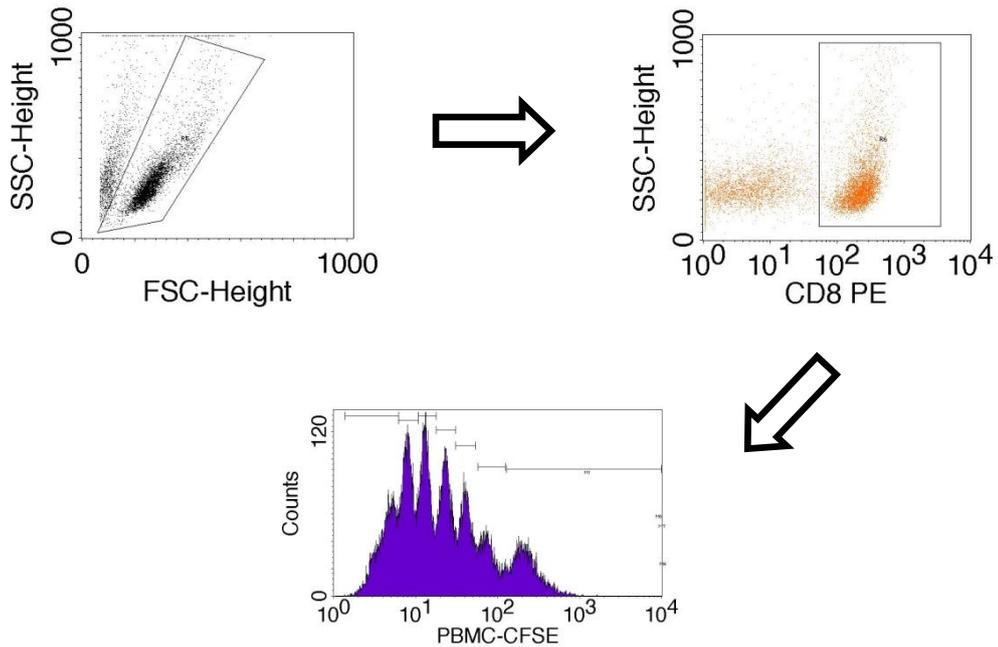
Figure 4.7: Plots of the flow cytometric analysis showing the gating process in order to calculate the proliferation index. First gate was made on living lymphocytes, second was on CD8$^+$ T population, then histogram plots were used to identify new generations of proliferating cells.
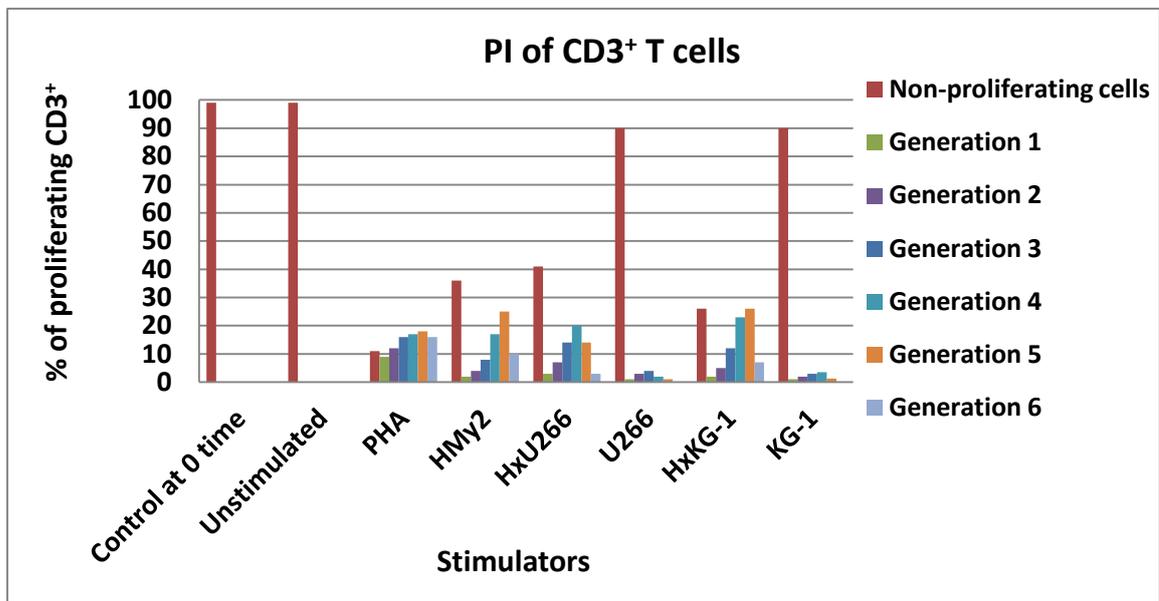
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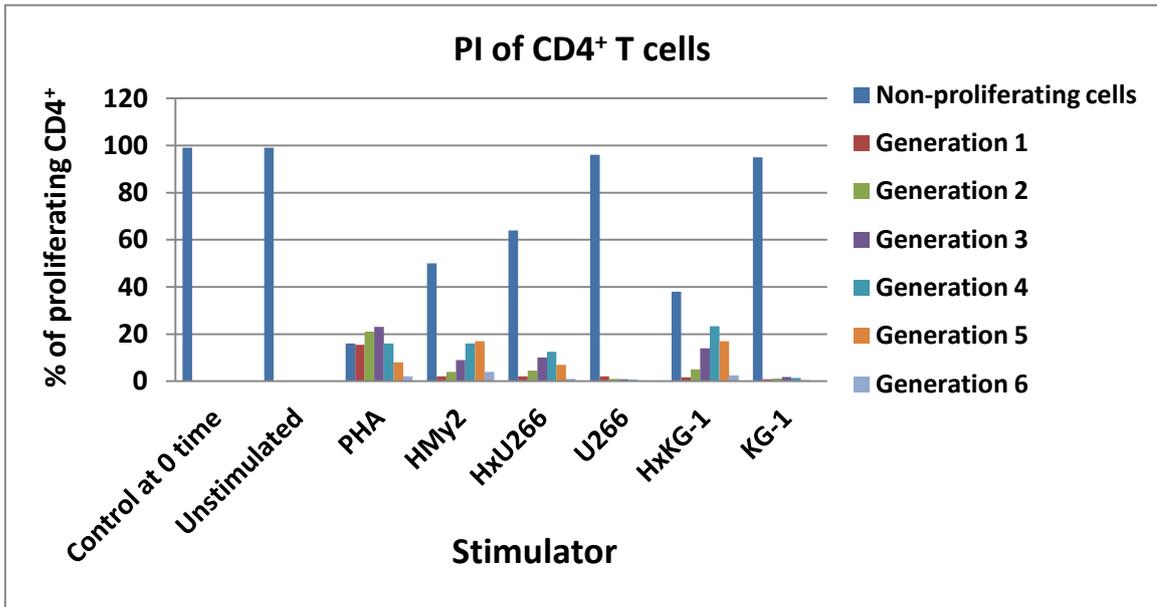
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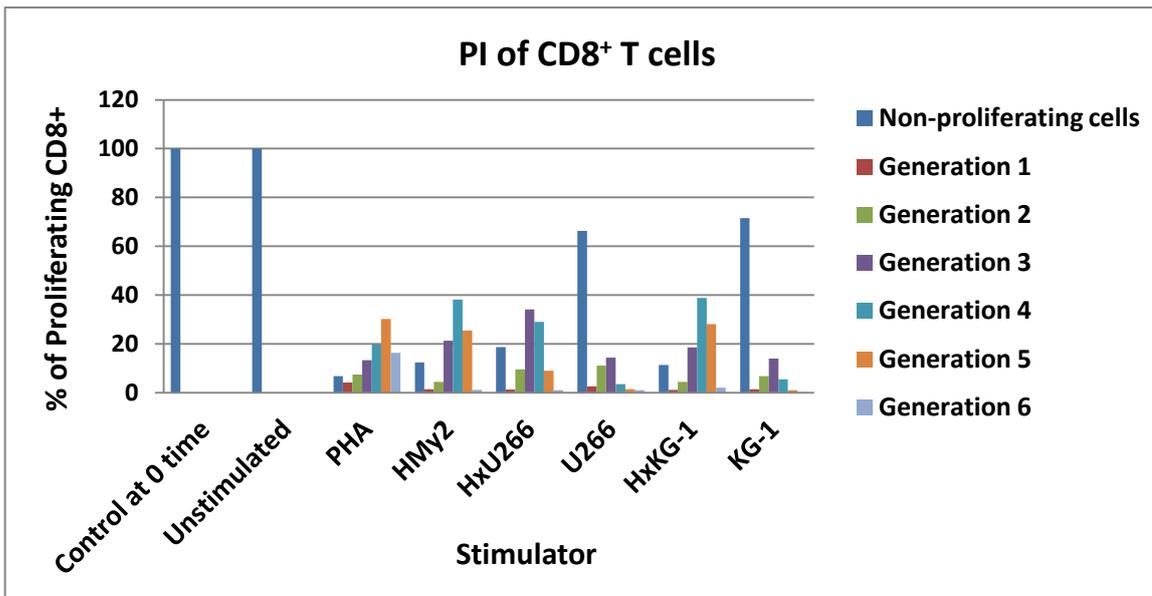
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Figure 4.8: Chart representation of proliferation index (PI; percentage of individual generations of the proliferating T cell subpopulation) induced by different stimulators, and of unstimulated PBMC at 0 and 6 day time points. Chart (A): represents CD3^{+}, chart (B): CD4^{+}, and chart (C): CD8^{+} T cell subpopulations.

4.2.1.5.3 Naive, memory, and effector T cell phenotyping in MLR

T cell populations can be divided into naive or memory cells. The naive cells, which have not encountered any antigens before, express both CCR7 (lymph node homing chemokine receptor) and CD62L (L-selectin), in addition to CD45RA. Naive T cells have the ability to circulate in the blood and penetrate to secondary lymphoid tissues using L-selectin and homing receptors, and once they encounter cognate antigen associated with sufficient co-stimulation, naive T cells proliferate and differentiate into either effector (characterised by expression of CD45RO/RA and absence of CCR7 and heterogeneous for CD62L expression) or memory (characterised by expression of CD45RO, CCR7, and CD62L) T cells. Naive T cells need two signals, provided by professional APC, for complete differentiation; the first is recognition of cognate peptide in the context of an appropriate HLA molecule, and the second is the appropriate co-stimulation provided by CD80/CD86 and other ligands. Effector memory (EM) cells are characterised by rapid onset effector responses, such as release of cytokines and cytolytic molecules. On the other hand, central memory (CM) cells are highly sensitive to antigenic stimulation through their TCR, and are less dependent on co-stimulatory signals than naive T cells. In addition, CM cells release IL-2, and in the secondary immune responses they differentiate to effector cells (Sallusto *et al.*, 2004). The normal composition of PBMCs contains all the previously mentioned T cell types, and upon allogeneic stimulation it was expected to see different levels of proliferation and differentiation affecting all or some of these T cell subsets, according to type and immunogenicity of the stimulator cells.

To investigate the proliferation and phenotype of allogeneic T cells before and after co-culturing with hybrid cell lines in MLR, CFSE-labelled cells were harvested and stained with mAb against the secondary lymphoid tissue homing-receptor, CCR7, and protein

tyrosine phosphatase receptor type C, CD45 isoform RA, in addition to anti-CD4 or CD8 mAb. Flow cytometric analysis also showed the reduction in CFSE level, to investigate the degree of proliferation of each phenotypically defined cell type.

Gates were made on live PBMC on FSC x SSC dot plot, and then on CD4$^+$ or CD8$^+$ populations on second dot plot. The third plot showed CCR7 on the X-axis and CD45RA on the Y-axis, where a quadrant gate was applied, (Figure 4.9).
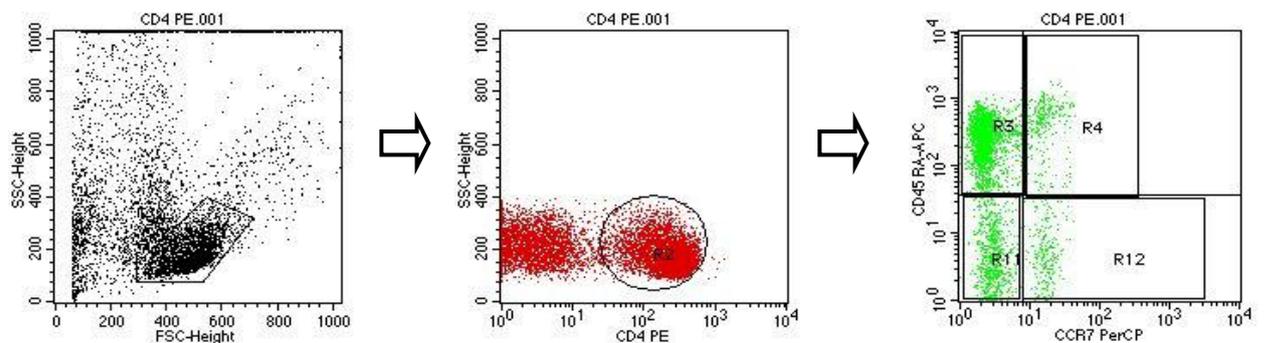
Figure 4.9: Three dot plots showing gating processes in flow cytometric analysis of the proliferating T cells' phenotype. From left to right: first plot showing FSC x SSC plot to gate on live PBMC; second plot showing CD4-PE x SSC-height to gate on CD4$^+$ population, last plot showing CCR7-PerCP x CD45RA-APC and containing quadrant marker of four gates: upper left (CD45RA$^+$/ CCR7$^-$), upper right (CD45RA$^+$/ CCR7$^+$), lower left (CD45RA$^-$/ CCR7$^-$), and lower right (CD45RA$^-$/ CCR7$^+$), to represent EMRA$^+$, naive, EM, and CM populations respectively.
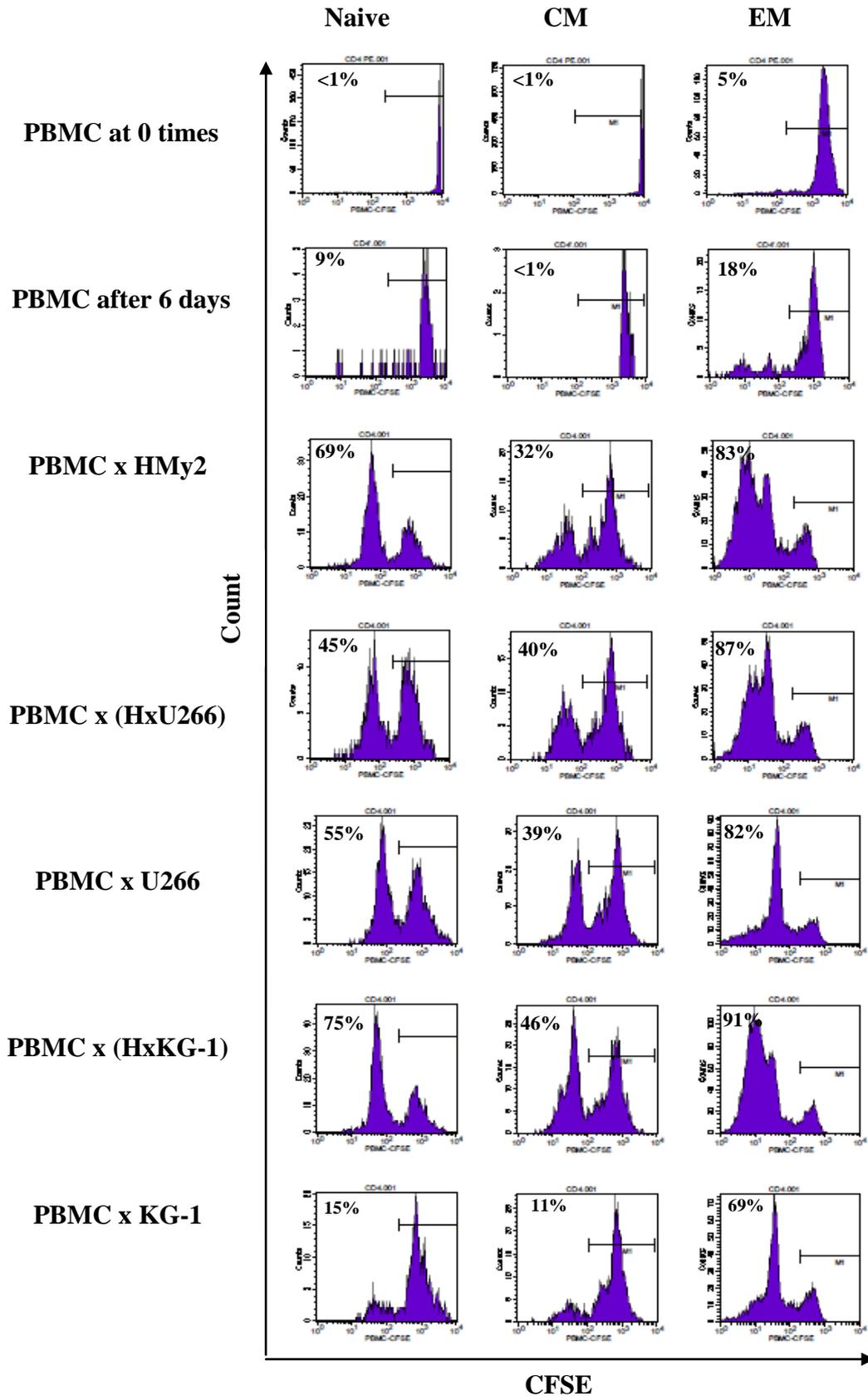
Figure 4.10: Flow cytometric analysis histogram plots showing proliferation of CD4$^+$ naive, CM (central memory), and EM (effector memory) populations with and without stimulation in MLR. The percentage values shown represent the proportion of proliferating cells of each phenotype under different stimulatory conditions.
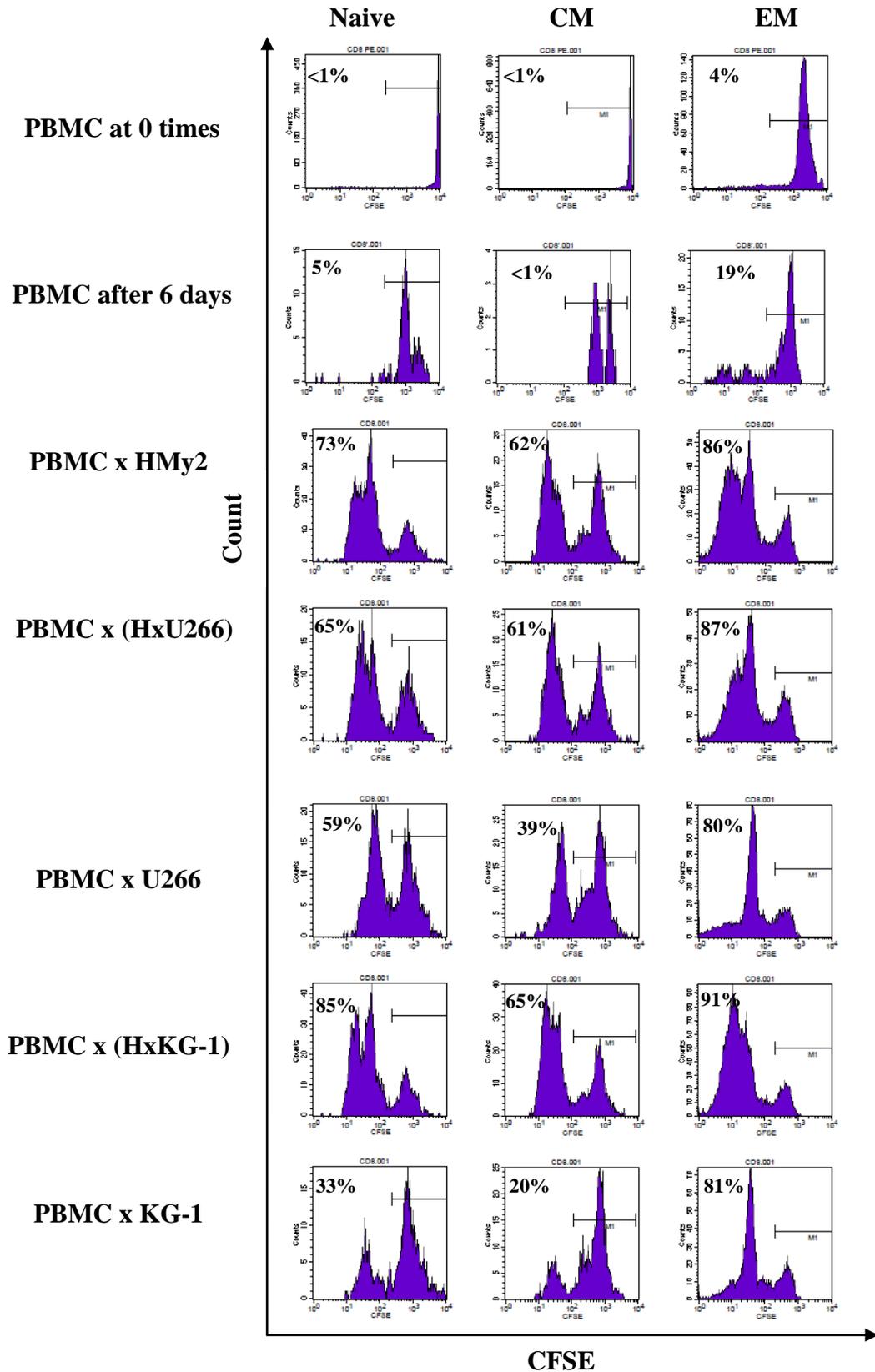
Figure 4.11: Flow cytometric analysis histogram plots showing proliferation of CD8^{+} naive, CM, and EM populations in response to different stimulators and unstimulated in MLR. The percentage values shown represent the proportion of proliferating cells of each phenotype under different stimulatory conditions.

The quadrant gate in Figure 4.9 showed four sub-groups of T cells according to expression pattern of CD45RA and CCR7. The upper left quadrant represents effector memory-RA$^+$ (T$_{EMRA}$; CD45RA$^+$/ CCR7$^-$), upper right represents naïve (Naïve; CD45RA$^+$/ CCR7$^+$), lower left represents effector memory (T$_{EM}$; CD45RA$^-$/ CCR7$^-$), and lower right represents central memory T cells (T$_{CM}$; CD45RA$^-$/ CCR7$^+$).

Figure 4.10 showed the proliferative responses of naive, CM, and EM CD4$^+$ T cells following stimulation with HMy2, hybrid cell lines, and relevant tumour cells. Unstimulated PBMC were used as negative control, in which no response was seen at zero time, however, weak responses were seen in naive and EM cells at day 6. HMy2 induced naive, CM and EM proliferative responses, although with relatively low CM proliferation level. Both of HxU266 and their parent tumour cells, U266, induced high proliferative responses in naive, CM and EM subtypes, with highest levels of proliferation seen in EM cells. On the other hand, HxKG-1 showed higher naive, CM, and EM responses than relative tumour KG-1 cell line. Figure 4.11 shows the same data but for CD8$^+$ T cells. Unstimulated cells showed low levels of proliferation after the incubation time. HMy2 stimulated high naive, CM, and EM proliferation rate, even higher than the corresponding CD4$^+$ response. The hybrid HxU266 cell line induced slightly higher proliferative responses than parent tumour cells (U266) in both of naïve and CM, whilst, similar EM responses were seen. The highest response was seen in EM population, and total CD8$^+$ responses were higher than corresponding CD4$^+$ T cells. HxKG-1 hybrid cell line was a stronger inducer for naive and CM cell responses than KG-1, but similar EM responses were seen. The overall CD8$^+$ responses were stronger than CD4$^+$ responses.

It can be concluded from these experiments that hybrid cell lines were able to induce allogeneic naive, EM, and CM T cell proliferative responses. These responses were stronger in hybrid-stimulated than parent tumour-stimulated T cells for HxKG-1 and KG-1, although U266 stimulated similar levels of proliferating T cells as HxU266. The data also confirmed that CD8$^+$ T cells were more susceptible to hybrid cell stimulation than CD4$^+$ T cells, a result which is consistent with that demonstrated in Section 4.2.1.5.1

4.2.2 IFN-γ Enzyme Linked ImmunoSpot (ELISpot) assays

Gamma interferon (IFN-γ) is one of the Th1 cytokine group. It is released *in vivo* by different immune cells, such as activated T lymphocytes, NK and NKT cells, in response to intracellular infection or tumour invasion. *In vitro* detection of IFN-γ on an individual cell basis was available using ELISpot assays to evaluate the number of responding lymphocytes on co-culturing with different stimulator cells.

Allogeneic healthy and tumour-bearing patients' PBMCs were used in IFN-γ ELISpot assays to quantitatively estimate their functional response by co-culturing with HMy2, hybrid and parent tumour cells for different time courses.

4.2.2.1 Short term IFN-γ ELISpot assays

In short-term ELISpot assays, responder PBMCs were co-cultured with the stimulator cells in triplicate wells in ratio of 3:1, and under optimised experimental conditions (mentioned in Section 2.5.1), for 48 hours. By the end of the experiment, developed spots were counted and the number of spots per 1x10^6 responder PBMCs was calculated and plotted for each stimulated cell line.

4.2.2.1.1 Allogeneic short term ELISpot assays

PBMC from allogeneic healthy donors were co-cultured with different stimulator cells such as HMy2, hybrid, and parent tumour cell/ cell lines in anti-IFN-γ mAb-coated 96-well plates as described in Section 2.5.1.
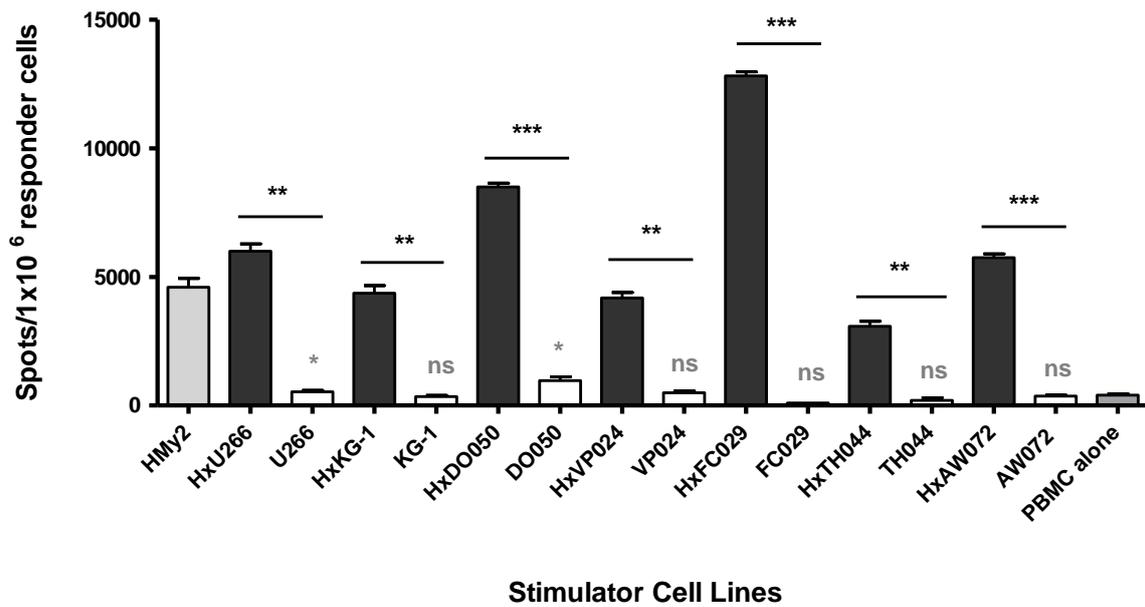
Figure 4.12: Short term allogeneic IFN-γ ELISpot assay using HMy2, a group of hybrid cell lines and their relevant parent tumour cells. Responder cells were mixed with stimulator cells in a ratio 3:1 respectively, and plates were incubated for 48 hours. The emerged spots were counted and the number of spots per $1x10^6$ responder cells was plotted. Data are presented as mean of triplicate wells ± SEM. Black stars show the degree of significance of difference between each hybrid and its parent tumour cell responses, while grey stars show the significance of difference between tumour cells and background control PBMC responses.

HMy2 and all the stimulator hybrid cell lines induced T cell IFN-γ-releasing responses to a significantly greater degree than the relevant parent tumour cells. On the other hand, most tumour cells did not induce IFN-γ releasing responses above background levels (Figure 4.12). The short incubation time used in the assays would not induce naive T cell response, but induced effector or memory T cell responses.

4.2.2.1.2 Autologous short term ELISpot assays

Tumour bearing patients' PBMCs were used as autologous responders in short term IFN-γ ELISpot assays, by co-culturing with the patient's own tumour cells, relevant hybrid cell line, or HMy2 cells, followed by incubation for 48 hours under the previously mentioned conditions (Section 2.5.1). Autologous PBMC from B-CLL (JR004 and AW072), AML (VP024 and DO050), and MCL (TH044 and FC029) patients were used as effector cells in the ELISpot assays. The autologous T cell IFN-γ releasing responses to relevant hybrid cells were significantly greater than the responses to respective matched tumour cells, in most cases, generating comparable responses to that seen in response to HMy2 cells. There was no evidence of significant autologous T cell IFN-γ releasing response to the patient's tumour cells *in vitro,* except in case of B-CLL patients (AW072 and JR004), where significant responses were observed (Figure 4.13). In all autologous responses, the number of IFN-γ-releasing cells is lower than relevant allogeneic response, which may be attributed to the low allo-MHC molecules, immune-suppressed state and low number of patient T cells.

These data indicate that in short-term IFN-γ releasing ELISpot assays, patients' T cells were immunocompetent in the presence of the appropriate co-stimulatory signals. In most cases, however, the patients showed no significant responses to their own tumour cells. Meanwhile, these data do not indicate the presence of tumour-specific T cell responses to the hybrid cell lines, but the enhanced responses may be attributed to the semi-allogeneic nature of hybrid cell lines (by expression of HMy2-derived HLA antigens).
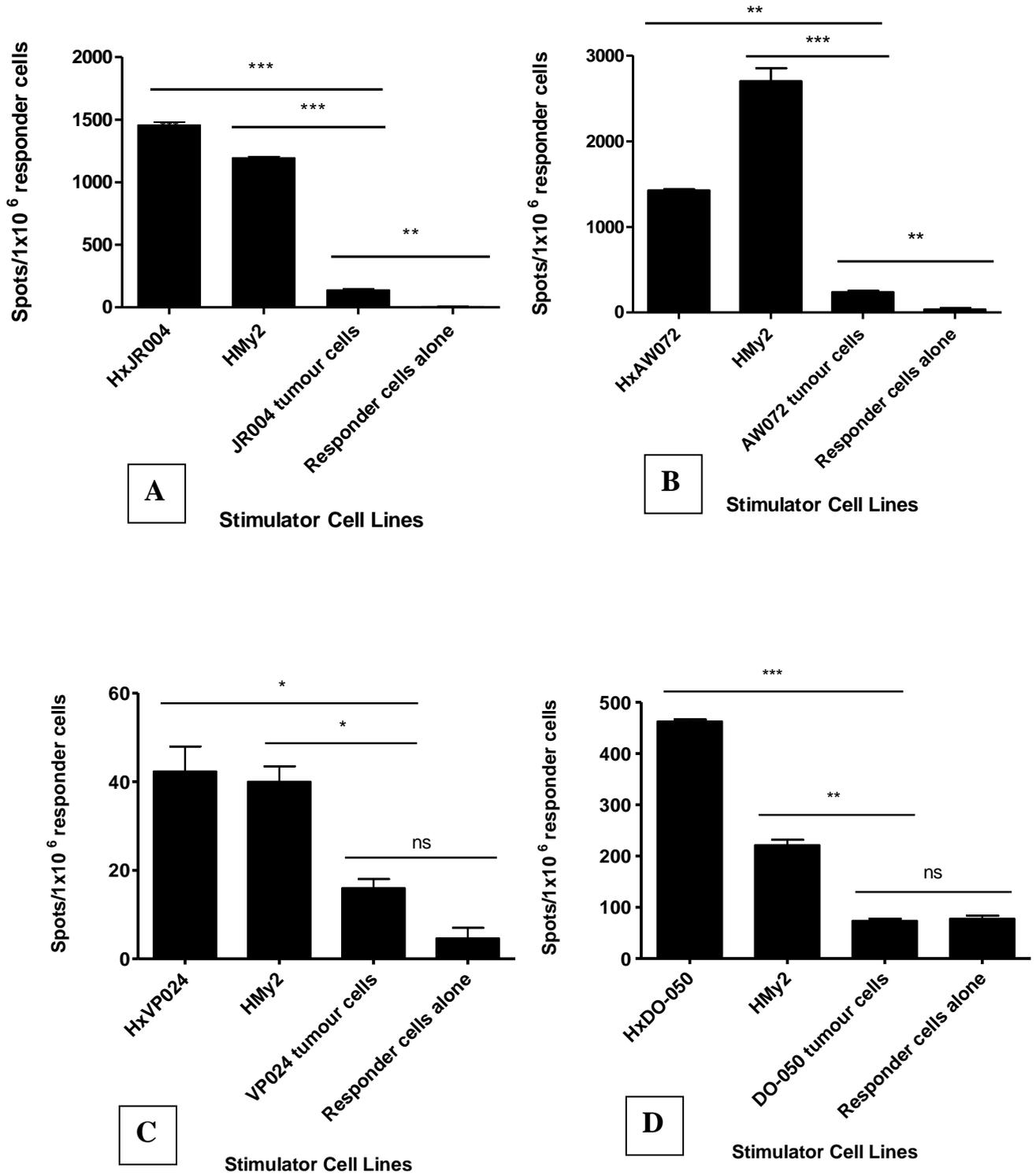
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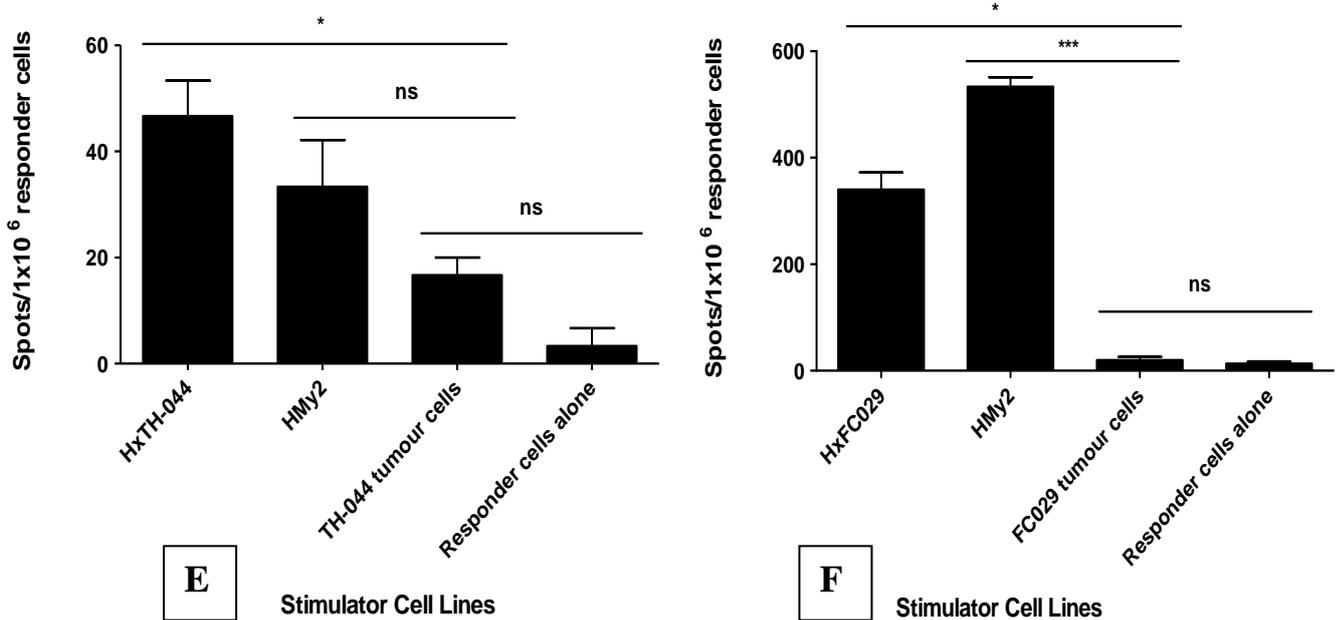
Figure 4.13: Short-term autologous IFN-γ ELISpot assays using HMy2, a group of hybrid and their relevant parent tumour cell/ cell lines as stimulators. Charts (A), (B), (C), (D), (E), and (F) represent the effect of hybrid cell lines and parent tumour cells of patients JR004, AW072, VP024, DO050, TH044 and FC029 respectively, in addition to HMy2 as a positive control, on induction of IFN-γ releasing responses by relevant, autologous PBMC cells. Relevant patient's responder PBMCs were co-cultured with stimulator cell lines in ratio 3:1 respectively and, incubated for 48 hours. The emerged spots were counted and number per 1×10^6 responder cells was calculated. Data are presented as mean of triplicate ± SEM.

4.2.2.2 Long term IFN-γ ELISpot assays

In order to investigate the ability of the hybrid cell lines and their parent tumour cells to induce primary T cell immune responses, long-term cultures of allogeneic (from healthy donors) and semi-autologous (from tumour bearing patients') PBMCs were stimulated *in vitro* for several rounds, each round of seven days, using new batches of Mitomycin-C-treated stimulator cells, as described in Section 2.5.3. HMy2 cells were used as positive control stimulators.

4.2.2.2.1 Allogeneic long-term IFN-γ ELISpot assays

Allogeneic PBMCs from healthy donors were stimulated for up to 4 rounds of stimulation, after which responder cells were harvested and used in 48 hour IFN-γ ELISpot assays by re-stimulation with stimulator cells, which include the relevant tumour cells and their respective hybrid cell lines, HMy2, K562 cells (as NK target), and (in some cases) an un-related tumour cell line. In addition, responder cells were cultured alone as background controls. Different hybrid cell lines, in addition to HMy2, were able to induce proliferation and differentiation of allogeneic PBMC in these cultures, whilst parent tumour cells failed to stimulate long-term allogeneic PBMC activation, with a progressive decline in responder cell numbers over 2 rounds of stimulation. I was therefore only able to perform ELISpot assays on PBMC cultures stimulated by HMy2 cells and hybrid cell lines.

4.2.2.2.1.1 Allogeneic responses to HMy2 and tumour cell line-derived hybrids (HxU266, and HxKG-1)

HMy2, HxU266, and HxKG-1 cell lines were used *in vitro* to stimulate allogeneic PBMCs from normal healthy donors (two different donors were stimulated with each cell line) for four consecutive rounds. The activated PBMCs were used as responders in setting up IFN-γ ELISpot assays by co-culturing with different stimulator cell lines, including HMy2, relevant hybrid and parent tumour cell lines, and an un-related tumour cell line, in addition to K562 as NK stimulant (Figures 4.14 - 4.16).
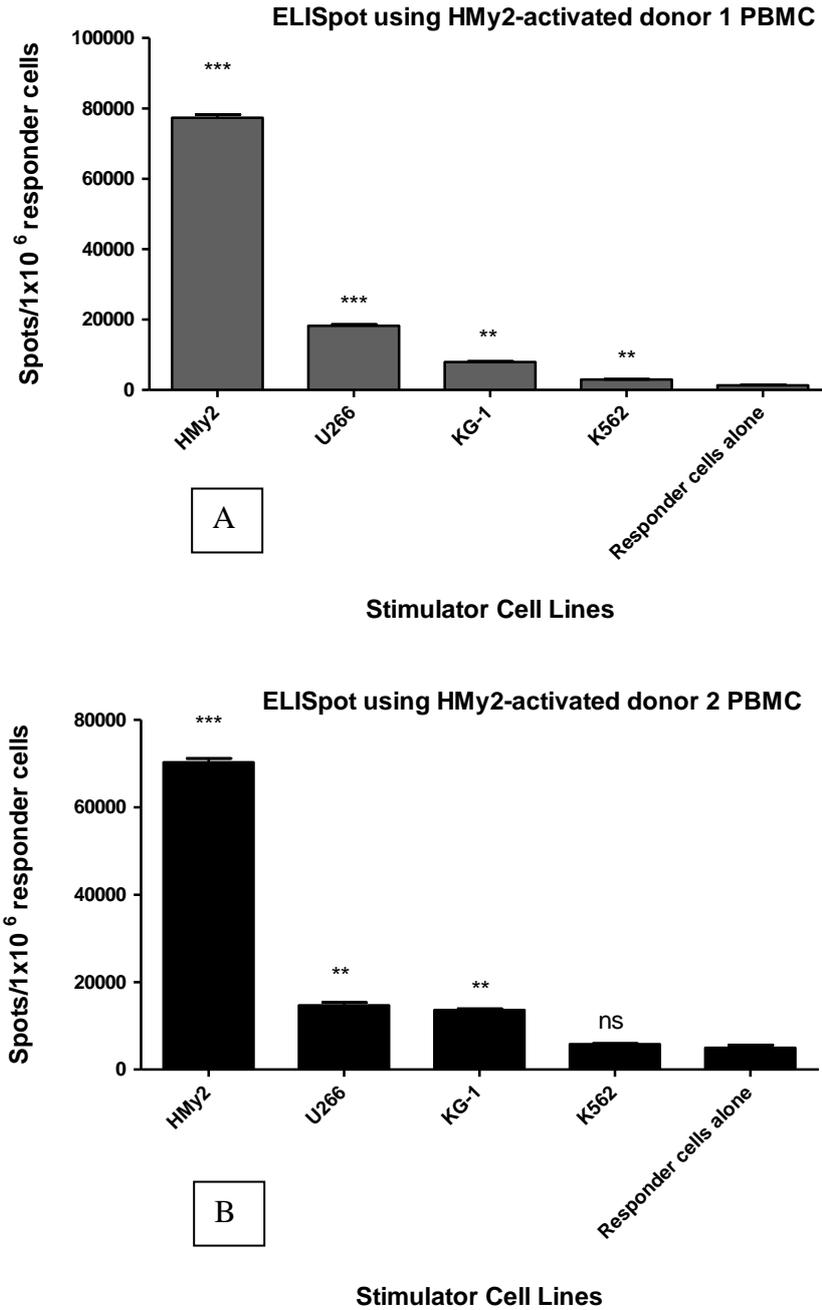
Figure 4.14: Long-term IFN-γ ELISpot assay. Responder cells (HMy2-activated allogeneic PBMC) were mixed with different stimulator cell lines (as labelled in the horizontal legend) in 48 hours ELISpot assays. Charts (A) and (B) represent responses of 2 independent, healthy allogeneic donors. Data are presented as mean ± SEM of triplicate wells.
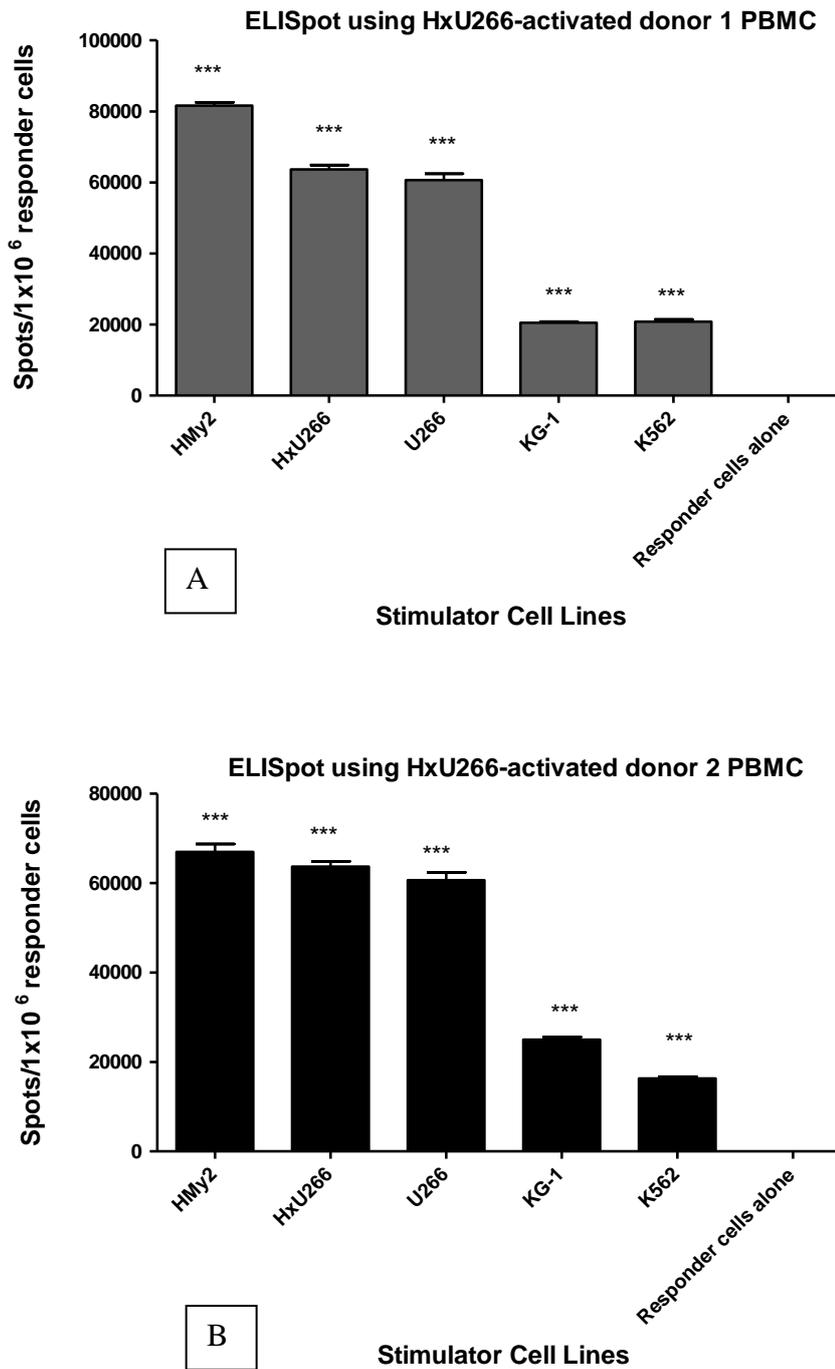
Figure 4.15: Long-term IFN-γ ELISpot assay. Responder cells (HxU266-activated allogeneic PBMCs) were mixed with different stimulator cell lines (as labelled in the horizontal legend) in 48 hours ELISpot assays. Charts (A) and (B) represent responses of 2 independent, healthy allogeneic donors. Data are presented as mean ± SEM of triplicate wells.
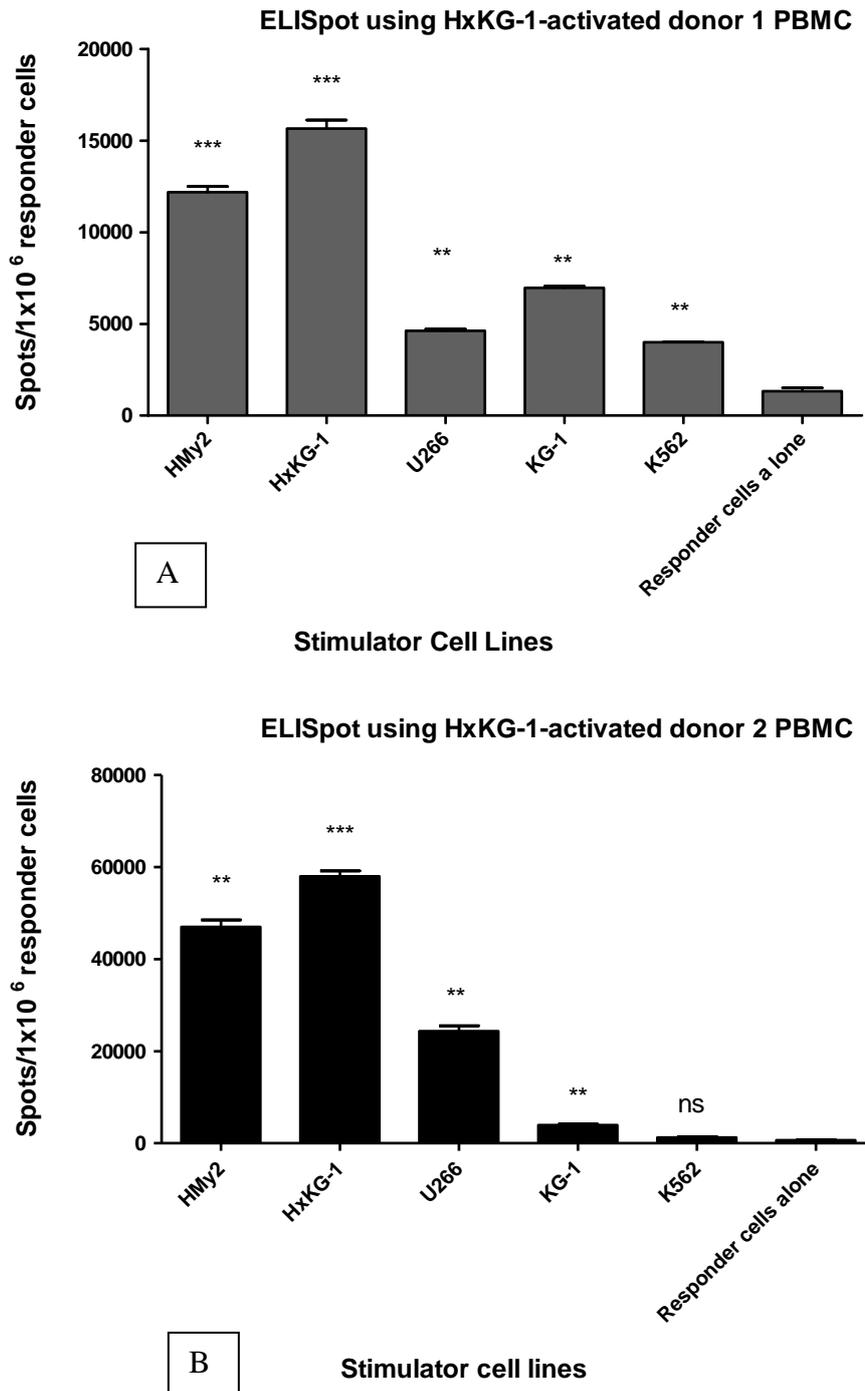
Figure 4.16: Long-term IFN-γ ELISpot assay. Responder cells (HxKG-1-activated allogeneic PBMCs) were mixed with different stimulator cell lines (as labelled in the horizontal legend) in 48 hours ELISpot assays. Charts (A) and (B) represent responses of 2 independent, healthy allogeneic donors. Data are presented as mean ± SEM of triplicate wells.

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HMy2-stimulated cultures showed strong IFN-γ releasing responses to HMy2 in both donors, with weak but significant responses to both of U266 and KG-1 tumour cell lines, and weak NK activity was seen in one but not in the other donor. These data suggest the presence of shared histocompatibility molecules between HMy2 and U266/ KG-1 tumour cell lines (Figure 4.14).

HxU266-stimulated cultures showed strong IFN-γ releasing responses to HMy2, HxU266, and U266 cell lines, significantly weaker responses to KG-1, and weak but significant K562-induced NK activities in both donors. The highly comparable responses to HMy2, HxU266, and U266 stimulation suggests induction of specific responses against allogeneic major and minor histocompatibility (MHC and mHC) molecules expressed by HMy2 and U266 as well as by the stimulator hybrid cell line (HxU266), and possibly against some tumour antigen-specific responses (Figure 4.15).

HxKG-1-stimulated cultures showed significant responses to HMy2 and HxKG-1 (strong in one donor but weaker in the other), significant but weaker responses to both U266 and KG-1 cell lines (KG-1 responses were weak in one and strong in the other donor), which suggested stronger induction of responses against MHC/mHC of HMy2 than KG-1, with some shared alloreactivity to U266. Moreover, induced NK activity was seen in one donor but not in the other (Figure 4.16).

4.2.2.2.1.2 Allogeneic response to ex vivo tumour-derived hybrid cell lines

Similar experiments were carried out using hybrid cell lines derived from *ex vivo* tumour cells as stimulators of allogeneic PBMCs in long-term cultures. T cell cultures, after four rounds of stimulation, were harvested and used as responder cells in 48 hour IFN-γ release ELISpot assays, using HMy2, K562, and relevant hybrid and parent tumour cells as stimulators (Figures 4.17 and 4.18). Hybrid HxIC038, HxTH044 and

HxDO050 cell lines induced long-term activated PBMC cell cultures, and by re-stimulating these cell cultures in ELISpot assays using relevant hybrid, parent tumour, and HMy2 cell lines, significant IFNγ releasing responses were seen. HxIC038-stimulated culture induced significantly enhanced responses to re-stimulation with HMy2, the relevant hybrid cell line and parent tumour cells. Similarly, HxDO050-activated cells induced significantly higher responses to HMy2, the relevant hybrid cells, and parent tumour cells. In contrast, HxTH044 induced significant responses to HMy2 and the hybrid cell line, but not to parent tumour cells. In all cases, results showed lack of NK activity in all cell cultures induced by these three hybrid cell lines (Figure 4.17A, B and C respectively).

In another set of long-term allogeneic ELISpot assays, two MM hybrid cell lines (HxRC056 and HxCM053) were used to induce long-term PBMC cell cultures. These activated cell cultures were used as responder cells to investigate the IFNγ releasing responses. Re-stimulation of responder cells with HMy2, relevant hybrid cells, and the MM tumour cell line (U266; as an alternative to parent tumour cells, which were not available), showed elevated responses to all relevant cells (including U266), and lack of NK activity in both cell cultures (Figure 4.18). The observed high responses to re-stimulation with HMy2 and relevant hybrid cell lines (stimulatory cell line) in all the activated cultures suggests induction of specific T cell responses against MHC/mHC antigens expressed by HMy2 and the stimulatory hybrid cell lines.
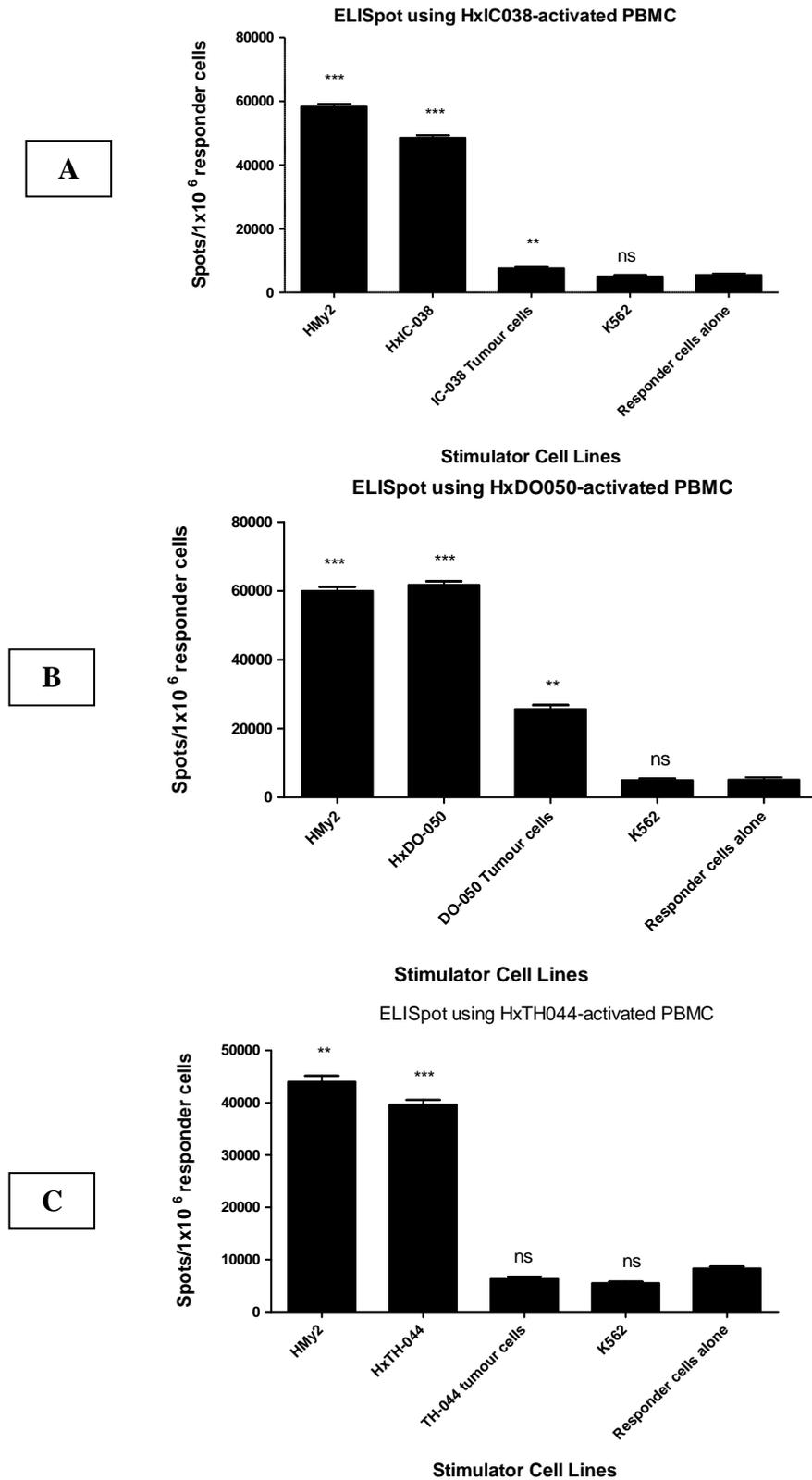
Figure 4.17: Long term allogeneic ELISpot assays using hybrid cell line-activated T cell cultures, charts (A), (B), and (C) representing HxIC038, HxDO050, and HxTH044-activated allogeneic PBMC as responder cells. HMy2, K562, and relevant hybrid and tumour cells were used as stimulator cells. Data are presented as mean ± SEM of triplicate wells.
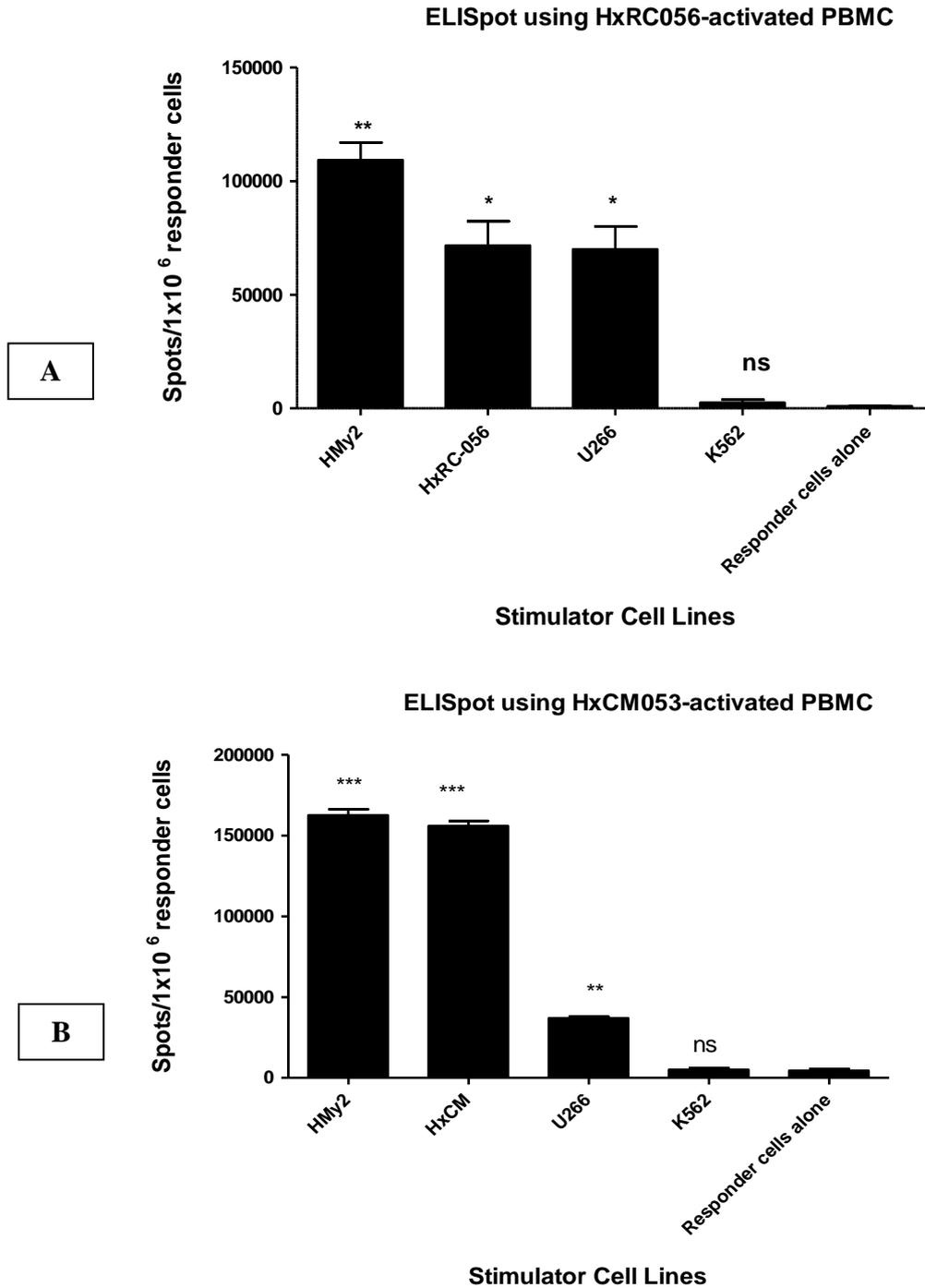
Figure 4.18: Long-term allogeneic ELISpot assays using different hybrid cell line-activated T cell cultures, charts (A), and (B) represent HxRC056 and HxCM053-activated PBMC as responder cells. HMy2, K562, and relevant stimulatory hybrid cells and MM-lineage tumour cells were used as stimulator cells. Data are presented as mean ± SEM of triplicate wells

The enhanced responses to parent tumour cells seen following stimulation with HxIC038 and HxDO050 hybrid cell lines may indicate the presence of T cell responses to tumour-specific elements/ as well as alloantigens expressed on parent tumour cells, although further experiments were required to establish the presence of tumour-specific T cell responses. Precise assessment of the presence of tumour-related immune responses within the long-term hybrid-activated cultures is presented in Chapter 5.

To conclude, long term stimulation of allogeneic PBMCs with hybrid cell lines induced T cell cultures with enhanced IFN-γ releasing responses to re-stimulation with HMy2 and the hybrid cell lines that were used for induction of the T cell cultures. In some cases, the activated T cell culture also showed significant responses to re-stimulation with the parent tumour cells (from which the stimulator hybrid cell line was derived), which may imply the presence of shared tumour- or allo-specific responses (such as response to U266, IC038, and DO050 tumour cells), which cannot be confirmed using this assay alone. Moreover, results showed little evidence of NK activity within the activated T cell cultures.

4.2.2.2.2 *Autologous long-term IFN-γ ELISpot assays*

PBMCs from two AML patients (DO050 and VP024) were used *in vitro* to induce long-term T cell cultures by stimulation with relevant hybrid cell lines (made from patient tumour cells) (Section 2.5.3). Stimulation of semi-autologous PBMCs by HxDO050 and HxVP024 hybrid cells for up to 8 rounds resulted in more than tenfold increase of IFN-γ releasing cells in response to re-stimulation with the relevant autologous parent tumour cells (DO050 and VP024), compared with results obtained from short-term autologous ELISpot assays.

In addition, several hundred folds increase in response to HMy2 and semi-autologous hybrid cell lines were observed, compared with short-term ELISpot assays (Figure 4.13 and 4.20). There was no significant IFN-γ response to K562 stimulation in both cultures, which indicates absence of NK activity within the activated T cell cultures. Furthermore, I did not have sufficient PBMCs from the other patients to perform further autologous long-term assays. Autologous ELISpot data suggest that at least a component of the T cells activated by the hybrid cell lines may be tumour-specific and able to recognise the autologous tumour cells *in vitro* (Figure 4.20).
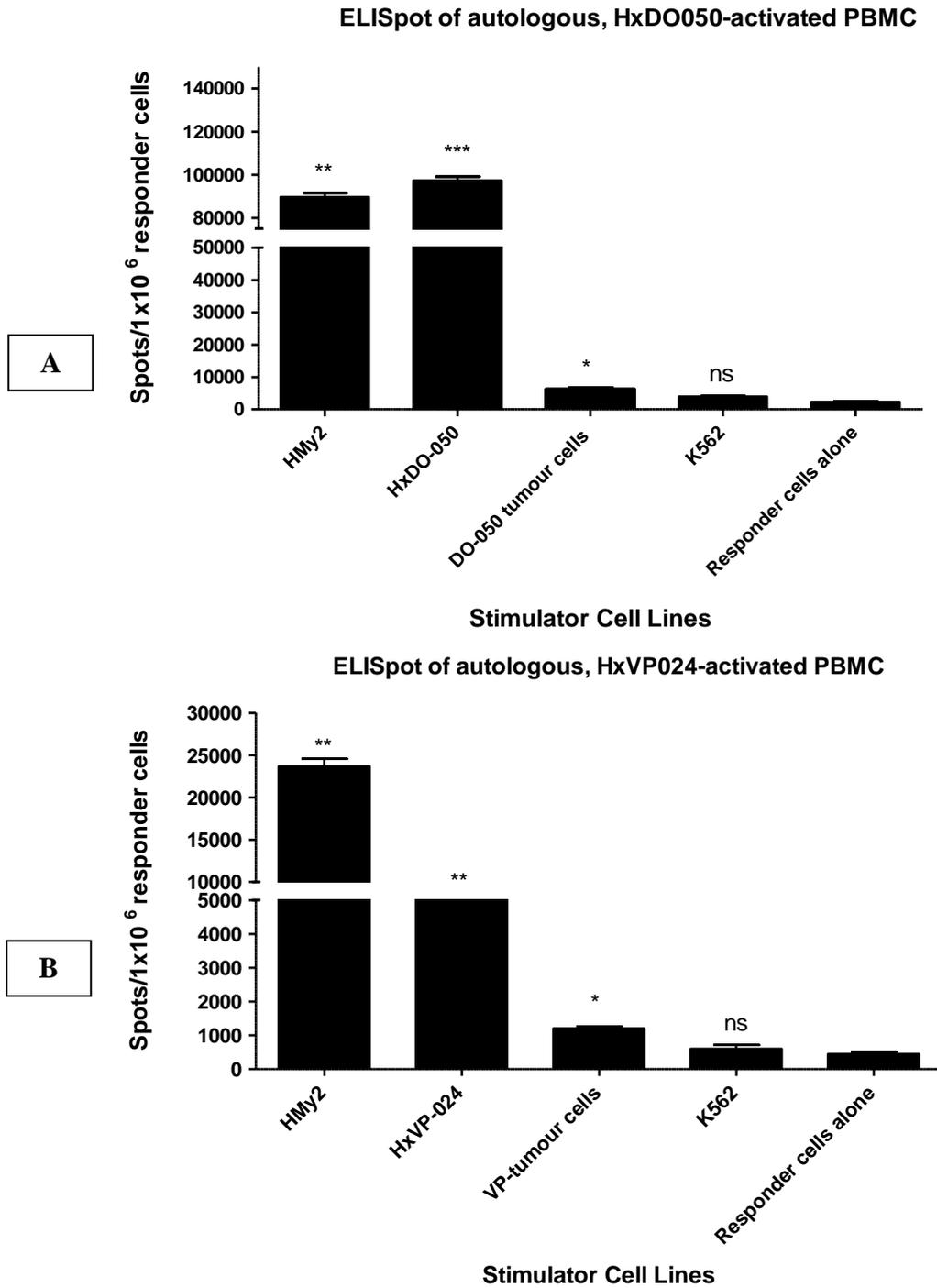
Figure 4.20: Long-term autologous ELISpot assays using HxDO050 and HxVP024-activated autologous PBMC after 8 rounds of stimulation. Activated cells were re-stimulated using their autologous tumour, semi-autologous hybrids, HMy2, and K562 cells. Chart (A) and (B) represent data of HxVP024 and HxDO050 activated autologous PBMC respectively. Results are presented as mean ± SEM for triplicate wells.

4.2.3 Induction of allogeneic cytotoxic T cell responses

Allogeneic T cell cultures were generated *in vitro* by stimulation of PBMC from healthy donors with different selected hybrid cell lines for 4 weeks in culture in the presence of rhIL-2 (Section 2.5.3.1). These cultures were investigated for their cytolytic capability against the parent tumour cells from which the stimulator hybrid cell lines were generated, and against the relevant hybrid cell line, HMy2, and K562 cell lines. Four hybrid cell lines were used (HxVP024, HxKG-1, HxU266, and HxTH044 cells), and in each of these cultures, cytolytic activities were investigated against the relevant tumour cells, as well as the stimulatory hybrid cell line and HMy2 cells, in addition to NK target cell line, K562 (Figure 4.21). The CTL responses against parent tumour cells were variable, ranging from more than 30% to 90% in responses of relevant culture cells to TH044, U266, KG-1, and VP024 tumour cells respectively, at an effector: target cell ratio of 30:1.

From these data, I can conclude the induction of responding CTLs against parent tumour cells, although the antigen-specificity of the responses was not demonstrated. These CTL lines may have been specific for major and minor histocompatibility antigens expressed by the stimulator hybrid cell lines and the parent tumour cells. However, in addition to allogeneic- responses, some TAA-specific CTLs may have been generated, and in order to test this, I generated HLA-A2-restricted TAA-specific CTLs, by stimulation of PBMC from healthy HLA-A2^{+} individuals and from 2 patients from whom hybrid cell lines were derived, by hybrid cell lines *in vitro* (Chapter 5).
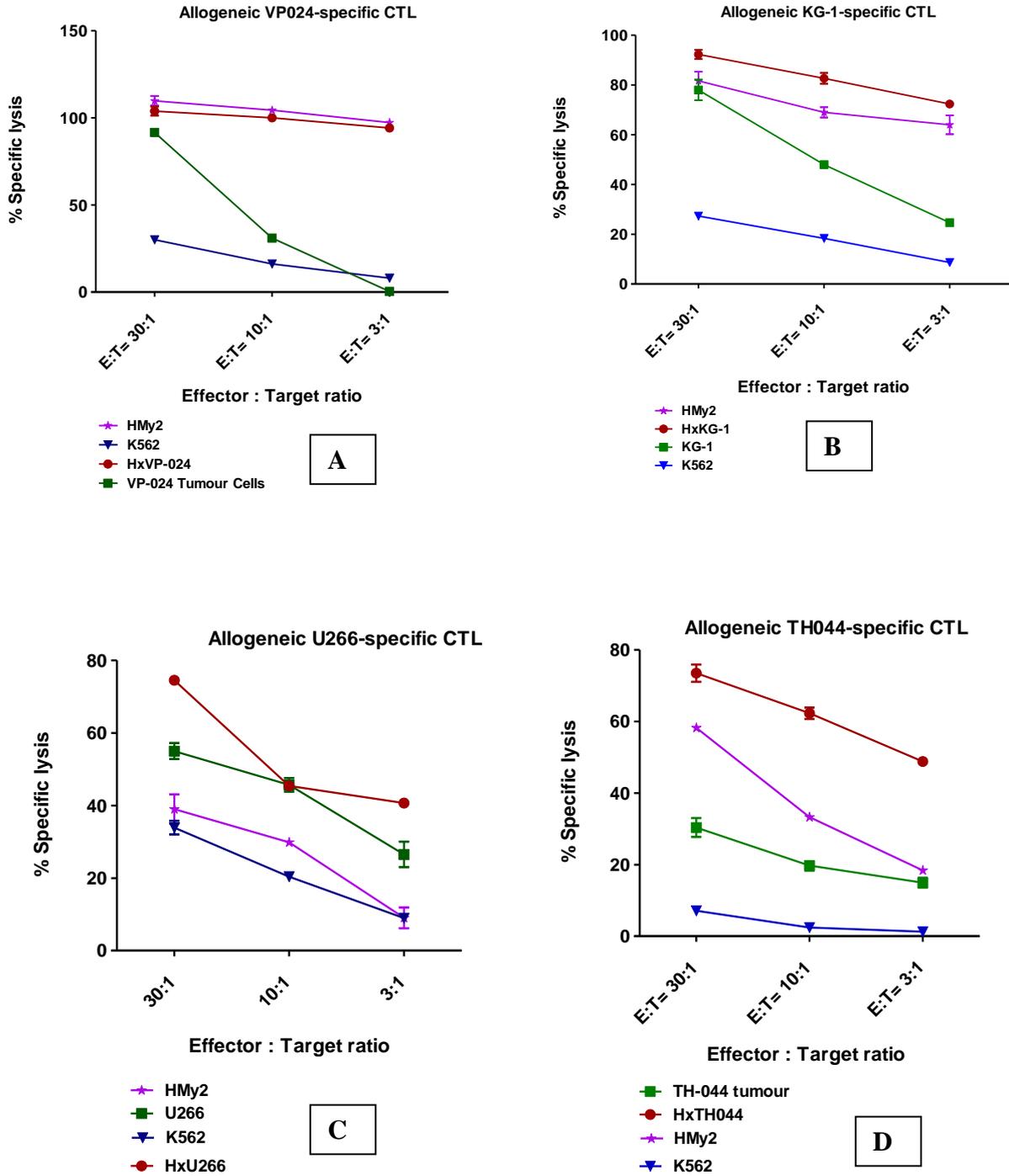
Figure 4.21: Allogeneic hybrid cell line-activated CTLs showing cytotoxic activity against parent tumour cells and cell lines from which hybrid cell lines were generated. Charts (A), (B), (C), and (D) show CTLs responses targeting VP024, KG-1, U266, and TH044 tumour cells respectively.

4.3 Discussion

The potential use of HMy2/tumour cell hybrids in tumour immunotherapy relies on their elevated immunogenicity and functional activity compared with their parent tumour cells. In the previous Chapter, the phenotype and tumour-antigen expression profile of the hybrid cell lines of this study showed the expression of a group of known TAAs, in addition to HLA class I and class II expression. T cell co-stimulatory molecules (CD80, CD86, and CD40) were shown to be expressed by all of the hybrid cell lines, although in variable levels. However, expression of HLA class I, class II, and co-stimulatory molecules does not, necessarily, indicate the hybrid cells' effectiveness as immunostimulatory agents. Therefore, several *in vitro* immunostimulatory and functional characterization assays had to be explored as a first step in the preclinical investigation of the hybrid cells as candidate immunotherapeutic agents. Although further *in vivo* studies are required as the second step to verify hybrid cell effectiveness in tumour immunotherapy, *in vitro* studies including mixed lymphocyte reaction of hybrids versus their relevant parent tumours (including the effect of HLA class I, HLA class II, and co-stimulatory molecules inhibition), and the ability of hybrid *versus* parent tumour cells to induce naive, central and effector memory CD4$^+$ and CD8$^+$ T cell responses (using CFSE-labelled responders in MLR), are important primary indicators of the hybrid cells' immunogenicity. Furthermore, IFN-γ ELISpot assays following short- and long-term stimulation of allogeneic and autologous PBMC with the hybrid and relevant parent tumour cell lines are a measure of the cellular immune responses, at the level of a relevant, Th1 cytokine.

Co-culturing of hybrid cell lines with allogeneic PBMCs from normal donors in MLR induced strong proliferative T cell responses as compared with resting lymphocytes. The lymphocyte proliferation response was correlated with the stimulator/ responder

cells ratios, and in all cases significantly higher levels of proliferative responses were achieved by stimulation with hybrid cell lines compared with their relevant tumour cells. The enhanced immunostimulatory ability of hybrid versus relevant parent tumour cells was attributed to their ability to induce primary naive, in addition to effector and memory, allogeneic T cell responses. The induction of naive allogeneic T cells requires provision of at least two signals, first is the recognition of processed peptide in context of HLA class I or HLA class II by relevant TCR of CD8$^+$ or CD4$^+$ T cells respectively, and second is co-stimulation provided by CD80 and CD86 molecules to the T cell's CD28 receptor. On the other hand, and unlike the induction of primary response, the stimulation of memory T cells in less dependent on the co-stimulatory signals. It was suggested that EBV-associated antigens expressed by HMy2, and inherited by hybrid cells (EBNA and LMP1/LMP2), were also a key factor in the enhanced immunogenicity of these hybrid cell lines. However, Dunnion *et al*. (Dunnion *et al.*, 1999) reported that stimulation of allogeneic PBMCs from EBV sero-negative or sero-positive individuals with HMy2 x tumour hybrids showed no differences in level of proliferation, which confirms inclusion of other factors, other than EBV-associated antigens, in the HMy2, and HMy2 x tumour hybrids stimulatory effects.

To investigate the involvement of HLA class I and class II and co-stimulatory ligands in T cell proliferation induced by the hybrid cell lines, blockade of these molecules was carried out using blocking monoclonal antibodies in modified MLRs. mAbs against HLA class I (W6/32) and class II (DR) (L243) were added in the MLR separately and in combination. Both mAbs significantly inhibited the T cell proliferative responses induced by the hybrid cell lines, whilst, addition of W6/32 and L-243 together inhibited the T cell proliferation nearly to the background level. These observations reflected the importance of allogeneic and tumour antigen processing and presentation in the context

of both HLA class I and class II by APC, and suggested the involvement of both CD8^{+} and CD4^{+} T cells in response to hybrid cell stimulation. In similar studies, the role of T lymphocytes as a main responder in MLR using HMy2 x haematological tumour hybrids was confirmed by Dunnion *et al*. (Dunnion *et al.*, 1999), who reported equivalent responses obtained on separated CD3^{+} (T cells) as well as on the whole PBMC, and showed that both purified CD4^{+} and CD8^{+} populations were activated by the hybrid cell lines, with higher responses shown in CD4^{+} than CD8^{+} T cells, by stimulation with the same hybrid cell line. In the same context, both of HLA class I and class II were reported by Walewska *et al*. (Walewska *et al.*, 2007) to be involved in allogeneic T cell responses to stimulation by HMy2/myeloma hybrid cell lines (which expressed both HLA class I and class II), and this role was inhibited by addition of W6/32, L243, or both mAbs to stimulator hybrid cells in MLR.

In a similar way, I investigated the role of co-stimulatory ligands (CD80 and CD86) in the induced lymphoproliferative responses in MLR, by addition of CTLA-4 Ig fusion protein to stimulator cells, before mixing with responder cells, to bind to and block the interaction of B7 (CD80 and CD86) on hybrid cells with its co-receptor (CD28) on responding T cells. The addition of CTLA-4 Ig significantly inhibited the lymphocyte proliferative responses as compared with a matched isotype control mAb. These results reinforce the importance of T cell co-stimulatory molecules in the induction of primary T cell immune responses by the hybrid cell lines. They may also explain inability of relevant parent tumour cells to induce such responses, as the parent tumour cells generally lacked expression of B7 molecules. Similar findings were reported by Dunnion *et al*. in studying haematological tumour hybrid cell lines (Dunnion *et al.*, 1999), and by Cywinski *et al*. (Cywinski *et al.*, 2006) who studied the CTLA-4 Ig blocking effects on lymphoproliferative T cell responses induced by HMy2 x

haematologic and HMy2 x solid tumours. Surprisingly, Cywinski noticed reduction in response to hybrid cells whatever the co-stimulatory ligand (CD80 and CD86) expression status of the hybrid cell lines (expressed by haematological, but not by solid tumour-derived hybrids). To explain these results, Cywinski removed the non-T cell fraction from allogeneic PBMC before mixing with solid tumour-derived hybrid cells, which lacked co-stimulatory ligand expression, a reduction of the allogeneic response to background level was observed, which was restored by re-addition of that fraction. She concluded the presence of bystander cells in PBMC, expressing co-stimulatory ligands, which provided co-stimulation, in a phenomenon called trans-co-stimulation (Ding &Shevach 1994), and this trans-co-stimulation was inhibited in the presence of CTLA-4 Ig. The role of co-stimulation was also investigated by Walewska *et al.* on a group of myeloma hybrid cell lines, in which a blocking effect of CTLA-4 Ig was noticed as a reduction in allogeneic lymphoproliferative responses (Walewska *et al.*, 2007). Several other *in vitro* and *in vivo* studies have evaluated the involvement of B7 (especially CD80) in immunogenicity of tumour cells, which was enhanced after transfection of the tumour cells with respective genes (CD80-gene). The transfected cells were able to induce tumour specific CTLs and were associated with clinical responses when used in human cellular vaccination studies (Schendel *et al.*, 2000, Raez *et al.*, 2004).

In a separate group of experiments using CFSE, which is an intracellular dye characterised by great protein binding and ability to be distributed equally between newly dividing daughter cells, I investigated the phenotype of the allogeneic proliferative responder T cells. CFSE-stained PBMC were stimulated in MLR by a member of MM and AML hybrid cell lines (HxU266 and HxKG-1), and their relevant parent tumour cell lines (U266 and KG-1) respectively (Section 2.4.4). PHA and HMy2-stimulated PBMCs were used as positive controls, and unstimulated PBMCs at

zero and 6 days time were used as background controls. The flow cytometric data were analysed by gating on CD4$^+$ or CD8$^+$ T cell subsets, followed by gating on naive, CM, or EM populations (using CD45RA and CCR7 expression). Hybrid cell lines induced proliferation of up to 70% of total T cells, compared with 10% by their respective tumour cells, and the majority of proliferating T cells in response to hybrid cell lines were CD8$^+$ (35-45% of total T cells), with lower CD4$^+$ (20-35% of total T cells) responding cells. In contrast, corresponding tumour cells induced very low levels of proliferation in both CD8$^+$ (5-7%) and CD4$^+$ (2-5%) of total T cells. By interpreting the percentage of cells in each subsequent generation induced during incubation with different stimulators (Proliferation Index; PI), six subsequent generations of the proliferating T cells were seen in response to PHA, HMy2, and hybrid cell line-stimulation. On the other hand, parent tumour cell lines (U266 and KG-1) showed much less number of generations and fewer cells in each generation of responder cells.

Further phenotyping of responder T cells as naive, central memory or effector memory cells was achieved by four colour flow cytometric analysis of the activated, CFSE-stained T cells, after IF staining with mAbs against CCR7, CD45RA, and CD4 (or CD8). Region gates were made on CD4$^+$ or CD8$^+$ T cell populations followed by quadrant gates on naive (CD45RA$^+$/ CCR7$^+$), EM (CD45RA$^-$/ CCR7$^-$), and CM (CD45RA$^-$/ CCR7$^+$) populations. Following stimulation with the hybrid cell lines, CD8$^+$ T cells showed the following pattern: EM cells were the highest population in terms of proliferation and differentiation, followed by naive and finally CM cells. The proliferating EM population induced by HMy2, HxU266, and HxKG-1 cell lines reached up to 85% of total EM cells, which was significantly higher than equivalent counterpart before stimulation (< 20%). On the other hand, lower level of responses were seen in naive and CM cells in response to HxU266 and HxKG-1 hybrid cell lines,

with an average rate of 63% and 75% respectively. The relatively lower naive and CM proliferation levels may be attributed to their differentiation into EM cells upon stimulation. Stimulation with U266 parent tumour cells was associated with high responses of the EM population, but in lower levels than corresponding hybrid cell line responses.

CD4^{+} populations showed similar patterns of CD8^{+} proliferation in response to HxU266 and HxKG-1 hybrid cell lines, but with lower percentage of proliferating cells. High relative proliferation was seen in EM, and lower levels were seen in naive and CM populations. Parent tumour cells (U266 and KG-1) induced much lower levels of proliferation compared with their hybrid counterparts.

To summarise the proliferating T cells' phenotyping results, CD8^{+} T cells were the main responders in MLR, although high levels of response were also observed with CD4^{+} T cells, as indicated from analysis of CFSE-stained PBMC. Within the lymphocyte subgroups, EM cells were the main responding population. The cellular phenotyping of allogeneic responder lymphocytes in MLR showed that hybrid cells formed by fusion of HMy2 x haematologic tumours were able to induce markedly enhanced proliferative T cell responses *in vitro*, as compared with their respective parent tumour cells, and the responding T cells included both CD4^{+} and CD8^{+} T cells, as well as naive, CM, and EM populations.

These data are consistent with the work of Dunnion *et al*. (Dunnion *et al*., 1999) who reported that both CD4^{+} and CD8^{+} T cell fractions were able to proliferate in MLR in response to stimulation by hybrid cell lines, to a greater degree than to parent tumour cells, depending on the level of co-stimulatory ligands expression by the hybrid cells. Similarly, Walewska *et al*. showed the ability of HMy2 x myeloma hybrids to stimulate

allogeneic PBMC, as well as untouched negatively purified fractions (CD3^{+}, CD4^{+}, or CD8^{+}), to considerably higher levels than respective parent tumour cells. Hybrid cells were also able to stimulate naive (CD45RA^{+}) and memory (CD45RO^{+}) T cell populations (Dunnion *et al.*, 1999). Cywinski *et al.* (Cywinski *et al.*, 2006) confirmed the lymphoproliferative responses to HMy2 x marrow derived tumour hybrids when incubated with purified, negatively separated T cell fractions (CD3^{+}, CD4^{+}, or CD8^{+}). Moreover, hybrids of HMy2 x solid tumour stimulated the respective T cell fraction depending on their pattern of HLA expression, e.g. HMy2 x 518.A2 melanoma hybrid (expressing HLA class I but not HLA class II) induced a CD8^{+} T cell response, but not CD4^{+} T cell response, while HMy2 x Ger143 hybrid (expressing both HLA class I and class II) induced CD4^{+} and CD8^{+} T cell responses to a greater degree than respective parent tumour cell lines.

Other *in vitro* and *in vivo* studies on APC x haematological and solid tumour hybrids showed their ability to stimulate tumour reactive CD4^{+} and CD8^{+} T cells (Avigan *et al.*, 2007, Rosenblatt *et al.*, 2011, Rosenblatt *et al.*, 2010, Zhou *et al.*, 2009). Rosenblatt *et al.* used autologous DC/MM tumour fusion to vaccinate 15 MM patients. 11 of 15 patients showed *in vivo* expansion of tumour reactive CD4^{+} and CD8^{+} T cells, and the majority of cases were associated with disease stabilization (Rosenblatt *et al.*, 2011). Avigan *et al.* (Avigan *et al.*, 2007) investigated a phase I/II study on allogeneic DC/ patient-derived RCC fusions. He used this model to vaccinate 24 RCC patients, and 21 of them were evaluated for immunological and clinical responses. 10 of 21 evaluated patients showed anti-tumour immune responses associated with elevated CD4^{+} and/ or CD8^{+} tumour-specific, *ex vivo* reactive T cells. These immunological responses were associated with partial clinical responses in 2 of 10 cases and stable disease in 8 of 10 cases. In a similar study, Zhou *et al.* (Zhou *et al.*, 2009) used the allogeneic DC/

patient-derived RCC fusion to induce *in vitro* immune (in the form of T cell proliferative, IFN-γ release, and CTL) responses, and clinical (in the form of partial and complete disease stabilisation in 70% of vaccinated patients) responses. These studies and others showed the ability of fusion vaccines to induce immunological and clinical responses *in vitro* and in clinical trials respectively. The results of allogeneic lymphoproliferative T cell responses in my experiments showed that the hybrid cell lines, but not their parent tumour cells, induced significant T cell activation *in vitro*.

The final set of experiments described in this Chapter investigated the ability of the hybrid cell lines to induce IFN-γ releasing T cell response in ELISpot assays. IFN-γ is a cytokine known to play an important role in protective immune responses, especially in tumour prevention and eradication (Shankaran *et al.*, 2001, Ikeda *et al.*, 2002). IFN-γ is produced by effector cells of both innate and adaptive immune systems. NK cells are a major source of IFN-γ, which is induced as a result of NK activation. NKT cells are another source of IFN-γ, as a result of activation of their invariant TCR by CD1d conjugated ligands (including α-GalCer). DCs and macrophages have also been reported to produce IFN-γ under certain stimulatory conditions. In addition, CD4$^+$ and CD8$^+$ T cells are important producers of IFN-γ during the adaptive immune response, especially in the Th1 response (Ikeda *et al.*, 2002). IFN-γ plays a crucial role in polarizing towards development of CD4$^+$ Th1 phenotype, through induction of IL-12 release by different APCs, and the up regulation of IL-12 receptor on CD4$^+$ T cells. Moreover, it inhibits the development of Th2 phenotype by inhibition of IL-4 synthesis by naive CD4$^+$ T cells, and by direct inhibition of Th2 T cell expansion (Ikeda *et al.*, 2002).

IFN-γ release ELISpot is an *in vitro* assay for identification of immune responses in various conditions including anti-tumour responses. Several assays have been used for IFN-γ-release evaluation, such as ELISA and flow cytometric analysis of intracellular stained cells. However, ELISpot offers the advantage of quantitative estimation of the number of IFN-γ-secreting cells, rather than the total amount of IFN-γ released. The *in vitro* IFN-γ release ELISpot assay is an indicator of the level of activated lymphocytes secreting IFN-γ in response to stimulation with stimulator cells. By including the NK sensitive cell line (K562) in the assay, IFN-γ ELISpot was able to distinguish NK from T cell responses, and to evaluate the level of activated, allogeneic or autologous T cells in the culture.

In short-term assays, allogeneic healthy PBMC showed higher levels of IFN-γ release in response to stimulation with hybrid cell lines than to their relevant parent tumour cells. In addition, parent tumour cells did not induce significant allogeneic IFN-γ response, compared with the background control PBMC, except in case of AML (DO050) and MM (U266) tumour cells. Autologous PBMCs showed variable responses to stimulation with relevant *ex vivo* tumour cells and hybrid cell lines in short-term assays. In this assay, the autologous T cell responses to hybrid cells were significantly greater than the responses to matched tumour cells, and in most cases responses were more or less equivalent to that seen to HMy2 cells. There was no evidence of significant autologous IFN-γ releasing response to parent tumour cells *in vitro,* except in case of B-CLL (AW072 and JR004) patients, where weak responses were observed to re-stimulation with relevant parent tumours. However, the highly significant responses to HMy2 and hybrid cell lines in short-term assay indicate the capability of these cell lines to induce responding cells to their overall antigen-profile (which may be MHC, mHC,

or TAA) compared with un-fused parent tumour cells. The low responsiveness of the patients' PBMC to relevant parent tumour cells may be attributed to the lack of co-stimulation or establishment of immunosuppressive environment induced by Treg and the associated cytokines.

In long-term ELISpot assays, the stimulation of allogeneic, healthy donors' PBMCs with individual hybrid cell lines for several consecutive rounds induced T cell cultures with enhanced IFN-γ releasing activity upon co-culturing with the stimulator hybrid cell lines, HMy2 cells, and (in most cases) relevant tumour cells, from which the stimulator hybrid cell lines were derived.

Two tumour cell line-derived hybrids, HxU266 and HxKG-1, and three *ex vivo* tumour-derived hybrids, HxIC038, HxDO050, and HxTH044 successfully induced allogeneic IFN-γ-secreting T cell cultures of enhanced activities against both the stimulatory hybrid cells and HMy2 cell line, which were significantly higher than the corresponding activities seen in short-term assays. In addition, all of these T cell cultures showed enhanced responses against parent tumour cells, from which the stimulatory hybrid cell line was generated, and these responses were significantly greater than background controls as well as the relevant responses seen in short term assays. The only exception was the HxTH044-induced T cell culture, in which significant responses were seen against HxTH044 and HMy2 cell lines, but not against the relevant parent tumour cells (TH044). The low responsiveness of long-term HxTH044-activated allogeneic T cell culture was consistent with the insignificant short-term allogeneic and autologous results, which may indicate an immune-suppressive effect or very low immunogenicity of the parent tumour cells. Two other MM hybrid cell lines (HxRC056 and HxCM053) successfully induced allogeneic, long-term T cell cultures of high IFN-γ releasing

activities against stimulatory hybrid cells, HMy2 cell line and against U266 cell line (a MM cell line, which was used due to shortage of relevant parent tumour cells).

To conclude, data from long-term allogeneic T cell cultures showed that, in most cases, several rounds of stimulation using selected hybrid cell lines induced T cell cultures of higher IFN-γ-releasing activities against the stimulatory hybrid cell line, HMy2, and against most relevant tumour cells/cell lines.

On the other hand, long-term autologous ELISpot showed significant increase in autologous activated T cell responses to VP024 and DO050 tumour cell, compared with the short-term autologous responses. These autologous, elevated responses suggest the induction of primary-induced T cell clones specific for tumour antigens expressed by the parent tumour cells (as no allogeneic histocompatibility antigens were expressed by parent tumour cells), and indicate that these antigens were also expressed by the relevant hybrid cell lines (which were used in activation process). In addition, significantly higher responses were seen to semi-autologous hybrid and to HMy2 cell lines in these assays. The autologous long-term responses lacked NK activity, as shown by the weak response to NK-sensitive cell line (K562). The induction of tumour antigen-specific responses by long-term activation of autologous patients' PBMC using relevant hybrid cell lines was also investigated using another approach (^{51}Cr release cytotoxicity assays) in Chapter 5.

Regarding the use of IFN-γ-releasing ELISpot assays in the assessment of immune responses, several reports elucidated the value of Th1 cytokine release as a tool for evaluation of fusion vaccines in immunotherapy. In a study by Kim *et al*., murine DC x fibrosarcoma hybrids were able to induce syngeneic IFN-γ releasing responses. This was confirmed by co-culturing responder cells with parent tumour cell line (Kim *et al*.,

2007). In a second study, Koido *et al*. investigated the ability of allogeneic DCs x breast cancer cell line hybrid cells to activate specific T cells, and the activated T cells were investigated using IFN-γ release assays with the parent tumour cells as stimulators (Koido *et al.*, 2007). Moreover, several preclinical and clinical studies reported the ability of APC x tumour cell hybrid vaccines to stimulate IFN-γ releasing effector T cells in renal cell carcinoma (Avigan *et al.*, 2007, Zhou *et al.*, 2009), hepatocellular carcinoma (Cao *et al.*, 2009), and multiple myeloma patients (Rosenblatt *et al.*, 2011).

To conclude, the *in vitro* functional characterization of the hybrid cell lines of this study was assessed by lymphocyte proliferation, CFSE labelling experiments, IFN-γ-releasing ELISpot, and cytotoxicity assays using allogeneic and autologous PBMCs as responder cells. As mentioned in Chapter three, the phenotypic characterization of hybrid cell lines showed dominant HMy2-like profiles, associated with expression of multiple TAAs derived from the respective parent tumour cells. In this Chapter, the phenotypic characterizations were reflected as enhanced immunogenicity and functional capability of the hybrid cell lines to induce naive, effector, and memory proliferative and functional, IFN-γ-secreting T cell responses, including both CD4$^+$ and CD8$^+$, of allogeneic and autologous T lymphocytes. In addition, cytotoxic T cell responses were induced in allogeneic cultures and were directed against parent tumour cells (from which stimulator hybrid cell lines were made), HMy2 and stimulator hybrid cells. Whilst demonstrating the induction of functional T cell responses, these experiments did not directly address whether the hybrid cell lines induced tumour antigen-specific (in addition to allogeneic) T cell responses. This question is addressed in the next Chapter, where tumour antigen-specific CTL responses were generated from normal donors' and tumour patients' PBMCs.   .

Chapter 5

Induction of tumour- and tumour-antigen specific CTLs

5 Induction of Tumour- and Tumour antigen-specific CTLs

5.1 Introduction

The immune system is well known to recognise and interact with malignant tumours through its different effector mechanisms. Most, if not all, tumour cells are characterised by expression of abnormal proteins or abnormal levels of self-proteins, which are normally expressed in certain developmental stages or by immune-privileged tissues. *In vivo*, DCs can process and present TAAs for recognition by antigen-specific T cells, including CD8$^+$ and CD4$^+$ subclasses, which are the main tumour-specific effector cells involved in tumour immunosurveillance. Based on that, in this Chapter, I investigated the ability of the HMy2/ tumour hybrid cell lines to induce tumour- and tumour antigen-specific CTL responses, through successive multiple rounds of stimulation of PBMC from healthy, normal individuals and from patients from whom hybrid cell lines had been derived.

5.1.1 Role of T cells in tumour immunotherapy

Different animal studies and clinical trials have demonstrated that tumour infiltration with immune cells was associated with improved prognosis in a variety of cancers (Eerola *et al.*, 2000). Other studies reported the significant suppression of tumour metastasis and post-treatment recurrence in patients where the primary tumour lesions were highly infiltrated with lymphocytes, especially CD8$^+$ T cells (Sato *et al.*, 2005). Recent reports have observed that the phenotype, but not the number, of T cells in the tumour environment, imposes good or bad prognosis. The presence of CD8$^+$ CTLs, but not CD4$^+$/ CD25$^+$ Treg, is a good sign of better prognosis (Curiel *et al.*, 2004).

In this Chapter, I investigated the induction of tumour and tumour antigen-specific CTL responses by long-term activation of normal healthy, and tumour bearing patients' PBMCs *in vitro,* using selected hybrid cell lines. The antigen-specificity and cytolytic functional activity of the induced lymphocytes were confirmed by IF staining with HLA-A2-restricted peptide pentamers, specific for a group of the highly expressed TAAs, followed by flow cytometric analysis of the $CD8^+$/pentamer positive populations, and by ^{51}Cr release cytotoxicity assays using relevant peptide-pulsed T2 cells as targets, and irrelevant peptide (*Plasmodium falciparum* peptide)-pulsed T2 cells as a negative control. In addition, K562 cells were used as an indicator of NK activity.

5.2 Results

To investigate the ability of the hybrid cell lines to induce tumour- and/ or tumour antigen-specific CTLs, a selected group of stimulator hybrid cell lines was chosen according to their expression of TAA-not expressed by HMy2 (Table 5.1), to establish *in vitro* long-term T cell lines by repeated, weekly stimulation of allogeneic and autologous HLA-A2$^+$ T cells. Seven hybrid cell lines (HxU266, HxCM053, HxRC056, HxKG-1, HxVP024, HxPG052, and HxTH044), all are HLA-A2$^+$, were able to establish *in vitro* long-term T cell cultures from normal healthy donors' T cells. HMy2 and two of the parent tumour cell lines were used in parallel experiments as stimulators. HMy2 cells and hybrid cell lines successfully induced long-term T cell lines, but parent tumour cells failed to establish T cell lines *in vitro*.

Stimulator Cell line	Tumour antigen expression (by qRT-PCR)					HLA-A2 expression
	NY-ESO-1	MAGE-A1	WT-1	PRAME	Survivin	
HMy2	Weak	Negative	Negative	Negative	Positive	Positive
HxU266	Positive	Positive	Negative	Positive	Positive	Positive
HxCM053	Positive	Positive	Negative	Negative	Positive	Positive
HxRC056	Weak	Positive	Negative	Positive	Positive	Positive
HxKG-1	Weak	Positive	Positive	Weak	Positive	Positive
HxVP024	Weak	Weak	Positive	Positive	Positive	Positive
HxPG052	Positive	Positive	Negative	Positive	Positive	Positive
HxTH044	Positive	Positive	Negative	Positive	Positive	Positive

Table 5.1: HMy2 and a group of selected hybrid cell lines that were used in induction of allogeneic tumour antigen-specific CTL, their HLA-A2 status, and TAA expression profile, as revealed by qRT-PCR expression criteria, which are summarised below. qRT-PCR results are presented in arbitrary units, derived from Table 3.2.

Criteria	NY-ESO-1	MAGE-A1	WT-1	PRAME	Survivin
Positive	$>2.5 \times 10^{-3}$	$>0.5 \times 10^{-2}$	$>1 \times 10^{-1}$	25×10^{-1}	>1
Weak	$(1-2.5) \times 10^{-3}$	$(0.2-0.5) \times 10^{-2}$	$(0.7-1)10^{-1}$	$(18-25) \times 10^{-1}$	NA
Negative	$<1 \times 10^{-3}$	$<0.2 \times 10^{-2}$	$<0.7 \times 10^{-1}$	$<18 \times 18^{-1}$	NA

In addition, I had sufficient PBMCs from two AML patients (DO050 and VP024) to establish autologous long-term T cell cultures, using their relevant hybrid cell lines HxDO050 and HxVP024 respectively.

5.2.1 Induction of allogeneic, tumour antigen-specific CTL lines

PBMC from healthy HLA-A2$^+$ individuals were stimulated for weekly rounds with hybrid cell lines, HMy2, or tumour cells *in vitro* in the presence of exogenous IL-2 (Section 2.6). After 4-6 weeks of successive stimulation, the established allogeneic T cell lines were assessed for the presence of antigen-specific CTL by staining with HLA-A2-peptide pentamers specific for TAAs known to be expressed by haematological and non-haematological malignancies (MAGE-A1, NY-ESO-1, PRAME, WT-1, and Survivin) followed by flow cytometric analysis, and by ^{51}Cr release cytotoxicity assays using relevant TAA and control peptide-pulsed T2 cells as targets.

5.2.1.1 HLA-A2-peptide pentamer analysis

Tumour antigen-specific CTLs were detected by staining T-cell cultures with PE-labelled Pro5$^®$ MHC class I pentamers and FITC-labelled anti-CD8, clone LT8. The used HLA-A2-peptide pentamers were listed in Table 2.9. Unstimulated PBMC from normal HLA-A2$^+$ donors were stained in parallel and used as negative controls. Following staining, the cells were analysed by flow cytometry to explore the presence and percentage of the tumour peptide-specific CTLs in the T cell cultures. Figure 5.1 shows the percentage of HLA-A2-restricted, tumour antigen-specific CTL from the total live PBMC populations in the stimulated or unstimulated (negative control) cultures, stained with HLA-A2-peptide pentamers specific for Survivin, MAGE-A1, PRAME, NY-ESO-1, or WT-1 antigens, in addition to anti-CD8 mAb. The unstimulated PBMC showed less than 0.25% pentamer-positive cell populations for each tested antigen, and HMy2-stimulated culture showed ~ 0.5% for unexpressed antigens. So, I arbitrarily determined a pentamer-positive population of more than 1% of total activated PBMC as representing the induction of a positive antigen-specific CD8$^+$ T cell response, and pentamer-positive population of between 0.5-1.0% as

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representing a weak antigen-specific response, with < 0.5% populations as a negative response (Table 5.2).

Following these definitions, HMy2 cells induced a positive Survivin-specific CD8$^+$ T cell response; and weak NY-ESO-1, MAGE-A1, and WT-1 responses; but did not induce antigen-specific T cell responses to PRAME antigen (Figure 5.1). All of the investigated multiple myeloma hybrid cell lines (HxU266, HxCM053 and HxRC056) induced positive CTL responses for Survivin and MAGE-A1. In addition, HxU266 cells induced positive responses for PRAME and NY-ESO-1, but a weak response was induced for WT-1 antigen. HxCM053 cells did not induce CTL responses for PRAME or WT-1, but they induced a response for NY-ESO-1 antigen. HxRC056 cells failed to induce WT-1 or NY-ESO-1 specific responses; however, they induced a weak response for PRAME. AML hybrid cells (HxKG-1 and HxVP024) induced positive CTL responses for all of Survivin, MAGE-A1, PRAME, and WT-1. In addition, HxVP024 also induced a positive response for NY-ESO-1, but HxKG-1 only induced a weak response for this antigen. Finally, HxPG052 and HxTH044 cell induced similar CTL responses, which were positive for Survivin, MAGE-A1, PRAME, and NY-ESO-1, but negative for WT-1 antigens.

According to that, all of the investigated hybrid cell lines induced positive CD8$^+$ T cell populations to multiple TAAs (Figure 5.1). There was a strong correlation between the TAA expression by hybrid cell lines, as indicated by qRT-PCR (Table 5.1), and their ability to induce relevant CTL populations, as revealed by HLA-A2 pentamer staining (Table 5.2).
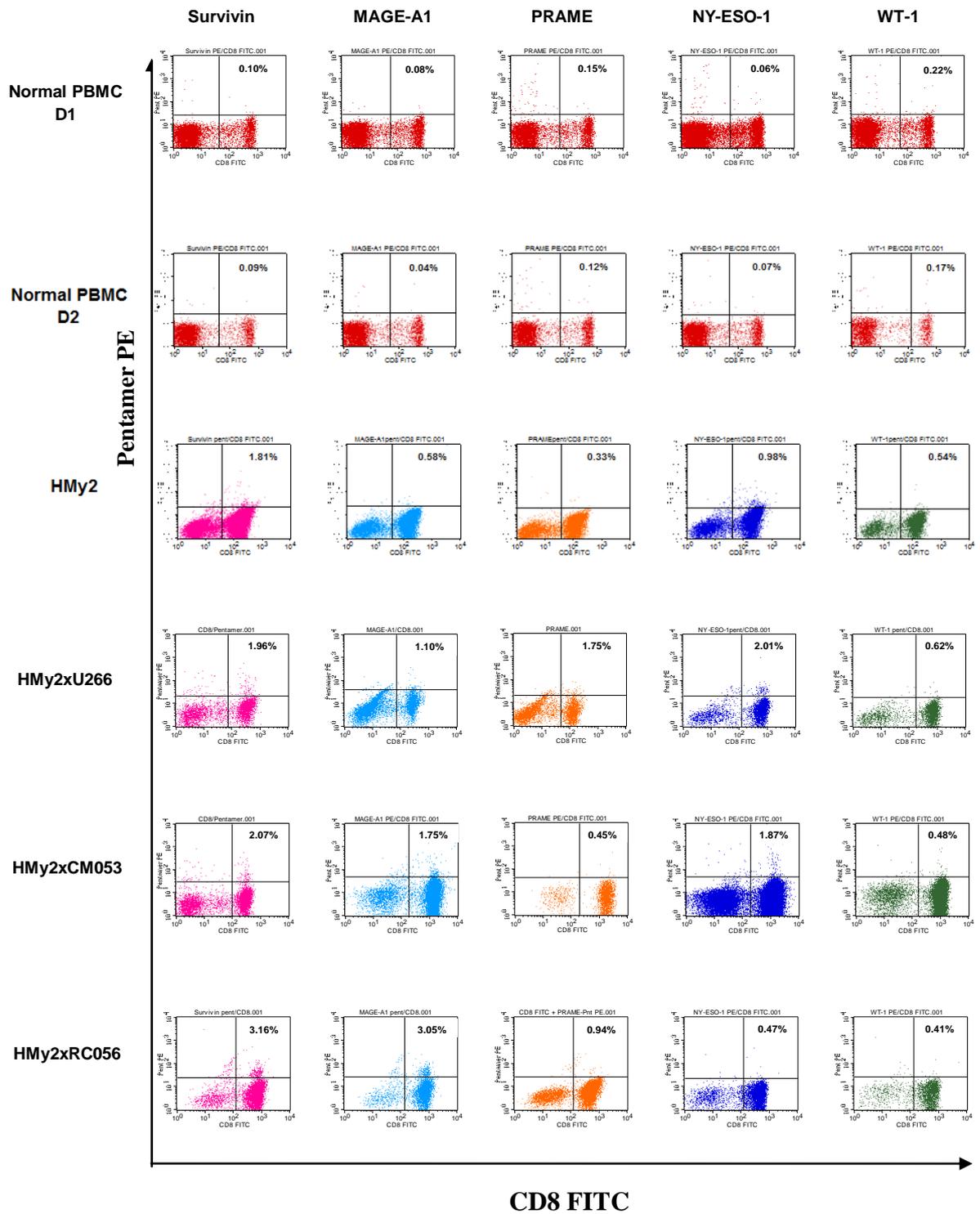
Figure is continued overleaf
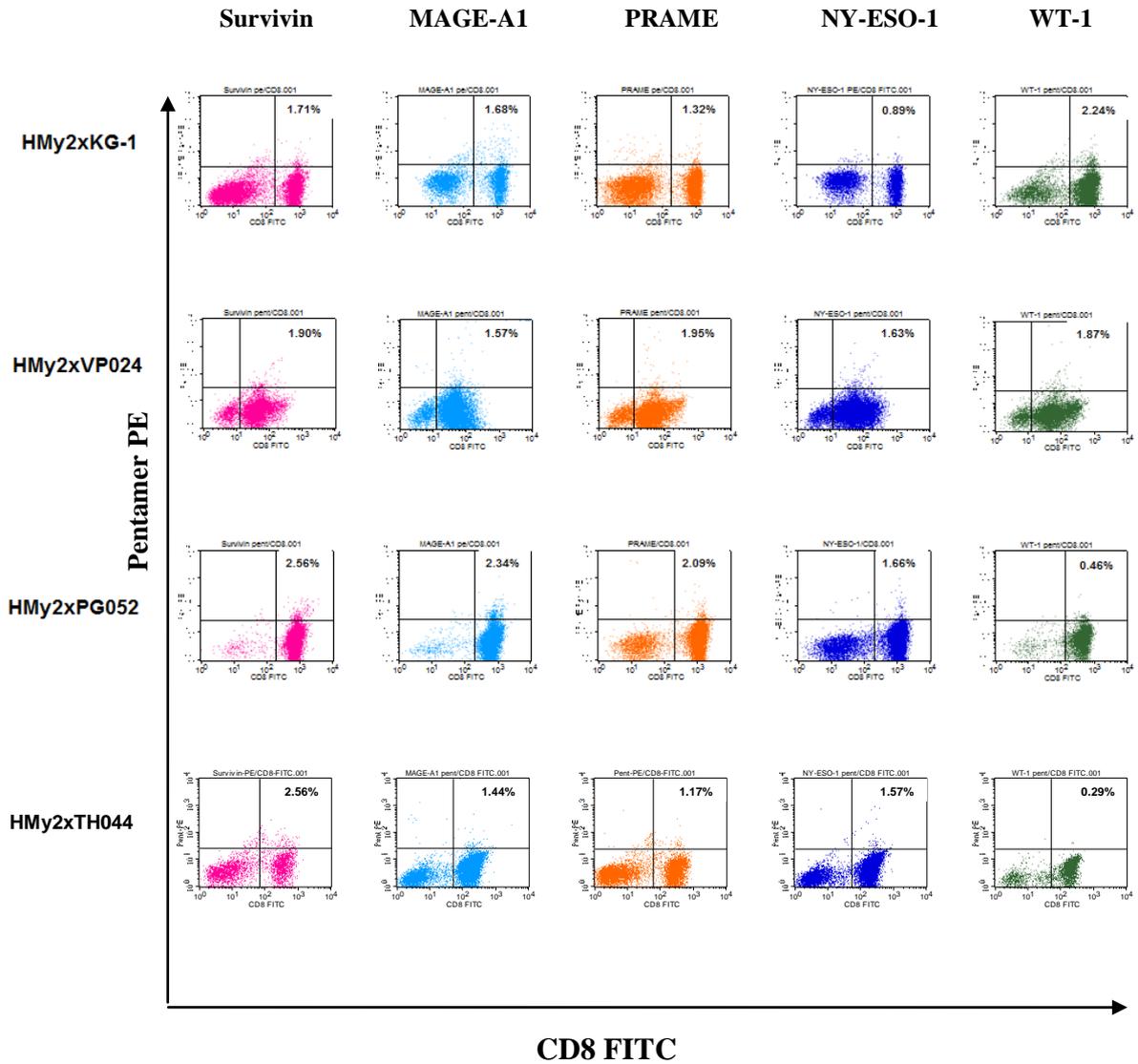
Figure 5.1: HLA-A2-peptide pentamer staining of T cell lines from healthy, HLA-A2[+] donors stimulated *in vitro* with hybrid cell lines. Dot plots are shown for CD8 (x-axis) and HLA-A2-peptide pentamer (y-axis) staining of unstimulated PBMC from 2 healthy, HLA-A2[+] donors (D1 and D2), or of T-cell lines derived from healthy, HLA-A2[+] donors cultures stimulated *in vitro* for 4 − 6 weeks by HMy2 cells, or by hybrid cells lines HxU266, HxCM053, HxRC056, HxKG-1, HxVP024, HxPG052 or HxTH044 cell lines. Percentage values (seen in the upper right quadrants) represent pentamer-positive populations as a proportion of total live-gated cells in the cultures.

Overall, for the 7 tested hybrid cell lines, there were 29 occasions where the hybrid cell lines expressed the relevant TAAs (positively or weakly by qRT-PCR), from which pentamer positive CD8$^+$ CTL populations (>1%) were induced on 26 occasions, which indicates a high positive correlation (around 90%, p<0.001) between TAA-expression (positive or weak) and induction of antigen-positive CD8$^+$ T cell populations (Figure 5.3). Moreover, on the remaining occasions, TAA-expression was associated with weak (0.5-1.0%) or negative (<0.5%) pentamer CD8$^+$ population (which were; PRAME and NY-ESO-1-specific responses in the case of HxRC056, and NY-ESO-1-responses in the case of HxKG-1-activated T cells). In all cases where the hybrid cell lines lacked TAA-expression (qRT-PCR negative), the pentamer-positive populations were <0.5% (Table 5.2), except for HxU266/WT-1, which was negative by qRT-PCR (although higher than the negative control expression level), but induced a weak pentamer-reactive population (0.62%).

5.2.1.2 ^{51}Cr release cytotoxicity assays

To investigate the cytotoxic capability of the pentamer-positive CD8$^+$ populations, ^{51}Cr release cytotoxicity assays were set up using T2 cells pulsed with either the relevant TAA-derived peptide (green squares), or with an irrelevant HLA-A2-restricted peptide (*Plasmodium falciparum* CSP; 334-342; red circles) as a negative control (full description of the used peptides is in Table 2.10). K562 cells (blue triangles) were used as a target to estimate NK activity of the stimulated cultures, and in some cases, un-pulsed T2 cells were used as an additional negative control.

Stimulator	Assay	NY-ESO-1	MAGE-A1	PRAME	WT-1	Survivin
Unstimulated PBMC	qRT-PCR	Neg	Neg	Neg	Neg	Neg
	pentamer	Neg	Neg	Neg	Neg	Neg
HMy2	qRT-PCR	Weak	Neg	Neg	Neg	Pos
	pentamer	Weak	Weak	Neg	Weak	Pos
	CTL	Neg	Neg	Weak	Neg	Pos
HMy2xU266	qRT-PCR	Pos	Pos	Pos	Neg	Pos
	pentamer	Pos	Pos	Pos	Weak	Pos
	CTL	Pos	Pos	Neg	Neg	Pos
HMy2xCM053	qRT-PCR	Pos	Pos	Neg	Neg	Pos
	pentamer	Pos	Pos	Neg	Neg	Pos
	CTL	Pos	Pos	Neg	Neg	Pos
HMy2xRC056	qRT-PCR	Weak	Pos	Pos	Neg	Pos
	pentamer	Neg	Pos	Weak	Neg	Pos
	CTL	Neg	Pos	NT	Neg	Pos
HMy2xKG-1	qRT-PCR	Weak	Pos	Weak	Pos	Pos
	pentamer	Weak	Pos	Pos	Pos	Pos
	CTL	Pos	Pos	Neg	Weak	Pos
HMy2xVP024	qRT-PCR	Weak	Weak	Pos	Pos	Pos
	pentamer	Pos	Pos	Pos	Pos	Pos
	CTL	Pos	Pos	Neg	Pos	Pos
HMy2xPG052	qRT-PCR	Pos	Pos	Pos	Neg	Pos
	pentamer	Pos	Pos	Pos	Neg	Pos
	CTL	Pos	Pos	Neg	Neg	Pos
HMy2xTH044	qRT-PCR	Pos	Pos	Pos	Neg	Pos
	pentamer	Pos	Pos	Pos	Neg	Pos
	CTL	Weak	Neg	NT	Neg	Neg

Table 5.2: Summary of the hybrid TAA expression by qRT-PCR, pentamer reactive populations, and specific cytotoxicity assays induced against relevant peptide pulsed T2 cells by HMy2-, hybrid-stimulated or un-stimulated allogeneic normal donors' T cells.

Criteria	Positive	Weak	Negative
qRT-PCR	See Table 5.1		
Pentamer staining	>1%	0.5-1%	<0.5% of live PBMC in culture
CTL significant difference at	2 or all 3 E:T ratios	1 of 3 E:T ratios	No significant difference (at any E:T) ratio

Cytotoxicity results are summarised in Table 5.2, and shown in Figure 5.2 (A-H) for Survivin, MAGE-A1, PRAME, NY-ESO-1, and WT-1 specific CTL responses investigated using relevant TAA peptide-pulsed T2 cells.

HMy2 cells induced a significant cytolytic response to Survivin, but no significant response to MAGE-A1, NY-ESO-1 or WT-1, and a weak cytolytic response was seen at 10:1 ratio for PRAME-pulsed T2 cells. HxU266 and HxCM053 induced positive cytolytic responses to NY-ESO-1, MAGE-A1, and Survivin; however, negative responses were seen to PRAME and WT-1-pulsed T2 cells. HxRC056 induced a positive CTL response to MAGE-A1 and Survivin, but was negative for NY-ESO-1 and WT-1-pulsed cells.AML hybrid cell lines (HxKG-1 and HxVP024) induced CTL of similar cytolytic activities, which were positive for NY-ESO-1, MAGE-A1, and Survivin, and negative for PRAME. However, HxVP024 cells induced a positive cytolytic response to WT-1, while HxKG-1 cells induced only a weak response to WT-1 peptide-pulsed T2 cells. HxPG052 cells induced positive cytolytic responses to NY-ESO-1, MAGE-1, and Survivin, but negative responses to PRAME and WT-1-pulsed cells. Finally, HxTH044 induced a weak response to NY-ESO-1, but negative responses for MAGE-A1, WT-1, and Survivin. Due to shortage of effector cells in HxRC056 and HxTH044, I could not carry out this assay for PRAME peptide-pulsed T2 cells.

In summary, hybrid cell lines induced antigen-specific CTL cytolytic activity broadly in relation with the presence or absence relevant-peptide pentamer positive T cell populations (Table 5.2 and Figure 5.2). Cytolytic activities of T cell cultures induced by HxCM053 and HxRC056 hybrid cells were 100% in correlation with pentamer staining results. However, in the rest of the hybrid cells, PRAME-specific cytotoxicity did not correlate with the PRAME-specific pentamer staining results, which were seen in HxU266, HxKG-1, HxVP024, and HxPG052 hybrid cells, where it failed to lyse T2

cells pulsed with the relevant PRAME peptide. Moreover, HxTH044 only induced a weak CTL cytolytic response to NY-ESO-1 antigen, in spite of expressing Survivin, MAGE-A1, PRAME and NY-ESO-1 antigens by qRT-PCR, and inducing pentamer-positive CD8$^+$ populations for these antigens. Different possible explanations for these discrepancies are discussed in Section 5.3. According to this summary, 6 of the 7 hybrid cell lines induced CTL functional activity against 2 or more of the tested antigens. Sometimes, there was a certain cytolytic activity directed toward negative control targets, this may be attributed to allo-reactivity to T2 cell MHC antigens.

Overall, on 19 out of 25 occasions where pentamer-positive (>1%) populations were present, the hybrid cell lines induced functional antigen-specific CTL, with significantly greater cytotoxicity against T2 cells pulsed with the relevant TAA-derived peptide than T2 cells pulsed with the irrelevant peptide (around 76% positive correlation, P<0.01), indicative of antigen-specific cytotoxicity (Figure 5.2 and 5.3, and Table 5.2).

In all cases where CD8$^+$ pentamer-positive T cells represented <0.5% of the total populations, they failed to induce significantly higher cytotoxicity for T2 cells pulsed with the relevant TAA-derived peptides than for T2 cells pulsed with an irrelevant peptide (Figure 5.2). Moreover, when compared with TAA-expression by the stimulating hybrid cells; cytolytic activities of activated T cells were significant in 20 out of 27 T cell cultures stimulated with TAA-positive hybrid cells (74% positive correlation, p<0.01), Figure 5.3. The total observations showed that the hybrid cell lines were able to induce *in vitro* functional, tumour antigen-specific CTL responses from PBMC of healthy HLA-A2$^+$ donors, and (in the majority of cases) the hybrid cell lines simultaneously induced CTL lines against multiple HLA-A2-restricted tumour antigens. In addition, all the investigated T cell cultures showed low to moderate NK activity (as indicated by lysis of K562 target cells), except HMy2 which showed high NK activity.

Survivin specific

*** (100:1) ns (30:1) ** (10:1)

T2- Survivin peptide pulsed
T-PF peptide pulsed
K562

MAGE-A1 specific

*** (30:1) *** (10:1) ** (3:1)

T2 MAGE-A1 pulsed
T2 PFP pulsed
K562

NY-ESO-1 specific

*** (30:1) ** (10:1) *** (3:1)

T2-NY-ESO-1 pulsed
T2-PFP pulsed
K562

WT-1 specific

ns (30:1) ns (10:1) ns (3:1)

T2- WT-1 peptide pulsed
T2- PF peptide pulsed
K562

B

PRAME specific

ns (30:1) ns (10:1) ns (3:1)

T2- PRAME peptide pulsed
T2- PF peptide pulsed
K562

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Survivin specific

MAGE-A1 specific

NY-ESO-1 specific

WT-1 specific

PRAME specific

E

Survivin specific

MAGE-A1 specific

- ■ T2 Survivin pulsed
- ● T2 PFP pulsed
- ▼ K562

- ■ T2 MAGE-A1 pulsed
- ■ T2 PFP pulsed
- ▼ K562

NY-ESO-1 specific

WT-1 specific

- ■ T2-NY-ESO-1 pulsed
- ● T2-PFP pulsed
- ▼ K562

- ■ T2-WT-1 peptide pulse
- ● T-PF peptide pulsed
- ▼ K562

F

PRAME specific

- ■ T2- PRAME PULSED
- ● T-PF peptide pulsed
- ★ K562

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Survivin specific

MAGE-A1 specific

NY-ESO-1 specific

WT-1 specific

Figure's legend is on the following page

Figure 5.2: Antigen-specific cytotoxic activity of T-cell lines from healthy, HLA-A2^{+} donors stimulated *in vitro* with hybrid cell lines. T2 cells were pulsed with peptides corresponding to known HLA-A2 epitopes of Survivin, MAGE-A1, NY-ESO-1, PRAME or WT-1 (green squares), or with an irrelevant HLA-A2-binding peptide (*P. falciparum* CSP; red circles), and used as target cells in ^{51}Cr-release cytotoxicity assays with T-cell lines derived from healthy, HLA-A2^{+} donors stimulated *in vitro* for $4 - 6$ weeks with hybrid cell lines. Additional control target cells were T2 cells without peptide (violet asterisks) and K562 cells (NK control; blue triangles). Data presented are for T-cell lines stimulated by (A) HMy2, (B) HxU266, (C) HxCM053, (D) HxRC056, (E) HxKG-1, (F) HxVP024, (G) HxPG052, and (H) HxTH044 cell lines. Data are shown as mean ± SEM of % specific lysis of triplicate tests at 3 different effector: target cell ratios. Statistically significant differences between T2 cells pulsed with cognate peptide *versus* T2 cells pulsed with irrelevant peptide are indicated with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).
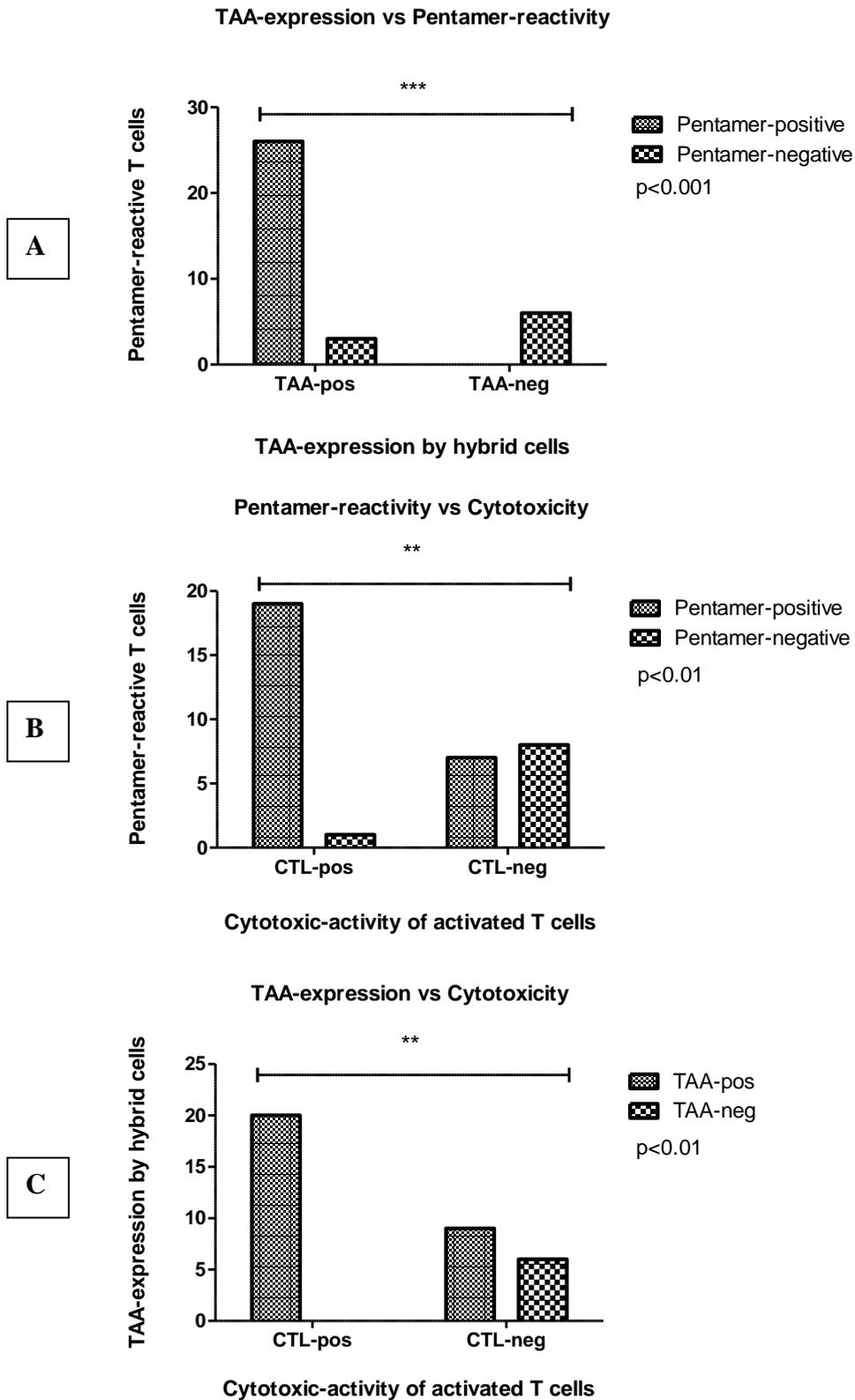
Figure 5.3: Presentation of significance of relation between; A- TAA-expression of the hybrid cells versus pentamer-reactivity of the generated CTL, B- Pentamer-reactivity versus cytotoxicity, and C- TAA-expression versus cytotoxicity. Data were analysed using Fisher's exact test.

5.2.2   Induction of autologous tumour antigen-specific CTL lines

PBMCs from two AML tumour-bearing patients from whom tumour cells were used in hybrid cell line generation, VP024 and DO050; both are HLA-A2$^+$, were available to be used in the establishment of long-term T cell lines by stimulation with semi-autologous hybrid cell lines, HxVP024 and HxDO050 respectively, for several rounds. The stimulated T cell lines were investigated for the presence of tumour antigen-specific CD8$^+$ T cells by HLA-A2-pentamer staining, and for cytolytic activity against peptide-pulsed T2 cells and autologous tumour cells, after 7-8 weeks of *in vitro* tissue culture stimulation, by ^{51}Cr release assays. Figure 5.3 shows the results of pentamer staining of the T cell cultures. Although it expressed all of the relevant tumour-antigens (as determined by qRT-PCR), HxVP024 cell line did not induce pentamer positive CD8$^+$ T cells to any of the 5 peptide pentamers tested. However, HxDO050 cells induced pentamer positive CD8$^+$ populations to PRAME and NY-ESO-1, and weak populations to Survivin and MAGE-A1 (Figure 5.4) (all of which TAAs were expressed by HxDO050 hybrid cell line).
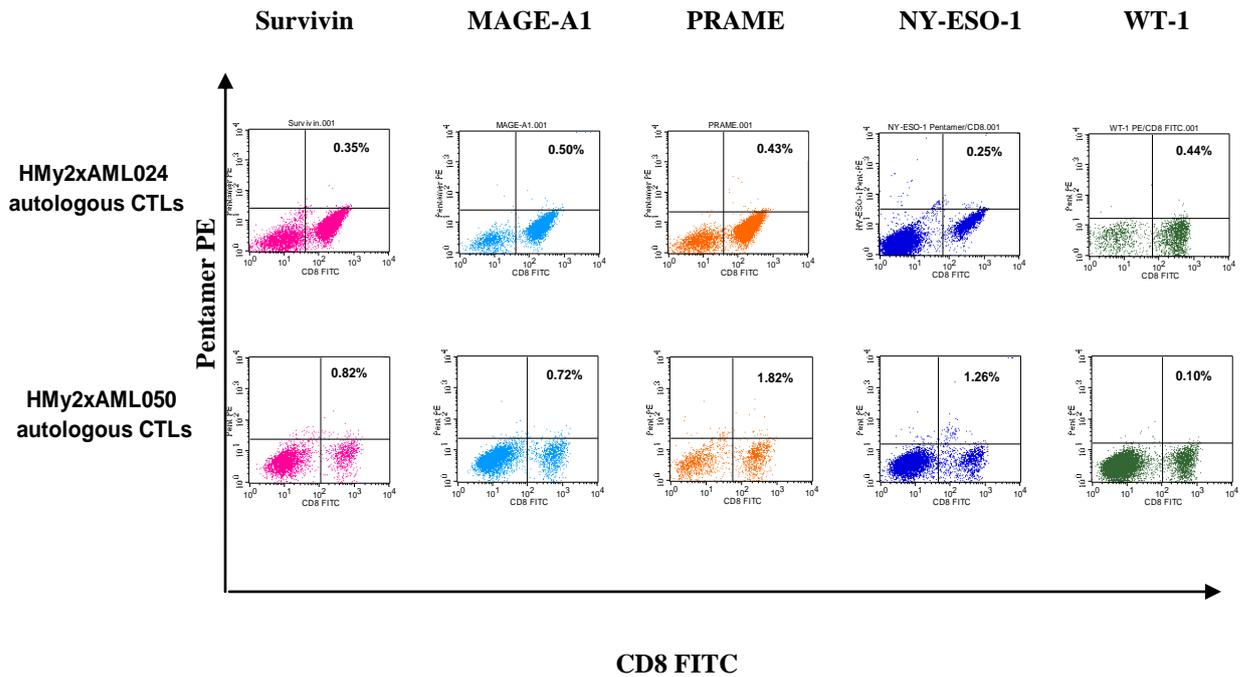
Figure 5.4: Flow cytometric analysis of HLA-A2-peptide pentamer and anti-CD8 mAb stained T cell cultures generated by long-term stimulation of autologous PBMC from two HLA-A2$^+$ tumour bearing patients (VP024 and DO050), from whom stimulator hybrid cell lines have been generated (HxVP024 and HxDO050 respectively). Cells were analysed for Survivin, MAGE-A1, PRAME, NY-ESO-1, and WT-1-peptide pentamers

As a consequence of pentamer-specific CTL data, cytotoxicity assays were performed using HxDO050-activated autologous T cell lines against peptide-pulsed T2 cells (as in Section 5.2.1.2). Results indicated presence of significant CTL cytolytic activity against MAGE-A1 and NY-ESO-1, a weak response against Survivin, but no activity was seen against PRAME-peptide-pulsed T2 cells (Figure 5.5).
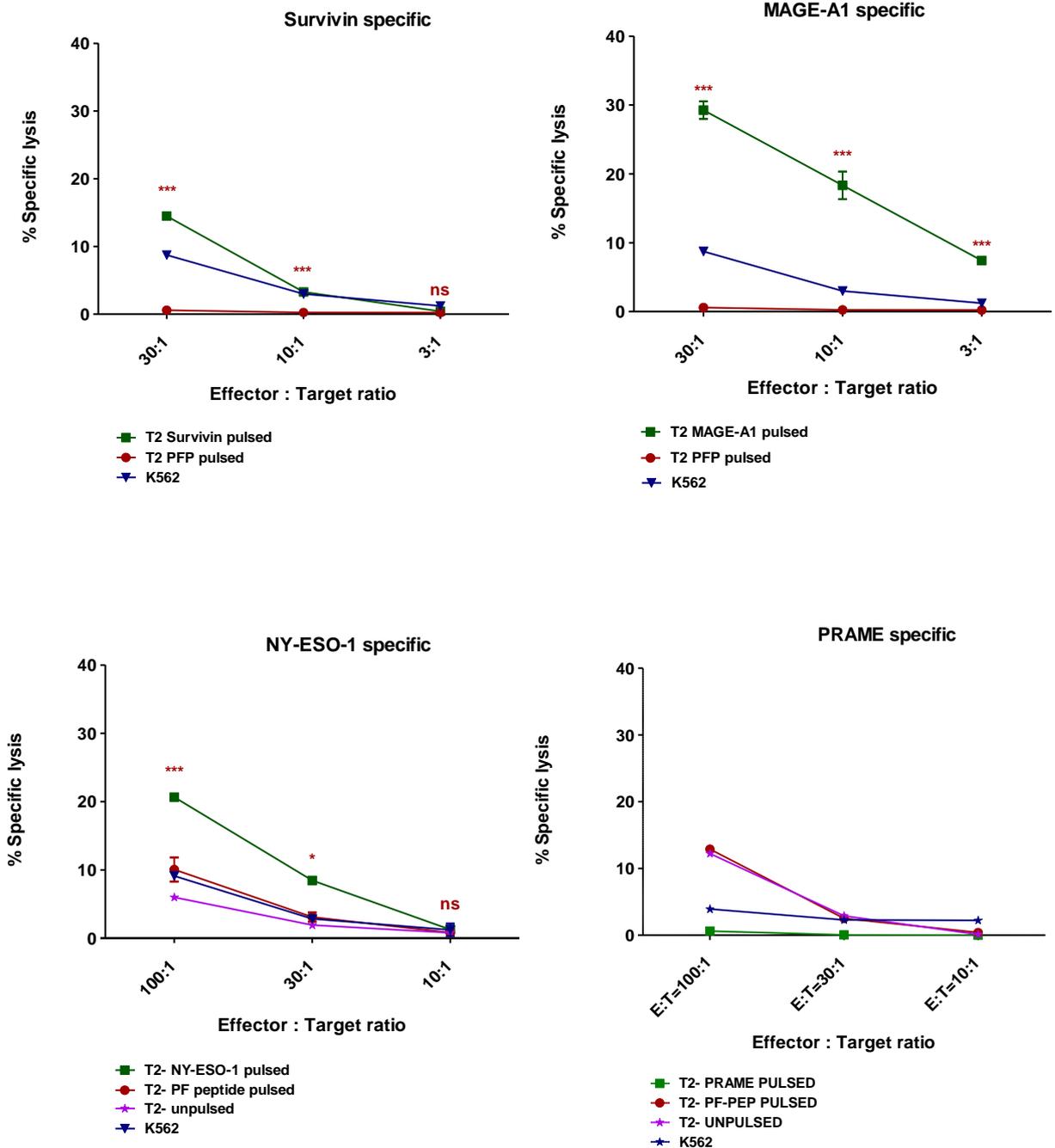
Figure 5.5: Antigen-specific CTL activity of T cells from HLA-A2⁺ AML-patient's (DO050) PBMC stimulated *in vitro* with semi-autologous hybrid cell line HxDO050. Target cells were T2 cells pulsed with peptide from relevant TAAs (Survivin, MAGE-A1, PRAME, and NY-ESO-1), *Plasmodium falciparum* peptide-pulsed T2 cells (as negative control), and K562 cell line as NK target.

5.2.3 Autologous tumour cell cytotoxicity within the stimulated CTL lines

[51]Cr release cytotoxicity assays were performed to investigate the tumour-specific cytotoxicity of T cell cultures generated by stimulation of VP024 and DO050 PBMCs using semi-autologous hybrid cell lines HxVP024 and HxDO050 respectively for up to eight weeks. The T cell lines showed high levels of cytotoxic activity against patient's own tumour cells, which indicates the presence of tumour-specific CTLs within the activated T cell cultures, as the effector CTL and target tumour cells are syngeneic, there was no possibility for allogeneic responses. In addition, high levels of cytotoxicity were also seen against the stimulating hybrid cells and against HMy2 cells. This may indicate the induction of T cell responses against allogeneic, HMy2-derived major and minor-histocompatibility antigens expressed by the stimulating hybrid cell line, or against TAAs expressed by both HMy2 and hybrid cell line (such as Survivin, MAGE-A3, h-TERT, or MUC-1) and presented through shared HLA class I molecules (such as HLA-A2) (Figure 5.6). Moreover, no NK activity was seen in the autologous activated T cell cultures (in both VP024 and DO050), as indicated by very low cytolytic activity for K562 cells (Figure 5.6). In addition, it will be worth in future work to investigate the avidity of tumour-specific T cells by doing T cell titrations.
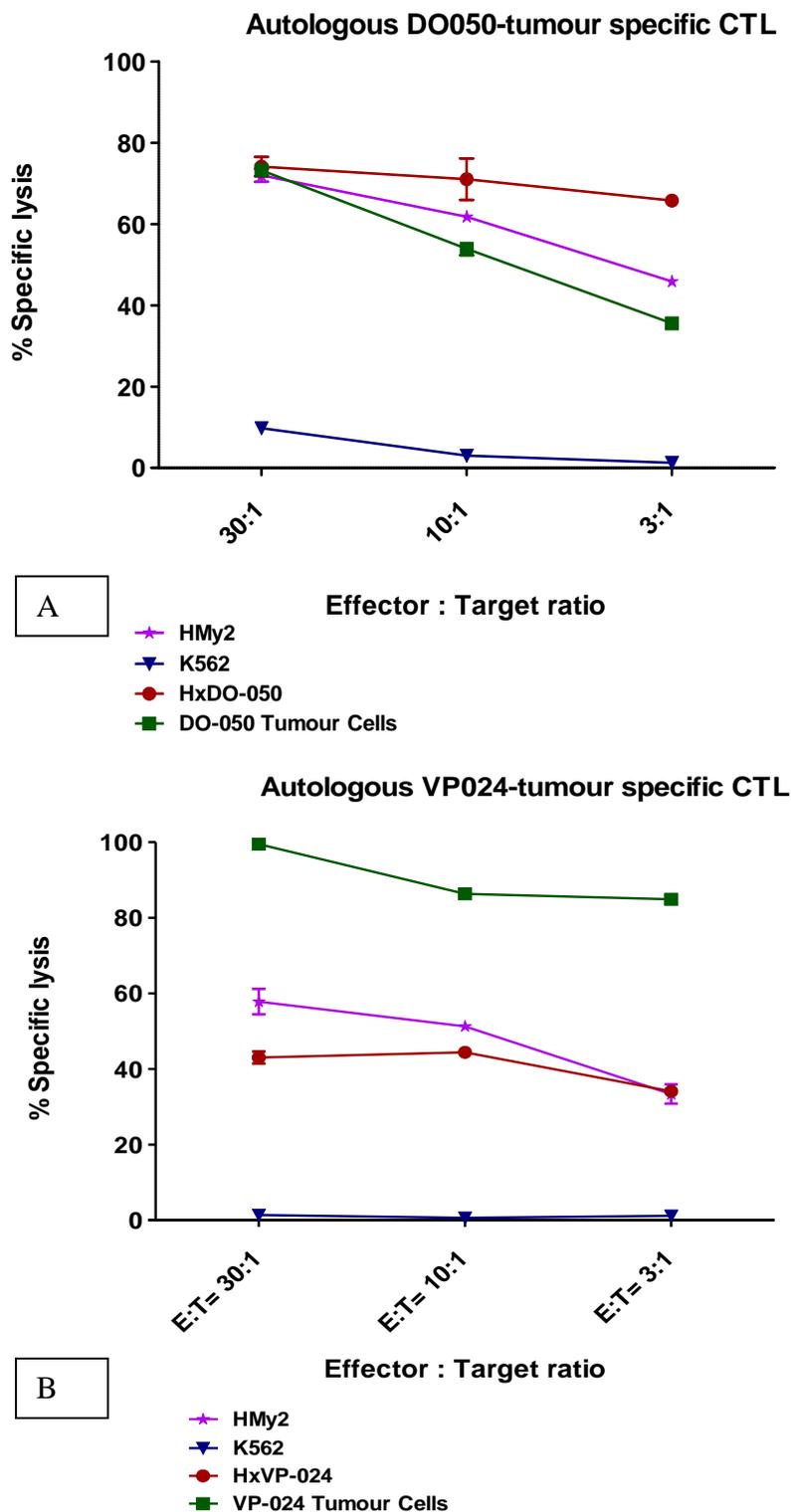
Figure 5.6: ^{51}Cr release cytotoxicity assay. Chart (A) and (B) represent autologous cytotoxic T cell activity of HxDO050- and HxVP024-activated patients' T cells against autologous DO050 and VP024 tumour cells (green squares) respectively. The stimulating hybrid cell line (red circles) and HMy2 (violet asterisks) cells were included as controls, and K562 (blue triangles) cells as NK activity indicator.

5.3 Discussion

In Chapter 3, I showed that most of the investigated hybrid cell lines expressed at least one TAA that was not expressed by HMy2 cells, and therefore, these TAAs were derived from the parent tumour partners. These included haematological malignancy-relevant antigens (such as WT-1) and other antigens expressed by a wide range of solid tumours such as PRAME, and MAGE-A1. Moreover, it was assumed that the hybrid cell lines may express other TAAs that have not been investigated in this study. The ability of the hybrid cell lines to express MHC class I, MHC class II, and co-stimulatory molecules (CD80 and CD86), and to induce allogeneic proliferative, IFN-γ releasing, and cytotoxic T cell responses of PBMC from normal healthy individuals was observed *in vitro*, and the data were presented in Chapter 4.

In this Chapter, I have shown that these hybrid cell lines had the ability to induce tumour- and tumour antigen-specific CTL responses *in vitro* from PBMC taken from normal HLA-A2$^+$ donors, or tumour-bearing patients from whom hybrid cell lines were derived. Seven hybrid cell lines were chosen, based on their expression of TAAs (Survivin, MAGE-A1, PRAME, NY-ESO-1, and WT-1) to investigate their ability to induce TAA-specific cytotoxic T cell responses *in vitro* using PBMC from HLA-A2$^+$ healthy donors. These cultures resulted in the induction of T cell lines specific for HLA-A2-restricted TAA-peptides of the candidate antigens.

The allogeneic CTL lines showed pentamer positive populations, which correlated with the TAA-expression profile of the stimulating hybrid cell lines in 90% of cases (p<0.001). The lack of correlation in the remaining 10% of cases (to NY-ESO-1 and PRAME in HxRC056; and NY-ESO-1 in HxKG-1 stimulated CTL) may be explained by the high sensitivity of NY-ESO-1 expression level in HxRC056 by qRT-PCR (where

weak antigen expression was not associated with induction of pentamer-positive population), or by the inability of HxRC056 and HxKG-1 cells to express the relevant antigens, on the protein level, and to present antigen-derived peptide in context of appropriate HLA class I allele, although the expression of these antigens on mRNA level by same cell lines.

In addition, the pentamer positive-CTL lines showed significant antigen-specific cytolytic activity against T2 cells pulsed with the relevant HLA-A2-restricted TAA-derived peptide epitopes, compared with T2 cells pulsed with an irrelevant HLA-A2-restricted peptide epitope (*Plasmodium falciparum* derived peptide). These results showed positive correlation between positive pentamer staining and peptide specific cytolysis in 76% of cases (p<0.01). The inconsistency in the remaining 24% was due to absence of cytolytic activity of the PRAME-specific pentamer-positive populations from HxU266, HxHxKG-1, HxVP024, and HxPG052-activated T cell cultures. This could be explained by the instability of PRAME-peptide binding to HLA-A2 antigen of T2 cells, or by a suboptimal concentration of peptide used in pulsing of these cells; accordingly, by increasing the peptide concentration (> 50μg/ ml) and/ or by increasing peptide stability on the surface of T2 cells, more significant cytotoxicity results would be achieved. A difficulty in loading of PRAME peptide was suggested to be the main cause of these discrepancies and by enhancing its solubility, a better correlation may be achieved. Another cause of inconsistency was the absence (or weak) cytolytic activity of TAA-specific pentamer-positive populations of the HxTH044-stimulated T cell culture to relevant peptide pulsed T2 target cells. These non-functional T cells may have been suppressed by action of activated Treg cells which might be produced in association with $CD8^+$ T cells by long-term activation with HxTH044. This assumption was supported by the lack of IFN-γ releasing ELISpot responses in short-term

autologous (Section 4.2.2.1.2), and in the long-term allogeneic T cell cultures (Section 4.2.2.2.1.2) in response to TH044 parent tumour cells. In addition, there was a great correlation between the TAA-expression by hybrid cell lines and their ability to induce functional TAA-specific CTL (74%, $p<0.01$).

In addition, PBMC from two HLA-A2$^+$ patients were used to establish autologous, tumour- and TAA-specific CTL lines, by stimulation with the respective semi-autologous hybrid cell lines. The T cells' antigen specificities were investigated by peptide-specific pentamer and anti-CD8 mAb staining, and by the cytolytic activity through ^{51}Cr release cytotoxicity assays.

The autologous T cell cultures (derived from tumour bearing patients) showed specific cytolytic activity against the relevant autologous tumour cells, as well as the stimulatory semi-autologous hybrid and HMy2 cell lines. In addition, one of the autologous T cell cultures (DO050 derived) showed HLA-A2-restricted, TAA-specific responses, as indicated by pentamer-positive CD8$^+$ populations, which were able to kill T2 cells pulsed with peptides derived from the following antigens (MAGE-A1, NY-ESO-1, and Survivin) to a significantly greater extent than control peptide-pulsed T2 cells. The other autologous T cells (VP024 derived) did not induce *in vitro* TAA-specific pentamer-positive populations, although the expression of most candidate antigens by the parent tumour cells, which may be explained by presence of anergic, non-functional T cells as a result of the immunosuppressive tumour microenvironment, or the inability to induce autologous CTLs against the investigated TAAs. Another support for this explanation was the inability of the same culture to respond, by IFN-γ release, to stimulation with parent tumour cells in short-term (Section 4.2.2.1.2) or in long-term (Section 4.2.2.2.2) autologous ELISpot assays.

These data indicate that the EBV B-LCL/tumour hybrid cell lines were proficient in inducing tumour- and tumour antigen-specific CTL responses *in vitro,* by stimulating PBMC from both haematological malignancy patients, and from normal (HLA-A2$^+$) donors. Furthermore, the hybrid cell lines were able to induce functional CTL against multiple TAAs that are known to be expressed by haematological malignancies (e.g. WT-1 and Survivin) and also against TAAs expressed by a wide range of solid tumour types (e.g. MAGE-A1 and NY-ESO-1). Moreover, while several studies showed the importance of CD4+ Th cells in maintenance of the proliferation and functionality of the effector cytotoxic CD8+ cell, my study showed induction of both T cell subtypes (Section 4.2.1.5). As a result, the hybrid cell lines of HMy2/ tumour cell provide candidate, T cell-stimulating agents for immunotherapy of not only haematological malignancies, but possibly also for other types of solid tumours which share the expression of some TAA and HLA molecules with the stimulatory hybrid cell lines. This model of hybrid cells could potentially be used for tumour immunotherapy, either in an active way as candidate cellular vaccines or a passive way by the *in vitro* induction and expansion of TAA-specific CTL lines derived from PBMC from HLA partially-matched patients or healthy donors.

Vaccination with APC/tumour cell fusions has been shown to induce immunological and clinical responses, ranging from protection against tumour challenges to rejection of established tumours in animal models of melanoma (Wang *et al.*, 1998), colorectal (Kao *et al.*, 2003) , breast (Xia *et al.*, 2003), oesophageal (Guo *et al.*, 2008), pancreatic (Yamamoto *et al.*, 2009), hepatocellular (Sheng & Zhang, 2007), lung (Savai *et al.*, 2006), laryngeal (Weise *et al.*, 2004), and renal cell carcinomas (Siders *et al.*, 2003), in addition to sarcoma (Kjaergaard *et al.*, 2003), myeloma (Gong *et al.*, 2002), and neuroblastoma (Iinuma *et al.*, 2006). Fusion cells may be fully allogeneic, semi-

allogeneic, or fully autologous, according to the source of APC and tumour cell partners. Allogeneic fusion vaccines were able to induce tumour-specific immune responses and to protect against tumour challenges, and a number of reports demonstrated that the allogeneic HLAs provided adjuvant effects for the tumour specific responses' initiation and maintenance (Cao *et al.*, 2009, Yasuda *et al.*, 2007, Nanni *et al.*, 2001, Toes *et al.*, 1996), and were not the detriment of the induced antigen-specific responses. My results in this Chapter are in agreement with this, as multiple tumour antigen-specific CTL lines were induced from PBMC of normal, allogeneic donors using allogeneic HMy2 x tumour hybrid cell lines as *in vitro* stimulators. Semi-allogeneic hybrid cell vaccines (in which either APC or tumour cells are taken from a different individual than the tumour bearing host) have been used in animal models and showed protective responses in several tumour types, such as melanoma, lymphoma, renal and colon cancers (Yasuda *et al.*, 2007, Siders *et al.*, 2003, Wells *et al.*, 2007).

Several preclinical trials reported the ability of human, semi-allogeneic APC x tumour hybrid cells to induce tumour- and tumour antigen-specific T cell responses from normal donors' PBMCs. Matsumoto *et al*. (Matsumoto *et al.*, 2006) developed hybrid cells by fusion of autologous DCs with gastric tumour cell lines. The semi-allogeneic hybrid cells expressed CEA, and were able to induce both CD4$^+$ T cell response, and CD8$^+$ HLA-A24-restricted CEA-specific CTLs *in vitro* from the same donor's PBMC. The CTL-specificity was investigated *in vitro* by tetramer staining and ^{51}Cr release cytotoxicity assays using the parent gastric tumour cell line and CEA-peptide pulsed T2 cells as targets, and a highly significant cytolysis was observed against these targets. In another study by Cao *et al*. (Cao *et al.*, 2009), to compare semi-allogeneic and autologous APC x tumour hybrid cells' ability to induce *ex vivo* immune responses,

DCs from patient and normal donors were fused to *ex vivo* autologous hepatocellular carcinoma (HCC) cells or a HCC cell line (HepG2; all cells and cell lines were HLA-A2$^+$/HLA-A24$^+$, and the tumour cells were AFP$^+$). Both autologous and semi-allogeneic hybrids made with patients' DCs induced CTL responses following co-culture with patients' PBMCs, and the resulting CTL were able to kill the autologous *ex vivo* HCC cells in *in vitro* cytotoxicity assays. Moreover, the semi-allogeneic hybrid-induced CTL specifically killed HepG2 and BEL7402 (HCC cell lines; both are AFP$^+$/ HLA-A2$^+$) in a specific, MHC class I-restricted manner. Cao concluded that, although the autologous hybrid cells provided known and un-identified TAAs which were best matched to the tumour bearing hosts, there were some drawbacks in using *ex vivo* tumour cells as fusion partners, such as scarcity of cells, time and labour intensiveness, lack of ability for long-term tissue culture maintenance, and the possibility of bacterial and fungal contamination of primary cultures. In contrast, tumour cell lines of the same tissue type provided a continuous source of cells, of well known and multiple tumour and allogeneic antigens, which upon fusion with APC could be presented in the context of MHC class I and class II and subsequently initiate innate and adaptive immune responses. The presence of allogeneic stimulation displayed by hybrid cells' MHC molecules induced additional immunostimulatory adjuvant signals to support the induction and maintenance of tumour-specific responses. Similarly, in another study using semi-allogeneic hybrid cells generated by fusion of DCs of colorectal cancer patients with allogeneic colorectal carcinoma cell line (COLM-6; CEA$^+$ and MUC-1$^+$) to induce tumour-specific T cell responses, Koido and his colleagues (Koido *et al.*, 2005) showed the ability of these hybrid cells to induce proliferative, cytokine releasing and antigen-specific CTL responses from autologous patients' PBMC. The generated CTLs were able to recognise and kill autologous colorectal tumour cells as well as

CEA/ MUC-1-expressing allogeneic cell lines in a HLA-A2-restricted manner. In the same context, Zhang *et al*. (Zhang *et al.*, 2007) reported the *in vitro* induction of T cell proliferative, IFN-γ releasing, and CTL responses from breast cancer patients' PBMC by activation with semi-allogeneic hybrid cells generated by fusion of patient derived DCs with MCF-7 breast cancer cell line. The generated CTLs induced cytolysis of autologous and HLA-A2$^+$/ CEA$^+$ matched cell lines *in vitro*. These studies support the assumption that allogeneic hybrid cell vaccines were able to express relevant TAA, and to induce proliferative and antigen-specific cytolytic T cell responses *in vitro,* and that the presence of allogeneic molecules enhanced the induction of tumour-specific responses.

The use of *in vitro* transformed B cells, EBV B-LCL (such as HMy2 cell line) as APC fusion partner, instead of *ex vivo* DCs, provides the advantage of parent APCs that are immortalised, easily cultured and manipulated *in vitro*, and capable of processing and presenting antigens through both MHC class I and class II pathways, with provision of co-stimulatory signals and accessory molecules for induction of naive as well as memory CD4$^+$ and CD8$^+$ T cell responses. Animal studies showed the feasibility of activated (but not the immortalised) B cell/ tumour cell hybrids in induction of cytotoxic anti-tumour immune responses (Guo *et al.*, 1994). In addition, activated B cells were reported to induce primary *in vitro* and *in vivo* immune responses (Schultze *et al.*, 2004, Lapointe *et al.*, 2003, von Bergwelt-Baildon *et al.*, 2002). Moreover, clinical trials have shown safety and immunological and/or clinical responses to hybrid cell vaccines generated by fusion of allogeneic, activated B cells with autologous patient tumour cells. The first trial in melanoma was reported by Trefzer *et al*. (Trefzer *et al.*, 2000), who observed clinical responses in 7 out of 16 melanoma patient receiving hybrid cell vaccines of allogeneic B cells x autologous melanoma *ex vivo* tumour cells,

with minimal side effects. Further trials were reported on glioblastoma (Moviglia *et al.*, 2008), which confirmed B cell hybrids' ability to induce clinical immunotherapeutic responses. Although the HMy2/tumour hybrid model represents a promising approach in tumour immunotherapy, it may be argued that as an EBV-transformed cell line which can be tumourigenic itself, it may not be applicable in EBV-seronegative patients, and alloreactivity may induce severe GvHD. So, an appropriate balancing between benefit versus harm is needed before using this model clinically.

Moreover, another important potential application of HMy2 x tumour hybrid cell lines is the *in vitro* induction and expansion of TAA-specific autologous (PBMC from the same individual as the parent tumour cells) or allogeneic (PBMC from an individual unrelated to the tumour cells, but with partial HLA matching) CTLs to be used in adoptive cell transfer (ACT) for treatment of tumour patients. ACT was firstly introduced as a new immunotherapeutic treatment for metastatic melanoma in 1988 (Rosenberg *et al.*, 1988), however, the efficacy of this approach was greatly enhanced after combining of pre-infusion conditioning of the patients by lymphodepletion. A chemotherapeutic agent was given to patients (to deplete regulatory and immunosuppressive cells) before introduction of tumour-reactive lymphocytes, which enhanced the growth, clonal expansion, and trafficking of effector T cells to different tumour bearing tissues *in vivo,* especially if co-administered with growth factors, such as IL-2 (Dudley *et al.*, 2002). Rosenberg observed a response rate of up to 50% in melanoma patients treated with autologous, *ex vivo* expanded TIL in combination with IL-2, however, an improved response rate of 72% was achieved by pre-infusion patient-lymphodepletion (Rosenberg & Dudley, 2009).

By way of improving ACT in the treatment of human cancers, several animal studies were carried out, which provided a better understanding of the processes of isolation, *in*

vitro expansion, and re-infusion of TIL into tumour bearing hosts to obtain the highest response with minimal toxicity. Early trials of ACT in animal models used the *in vivo* generated tumour-specific leukocytes. Delorme was first who reported *in vivo* clinical responses following infusion of effector leukocytes in a rat fibrosarcoma model (Delorme & Alexander, 1964). This observation was followed by a similar report of successful use of a combination of spleen cells from sarcoma-immunised mice along with chemotherapy to eradicate syngeneic sarcoma in tumour bearing mice (Fefer, 1969). Secondary sensitised, *in vivo* immune cells (in association with chemotherapy) were able to neutralise and eradicate intraperitoneal lymphoma in mice after adoptive transfer (Cheever *et al.*, 1977). In addition, assessment of the ability of long-term expanded immune cells in IL-2 to eliminate disseminated syngeneic tumour, as observed in a murine model after total body irradiation of treated animals, was reported by Eberlein *et al.* (Eberlein *et al.*, 1982). Moreover, the co-administration of growth factors, such as IL-2, with immune cells was reported to enhance the clinical response, especially the lymphocyte-induced response, in a murine disseminated lymphoma model (Donohue *et al.*, 1984). Further improvement of immunological manipulation of large disseminated murine tumours was achieved by combining ACT with TAA-specific vaccination and IL-2 administration. Overwijk *et al*. introduced tumour antigen-specific vaccination and IL-2 administration to enhance functionality of the administered tumour specific T cells in the treatment of murine metastatic melanoma. This triple therapy reactivated the anergic CD8$^+$ CTL, and succeeded in eradicating all tumour masses completely, although the treatment was associated with the induction of autoimmune reactions (vitiligo) at levels that were considered clinically tolerable (Overwijk *et al.*, 2003). These data confirm the synergistic effects of the anti-tumour

vaccines, ACT and growth inducing factors (e.g. IL-2) in treatment of advanced tumours, with minimal side effects.

Further progress in ACT mediated responses was reported after depletion of the host suppressor lymphocytes (Gattinoni *et al.*, 2005); the explanation of this progress was attributed to elimination of the anti-tumour inhibitory cellular mechanisms, as well as enhancing the adoptively transferred CTL stimulatory and homeostatic effects by the endogenously-released IL-7 and IL-15 cytokines (Gattinoni *et al.*, 2005). The more lymphodepleted the host, the more effective was the transferred anti-tumour $CD8^+$ T cells, as reported by Wrzesinski *et al*. (Wrzesinski *et al.*, 2007). In addition, the adoptively transferred T cells' phenotype was also an important factor, as central memory populations were reported to be more effective and persisted longer *in vivo* in animal models. Therefore, it was supposed that CM phenotype is most effective in ACT (Gattinoni *et al.*, 2005).

In the first human ACT clinical trial performed on melanoma bearing patients, Rosenberg *et al*. adoptively transferred *ex vivo* expanded autologous melanoma TIL in 20 melanoma patients after chemotherapy-induced lymphodepletion. The regime involved co-administration of IL-2 at the same time as the adoptive transfer, and clinical results showed complete tumour regression in multiple organs with minimal autoimmune side effects in most cases (Rosenberg *et al.*, 1988). In another study, ACT in combination with IL-2 administration and pre-infusion lymphodepletion, was shown to be clinically beneficial in melanoma in up to 50% of enrolled patients in a phase II clinical trial (Besser *et al.*, 2010). Recently, a phase I/II trial on 54 myeloma patients was reported, in which patients received autologous stem cell transplantation (ASCT) together with vaccination using tumour antigen (h-TERT and Survivin) vaccines and/ or vaccination with polyvalent pneumococcal conjugate vaccine (PCV), followed by

infusion of autologous, *in vivo* vaccine-primed, and *in vitro* expanded T cells. The study showed that adoptive transfer of autologous TAA-specific T cells together with TAA/ PCV vaccination of the myeloma patients receiving ASCT, induced cellular and humoral immune responses. These responses were associated with improved overall survival in a group of the treated patients (Rapoport *et al.*, 2011).

Furthermore, culturing and expanding TIL for therapeutic use from different tumour types (melanoma, RCC, colorectal cancer, breast cancer and sarcoma) was reported *in vitro,* with an average success rate of 71% from fresh and cryopreserved samples. The phenotypic characterization of expanded lymphocytes revealed a variety of T lymphocyte subtypes (Schiltz *et al.*, 1997). Further clinical observations were reported on ACT of different malignant diseases with great promise. Haque *et al*. (Haque *et al.*, 2007) reported the use of EBV-specific CTL from healthy, EBV-seropositive donors in adoptive allogeneic, cytotoxic T cell transfer of post transplantation lymphoproliferative diseases (PTLD). The CTL infusion showed no side effects in 33 enrolled patients, with a total response rate of 62% after 5 weeks follow up and 52% after 6 months. The data from this study confirm the ability of allogeneic CTL to induce safe, therapeutic responses in PTLD without the need for developing personal CTL for each patient. These results were confirmed by another study carried out by Heslop *et al* (Heslop *et al.*, 2010), where 114 post transplant patients received allogeneic CTL specific for EBV for PTLD prophylaxis. Results showed minimal side effects, in the form of localised swelling; however, none of 101 patients receiving adoptively transferred CTLs developed EBV[+] LPD, whilst 11 of 13 patients with established PTLD achieved sustained complete remission following adoptive CTL transfer.

Other clinical trials of ACT were performed on myeloma (Rapoport *et al.*, 2005), and acute and chronic leukaemia (Marijt *et al.*, 2007), and showed the feasibility, safety,

and therapeutic outcomes of *in vitro* generated CTLs, produced in accordance with GMP standards, in ACT of different tumour types, including haematological malignancies. A further study investigated the feasibility of using hybrid cells in the induction of human tumour-specific CTL for use in ACT to a SCID mouse model (Koido *et al.*, 2007). The author observed the *in vitro* induction of CTL responses from normal HLA-A2$^+$ donors' PBMC, by co-culturing with hybrid cells generated by fusion of donor-derived DCs with the allogeneic HLA-A2$^+$ breast cancer cell line MCF-7. The generated CTLs showed HLA-A2-restricted TAA-specific cytolytic activity *in vitro*. Koido and co-workers reported that DC/MCF-7 hybrid cells induced tumour-specific CTL from normal donors' PBMCs, which were specific for the parent tumour cells and induced complete tumour regression with prolonged tumour-free survival following adoptive cell transfer to 7day MCF7 tumour SCID mice.

In conclusion, in this Chapter I have shown the ability of HMy2/tumour cell hybrids to induce tumour and tumour antigen-specific CTL lines by multiple rounds of *in vitro* stimulation of PBMC from patient and normal (HLA-A2-matched) donors. These induced tumour-specific CTL lines were able to recognise and kill relevant autologous parent tumour cells and TAA-peptide-pulsed T2 cells *in vitro* in a HLA-A2-restricted manner.

These results suggest that HMy2-derived hybrid cell lines have the potential as cancer immunotherapeutic agents, either through active immunisation of patients, or as *in vitro* inducers of tumour-specific CTL for use in adoptive immunotherapy protocols.

Chapter 6

General Discussion and Future Work

6 General discussion and future work

6.1 General discussion

It is generally accepted that most, if not all, tumour cells express abnormal self proteins or abnormal levels of self proteins which are normally expressed only by privileged tissues or during certain developmental stages. The immune system is able to recognise these aberrantly expressed proteins as foreign antigens, presented in the context of MHC class I and class II on the surface of host tumour cells, or cross presented by host professional APCs, through recognition by TCR of $CD8^+$ and $CD4^+$ T cells respectively. Although innate and humoral responses are induced against growing tumours in most immunocompetent hosts, cellular immune responses, including non-specific killers (NK and NKT cells), and antigen-specific $CD8^+$ and $CD4^+$ T cells are the main effector anti-tumour mechanisms. *In vivo* activation of anti-tumour T cell immune responses was discussed in Section 1.2.2. In addition, experimental and clinical evidences support the role of the hosts' T cell immune responses in protection against tumour initiation and progression in immunocompetent hosts (Section 1.1). However, anti-tumour T cell immune responses ultimately fail to protect against most immune-resistant transformed cells due to the development of potent immune-escape and regulatory mechanisms (Section 1.4), leading to immune evasion by the tumour cells. Manipulation of the immune system to reinforce pre-existing responses or to initiate strong *de novo* anti-tumour immune responses which are able to eliminate developing tumour masses and to protect against further recurrence, has been investigated over the last two decades. The conventional anti-tumour therapeutic approaches include surgical removal of operable masses, courses of radiotherapy or chemotherapy, and hormonotherapy. The main side effects of conventional therapies arise from their low specificity and short-term activity, which are potentially avoidable in the new

immunotherapeutic approaches. The immune-mediated anti-tumour responses aim to target tumour cells more specifically, so that the normal tissues are not affected, and to mediate long-lasting memory effectors for protection against tumour recurrence.

The recent advances in identification and classification of tumour antigens recognised by T cells (Machiels *et al.*, 2002) have led to investigation of several new tumour-derived cellular and peptide-based approaches for cancer immunotherapy. These include immunisation with synthetic peptide alone or with adjuvant (Machiels *et al.*, 2002, Parmiani *et al.*, 2002), peptide-pulsed APC (Aloysius *et al.*, 2009, Nakai *et al.*, 2009), mRNA-pulsed APC (Bonehill *et al.*, 2009), or immunisation with naked DNA or protein encoding plasmid (Anderson & Schneider, 2007). Other vaccination approaches have included tumour cells genetically modified to express immunostimulatory cytokines (such as IL-12, IFN-γ or IL-2) (Yoshida *et al.*, 1999), co-stimulatory molecules (such as CD80 and CD86) (Chan *et al.*, 2005), or MHC class II molecules (Lu *et al.*, 2003). The use of professional APCs (such as DCs or activated B cells) to induce primary specific anti-tumour CD8$^+$ cytotoxic and CD4$^+$ Th1 responses has been widely employed, and therefore, several approaches have been described to load professional APCs with TAAs for anti-tumour immunotherapy (Section 1.5.5).

Fusion of professional APCs (especially DCs) with tumour cells is an approach which combines the full antigenicity of the parent tumour cells with the antigen processing and presenting, and immune stimulatory capacities of APCs, in order to induce strong anti-tumour cellular immune responses, especially by using allogeneic APCs, which express allogeneic MHC molecules (in association with other molecules) and enhance induction and maintenance of anti-tumour CTL and Th1 proliferation and differentiation as well as cytokine releasing and cytolytic responses.

The description of effective hybrid cells, generated by fusion of rat HCC tumour cells with activated B cells by Guo *et al*. (Guo *et al.*, 1994), and the promising *in vivo* results of this approach in inducing tumour rejection and protection against subsequent challenges in immunised rats, opened the door for further *in vitro* and clinical studies of hybrid cell vaccines generated by fusion of different types of tumours with DCs and other professional APCs. In laboratory animal models, DC/tumour hybrids have been reported to induce protective immune responses to tumour challenges and, in some cases, rejection of established tumour masses in different animal models, such as melanoma and lymphoma (Wang *et al.*, 1998), multiple myeloma (Gong *et al.*, 2002), neuroblastoma (Iinuma *et al.*, 2006), colon adenocarcinoma (Yasuda *et al.*, 2007), and oesophageal carcinoma (Guo *et al.*, 2008). In humans, numerous phase I/II clinical trials have been described, showing evidences of clinical and/ or immunological responses to hybrid cell vaccination in a number of different tumour types, including metastatic melanoma (Banchereau *et al.*, 2001, Krause *et al.*, 2002, O'Rourke *et al.*, 2003, Trefzer *et al.*, 2005), RCC and breast cancer (Avigan, 2004, Avigan *et al.*, 2004, Zhou *et al.*, 2009), glioma (Kikuchi *et al.*, 2004), pancreatic cancer (Yamamoto *et al.*, 2009), and multiple myeloma (Rosenblatt *et al.*, 2010). Several *in vitro* studies on haematological malignancies were conducted using tumour cell/APC fusions, such as AML/DC fusions, which induced more potent CTL responses than DC pulsed with tumour cell lysate (Galea-Lauri *et al.*, 2002). Klammer *et al*. induced tumour specific CTL responses *in vitro* using autologous DC/AML hybrids (Klammer *et al.*, 2005), whilst Vasir *et al*. and Raje *et al*. confirmed the ability of DC/MM hybrids to induce *in vitro* cellular responses from autologous T cells to a greater degree than tumour cells or DCs alone (Raje *et al.*, 2004, Vasir *et al.*, 2005). Further details about trials involving DC-derived hybrids were mentioned in Sections 1.5.5.4 and 1.5.5.5.

However, the use of DC as an APC partner in hybrid cell generation has several limitations, which require further efforts for improvement or finding an alternative approach for therapeutically effective hybrid cell production. The DC-derived fusions are labour intensive, need to be tailor-made for each individual patient, have limited replicative and culturing capacities, and low fusion efficiencies with little chance for post-fusion standardisation of the fusion products (Siders *et al.*, 2003, Cao *et al.*, 1999, Liu *et al.*, 2002, Phan *et al.*, 2003, Raje *et al.*, 2004). In addition, DCs can only be separated *ex vivo* from PBMC or bone marrow in low numbers, so a large volume of blood is required to obtain the sufficient number for hybrid production.

Nevertheless, the generation of stable APC/tumour hybrid cell lines, which can be grown and expanded in tissue culture, able to process and present tumour associated antigens in the context of MHC class I and class II, and retain the ability to induce *in vitro* and *in vivo* antigen-specific T cell responses, would overcome most of these problems. The HMy2 cell line provides a suitable APC partner for generation of effective and stable hybrid cell lines (Edwards *et al.*, 1982), by providing key features of professional APC, such as expression of MHC class I and class II, and co-stimulatory molecules CD80 and CD86. In addition, HMy2 offers double chemical selection (HAT-sensitivity and ouabain-resistance of the hybrid cells), which provides a potent mechanism to ensure that only the heterokaryons (HMy2 x tumour hybrid) can survive in culture, as the unfused or homokaryons of HMy2 cells are sensitive to HAT, and the unfused or homokaryons of parent tumour cells are sensitive to ouabain, and therefore do not survive in the culture.

Therefore, I investigated the potential of a panel of previously generated hybrid cell lines, formed by fusion of HMy2 cells with a group of *ex vivo* haematological tumour cells or cell lines (including MM, AML, ALL, CLL, FCL, and MCL), to produce stable,

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self replicating cell lines, and assessed their potential to process and present TAAs, and stimulate proliferative, functional, and tumour antigen-specific T cell responses *in vitro* by stimulating PBMC from tumour bearing patients or normal donors. My study has shown that HMy2 fusion with haematological tumour cells produced stable hybrid cell lines that grew continuously in tissue culture, were phenotypically stable, could be frozen and thawed without loss of their phenotypic or immunostimulatory properties, and were able to multiply to large numbers in a short period of time in tissue culture (mostly, with a doubling time of less than 36 hours). Phenotypically, like the parent HMy2 cells, the hybrid cell lines expressed high levels of tumour antigen, costimulatory, and accessory molecules (such as MHC class I, class II, CD40, CD80 and CD86 molecules), comparably higher than the respective parent tumour cells/cell lines. Functionally, the hybrid cell lines showed a markedly enhanced ability to stimulate proliferative and IFN-γ-secreting allogeneic T cell responses *in vitro*, as compared with their respective parent tumour cells. In most cases, the parent tumour cells failed to stimulate significant T cell responses above background levels under the same *in vitro* conditions. Moreover, the enhanced T cell proliferative responses induced by the hybrid cell lines were dependent on the expression of CD80 and CD86, as indicated by the ability of CTLA-4 Ig fusion protein to block the responses almost to their background levels. Furthermore, the hybrid cell lines directly stimulated both CD4$^+$ and CD8$^+$ T cells, and these responses were inhibited in the presence of monoclonal antibodies to both MHC class I and class II antigens, indicating a role for both MHC class I and class II in antigen presentation by the hybrid cell lines. Moreover, the T cells responding to hybrid cell line stimulation included not only central and effector memory cells, but also naive T cells. Through long-term stimulation, hybrid cell lines induced IFN-γ-releasing T cell responses to a greater

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extent than in short-term stimulation, and in both cases the responding T cell number was significantly higher when re-stimulated with hybrid than with respective parent tumour cells, in both allogeneic and autologous cultures.

By investigating TAA expression at the mRNA level, most hybrid cell lines expressed at least one of TAAs that were not expressed by HMy2 cells, and were therefore derived from parent tumour cells. The expressed antigens were of relevance for haematological malignancies (such as WT-1), as well as of relevance to a wide range of solid tumours (such as MAGE-A1, NY-ESO-1, and PRAME). In addition, a group of TAAs was expressed by all hybrid cell lines as well as HMy2 (such as Survivin, MAGE-A3, h-TERT, and MUC-1). A high degree of consistency was shown between qualitative and semi-quantitative RT-PCR in TAA-expression, for the hybrid cell lines. In all cases normal PBMC did not express any of the investigated TAAs. However, other TAAs might have been expressed by hybrid cell lines, as few antigens were investigated.

The type and level of TAA expressed and presented by hybrid cells are limiting factors for their use in immunotherapy. In my study, a group of the investigated hybrid cell lines expressed a number of TAAs that are highly immunogenic, tumour specific, widely expressed in different tumour types, and essentially important for tumourigenesis, which are important factors for stimulation of strong anti-tumour effector mechanisms (Mocellin *et al.*, 2004). This group were selected for induction of specific T cell responses *in vitro*.

Most importantly, I have shown that the hybrid cell lines elicited tumour- and antigen-specific CTL responses *in vitro,* from peripheral blood T cells taken either from patients from whom-tumour hybrid cells were derived, or from healthy (HLA-A2[+]) individuals,

by long-term (up to 8 rounds, each of 7 days) stimulation of responding PBMC by weekly pulsing with new batches of Mitomycin-C-treated stimulator hybrid cells, in the presence of exogenous IL-2. Based on their antigen expression profiles, seven hybrid cell lines were selected to investigate their capacity to induce antigen-specific CTL responses *in vitro*, using PBMC from (HLA-A2$^+$) healthy donors. The established cultures resulted in expansion of CD8$^+$ T cell clones specific for HLA-A2-restricted peptide epitopes from relevant TAA (Survivin, MAGE-A1, PRAME, NY-ESO-1 and WT-1). Although the expression of EBV-specific antigens (which may be immune-inhibitory) by HMy2, its derived hybrids showed high allogeneic immunogenicity and ability to induce allo- and antigen-specific T cell responses. In addition, cells from these cultures showed antigen-specific cytolysis of T2 cells pulsed with TAA-derived, HLA-A2-binding peptides, but not T2 cells pulsed with an irrelevant HLA-A2-binding peptide. I also established T cell lines from PBMC of 2 patients (both of whom were HLA-A2 positive) from whom hybrid cell lines had been generated. After several rounds of stimulation of the patients' PBMC *in vitro*, cytotoxic T cell activity directed against the patients' own tumour cells was detected. In one of these cultures, I showed the presence of CTL lines against specific TAAs that were expressed by the patient's tumour cells; in addition, the CTL lines from this patient showed significantly higher cytolytic activity against relevant peptide epitope-pulsed T2 cells compared with irrelevant peptide-pulsed T2 cells.

These data indicate that the hybrid cell lines were capable of inducing tumour- and antigen-specific CTL responses *in vitro*, from PBMC from both patients with haematological malignancies and from normal individuals of partially matched HLA type. None of the parent tumour cells were able to establish T cell lines by stimulation of autologous or allogeneic T cells under identical culturing conditions.

The HMy2/tumour hybrid cell lines simultaneously induced functional CTLs to multiple TAAs, suggesting that HMy2-derived hybrid cell lines represent promising candidates as T-cell-stimulating agents for potential use in immunotherapy of haematological malignancies. Furthermore, the ability of the hybrid cells to induce antigen-specific CTL responses to TAAs (such as MAGE-A1, Survivin and NY-ESO-1) that are expressed by a broad range of tumour types suggests that the potential of HMy2/tumour hybrid cell lines in cancer immunotherapy may not be restricted to haematological malignancies, but may apply to certain solid tumours expressing shared tumour antigens as well.

Hybrid cell lines, such as those described in this study, when produced under GMP protocols, could be used as candidates for cellular cancer vaccines in patients with haematological malignancies (semi-allogeneic), and special consideration would be given to AML-hybrids (future inter-assay variability would be essential before employing them as cancer vaccines), which showed induction of robust tumour antigen-specific T cell responses (mainly CTLs). HxVP024 hybrid cell line, in particular, showed higher tumour-specific response with moderate allogeneic cytolytic response, which helps to eliminate tumour cells without induction of GvH autoimmune side effects. The cellular cancer vaccine might be combined with other immune-modulators such as anti-CTLA-4 mAb, IL-2, or ACT.

Another possibility is to be used as stimulators for *in vitro* induction of tumour antigen-specific CTL for adoptive cellular transfer, in the context of either semi-autologous recipients (from whom tumour cells hybrids have been made), or allogeneic recipients (unrelated, but with partial matching in TAA and HLA expression).

Vaccination with allogeneic hybrid cells has been reported to induce tumour antigen-specific immune responses, and protection against tumour challenges; furthermore, several studies have shown that the allogeneic responses do not overwhelm tumour-specific responses (Trefzer & Walden, 2003, Schultze *et al.*, 1997, Yasuda *et al.*, 2007, Nanni *et al.*, 2001). My results support this, as my data demonstrate the ability of the allogeneic hybrid cell lines to induce polyclonal antigen-specific CTL responses to known TAAs *in vitro* from PBMC derived from un-related normal individuals. Similarly, semi-allogeneic hybrid cell vaccines have been shown to induce anti-tumour immunity and protective responses in murine models of lymphoma (Wang *et al.*, 1998), melanoma (Li *et al.*, 2001, Cao *et al.*, 1999), multiple myeloma (Gong *et al.*, 2002), and colon cancers (Yasuda *et al.*, 2007). In some of these studies, the expression of allogeneic MHC by hybrid cell lines was associated with the induction of stronger anti-tumour immunity and protection against tumour challenges than syngeneic fusion cells (Yasuda *et al.*, 2007, Haque *et al.*, 2007), although this finding has not been universal. Several preclinical and clinical studies have reported the immunological and clinical values of hybrid cell vaccination in different types of malignancies, and are mentioned in more detail in Section 5.3.

An alternative proposed immunotherapeutic approach would be the use the hybrid cell lines to stimulate and expand tumour antigen-specific CTL *in vitro*, from PBMCs from patients or normal individuals (with partially matched HLA), for autologous or allogeneic adoptive T cell transfer. The first introduction of ACT in treatment of cancer was reported in 1988 in melanoma immunotherapy (Rosenberg *et al.*, 1988), and further success was achieved by pre-infusion patient-conditioning (lymphodepletion; to eliminate the immunosuppressive cells), and co-administration of lymphocyte growth factor e.g. IL-2 (Dudley *et al.*, 2002).

Clinically, adoptive CTL transfer has been investigated in a number of malignant diseases, with great benefits. Haque *et al*. (Haque *et al.*, 2007) reported the successful use of EBV-specific CTL from EBV-seropositive donors in adoptive, allogeneic cytotoxic T cell therapy of post-transplantation lymphoproliferative disease (PTLD). These results were confirmed by further reports where CTLs were used in tumour therapy. Heslop *et al*. and Rooney *et al*. successfully used adoptive allogeneic CTL transfer to post-transplant patients for protection against EBV-mediated lymphoproliferative diseases (Heslop *et al.*, 2010, Rooney *et al.*, 1998). More clinical trials were carried out on adoptive CTL transfer for treatment of EBV[+] Hodgkin's lymphoma (Bollard *et al.*, 2004), nasopharyngeal carcinoma (Louis *et al.*, 2010), multiple myeloma (Rapoport *et al.*, 2011), acute leukaemia (Marijt *et al.*, 2007, Warren *et al.*, 2010). Methods for the culture of multiple, tumour-specific, clinical grade CTLs to good manufacturing practice (GMP) standards in large scale with high viability, and minimal risk of contamination were described by Turin *et al*. (Turin *et al.*, 2007), and Meehan *et al*. (Meehan *et al.*, 2008). In addition, studies of ACT clearly reported the avidity and efficacy of suitably selected anti-tumour CTL targeting identified tumour antigens in mediating clinical responses and tumour regression of different late-stage human cancers. Although requiring a personalised strategy to develop patient specific autologous CTLs (which are time and labour intensive), further development is ongoing to overcome these obstacles (Rosenberg *et al.*, 2008).

In summary, I have shown that fusion of the B-lymphoblastoid cell line, HMy2, with tumour cells from patients with a range of haematological malignancies can generate stable, self replicating hybrid cell lines that induced antigen-specific CTL responses *in vitro* in T-cells from both patients with haematological malignancy and normal individuals. HMy2/ tumour hybrid cell vaccines generated in this way may therefore

represent novel agents that offer an alternative to DC-based strategies for use in active immunization or adoptive T cell immunotherapy of haematological malignancies, and possibly in other forms of cancer as well.

6.2 Future work

If continuing my PhD project, I would plan to continue looking for induction of more TAA-specific CTL clones *in vitro*, following GMP protocols, and to separate the individual peptide epitope-specific, clinical grade CTL clones for adoptive T cell transfer. Additional candidate TAAs may be investigated, and antigen positive hybrids would be used in induction of more specific CTL, such as EBV-specific clones for use in PTLD treatment by ACT. In addition, TAA-expression of hybrid cells on the protein level may provide an additional positive correlation with the ability to induce specific T cells *in vitro*.

Moreover, I would assess the feasibility of fusion of EBV B-LCL to *ex vivo* tumour cells from different solid tumours (such as RCC, HCC, colon and gastric carcinoma), and investigate the generated fusion cells' ability to induce new TAA-specific T cell responses.

In addition, I would investigate the *in vivo* efficiency of the previously generated CTLs in animal models, after adoptive transfer, to reject experimentally established human tumours in the immunodeficient NOD/SCID mouse model. Also, the animal model would allow investigation of toxic and autoimmune drawbacks of allogeneic CTL in adoptive cellular therapy.

Chapter 7

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7 Bibliography

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