NOTTINGHAM TRENT UNIVERSITY

Investigating the potential of the Metastasis Associated Antigen 1 (MTA1) for cancer immunotherapy in a murine model

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A thesis submitted in partial fulfilment of the requirements of Nottingham Trent University for the degree of Doctor of Philosophy

2007

Acknowledgements

Firstly, I would like to thank Bob for giving me an opportunity to pursue a PhD in his group. I am very grateful to the “dynamic duo” of Steph and Bal for their guidance, advice and friendship for the past 4 years and it would have been really difficult without their help (also for allowing me to use the office for coffee, gossiping and some work!!!!). I am also thankful to Murrium for her help with the animal work. I would also like to thank my other supervisors Selman and Geng. I am also grateful to Amanda for her help with the molecular biology aspect of my project. Along with everybody else in the lab (Morgan, Catherine, Hossein, Krish, Alistair), who made it a great place to work (at most times!), I wish to thank Steve and Rob for their technical assistance, ordering and keep the lab in one piece. Sincere thanks to Ian and Emma for their efforts in breeding and taking care of the mice during this work.

Finally, I could not have done this without the support and belief of my family and friends.

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Abstract

Immunotherapeutic approaches to target antigens associated with metastasis could provide a valuable means of targeting metastatic cells specifically. Metastasis Associated Antigen (MTA1) is one such relatively novel antigen, which has been associated with aggressive tumours, and shown to be over expressed in breast, oesophageal, colorectal, gastric and pancreatic cancer, amongst others. Various studies have indicated that MTA1 is essential for the transformation of cells and hence targeting it is unlikely to generate antigen loss variants. This study proposed to investigate MTA1 as a potential target for immunotherapy in a murine tumour model. We have shown that murine MTA1 (mMTA1) mRNA is highly expressed in most of the tumour cell lines as compared to normal tissues, which express mMTA1 at very low levels. Furthermore, to rule out any post-transcriptional modifications, MTA1 protein levels were also confirmed by western blotting. It was observed that most of the cell lines expressed MTA1 at high levels, whereas no protein expression was detected in the normal tissues by western blotting. Next, we decided to identify MHC class I and II restricted immunogenic peptides from murine and human MTA1 gene for syngeneic and transgenic mice respectively. Three MHC class I immunogenic peptides for Balb/c mice and two for C57BL/6 syngeneic mice were identified but none of those peptides were found to be naturally processed. Similarly, HLA-A2 and HLA-DR4 restricted immunogenic peptides were also identified using transgenic mice, but proved to be not endogenously processed. We hypothesised that, MTA1 being a ubiquitously expressed self-antigen, central and peripheral tolerance mechanisms might have a vital role in non-availability of high affinity T cell repertoire against MTA1 and therefore peptide vaccination might be unable to break tolerance to mMTA1 on its own. Hence we decided to investigate more potent strategies such as DNA vaccination, viral vaccination and xenogeneic vaccination to overcome this issue.

Syngeneic or xenogeneic plasmid DNA vaccination was unable to generate an immune response to MTA1 in a mouse tumour model, even in combination with low dose cyclophosphamide for regulatory T cell depletion. Finally, semliki forest virus particles encoding for MTA1 did suggest weak immune reactivity against MTA1, as seen by delay in tumour growth in immunised animals. Further work will have to be done to optimise vaccination protocol to generate immune reaction against MTA1 and other such ‘self-antigens’. Moreover, recent studies have suggested a presence of Treg repertoire against such SEREX defined self-antigens and immunisation with such antigens may actually be immunosuppressive. Such a possibility might exist for MTA1 and needs to be investigated.

Abbreviations

ADCC	Antibody dependant cellular cytotoxicity
ALL	Acute lymphatic leukaemia
AML	Acute Myeloid Leukaemia
APC	Antigen Presenting Cells
Bgal	Beta-galactosidase
CEA	Carcino-embryonic antigen
CIITA	MHC class II transactivator
CML	Chronic Myeloid Leukaemia
CNS	Central Nervous system
CPM	Cyclophosphamide
CT	Cancer Testis
CTL	Cytotoxic T Lymphocytes
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
DTH	Delayed type II hypersensitivity
EBV	Epstein Barr Virus
EGF	Epidermal growth factor
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony stimulating factor
HDAC	Histone deacetylase complex
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
HSC	Haematopoietic stem cells
HSP	Heat shock proteins
hTERT	human Telomerase reverse transcriptase
HTLV	Human T cell leukemia virus
ICAM	Intercellular cell adhesion molecule
IDO	Indoleamine 2,3-Dioxygenase
IFN	Interferon
IGF	Insulin like growth factor
IL	Interleukin
KIR	Killer immunoglobulin-like receptors
LPS	Lipopolysacchride
MHC	Major Histocompatibility Complex
mRNA	Messanger RNA
MTA	Metastasis Associated Antigen
NK	Natural Killer Cells
NuRD	Nucleosome remodelin
PDGF	Platelet-derived growth factor
PPAR	Pathogen pattern recognition receptors
PSA	Prostate specific antigen

PSMA	Prostate membrane specific antigen
Rb	Retinoblastoma
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SEREX	Serologic Analysis of Recombinant cDNA expression libraries
SFV	Semliki forest virus
TAM	Tumour associated macrophages
TAM	Tumour associated macrophages
TAP	Transporter Associated Protein
TCR	T cell Receptor
Th	Helper T cells
TLR	Toll like receptors
TNF	Tumour Necrosis Factor
TRAIL	TNF-related apoptosis-inducing ligand
TSG	Tumour suppressor gene
VEGF	Vascular Endothelial growth factor
WHO	World Health Organisation

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1.1 Cancer

Cancer is a disease of varied spectrum and stages and can be defined as "uncontrolled cellular proliferation which at its advanced stages spreads to distant organs of the body". According to the latest figures of World Health Organisation (http://www.who.int/cancer/en/), cancer is the second largest cause of mortality in the world; approximately 11 million new cases are diagnosed every year and 7 million deaths a year are cancer related. Various etiologies, such as environmental, biological and chemical agents, have been implicated in cancer causation. Smoking is a very well known risk factor for cancer. Inhalation of minerals such as asbestos and silica during mining also presents a serious risk of mesothelioma, either on its own or synergistically with smoking (Kurihara & Wada, 2004). Similarly, exposure to the ultra-violet radiation of sunlight is the main risk factor for melanoma, the commonest type of skin cancer (Ivry *et al*., 2006). Experimental and epidemiological studies have also associated various chemicals to cancer and epidemiological studies have linked obesity to cancers such as adenocarcinoma of the oesophagus, colon cancer, breast cancer, endometrial cancer and renal carcinoma (Calle & Kaaks, 2004). Studies have also suggested an association between obesity and pancreatic, liver and gall bladder cancer (Calle & Kaaks, 2004). Among biological agents, several viruses have been shown to be directly and indirectly responsible for cancer. Epstein Barr virus has been linked to at least four different types of human cancer, including Burkitt's lymphoma and nasopharyngeal carcinoma. Similarly, Hepatitis B virus is shown to cause hepatocellular carcinoma (zur Hausen, 1991). Pathological examination of more than 98% cervical cancers show infection by human papilloma virus and thus provides a very strong evidence of this virus involvement in the disease process. Apart from these, cells can also acquire spontaneous DNA damage during normal cell division, leading to cancer.

1.1.2 Cell Cycle and Cancer

The human body consists of approximately 10^{15} cells and each cell of the body has to faithfully duplicate its genomic contents before dividing. Existence of haematopoietic stem cells (HSC), which have the capability of self-renewal and multi-lineage differentiation potential, is well known now. Recently, tissue specific stem cells have also been identified, which can divide and repopulate the mature organs (Huntly & Gilliland, 2005). Thus, approximately 10^{12} divisions in the stem cell compartment alone occurs daily (Bertram, 2000). Each cell cycle consists of four

main stages G1,S, G2 and M (Fig 1.1).

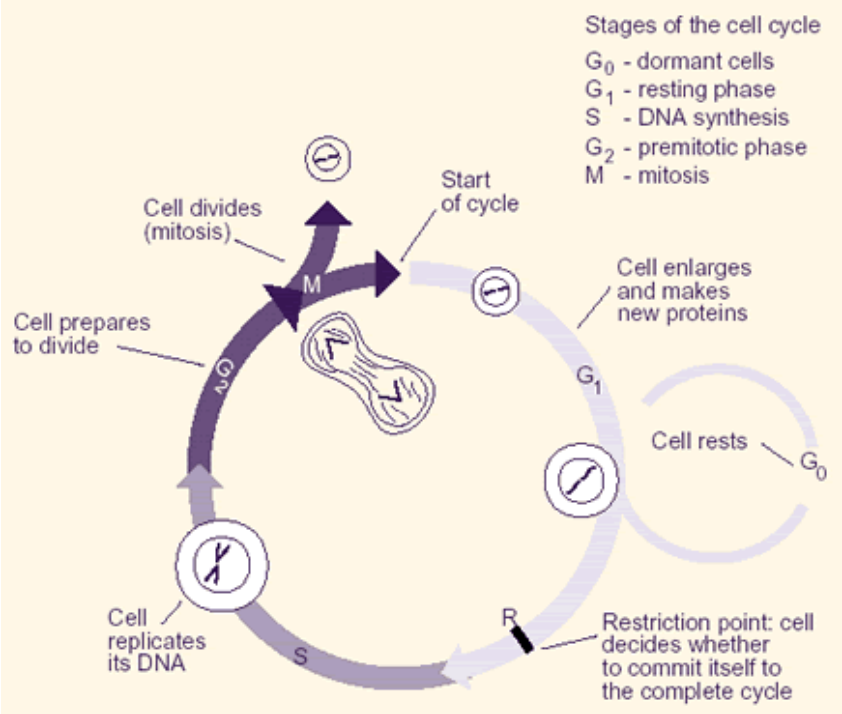


Figure 1.1:- Cell cycle *(Taken from www.cancerline.com)*

Various proteins control these cell cycle stages and to ensure efficient cell division, cells have evolved cell cycle check points to ascertain the integrity of the DNA and DNA mutations and chromosomal abnormalities are identified and repaired at these check points. If the DNA damage incurred by the cell is irreparable, the complex pathway of programmed cell death (apoptosis) is activated, thus ensuring that the damaged DNA is not duplicated and the mutation does not become fixed. Apoptosis, a programmed cell death mechanism, has evolved to induce cell suicide in cells and occurs via either extrinsic or intrinsic pathways. Extrinsic pathway is activated in response to engagement of death receptors on the cell surface i.e binding of Fas ligand and tumour necrosis factor (TNF) to their respective receptors. Intrinsic apoptotic pathway is activated in response to various intracellular and extracellular stresses such as DNA damage, growth-factor withdrawal and hypoxia. Both these distinct pathways, through complex cascade reactions, lead to activation of caspases, which exist in normal cells as pro-caspase precursors. Effector caspases on activation cleave other substrates in the cell which in turn cause several biochemical and morphological changes in the cell, eventually leading to cell death (Okada & Mak, 2004). One of the several genes involved in this complex process is p53, also known as the 'guardian of the genome'. Considering the complex machinery operating in the cell to prevent DNA damage and division, it is not surprising that most of the mutations observed in the cancer cell occur in the proteins controlling these cell cycle pathways and check-points.

1.1.3 Cancer-A multistep process

In spite of the numerous cell divisions taking place throughout life, the occurance of a cancer cell is still a relatively rare event. This is due to the fact that a single cell has to acquire multiple mutations to attain the cancerous phenotype. In fact, it is estimated that in humans, 5-6 mutations are required by the cell to become cancerous. Each of these mutations provides the cells with a selective advantage and endows them with properties such as independence of growth factors, non-responsiveness to growth inhibitory signals, infinite replicative potential, resistance to apoptosis, angiogenic potential and ability to metastasise; which are the essential hallmarks of cancerous cell (Fig 1.2) (Bertram, 2000). However, this is not strictly true as evident by certain childhood cancers, such as retinoblastoma (Rb), where in most cases, loss or mutation of both alleles of the Rb gene can cause cancer. Any DNA damage, which goes undetected by the cellular machinery becomes fixed and is passed on to daughter cells, which has to then acquire further mutations.

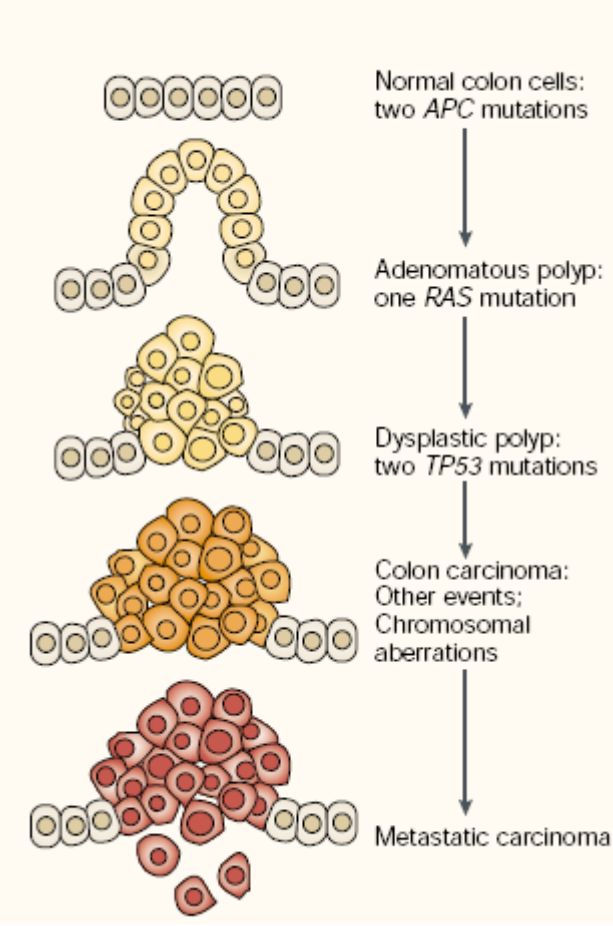


Figure 1.2:- Multistep progression of tumours in colon carcinoma *(Taken from Knudson, 2001)*

Thus, cancer cell acquire sequential mutations, each providing them with a selective advantage over other cells in a 'Darwinian' evolution manner, helping them to overcome the natural defenses protecting against cancer.

1.1.4 Oncogenes and Tumour Suppressor Genes

Almost all the agents implicated as a cause of cancer, lead to mutations in the genes controlling the cell cycle or cause disruption of these pathways or in case of viruses, can introduce oncogenes in the cell genome i.e. E6/E7 by human papilloma virus. Most of these genetic changes have been discovered in genes that are classified into either oncogenes or tumour suppressor genes.

1.1.4.1 Oncogenes

Initially, viruses were implicated as the causative agents for cancer and they were discovered to carry certain genetic sequences, 'oncogenes' which result in transformation of infected cells. Indeed, viruses such as Epstein-Barr virus, hepatitis B virus, human papilloma virus etc. have been implicated as causative agents for cancer (zur Hausen, 1991). Oncogenes carried by these viruses can integrate into the human genome and lead to uncontrolled cell proliferation. However, it was later identified that normal cells also contain these genes, known as 'proto-oncogenes'. Proto-oncogenes encode for proteins which are involved in pathways of growth regulating signal transduction and hence any disruption of this cellular signaling could lead to cancer. Various genetic events such as chromosomal translocations, gene amplification and point mutations can lead to oncogenic activation. One of the most commonly known chromosomal abnormalities is the translocation between chromosomes 9 and 22, which juxtaposes the ABL (tyrosine kinase) and BCR (break point cluster region), giving rise to Philadelphia chromosome and this abnormality is seen in 90% of the CML patients (Ren, 2005).

Proteins encoded by oncogenes include growth factors, growth factor receptors, cytoplasmic protein tyrosine kinases, signal pathway transduction mediators and transcription factors (Table 1) (Fig 1.3)(Peters and Vousden, 1997).

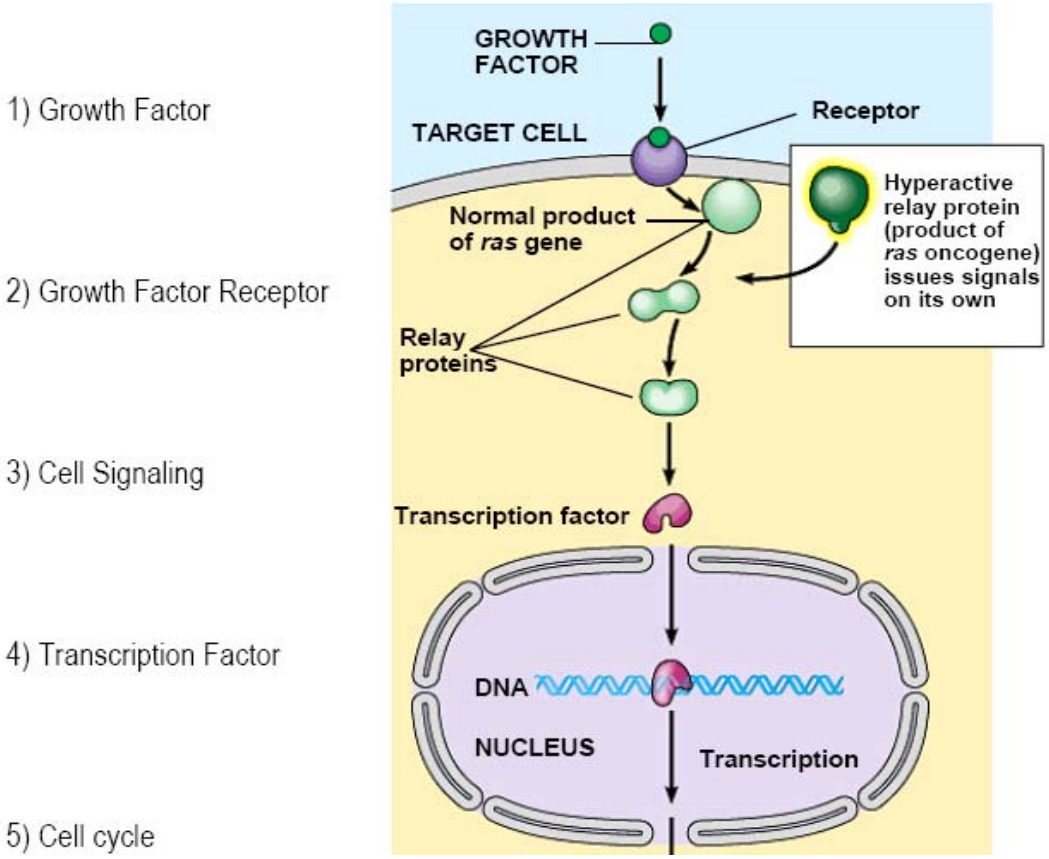


Figure1.3 Different levels of cell cycle being affected by oncogenic mutations *(modified from www.nature.com/nature/journal/v411/n6839/images/)*

Growth factors mediate cellular signaling by binding to their receptors on cell surface and can initiate cell differentiation, migration and activation of cell multiplication. Growth factors of the fibroblast growth factor, epidermal growth factor, platelet derived growth factor, WNT family, transforming growth factor β (TGFβ), insulin like growth factors and hepatocyte growth factors have been shown to be involved in cellular transformation (Table 1) (Peters and Vousden, 1997). Growth factors, such as vascular endothelial growth factor (VEGF) can induce endothelial cell proliferation and chemotaxis, thereby promoting angiogenesis of tumour cells (Folkman, 1995). Moreover, they may also be involved in extracellular matrix degradation and facilitate invasion and metastasis (Radinsky, 1991). Apart from the growth factors secreted by the tumour cells, stromal cells also support the tumour growth by secreting other factors, although their role is not fully understood.

Growth factor receptors can be mutated or present in high amounts leading to their constitutive activation in the absence of or low amounts of growth factors. EGF receptors, angiogenic receptors, PDGF receptors, IGF receptor and neurotropin receptor family are some of the receptor families implicated in cancer. EGF receptor is one of the most well studied receptor and

is found to be frequently altered in certain types of tumours such as glioblastomas. Mutations in the growth factor receptors can lead to a constitutively activated receptor, without a need for ligand binding. Furthermore, over-expression of normal human EGF receptors is also sufficient for transformation of fibroblasts *in vitro* (Velu *et al*., 1987). EGF receptor downregulation by anti sense RNA is able to reverse the transformed phenotype of the human epidermoid carcinoma cell line (Moroni *et al*., 1992).

Table 1.1:- Growth factors and Growth factor receptors implicated in cancer development

Growth Factors	Growth Factor Receptors
Fibroblast growth factor family	ERBB2/3/4
Epidermal growth factor family (EGF)	Epidermal growth factor receptor
Platelet-derived growth factor family (PDGF)	PDGF α and β receptors
WNT family	Stem cell factor receptor (KIT)
Transforming growth factor beta family	Colony stimulating factor-1 receptor (FMS)
Hepatocyte growth factor	Hepatocyte growth factor receptor
Insulin-like growth factors (IGF)	Insulin-like growth factors I receptor
	Neurotrophin receptor family
	Angiogenic receptors
	Erythropoietin receptor
	Thrombopoietin receptor

Protein tyrosine kinases are responsible for cell growth and differentiation, cell motility and secretion. Receptor tyrosine kinases are associated with growth factors. Of the seven different classes of cytoplasmic tyrosine kinases, members of the SRC, FPS and ABL families have so far demonstrated oncogenic potential. Many of the cytoplasmic tyrosine kinases can be activated by different growth factors such as PDGF, GM-CSF and IL-3. However, overexpression of oncogenic kinases generates signals which are sufficient for cell growth without the need for either a growth factor or receptor. These oncoproteins can have multiple roles in cell mitosis; activation at the receptor level, phosphorylation of the membrane docking proteins as well as direct interaction with signalling proteins (Peters and Vousden, 1997).

Downstream effectors of the growth factors include the signal transduction molecules of the RAS/RAF/ERK families, which are mutated in a number of tumours. Mutant version of the normal H-RAS was the first oncogene to be identified (Parada *et al*., 1982). It is now well known that RAS oncogene regulates multiple pathways in the cells and constitutive activation of this H-RAS can deregulate the JNK and/or ERK pathways, giving rise to biochemical and cytoskeletal changes in the transformed cells (Peters and Vousden, 1997). Being involved in transcription of protein genes, it is not surprising that transcription factors are frequent targets of the mutagenesis leading to cancer. Members of FOS, JUN and MYC family are the main transcription factors

involved in cancer. These transcription factors regulate gene expression of a number of molecules involved in the cell cycle. MYC oncogene is involved in regulation of p53, cyclin A and cyclin E among others (Peters and Vousden, 1997). Deregulation of the cyclins promotes entry of the cells in the cell cycle and cell division. Similarly, MYB may be involved in blocking cell differentiation (Patel, 1993)

1.1.4.2 Tumour Suppressor Genes (TSG)

Normal genes suffer numerous insults from environmental factors but only a small number of these are propagated, eventually leading to cancer (Table 2). Tumour suppressor genes, are involved in controlling the growth of cells which have sustained DNA damage. These genes are involved in various functions such as DNA repair, inhibition of cell growth, induction of cellular senescence, differentiation and death (Jakobisiak *et al*., 2003). Loss of tumour suppressor gene activity due to 'loss of function' via mutations predisposes the cells to various kinds of cancers. Two of the most important TSG are the retinoblastoma gene (Rb) and p53. Rb gene was the first TSG to be discovered (Friend *et al*., 1986).

Table 1.2:- Tumour Suppressor genes involved in cancer

Tumour Suppressor Genes	Cancers
Retinoblastoma Tumour suppressor gene (pRB)	Retinoblastoma
p53	Many Cancers
BRCA1/BRCA2	Breast and Ovarian cancers
p16INK4a	Esophageal squamous cellcarcinoma
THY1	Nasopharyngeal carcinoma
Neurofibromatosis type 1 (NF1)	Many Cancers

During the cell cycle, phosphorylation level of Rb is periodically regulated, with hypophosphorylated Rb being involved in negative cell growth. Rb mediated cell growth suppression is overcome by increased phosphorylation. It was initially identified in pediatric tumour retinoblastoma but has since been shown to be mutated in a large number of cancers. Rb interacts with a number of proteins in the cell, such as E2F. E2F functions as a transcription factor for several molecules involved in cell cycle. Hyper phosphorylation of Rb inhibits the interaction between E2F and Rb, resulting in unchecked activity of E2F. Other regulatory proteins shown to interact with Rb are cyclins D1, D2, D3, c-MYC, MDM2, c-ABL etc (Peters and Vousden, 1997).

p53 is the most frequently mutated protein in the cancer cells and serves various biological functions in the cell, including the maintainance of G1 checkpoint in the cell cycle. Delay in the

G1 phase allows the cell to repair the DNA damage, failing of which may lead to p53 mediated apoptosis. It is also involved in cellular differentiation, DNA replication and repair. It is known as the 'guardian' of the genome and the importance of its role is underlined by the fact that p53 gene is mutated in more than 70% of all cancers (Bertram, 2000). Analyses of these mutations have revealed that they are concentrated in specific regions of the gene, known as 'mutational hotspots', mainly in the DNA/protein interacting region of p53. Thus these mutations disrupt the association of p53 with DNA molecules (Bertram, 2000). Interestingly, different environmental insults can cause different mutations in p53, although it may lead to the same cancers. For example, mutational spectrum of p53 in hepatocellular carcinoma in China is different from those in Western countries (Aguilar *et al*., 1994). Moreover, both mutated and wild-type p53 can exist in the same cells and not only does the mutant form have a longer half-life than wild type but can also be dominant over wild-type p53. Fortunately, frequent occurrence of certain mutations, provides an opportunity to target these mutant p53 expressing cancer cells.

1.1.5 Cancer Antigens and Their Classification

Mutated or non-mutated, oncogenes and TSG, can be targeted for therapy and are known as tumour antigens. Hundreds of tumour antigens have been identified in the past 15 years, since the identification of first tumour antigen MAGE-1 (van der Bruggen *et al*., 1991). The techniques used to identify these antigens are explained in chapter 5. Different tumour antigens have been classified as follows:-

1. ***Cancer Testis Antigens*:** - CT antigens are found to be overexpressed in a number of different tumours but amongst normal tissues, they are mainly expressed in testis, ovary and rarely in placenta. However, these normal tissues are generally immuno-privileged since testis does not express MHC class I molecules and is not a target for CD8+ T cells. Expression of these genes in cancers and testis is due to the demethylation of genes which are generally transcriptionally silent in the normal tissues. Functions of most of the CT antigens are still unknown. These represent attractive targets for immunotherapy due to their widespread expression in cancer cells. Indeed, CT antigens are amongst some of the most extensively investigated antigens in animal models and clinical trials. MAGE was the first antigen to be discovered in this group following which a large number of others have been added to the list such as GAGE, BAGE, LAGE and NY-ESO-1 (van der Bruggen *et al*., 1991; Chen *et al*, 1997; Simpson *et al*., 2005).

2. ***Differentiation Antigens:*** - These antigens are generally shared between cancer cells and their normal counterparts, and might be specific for a particular lineage of cells or a point of differentiation. Melanoma associated antigens are mostly expressed in melanomas and normal melanocytes. Some of these antigens are involved in the biosynthesis of the pigment melanin. The most well studied antigens from this group are tyrosinase, TRP-1, gp100, MART-1, prostate specific antigen (PSA), carcino-embryonic antigen (CEA) and prostate membrane specific antigen (PSMA) (Kawakami *et al*, 1994; Yao and Bachich., 2006). Since these antigens are also expressed at low levels in normal tissues, targeting them involves the risk of autoimmune reactions like vitiligo, as observed in some clinical trials (Schreiber *et al*., 1999; Trefzer *et al*., 2000). Moreover, being self antigens, generating immune response to them will require breaking tolerance to these antigens and potent immunisation strategies, as it is generally agreed that only the low avidity T cells for these antigens would have escaped the thymic tolerance mechanisms.
3. ***Tumour Specific unique antigens:*** - These represent the most attractive immunotherapeutic targets as they are unique to each individual tumour or shared between specific tumour types. Bcr/abl is a well recognised antigen in this group, a fusion protein which is formed due to translocation between chromosomes 9 and 22 leading to chronic myeloid leukaemia (CML) (Ren, 2005). Other unique antigens are generated due to point mutations or gene fusions in some of the essential oncogenes or tumour suppressor genes such as k-ras, β-catenin, CDK4 and p53 (Yang & Cristofanilli, 2006; Ruiz-Godoy *et al*, 2006). Some research groups consider tumour specific antigens as the best option for therapy as they represent the most immunogenic epitopes. Tailor-made individualised therapies are seriously being considered as an option by certain immunologists and this group of antigens is their priority targets.
4. ***Over-expressed self antigens:*** - These antigens are widely expressed in normal tissues are over-expressed in wide range of tumours as well. Unfortunately, most of the tumour antigens identified till date belong to this group, and represents the most challenging aspect of cancer immunotherapy. Like differentiation antigens, tolerance to these antigens seems to be the biggest concern as well as the challenge of finding the "narrow therapeutic window" between immunity and autoimmunity. However, recent evidence suggests that normal tissues are more robust in dealing with immune responses than their cancerous counterparts and/or the level of antigen expression in them is below the threshold of T cell recognition and activation (Hodge *et al*, 2003). Their ubiquitous expression suggests an essential role for them in cell growth and proliferation, which

reduces the possibility of generating antigen loss variants while targeting these antigens. Amongst the interesting antigens in this group are survivin, p53 and hTERT. Metastasis Associated antigen 1 (MTA1) also belongs to this category of antigens (Toh *et al*, 1995).

5. ***Viral antigens:*** - Viral infections are responsible for a number of human cancers such as Epstein Barr virus (EBV) for Burkitt's lymphoma, hepatitis B virus for hepatic carcinoma, human papilloma virus (HPV) for cervical cancer and human T cell leukemia virus (HTLV) for adult T cell leukemia (zur Hausen, 1991). Although prevention of these viral infections offers the most promising chance of decreasing these cancer incidences, for established cases, therapeutic vaccination might also hold potential. Tissues infected with virus would generally also express viral antigens offering the T cells an opportunity to recognise and destroy them. Moreover, viral antigens are highly immunogenic as the immune system has not encountered them previously and has evolved to clear microbial infections. Several clinical trials have been undertaken to target these viral antigens. For example, the antigens E6 and E7 of the HPV have been the focus of clinical trials for cervical cancer and have generated encouraging results (Govan, 2005).
6. ***Post-translationally modified antigens:*** - Altered glycosylation appears to be a constant phenomenon associated with oncogenic transformation in essentially all types of naturally occurring human cancers. Most of the biochemical or, more recently, immunological methods used to identify tumour-associated antigens have resulted in the isolation of glycolipids or glycoproteins (mucins) with altered glycosylation patterns (Taylor-Papadimitriou & Epenetos, 1994). Mucins are large (> 200 kDa) glycoproteins with a high carbohydrate content. They are expressed by a variety of normal and malignant epithelial cells.
7. ***Oncofetal antigens:*** - The oncofoetal antigens are "self" proteins normally expressed during foetal development and then undetectable or at low levels in normal healthy adult tissues. On the other hand these antigens have been detected in the sera of patients with gynaecological cancer. Carcinoembryonic antigen (CEA) is one of the first known tumour markers. Since then, many more have been described, but CEA remains alone or in combination with others the most commonly used biomarker for cancer progression. CEA is not organ specific and abnormal values may be found in a wide range of carcinomas (Ballesta *et al*, 1995). One of the most useful applications of this marker is in post surgical prognosis in the treatment of neoplasms. Any elevation of this marker after conventional treatment of neoplasms has been correlated with a recurrence of cancer (Holubec *et al*, 2000).

Table 1.3 Classification of Tumour Antigens

Group	**Tumour Antigens**	**Cancer expressing the antigen**
Cancer Testis	MAGE1-3 and -12, BAGE, GAGE, HAGE, NY-ESO-1	Melanoma, breast Head/neck, bladder, gastric and lung
Differentiation antigens	Tyrosinase, gp-100, TRP-1 and -2, MART-1	Melanoma
Tumour Specific Antigens	p53 (mutated) Ras (mutated) CDK4 Caspase-8 b-catenin BCR/ABL	Breast, colon, other cancers CML, ALL, AML, Melanoma Head/neck Melanoma CML
Over-expressed/ Mutated antigens	HER-2/neu MUC-1 p53 (non-mutated) WT-1 Proteinase-3, PAP, PSA, PSMA	Breast, ovary, lung Breast, adenocarcinoma colorectal, lung, bladder, head/neck Pancreatic, colon, lung CML Prostate
Viral antigens	EBV HCV HPV	Burkitt's lymphoma, Hepatocellular carcinoma Cervical and penile cancer
Post-translationally modified antigens	Mucin	Oesophageal, pancreatic cancers
Onco-foetal antigens	CEA α-Fetoprotein 5T4 Onco-trophoblast glycoprotein	Colon, breast, pancreatic caner Liver cancer Many carcinomas Many carcinomas
Idiotype antigens	Ig Idiotype	B-cell NHL, MM

8. ***Idiotypic antigens*: -** B cell lymphoma arises as a clonal event. Specific immunoglobulins (Ig) are generated by a unique combination of gene segments during B cell differentiation

and these are expressed throughout the development of the tumour. The Ig idiotypic determinants comes from a unique heavy (VH) and light (VL) chains and provide a unique tumour associated antigen to target for immunotherapy against lymphoma.

1.1.6 Cancer Stem Cells

In recent years, the concept of cancer stem cells has gained widespread acceptance. Stem cells were first identified in haematological malignancies but since then they have also been isolated from CNS, breast and prostate cancers (Collins *et al*., 2005; Huntly & Gilliland, 2005). Cancer stem cells are defined as cells with self-renewal capability and multi-lineage differentiation potential and are similar in nature to normal tissue stem cells. Current evidence from studies in animal models also suggests that tumours arise from a single cancer stem cell and transplantation of even a single cancer stem cell can lead to tumour formation (Polyak and Hahn, 2006).

Several hypothesis regarding the origin and the role of cancer stem cells have been suggested. One possibility is that these stem cells are the primary cells in which cancer initiation occurs. Tissue stem cells persist throughout adult life and undergo frequent cell divisions making them more susceptible to accumulate the required number of genetic hits (Polyak & Hahn, 2006). Stem cells form a tiny compartment of the normal tissues and most cancers consist of heterozygously differentiated cells. It is possible that initial mutations affecting genetic instability occur in the tissue stem cells followed by accumulation of more genetic mutations in the more differentiated cells arising from such stem cells. Fusion of stem cells with other cells which have accumulated additional mutations might also be responsible for providing these cells an immortalized phenotype with tumour forming potential (Bjerkvig *et al*., 2005). Lastly, cancer stem cells might represent the dedifferentiated cells of the tumour. Thus, tumour cells sustaining genetic alterations affecting their differentiation process might cause the cell to be de-differentiated and mimic stem cell behavior (Polyak & Hahn, 2006).

The increased interest in these stem cells has arisen due to their importance in cancer therapy. Inability to target these stem cells might be one of the reasons for the failure of chemotherapeutic drugs. Most chemotherapy drugs target rapidly dividing cells and may not affect the slow growing stem cells, which in future can repopulate tumours. However, this does not apply to all the cancers as certain chemotherapy drugs provide a distinct therapeutic affect, especially in hematopoietic malignancies, which arise from such stem cells. Further research will be needed to

determine the real extent of cancer stem cell involvement in tumour growth and resistance to therapy.

1.1.7 Conventional Modalities of Treatment and their Limitations

Various methods of treatment are currently used for the treatment of cancer.

(i) Surgery: - Surgical removal of the cancerous tissue is probably the most favoured treatment as it causes the least damage to the surrounding normal tissue and have the least side effects. With modern surgical techniques, it is possible to remove tumours from almost any part of the body. However, it can only be applied in initial stages of cancer as once the cancer cells have metastasised, removal of primary tumour is generally palliative. Surgery can be used in combination with either chemotherapy or radiotherapy to initially decrease the tumour burden.

(ii) Chemotherapy: - Chemotherapy is essentially treating cancer with cytotoxic drugs, which act on the rapidly dividing cells, and inhibits their proliferation or kills them. Although reasonably successful, chemotherapy has been associated with severe adverse effects and superseding their benefits. There are over 50 chemotherapeutic drugs; while some are given alone, most of them are given in combinations. Methotrexate is one of the main drugs used for treatment of acute lymphatic leukaemia (ALL) as well as certain lymphomas, osteosarcoma and choriocarcinoma (Chabner & Roberts, 2005). Similarly vincristine, a vinca alkaloid which inhibits microtubule polymerisation and cell division, is used in a combination regime for treatment of ALL, hodgkin's and non-hodgkin's lymphoma (Moxley *et al*, 1967). Chemotherapy is generally used at high doses in combination regimes to prevent the generation of resistant cells to one drug, which can also increase adverse effects. Usually chemotherapeutic drugs are administered at intervals of 3 weeks to allow the hematological cells of the bone marrow to proliferate and re-constitute the immune cells, thereby decreasing the likelihood of infections. However, chemotherapy is most effective only on the well perfused and rapidly proliferating cells, a drawback which allows the tumour cells distant to the blood supply to escape their cytotoxic effects and repopulate.

(iii) Radiotherapy: - Cancerous tissues are targeted by ionizing radiation, which is usually administered in small doses of 1.8-2.0 Gray and is given on the weekdays for 5-7 weeks (Kim and Tannock, 2005). No therapy is given on the weekends to allow the normal tissues of the body to recover from sub-lethal irradiation. This ionizing radiation causes lethal damage to the DNA of the tumour cells and thereby causing their death. However, radiotherapy is most effective on well-oxygenated cancerous cells and thus is unable to kill the hypoxic tumour cells

allowing their re-population. There is also increasing evidence that in some cases radiotherapy and chemotherapy regimes actually lead to increase in the growth rate of the tumour (Kim & Tannock, 2005).

(iv) Hormonal therapy: - Cancers such as breast and prostate are dependent on the hormones produced by the body and can be controlled by hormonal therapy. Prostate cancers are usually dependent on testosterone, and hence therapies aimed to reduce serum testosterone levels have been used in the past with reasonable success. However, many of these cancers will eventually become resistant and these therapies are not suitable for hormone refractory tumours and for advanced cancers (Denmeade & Isaacs, 2002).

Thus, in spite of the various advances in the diagnosis and treatment of cancer, the conventional modalities of treatment have been unable to significantly decrease the mortality rate in patients diagnosed with advanced disease. This has resulted in a renewed interest in the field of immunotherapy i.e. activating the immune system of the body to fight against cancer cells.

1.2 Immune System and Cancer

The immune system consists of various cells which have evolved to protect us against dangerous pathogens and microbes. However, its role in cancer prevention was realised when an increase in cancer development was observed in immuno-deficient animals and transplant patients on immunosuppressive therapy (Dunn *et al*, 2004). It was argued that the observed increase was due to the inability of the body to combat the oncogenic viruses, which are the real cause of these cancers. However, this argument has been countered by several lines of evidence in the recent years. Firstly, significant increase in the spontaneous tumour development has been shown in the various animal models with no apparent viral etiology (Dunn *et al*, 2004). Secondly, various tumour antigens have been identified using SEREX technique (Serological Analysis of antigens by recombinant expression cloning), whereby antibodies from serum of cancer patients is used to screen the cDNA library from the testis or cancer cells, which provide conclusive evidence of immune reactivity against cancer (Li *et al*, 2005). Lastly, immune cell infiltration has been observed in many human and murine tumours and the prognosis correlated with their numbers and activity (Dunn *et al*, 2004). Thus, it is clear from these findings that the immune system has the ability to react against the tumour cells.

How then does the tumour develop despite the presence of the active immune system and can it be activated to identify the presence of transformed cells and eliminate them? The following

sections describe the development of the immune system, the cells involved in immunity and their mechanism of action to deal with cancer, including the mechanisms evolved by the cancer cells to evade the immune system. Moreover, evidence of immune system's role in shaping the phenotype of the tumour cells will also be discussed, termed as 'immunoediting'.

Immune System Development

All cells of the immune system develop from the pluripotent stem cells in the bone marrow; differentiate into common myeloid and lymphoid progenitors. Further development of the myeloid progenitors gives rise to cells of the innate immune system such as eosinophils, neutrophils, basophils, macrophages and dendritic cells, whereas lymphoid progenitors differentiate into cells of the adaptive immune system, B lymphocyte and T lymphocyte (Janeway *et al*, 2001) (Fig 1.4).

1.2.1 Innate Immune System

Innate immune system provides the first line of defence against the pathogens. It is characterised by non-specific response, no immunological memory and it is constitutively present in the body, in contrast to adaptive immune cells, which require some time to be mobilised and are antigen specific.

The immune system has evolved to protect living organisms against various microbes and infectious agents. This is achieved by recognition of the pathogens through various receptors present on the surface of the immune cells. Cells of the innate immune system detect the presence of a microbe in the body through a limited number of germline encoded receptors known as pathogen pattern recognition receptors (PPAR) (Janeway *et al*, 2001). These receptors are specialised to recognise the conserved molecules present in different bacteria, viruses and other pathogens. Activation of these receptors on the surface of the antigen presenting cells such as dendritic cells and macrophages can lead to either phagocytosis of the pathogens and activation of other cells of the innate and adaptive system. Toll Like receptors (TLR) are one of the major pattern recognition receptors and are responsible for activation of antigen presenting cells and subsequent activation of the adaptive immune system.

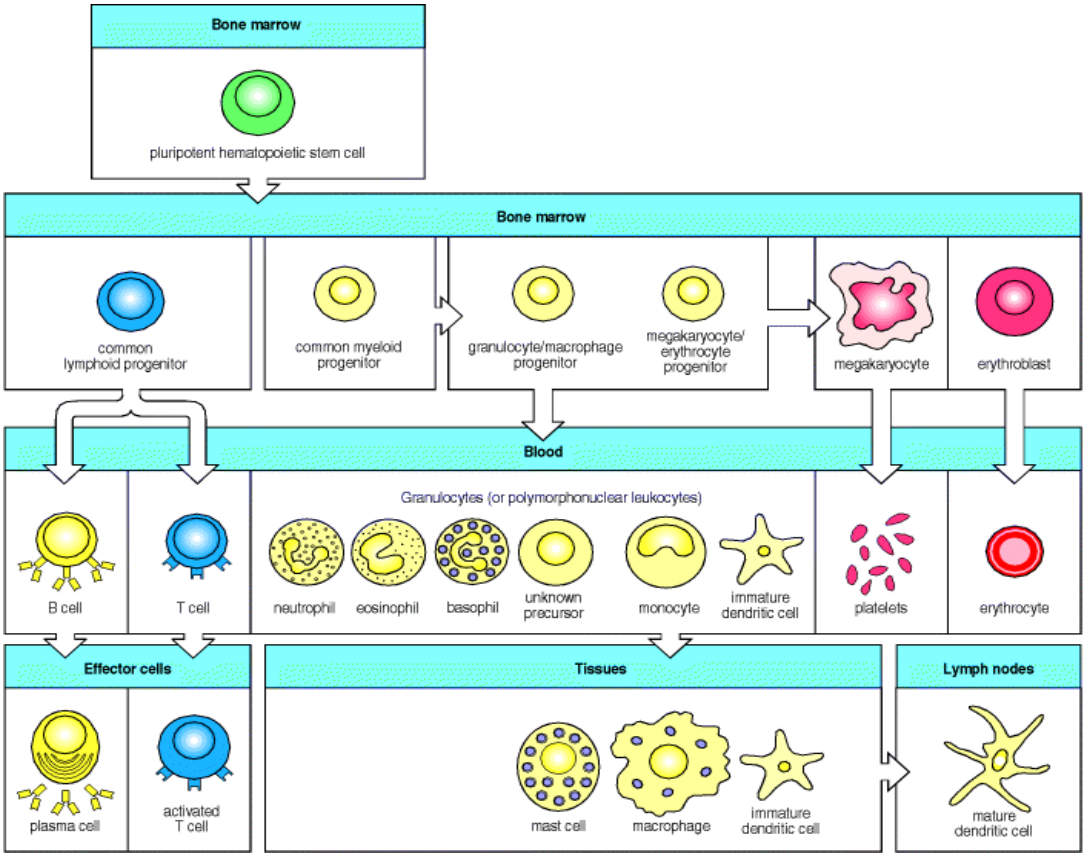


Figure 1.4:- Development of the immune system cells from common progenitor cells arising from the bone marrow (*Taken from Janeway et al, 2001).*

(a) Neutrophils

Neutrophils form the majority of white blood cells in our body, as they might have evolved to provide the first line of defence against most commonly occurring viral infections and other pathogens. They are the chief mediators of inflammation, they are considered as pro-tumour due to their role in chronic inflammation, which may lead to cancer development (De Larco *et al*., 2004). Neutrophils produce cytotoxic mediators and cytokines such as reactive oxygen species (ROS), Interferons, TNF-α, IL-1β, proteases and membrane perforating agents (Di Carlo *et al*., 2001). Neutrophils can recruit APC to site of tissue damage, influence their differentiation, and can also drive the proliferation of T and B cells (Nathan, 2006). Although neutrophilic ROS can suppress T cell and macrophage function, they can also lyse tumour cells (Di Carlo *et al*., 2001). Inspite of their limited ability to secrete cytokines, the fact that these cells form up to 70% of total leukocytes suggests that they can form a reckoning force to mediate tumour cell destruction.

(b) Eosinophils

Eosinophils are considered as the main mediators of allergic reactions due to release of cationic proteins by them during asthma, but they are also known to interact with T cells and DC and lead

to Th2 polarisation of immune response (Odemuyiwa *et al*., 2004). Eosinophil infiltrates have been observed in a number of different tumours, however, their role in cancer is still debatable. Earlier studies indicated that eosinophil infiltrates in tumour cells correlated with a good prognosis (Iwasaki *et al*., 1986). However, a recent study demonstrates that they are responsible for producing Indoleamine 2,3-Dioxygenase (IDO) enzyme, which has been implicated as one of the tumour escape mechanism (Odemuyiwa *et al*., 2004).

(c) Macrophages

Macrophages are tissue resident antigen presenting cells derived from monocytes. Apart from phagocytosis of dead/dying or infected cells and their debris, they are also involved in antibody mediated cellular cytotoxicity (ADCC). Although some animal models have demonstrated anti-tumour activity of the macrophages, most studies seem to suggest that tumour cells can manipulate tumour associated macrophages (TAM) for their own benefit. Initial studies reported macrophage associated tumour cell killing through secretion of cytotoxic cytokines likeTMFα and IL-1, reactive oxygen intermediates, proteases and nitric oxide. Macrophages also recruit secondary inflammatory cells, like neutrophils, to tumour site and are involved in antigen dependent cellular cytotoxicity (ADCC) of tumour cells (Foss, 2002). TAM have been shown to be manipulated for tumour invasion, growth, metastasis, angiogenesis and immuno suppression. In different areas of the tumour such as the stroma, perivascular sites and hypoxic/necrotic areas macrophages may be educated by their microenvironments to perform different functions (Lewis & Pollard, 2006). Supporting this hypothesis, macrophages derived from healthy tissues are capable of lysing tumour cells, whereas the ones isolated from tumours are unable to do so (Lewis & Pollard, 2006).

(d) Mast Cells

Mast cell accumulation at the edge of the tumours was observed in a number of studies and their degranulation is often associated with later stages of tumour proliferation (Cawley & Hoch-Ligeti, 1961; Hartveit *et al*., 1984). A significant increase in tumour growth rate in mast cell deficient mice and their ability to produce TNF-α suggests that they might have some anti-tumour function (Dimitriadou & Koutsilieris, 1997). On the contrary, mast cells can also promote tumour angiogenesis through release of mediators such as heparin, bFGF, TGFβ, proteases and histamine (Dimitriadou & Koutsilieris, 1997).

(e) Natural Killer Cells

Natural killer (NK) cells consist of large granular lymphocytes and are considered as one of the most important defence mechanism of immunosurveillance against virally infected cells and tumour cells. Being an innate "immune" cell, the NK cell does not require prior priming and expansion, and does not kill in an antigen specific manner. NK cells are capable of detecting virally infected or tumour cells which have down-regulated their MHC class I molecules and mediating their killing ('Missing Self" hypothesis) (Kumar & McNerney, 2005). On the other hand, they maintain a status of self-tolerance towards the normal cells of the body owing to the fact that almost all nucleated cells of the body express MHC class I molecules. This property of the NK cells is due to the presence of inhibitory receptors on their surface, which belong to two families, immunoglobulin like receptors and C-type lectin-like receptors. Killer immunoglobulin-like receptors (KIR) belong to the previous category and specifically recognise HLA-A, HLA-B and HLA-C alleles (Papamichail *et al*., 2004). Apart from the inhibitory receptors, NK cells also express an activating receptor, NKG2D, which recognises the human non-classical MHC molecules MIC-A and MIC-B. MIC molecules are stress inducible molecules that are frequently expressed by the transformed cells (Papamichail *et al*., 2004). NK cells can not only kill transformed cells through granzyme-perforin and apoptosis inducing receptors (FasL, TNF and TNF related apoptosis inducing ligand [TRAIL]), but they can also secrete huge quantities of cytokines such as IFN-γ, TNF-α, IL-10 and GM-CSF (Lehmann *et al*., 2001; Mori *et al*., 1997; Takeda *et al*., 2005). Thus, these cells might also play an important role in directing the immune response and in the process establish an important link between the innate and the adaptive immune response. Indeed, it has been observed that NK cells and DC might be involved in a complex crosstalk to enhance the immune reaction (Zitvogel, 2002; Fernandez *et al*., 1999). Contrary to NK cell's established role in immunosurveillance, NKT cells have recently generated very conflicting results. NKT cells express markers of both NK and T cells and are marked by production of large quantities of cytokines, mainly IL-13. Several studies have established their role as immunosuppressive cells and down regulators of immunosurveillance (Terabe *et al*., 2000; Moodycliffe *et al*., 2000). However, other studies have argued that they are actually responsible for rejection of tumours (Cui *et al*., 1997; Godfrey *et al*., 2000; Crowe *et al*., 2002).

(f) Antigen Presenting Cells

Cells such as macrophages, B cells and dendritic cells (DC) have been endowed with specialised functions of antigen capture and presentation to the other immune cells in order to initiate an immune response against these antigens. Although B cells and macrophages are competent antigen presenters, dendritic cells are the most potent and important APC in the body. DC are located at the sites of antigen entry in the body and are efficient in antigen capture, processing and presenting it to T cells along with co-stimulatory molecules (Itano & Jenkins, 2003). Thus, it is not surprising that a lot of research has been devoted to unraveling the different subsets and the roles of these cells in various microbial infections and cancer.

DC, along with other APCs possess evolutionarily conserved sets of receptors known as pattern recognition receptors (PRRs), which are involved in cell activation. PRR also include molecules mediating opsonisation, endocytosis, activation of complement and coagulation cascades, activation of inflammatory signaling pathways and/or induction of apoptosis (Kapsenberg, 2003). One of the most important PRRs are the toll like receptors (TLR), of which ten different subtypes in humans and nine in mice have been identified. These TLR are specialised in recognition of pathogen associated molecular patterns (PAMP), which are shared by various microbes and pathogens. Different TLR receptors have different microbial ligands and can induce different intracellular signalling pathways. Various PAMP recognised by TLR include lipopolysaccharide (TLR4), bacterial lipoproteins and lipoteichoic acids (TLR2), flagellin (TLR5), unmethylated CpG DNA of bacterial and viral origin (TLR9), double stranded RNA (TLR3) and single stranded viral RNA (TLR7) (Iwasaki & Medzhitov, 2004). Thus, TLRs 1,2,4,5, and 6 bind to bacterial products whereas TLRs 3, 7 and 9 are involved in viral pattern recognition. Apart from these microbial products, DC can also be activated by inflammation and stress induced tissue factors such as inflammatory cytokines, eicosanoids, heat shock proteins (HSPs), extracellular matrix components, cell-surface bound molecules and necrotic cell lipids (Gallucci *et al*., 1999). Initial environmental factors during DC activation, through different receptors, can later dictate their ability in directing polarisation of the immune response.

Dendritic cells constitute about 0.3% of the entire circulating blood-leukocyte population and also reside in various tissues of the body in an immature state (Dallal & Lotze, 2000). On being activated in the periphery, DC engulfs the antigens released by the damaged or infected cells, through several mechanisms including macropinocytosis and receptor mediated endocytosis via

mannose and Fc receptors. After antigen uptake these DC migrate to the neighboring lymph node and on route they mature and process the captured protein antigens into peptides. These peptides are bound to the MHC molecules and are presented to naïve T cells in the lymph node. On maturation, the phagocytic ability of DC decreases considerably, and is replaced by enhanced expression of MHC and co-stimulatory molecules on their surface for optimum antigen presentation. Maturation state of the DC is highly important in generating stable T cell synapse and their activation (Benvenuti *et al*., 2004). Immature DC are unable to form long-lasting interaction with naïve T cells, which could lead to T cell anergy (Benvenuti *et al*., 2004). Usually, captured antigen is delivered to the MHC class II compartments where it is processed and presented on the cell surface through MHC class II receptors and serves to elicit CD4+ T cell responses. However, DC can also present exogenous antigens on MHC class I molecules by directing exogenous antigens to the endoplasmic reticulum through 'cross-priming'. Thus, DC can also present antigens to the CD8+ T cells and lead to their activation. Even a single peptide-MHC complex can be recognised by the T cells on the APC surface (Krogsgaard & Davis, 2005). Moreover, DC have also been shown to interact and activate other innate cells of the body such as natural killer (NK), natural killer T lymphocytes (NKT) cells and B cells (Fernandez *et al*., 1999). Hence, antigen presentation by the DC can lead to activation of both innate and adaptive immune responses.

Optimal activation of T cells by the DC requires two signals. Signal one is provided by the ligation of the T cell receptor (TCR) to the MHC-peptide complex on the surface of the DC. Along with this, co-stimulatory signals (signal 2) are vital, as signal 1 in absence of signal 2 can lead to T cell anergy. Signal 2 is provided by activation of CD28 molecules on T cells by binding of CD80 and CD86 molecules on the APC. Recently, it has been suggested that a third signal in the form of cytokines secreted by DC is also required for polarisation of the T cell response (Kapsenberg, 2003). Cytokines such as IL-12, IL-23, IL-27 and type I interferons are considered as Th1 polarising whereas IL-10, TGF-β and monocyte chemotactic protein 1 (MCP1) are Th2 polarising. Various subsets of DCs have been discovered in humans and mice, and it is unclear at the moment whether different subsets of DC possess different capability in generating immune response and tolerance (Shortman & Liu, 2002).

1.2.2 Adaptive Immune System

Unlike innate system, cells of the adaptive immune system use highly diverse and stringently selected somatically generated antigen-specific receptors. Adaptive immune reaction is mediated by either humoral (antibody) mediated or cell mediated mechanisms. Although both types of immune response have an essential role in immune recognition and elimination, cell-mediated response is considered as the chief architect of immune responses against cancer.

1.2.2.1 Humoral immune response and Cancer

Antigen recognition receptor on the B cells is called Immunoglobulin receptor and consists of two heavy and two light chains. These heavy and light chains are arranged in a Y shape and are bound to each other by disulphide bonds. Both the chains contain a hyper variable region and a constant region. Different combinations of the light and heavy chains, combined with the diversity of the hyper variable regions, due to somatic rearrangement of the segments forming these regions, generate a highly diverse set of receptors (Janeway, 2001).

Identification of numerous tumour antigens through SEREX (Serological analysis of tumour antigens by recombinant cDNA expression cloning) suggests that the immune system is quite capable of mounting an antibody response to most tumour antigens. In contrast to the CD8+ T cells, self reactive CD4+ and B cells may not be subjected to intense scrutiny and deletion during development and hence they are easily reactivated against cancer cells. Depending on the environment at the initial DC activation, they can direct the ensuing immune response to a Th2 response, which mainly leads to antibody production. However, a Th2 response has traditionally been considered as detrimental to effective anti-tumour immune response (Kao *et al*., 2006; Osawa *et al*., 2006). It has been suggested that a proper Th1/Th2 balance in the favour of a Th1 is essential for tumour rejection. In fact, tumour cells might themselves skew the immune system towards a predominant humoral response to avoid CTL generation (Kao *et al*., 2006). Furthermore, several studies have shown that rejection of tumour cells does not require B cells (Lindencrona *et al*., 2004).

Contrary to the above studies, several other tumour models have demonstrated an effector role of antibodies in mediating tumour rejection (Renard *et al*., 2003; Simon *et al*., 2002). Antibodies alone or in a combination with Th1 response are also capable of mounting tumour rejection. In fact, several monoclonal antibodies have already been approved for the treatment of various

cancer and several others are in clinical trials. Moreover, tumour cells often down regulate MHC class I molecules to evade CTL response, and in such cases antibodies might be the appropriate therapeutic option. Thus, effective tumour rejection might require activation of multiple arms of the immune system such as CD4+, CD8+ and B cells.

1.2.2.2 Cell mediated immune response and Cancer

The T cell receptor (TCR) is generated in a similar manner to receptors expressed on B cells, however, there are certain crucial differences. T cell receptors consist of a heterodimer formed by a single α and β chain or γ and δ chain which are linked by disulphide bond. Unlike B cell receptors which can be secreted as antibodies, T cell receptors are membrane bound (Janeway, 2001). Both the α and β chains consist of the variable domains formed by the somatic rearrangement of the genes forming these segments, thus generating a highly diverse set of receptors enabling each T cell with a unique specificity. However, this is not strictly true in all instances, as explained later. These highly diverse repertoires of receptors enable the cells of the adaptive system to react specifically against different pathogens.

Although the majority of the T cells in the body bear αβ TCR, less than 5% of them have γδ TCR. These γδ T cells arise from a common progenitor as αβ T cells, but have a distinct role in the body. They are found mostly in the gastrointestinal mucosa and are adapted to identify antigens such as mycobacterial ligands and heat shock proteins (Chen & Letvin, 2003). Moreover, they can identify antigens presented by non classical MHC molecules (Spada *et al*., 2000). Function of γδ T cells is not entirely clear at the moment, although several groups have suggested that certain subtypes of these cells are capable of generating an adaptive immunity and might have an important role in some cancers (Chen & Letvin, 2003).

(a) Major Histocompatibility Complex and its polymorphism

Tissue grafting experiments suggested the existence of genes controlling graft acceptance and rejection. The genetic complex devoted to this function was named as major histocompatibility complex (MHC) in mice and human leukocyte antigen region (HLA) in humans (Fig 1.5). The genes encoded by the MHC are involved in processing of proteins into peptides, their transport and their presentation on the surface of cells for recognition by T cells. T cells only recognise peptides when they are presented to them as a complex with the MHC molecules on the surface of cells.

The murine MHC is located on chromosome 17 and that of humans on chromosome 6 (Klein, 1979; Erlich *et al*., 1983). Two of the major complexes encoded by MHC are the MHC class I and class II molecules, which present peptides to CD8+ and CD4+ T cells respectively. MHC class I molecule is heterodimer, made up of highly polymorphic integral membrane glycosylated α chain which is non-covalently associated with β-2 microglobulin (β2m) (Rajagopal *et al*., 2004). Interestingly, gene coding for β2m is located on chromosome 15, separate from rest of MHC genes. The α chain is folded into three domains α1, α2 and α3. Folding of the α1 and α2 domain creates a groove, which is the site where the peptides are bound to the MHC molecules and are displayed on the surface (Fig 1.6) (Janeway *et al*, 2001). MHC class II molecules are heterodimer molecules consisting of a non-covalently linked, trans-membrane glycoprotein α and β chain. Each chain is folded into two domains. The α1 and β1 domains form the groove for the peptide binding and unlike MHC class I molecule, it is open at both ends thereby allowing binding of longer peptides (Fig 1.7).

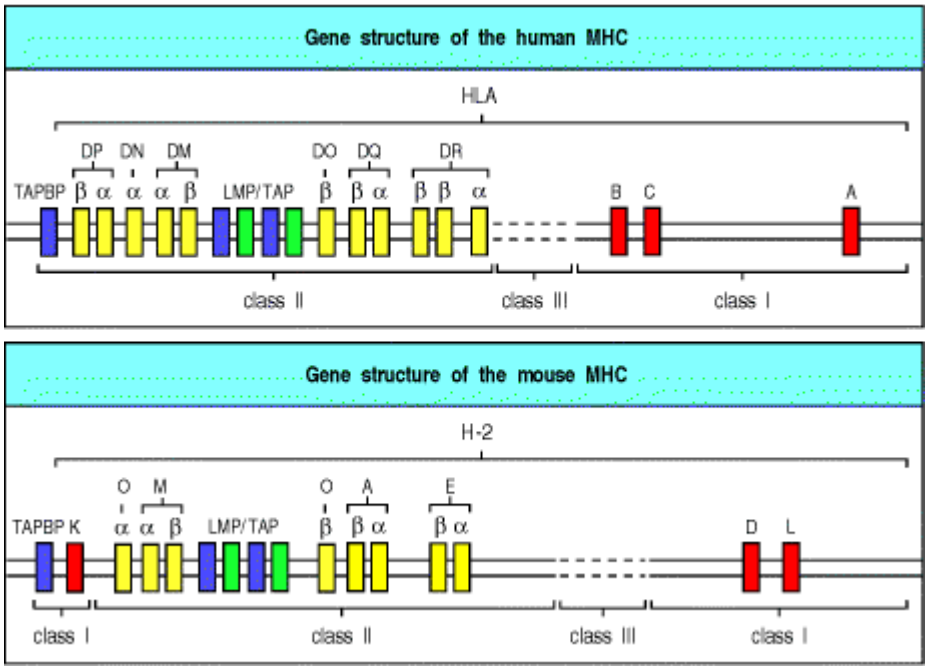


Figure 1.5:-Distribution of MHC genes in humans and mice *(Taken from Janeway et al, 2001).*

Because antigen recognition by T cells critically depends on peptide presentation by MHC molecules, they are polygenic and highly polymorphic, providing a broad range of peptide binding molecules. In fact, MHC molecules are the most polymorphic genes in the human body. Several genes encode for MHC class I and II molecules and every individual possess a set of MHC molecules with different range of peptide binding specificities (Janeway *et al*, 2001). In the MHC complex there are three MHC genes for class I called HLA-A, B and C; and 3 pairs of

MHC class II α and β genes called HLA-DR, DP and DQ (Fig 1.5). Moreover the DR region contains an extra β chain which can pair with DR α chain, thus generating two different DR molecules. At each gene locus, there are considerable variants which can give rise to different alleles and there are more than 200 different MHC class I and II alleles. Moreover, expression of MHC alleles is co-dominant, so each individual expresses six sets of MHC class I and eight types of MHC class II molecules. Each allele differs from the other by up to 20 amino acids which are concentrated in the peptide binding groove of the MHC molecule. This provides the diversity to the MHC molecule to bind to numerous peptides with different specificities and affinities. This is helpful in predicting the peptides from an antigen which are likely to bind to a given MHC molecule with high affinity and could be used as peptide vaccines. Importantly, MHC class I molecules are expressed by all nucleated cells of the body except testis, whereas MHC class II molecules are normally expressed by only professional antigen presenting cells (DC, B cells and macrophages).

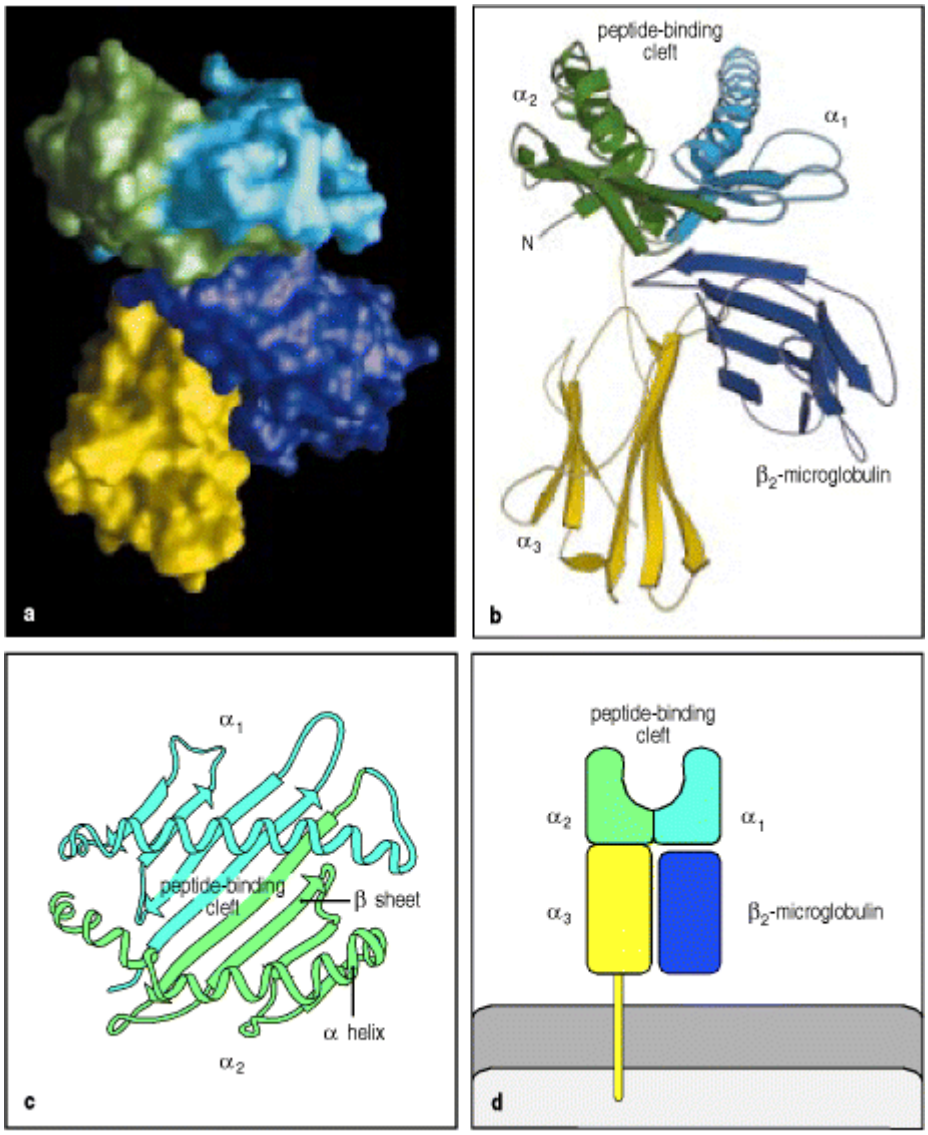


Figure 1.6:- MHC class I molecule structure as determined by x-ray crystallography
(Taken from Janeway, 2001; Fremont et al, 1992)

Apart from the MHC class I and class II molecules, the MHC also encodes for other proteins such as LMP 2/7, TAP, Tapasin, HLA-DM and HLA-DO, which are required for the antigen processing and presentation. On exposing cells to IFN-γ, which would be produced in viral infections, there is a marked increase in transcription of MHC class I and β2m genes along with proteasome, tapasin and TAP genes (Khan *et al*., 2001). Similarly MHC class II genes are also regulated by IFN-γ via production of a transcriptional activator known as MHC class II transactivator (CIITA) (Boss, 1997). This positive regulation of these MHC class I and II genes is essential for the immune cells to cope with increased antigen processing during any pathogenic infections.

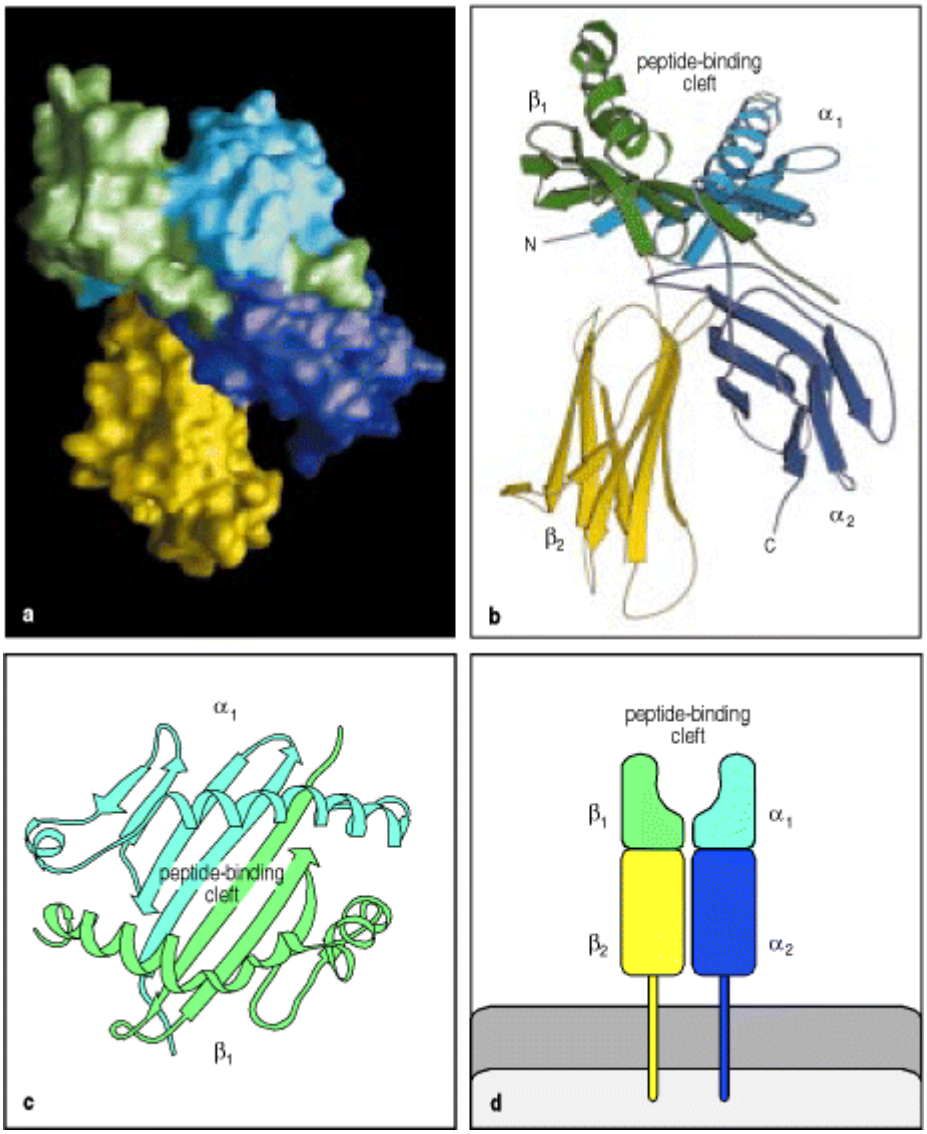


Figure 1.7:- MHC class II molecule structure as determined by x-ray crystallography
(Taken from Janeway et al, 2001; Dessen et al, 1997).

(b) Antigen Processing

As discussed previously, T cells can only be activated when they bind to peptide-MHC complexes on the surface of APC. CD4+ T cells bind to peptides presented in complex with MHC class II molecules, whereas CD8+ T cells recognise the peptides in complex with MHC class I molecules. MHC class I molecules are expressed by all nucleated cells of the body, whereas MHC class II molecules are expressed only on the surface of certain specialized antigen presenting cells like B cells, macrophages and dendritic cells. So how are these MHC peptide complexes formed and displayed on the surface of the cells?

MHC class I antigen processing

All proteins produced by the cell have to be degraded by various mechanisms once their function is completed. Initially recognition of the proteins to be degraded is required and the most common and clearly understood mechanism is by poly-ubiquitinylation, where enzymes such as E1, E2 and E3 activate ubiquitin molecules and covalently link them to the lysine residues of the proteins to be degraded. This tagging of the proteins allows the cytosolic proteasomes to distinguish the proteins to be degraded and is likely to be involved in the unfolding of the proteins. The proteasomes mediate the degradation of the majority of cytosolic proteins. Without proteasomal degradation, the MHC I molecules remain starved in the endoplasmic reticulum (ER) and are themselves eventually degraded (Pamer & Cresswell, 1998). Moreover, in the absence of the peptides, MHC class I molecules can assemble but are unstable at body temperature and recycled rapidly (Ljunggren *et al*., 1990).

Proteasomes are multicatalytic units, composed of approximately 28 subunits arranged in four stacked rings of 7 subunits each and consisting of a hollow core lined with the proteolytic subunits. Eukaryotic proteasomes contain 3 Interferon-γ inducible units, two of which are encoded by the MHC complex, LMP 2 and LMP 7. Usually, cells express constitutive proteasomes and exposure to IFN-γ causes LMP 2 and 7 to replace two β subunits. The third subunit recently identified, MECL-1 replaces proteasome subunit Z. This proteasome with IFN-γ inducible subunits is known as 'Immunoproteasome' and is found to be constitutively expressed in antigen presenting cells (Janeway *et al*, 2001). Expression of these subunits alters the specificity of the proteasome. Specifically, LMP-2 and LMP-7 expression enhances the cleavage after hydrophobic and basic amino acids whereas cleavage after acidic residues is inhibited. This proteasomal machinery generates numerous peptides, the majority of which are believed to be degraded in the cytosol and only a few survive to become ligands for MHC class I. Although the majority of the class I peptides are generated from the endogenous antigens, it has been shown that often antigens acquired from extracellular spaces are also displayed on MHC class I surface; a process known as 'Cross Priming' (Rock *et al*., 1990; Kovacsovics-Bankowski & Rock, 1995). The precise mechanism of this process is unknown although occasional rupture of the phagosome carrying the antigen might be responsible for release of the antigen into the cytosol.

Peptides resulting from proteasome cleavage are transported to the ER through certain chaperones like heat shock proteins (HSP) and are translocated across the ER membrane through

the means of a heterodimer TAP, consisting of two subunits TAP.1 and TAP.2, encoded by genes within the MHC (Fig 1.8). Numerous studies have confirmed the requirement of both subunits of TAP to be expressed for efficient translocation of the peptides. Also, TAP is able to translocate longer peptides into the ER, where NH_2 terminal trimming is thought to occur by means of aminopeptidase enzymes.

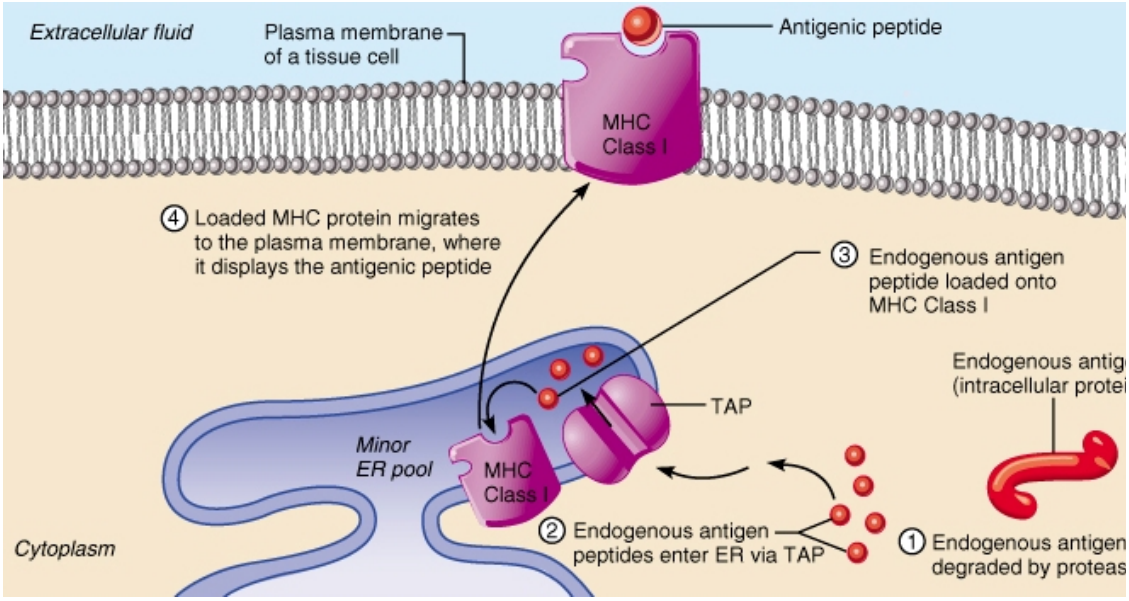


Figure 1.8:- Schematic diagram of MHC class I antigen processing *(Taken from www.innovitaresearch.org)*

MHC class I heavy chains, after being synthesized in the ER, bind to a protein called Calnexin. On binding of the heavy chain to the β2 microglobulin chain (β2m), calnexin is exchanged for a similar protein called calreticulin. β2 microglobulin is an obligate subunit of the MHC class I complex as its absence causes misfolding of the MHC class I molecule and its degradation. This complex of MHC class I heavy chain, β2 microglobulin and calreticulin associates with TAP molecules through another transmembrane protein called Tapasin. Tapasin is thought be responsible for coordinating peptide translocation and protection of class I- β2 m dimers from degradation. Finally the peptides translocated by TAP form a complex with class I- β2m dimers leading to dissociation of the complex from calreticulin and tapasin. This complex is then transported from the ER through the Golgi apparatus to the plasma membrane.

Studies have shown that on average, 1.8×10^6 substrates undergo degradation per minute in a cell and a single MHC-peptide complex generation requires about 450-3000 substrates to be degraded (Rajagopal *et al*., 2004).

MHC class II antigen processing and Presentation

Peptides presented by MHC class II molecules are generated in the endocytic vacuoles rather than by the proteasomes (Fig 1.9). Extracellular pathogens are internalised by antigen presenting cells through endocytosis or phagocytosis and become enclosed into endosomes. These endosomes become more acidic as they progress to the interior of the cell and fuse with lysosomes. The proteins produced by these pathogens are eventually degraded in this acidic environment by certain acid proteases such as cathepsins B, D, S and L (Janeway, 2001).

Similar to MHC class I molecules, MHC class II molecules are generated in the ER but are prevented from binding to the pool of peptides due to the association with a protein known as the MHC class II associated invariant chain (Ii). The invariant chain forms a trimer with the MHC clas II α:β dimers. Moreover this chain might also be responsible for targeting class II molecules to the low pH endosomal compartment, where the MHC class II molecules are retained for 2-4 hours (Janeway *et al*., 2001). During this time the invariant chain is cleaved in several steps. Initial cleavage generates a truncated form of the invariant chain, which still remains bound to the MHC class II molecule. Subsequent cleavage releases it from the membrane bound form of Ii and leaves a smaller fragment called CLIP to the peptide binding groove of the MHC class II molecule. Late in the endosomal pathway, these MHC class II:CLIP complexes enter into a specialised MHC class II compartment, where it is believed that the actual loading of the MHC class II molecule takes place after the dissociation of CLIP. The loading of peptide and release of CLIP from MHC class II molecule is facilitated by an MHC like molecule known as HLA-DM. HLA-DM also catalyses the release of unstably bound peptides to MHC class II molecules and in the process ensures that the MHC class II:peptide complexes delivered on the cell surface are stable and can survive on the surface for a few days.

Recently, a TAP and proteasome dependent antigen processing pathway was shown to exist for MHC class II peptides, however this pathway was only demonstrated in dendritic cells having a permeable endosome (Tewari *et al*., 2005). Moreover, endogenous antigens can also enter the MHC class II pathway and generate T-helper responses (Bogen *et al*., 1990). Cells produce excess of MHC class I and II molecules so that when the cell is infected by the pathogen, the peptides generated from the foreign antigens have sufficient empty MHC molecules to bind to.

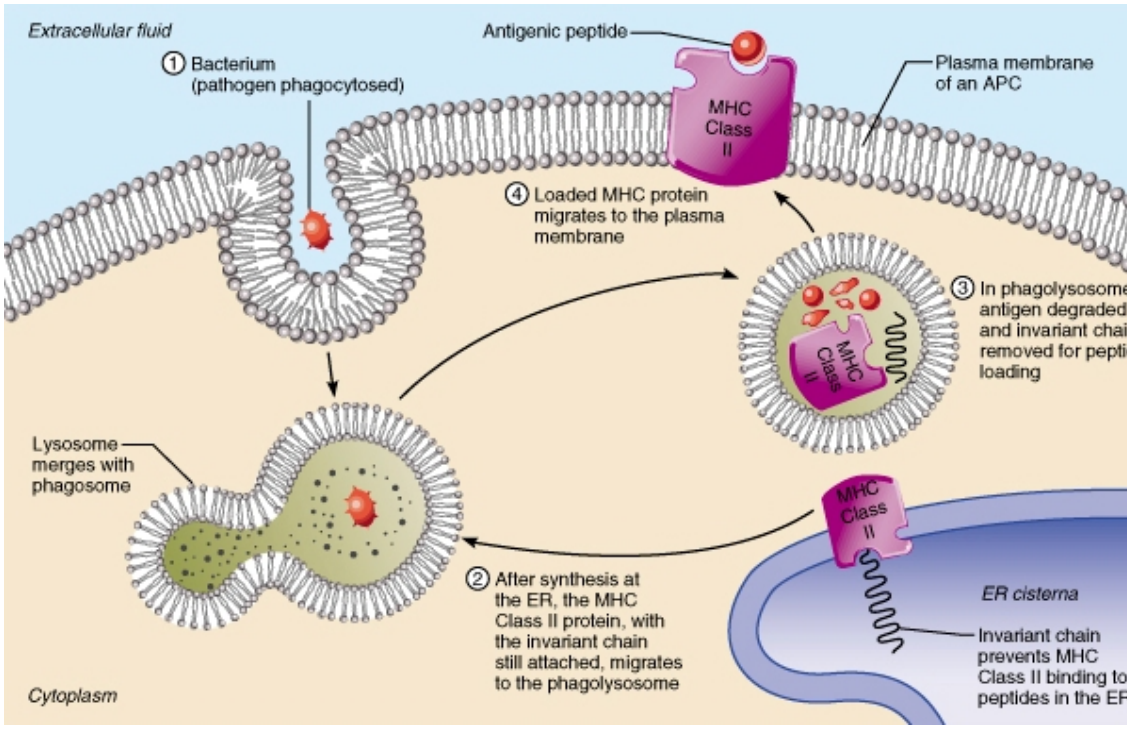


Figure 1.9:- Schematic diagram of MHC class II antigen processing *(Taken from www.innovitaresearch.org)*

(c) CD4+ T cells – Helpers and Co-ordinaters of the immune response

On reaching the lymph nodes, mature DC present processed antigens to CD4+ T cells through MHC class II molecules. Recognition of an epitope on the DC surface by the T cells leads to a formation of an immunological synapse and cross-talk between the CD4+ T cells and the DC. This cross-talk is essentially through the CD40-CD40L interaction, which licenses DC to optimally activate antigen specific CD8+ T cells and on the other hand activates the CD4+ T cells and polarises them towards either Th1 or Th2 cells. T helper 1 cells provide cytokines which support for the development and maintenance of the CTLs from CD8+ T cells. On the contrary, Th2 cells secrete cytokines such as IL-4 and IL-5 and lead to development of an antibody response towards the antigen. Recently, Xiang *et al* (2005) have demonstrated the ability of the CD4+ T cells to acquire MHC class I peptide complexes from DC on initial encounter. These CD4+ T cells can then act as APC themselves in activating CD8+ T cells leading to anti-tumour immune response.

Apart from this direct help in CTL generation, CD4+ T cells have been shown to possess multiple functions. CD4+ T cells can also recruit other immune cells such as macrophages and eosinophils to the tumour site (Wang, 2001). In several animal models, CD4+ T cells have been shown to mediate tumour rejection on their own (Daniel *et al*., 2005; Dudley *et al*., 2001; Lundin

et al., 2004). Indirect anti-tumour effects of the CD4+ T cells are mostly mediated by the secretion of IFN-γ, which has a pro-apoptotic and anti-proliferative effect on them; it also inhibits angiogenesis and by inducing upregulation of MHC class I molecules, makes tumour cells more susceptible to CTL mediated killing (Ikeda *et al*., 2002)). Several studies have also demonstrated the ability of CD4+ T cells to mediate tumour cell killing directly (MHC restricted) through Fas-FasL, granzyme-perforin and TRAIL pathways, generally employed by CTLs (Schattner *et al*., 1996; Thomas & Hersey, 1998a; Thomas & Hersey, 1998b). However, this is unlikely to be their main role as most of the tumours are MHC class II negative.

Adoptive transfer clinical trials have reinforced the role of CD4+ T cell in the generation and maintenance of the CTL response. Dudley and Rosenberg have recently showed that adoptive transfer of CD8+ T cells alone provided little clinical benefit to the patients. However, when CD4+ T cells were included in the adoptive transfer, this led to objective clinical regression in more than 50% of the patients (Rosenberg & Dudley, 2004)). One of the most intriguing questions for immunologists has been the nature and mechanism of help provided by the CD4+ T cells to CTLs. Several studies have argued that T cell help is only required during the priming phase of the CTL response whereas other models have showed their requirement for the maintenance of the memory cells (Bevan, 2004; Buller *et al*., 1987; Cassell & Forman, 1988; Shedlock & Shen, 2003; Sun & Bevan, 2003). These discrepancies might be explained by the different models used in these studies and the nature of the antigen. Perhaps, pathogenic infections provide stronger danger signals to the DC, bypassing the need of CD40L provided by the CD4+ T cells, whereas cancer being a chronic disease would require this CD4+ T cell mediated help. Moreover, CD4+ T cells can provide help for DC and CTL induction in a CD40 independent manner as well. This could occur either by cytokine release or by a contact dependant mechanism (Bachmann *et al*., 1999; Lu *et al*., 2000).
Thus, CD4+ T cells control multiple arms of the immune response against cancer; including providing help in CTL induction and maintenance, recruiting other immune cells, mediating direct and indirect tumour cell killing.

(d) CD8+ T cells - The Effector cells

CD8+ T cells are considered as the main effector cells in defence against virally infected cells and tumour cells. After being licensed by the CD4+ T cells, DC are optimised to present antigen to CD8+ T cells in an MHC class I restricted manner. Several studies have argued that for optimal CTL activation, both CD8+ and CD4+ T cells have to recognise antigen on the same

APC (Ridge *et al*., 1998; Smith *et al*., 2004). Moreover, there is also evidence to suggest that CD4+ and CD8+ T cells need to be in contact with the APC at the same time. However, antigen carrying APC coming in contact with rare antigen specific CD4+ and CD8+ T cells at the same time seems unlikely. Recently, Castellino *et al* have provided support to this argument by showing that interaction of naïve CD4+ T cells with DC in the lymph nodes leads to chemokine secretion by CD4+ T cells (CCL3 and CCL4) and this chemokine gradient permits active recruitment of the naïve CD8+ T cells through the CCR5 chemokine receptor (Castellino *et al*., 2006). Therefore, a two stage model, where APC activates CD4+ T cell first and then rendezvous with the antigen specific CD8+ T cell, seems more realistic (Ridge *et al*., 1998).

Following activation, CD8+ T cells differentiate into specialised killer cells (CTLs) and migrate to the area of infected cells or to the tumour site. On recognising the same antigen on the surface of the cells, CTLs seem to form a stable synapse with the target cells and mediate their apoptosis by multiple mechanisms. The most common and important pathway employed by the CTL is the perforin-granzyme pathway. After 24-48 hours of T cell activation, granules of proteins are synthesised in the CTL and on junction formation with the target cells these granules are orientated towards the contact site (Yannelli *et al*., 1986). Initially the granules release perforin, a molecule found exclusively in the cytotoxic lymphocytes (CTL and NK cells). In the presence of calcium, perforin polymerises in the cell membrane of the target cells creating conduits for the clear passage of other CTL granular proteins (Stinchcombe *et al*., 2001). This is followed by release of other cytotoxic granules such as the granzymes by the CTLs. Granzymes are a family of serine proteases, of which about 11 types have been identified in humans and mice. Of these granzyme A, B, H and K are ubiquitously found in CTLs and only granzyme A and B are considered to play a major role in mediating cell death (Barry & Bleackley, 2002). It was earlier considered that perforin only performs the role of creating an entry for other granules. However, it has now been shown that in the absence of the perforin, granzymes remain trapped inside the vesicles of the target cells after entering them and perforin addition helps in the release (Pinkoski *et al*., 1998). Granzyme B on being released acts on the downstream pro-apoptotic molecules of the target cells such as caspase 8 and caspase 3, and ultimately leads to the apoptosis of the target cell (Darmon *et al*., 1995). Granzyme B is also capable of mediated caspase independent mitochondrial collapse of the target cells with release of cytochrome C, leading to cell death (Heibein *et al*., 1999). Up to 90% of the CTL mediated cytotoxicity is mediated by perforin-granzyme pathway (Graubert & Ley, 1996).

Another important death mechanism is mediated through the Fas-FasL pathway. FasL is expressed on the T cells a few hours after activation (Shresta *et al*., 1998). This FasL acts as a ligand for the Fas receptors, a member of the tumour necrosis factor family of death receptors on the surface of the target cells. Engagement of the Fas receptors leads to aggregation of the intracellular death domains and ultimately formation of a death inducing signalling complex (Shresta *et al*., 1998). Further downstream substrates of this pathway causes activation of the caspases and instructs cells to commit suicide. Another contact dependant, although less important, killing by CTL is by TRAIL (TNF related apoptosis inducing ligand). TRAIL is expressed by various immune cells of the body including CTL and binds to its receptor on the target cells mediating their apoptosis similar to FasL (Hersey & Zhang, 2001).

1.3 T cell Development and Tolerance – Relevance to Cancer

Cells destined to become T cells differentiate from the pluripotent stem cells and leave bone marrow to travel to the thymus where further development and selection of T cells occurs. T cell receptor is formed by combination of α and β chains or γ and δ chains, which is similar to combination of light and heavy chains of immunoglobulin molecules. Both chains consist of a variable region (V) and a constant region (C). The genes encoding the variable regions consist of V and J segments for α chain and V, D and J regions for β chain. Each of the individual segments is encoded by multiple genes and recombination of these different gene segments results in the extraordinary diversity of the TCR. Recombination of the non-contiguous segments encoding the variable region of the T cell receptor, imprecise joining of "nicked" segments, addition of non-germline nucleotides by DNA repair machinery and different pairing of the TCR α and β chains results in generation of more than 1×10^{15} αβ TCRs (Zuniga-Pflucker, 2004). However, this repertoire undergoes selection process in the thymus which reduces the diversity of the TCRs by 3 to 100 fold (Zuniga-Pflucker, 2004). Lymphoid progenitors entering the thymus are double negative (DN) (CD4-CD8-) and following various stages of development, selection and lineage commitment, become single positive (SP, CD4+ or CD8+) and enter the medulla where they undergo central tolerance mechanisms before exiting to form the peripheral T cell repertoire (Fig 1.10).

Some of the TCRs on these thymocytes never bind to any MHC-peptide complex and are deprived of the survival signal provided by this interaction and thus undergo death by neglect. Thymocytes expressing TCR having very high affinity for any of the peptide-MHC complex are

eliminated by negative selection as these could lead to auto-immune reaction. However, the negative selection of T cells is not perfect and sometimes potentially auto-reactive T cells are released into the periphery (Anderton & Wraith, 2002). Lastly, the cells bearing TCR which binds to MHC-peptide complex with medium affinity receive a survival signal and these are positively selected. This T cell selection and the following lineage commitment occur in the thymic cortical region. Following lineage commitment, CD4+ and CD8+ T cells venture into the medulla where the stringent central tolerance mechanisms ensures that most of the tissue self-antigen reacting T cells are deleted. Previously it was assumed that thymic cortical epithelial cell (TECs) do not express tissue specific antigens (TSA) and T cells reactive against them are dealt by other mechanisms i.e. regulatory T cells in the periphery. However, it has now been shown that the TECs, mainly medullary TEC (mTEC), normally express various TSA, a phenomenon termed as 'promiscuous gene expression' (Kyewski *et al*., 2002). By virtue of promiscuous gene expression, mTEC are perhaps the most important cells involved in the central tolerance, as they not only select the T cell repertoire against various self-antigens generally expressed in all cells but also eliminate T cells against numerous tissue specific antigens. Finally, after stringent quality control mechanism these CD4+ and CD8+ T cells migrate to the periphery and constantly analyse the peptides presented by the antigen presenting cells as well as other cells to identify any pathogenic invasion.

Thus during the development of T cells, various mechanisms ensure to eliminate or anergise T cell clones with a potential to react against self antigens. And since most commonly shared tumour antigens are 'over-expressed' self antigens, to overcome tolerance and generate an immune response to these, remains a formidable challenge.

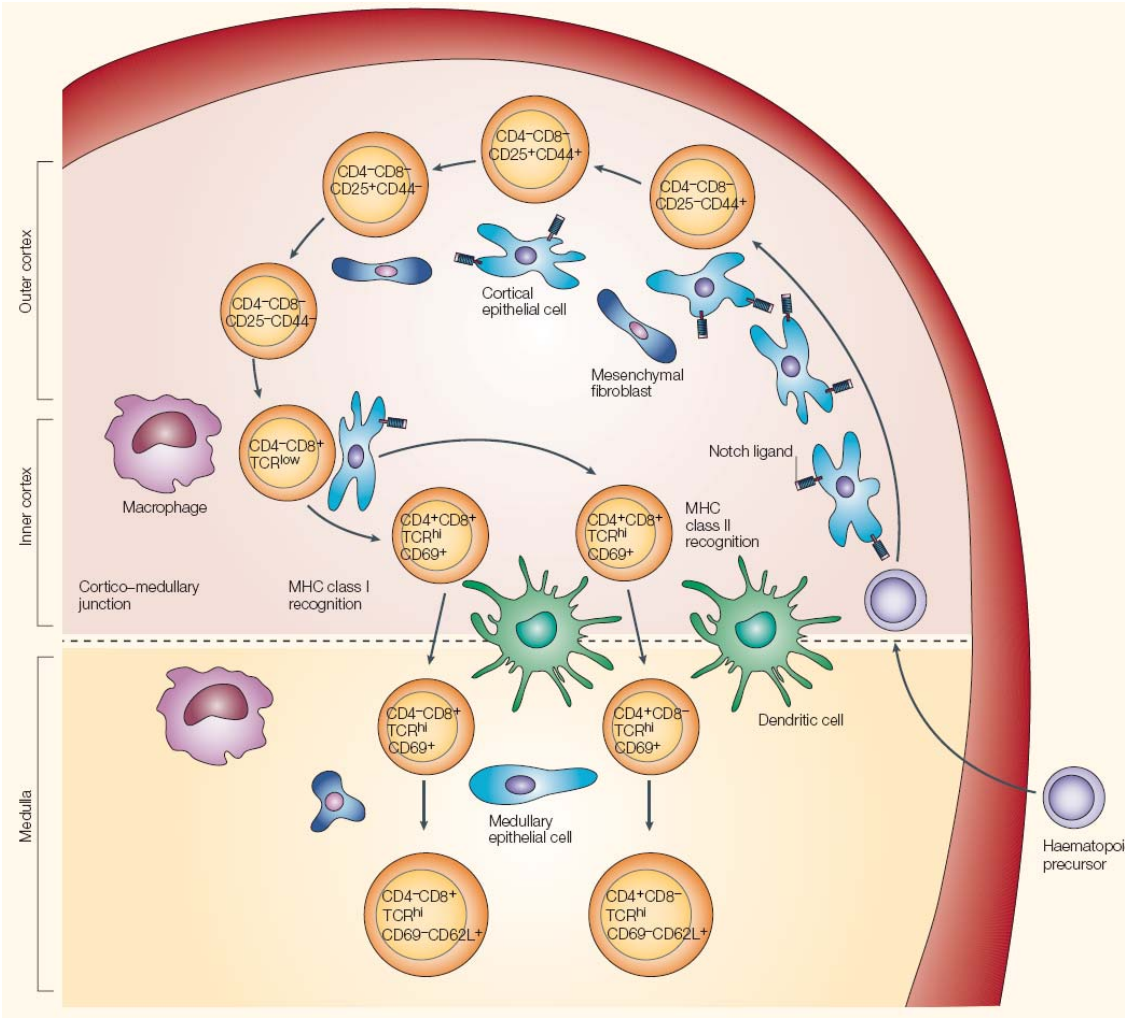


Figure 1.10:- Development of the lymphoid progenitor cell into SP T cells in the thymus
(Taken from Zuniga-Pflucker, 2004)

1.4 Cancer Vaccines

Prevention of infectious diseases through vaccination has been immensely successful for diseases such as small pox, polio, tuberculosis, mumps etc. Traditionally vaccination is considered as a prophylactic therapy, where vaccination generates a memory response and prevents future infections by the infectious organisms. However, cancer vaccination can be subdivided into prophylactic and therapeutic vaccines.

Data from numerous animal studies have shown the ability of different vaccines to generate antigen specific immune response and preventing the growth of tumour (expressing the particular antigen) in animals challenged. Prophylactic vaccines, although successful are not applicable in most cases of cancer, as it is difficult to predict cancer occurrence in humans. In cancers of known viral etiology, immunisation is able to prevent viral infection and consequent cancer development as observed in remarkable success of prophylactic human pappilloma virus

vaccines against cervical cancer (Harper *et al*, 2006; Koutsky *et al*, 2002). Similar vaccines may also be applicable for other virus induced cancers, such as hepatic cancer (hepatitis B/C virus). However, most human cancers are caused by mutations in normal cellular genes and predicting these mutations is complex. It may be possible in the future to base on the epidemiological data and genetic analysis, predicting a person's susceptibility to particular types of cancer would be accurate enough to justify their prophylactic immunisation targeting common oncogenes. However, immunisation against known 'self-antigens' can induce auto-immune response and risk-benefit ratio will have to be properly evaluated in such cases.

Thus far, most of the cancer vaccination studies have targeted their use for therapeutic application in humans. Unless stated otherwise, the term cancer vaccines will mean therapeutic vaccines.

(i) Whole Cell Vaccines:- Whole cell vaccines consist of irradiated autologous or allogeneic tumour cells, with or without any *in vitro* modifications and many clinical trials have explored this method of cancer vaccination. Earlier clinical trials using autologous or allogeneic tumour cells, without any modification met with limited success. Subsequently, various approaches have been used, where the tumour cells have been modified *in vivo* to express either co-stimulatory molecules or secrete cytokines to enhance their immunogenicity (Ali *et al*., 2000; Palmer *et al*., 1999). In a phase I clinical trial of prostate cancer patients vaccinated with irradiated autologous prostate tumour cells engineered to secrete GM-CSF, most patients generated prostate antigen specific T and B cell response (Simons *et al*., 1999). In metastatic melanoma patients immunised with autologous melanoma cells modified to secrete IL-12 cytokine, thus favouring the generation of a Th1 response and activation of the innate and adaptive immune system (Sun *et al*., 1998; Trinchieri, 2003), two of the seven patients developed DTH reaction to the autologous tumour cells, whereas one patient had a minor clinical response and generated tumour specific CD4+ and CD8+ T cell infiltrates into the metastases (Sun *et al*., 1998). This approach is restricted by the inability to generate large number of autologous tumour cells and is clinically impractical for larger clinical trials.

Most of the recent clinical trials have been conducted using modified allogeneic tumour cell lines as they can be genetically modified and cultured to large numbers prior to injection. Moreover cross presentation of antigen to T cells by the APCs does not require the tumour cells to be MHC matched with the recipient. One concern regarding the long term cultured cell lines relates to

changes of their antigenic profile, immunogenicity and gene expression; thus quality control and consistency of the vaccine is a central issue.

(ii) Heat Shock Protein Vaccines:- Heat shock proteins are a large family of ubiquitously and inducible proteins involved in transport of intracellular peptides and assist in the folding and unfolding of proteins along with a host of other functions (Srivastava & Amato, 2001). Rejection of autologous tumours by animals immunised by HSP derived from the tumours, led to the identification of their role as a promising approach to cancer immunotherapy. The ability of tumour derived HSP to generate anti-tumour responses has been demonstrated for hsp gp96, hsp70, hsp90, hsp110, grp170 and calreticulin (Binder *et al*., 2004). Because HSP are involved in peptide transport to the endoplasmic reticulum, immunisation with purified HSP would potentially generate an immune response against all the peptides in the cell, bypassing the need for individual epitope determination as well as MHC restriction. HSPs seem to have an intrinsic adjuvant property and binding to specific receptors on APC results in their maturation and internalisation of the HSP-peptide complex followed by internal processing and presentation of HSP-associated peptides on MHC class I and II molecules (Singh-Jasuja *et al*., 2000). This property of HSPs could be extremely important as not only are they the first and only "natural" adjuvants of mammalian origin but they also fail to elicit an immune response to themselves and can generate a response to very low levels of bound peptide compared to conventional peptide immunisation (Nieland *et al*., 1996). HSP have been referred to as the 'swiss army knives' of the immune system due to their multiple roles in CTL and antibody generation as well as activation of DC and NK cells (Schild & Rammensee, 2000; Tamura *et al*., 1997). It has also been suggested that HSPs might be able to overcome tolerance to tumour cells by virtue of their ability to activate APC, and generating co-stimulatory signals (Li *et al*., 2002). In most of the HSP clinical trials vaccination was performed, following surgical excision of the primary tumour, HSP-peptide complexes were purified from the tumour and the autologous complexes were injected back into the patient. In one such trial, out of sixteen renal cell carcinoma patients immunised with 25µg of autologous tumour derived hsp gp96, three patients demonstrated complete tumour regression, three had partial responses and three showed prolonged stabilization of the disease (Amata *et al*, 1999, Srivastava, 2005).

Table1. 4 Examples of different Types of Cancer Vaccines used in clinical trials

Vaccine Type	Trial Details	Results	Reference
Whole Cell Vaccines	Prostate cancer patients immunised with irradiated autologous cells secreting GM-CSF	Phase I clinical trial of 8 patient 7/8 DTH responses, antibodies detected in 3/8 patients. No significant clinical response	Simons *et al*, 1999.
	Melanoma patients, autologous cells secreting IL-12	2/7 DTH responses, 1 minor clinical response	Sun *et al*, 1998.
	Prostate cancer patients injected with 3 ID doses of allogeneic prostate cancer cells with BCG adjuvant	PSA levels decreased in 11 of 26 patients. Increased median time for disease progression of 58 weeks vs 28 weeks for controls	Michael *et al*, 2005.
Heat Shock Proteins	300 Stage IV melanoma patients	Increased survival observed in vaccinated patients	Srivastva PK, 2006.
	HSP (gp96) vaccine prepared for 17/20 non hodgkins lymphoma patients enrolled in phase II clinical trial	Stable disease in 8 patients from 6 to 19.8 months. 1 patient had partial response.	Oki *et al*, 2007.
	16 renal cell cancer patients injected with 25µg of Autologous HSP vaccine	3 complete tumour regression, 3 partial responses and 3 disease stabilisation	Amata *et al,* 1999.
Peptide Vaccines	23 melanoma patients immunised with either gp100 peptide alone or with IL-2	Post vaccination peptide specific T cell frequency increased in most pateitns but no tumour regression was observed	Lee *et al*., 1999.
	13 advanced melanoma patients immunised with modified peptide plus IL-12	Clinical regression of metastases observed in 13 out of 31 patients	Rosenberg *et al*, 1998.
	Stage IV melanoma patients immunised with peptides from melanoma antigens	No objective clinical response although few disease stabilisations	Hersey *et al*, 2005.
DNA vaccines	Stage IV melanoma patients injected intranodally with plasmid vaccine	11 out of 26 immunological responses with longer survival in 50% patients	Tagawa *et al*, 2003.
	22 metastatic melanoma patients were administered gp100 plasmid vaccine either ID or IM	1 partial response, rest all had progressive disease. No patient showed T cell responses against gp100.	Rosenberg *et al*., 2003.
	Phase I study of MART-1 plasmid vaccine in patients with resected melanoma	No immunological or clinical responses observed	Triozzi *et al*, 2005
Dendritic cell vaccines	16 melanoma patients immunised with DC pulsed with cell lysates or cocktail of peptides	5 objective clinical responses (2 complete and 3 partial)	Nestle *et al*, 1998.

	17 metastatic renal cell carcinoma immunised with autologous tumou to DC	Objective clinical response in 7 out of 17 patients (41%)	Kugler *et al*, 2000
	Phase I trial of 13 metastatic prostate cancer patients, DC transfected with autologous tumour derived mRNA were injected	In 6 patients, transient decrease of serum PSA was observed, although no long term clinical benefits	Heiser *et al*., 2000.
Recombiant viral vaccines	Melanoma patients immunised with canary pox virus encoding gp100	44% patients generated antigen specific response on virus vaccination but not peptide vaccine. No objective clinical response	Spaner *et al*, 2006.
	16 melanoma patients immunised with recombinant adenovirus expressing MART-1	One complete response. Other patients had non-specific clinical responses due to IL-2	Rosenberg *et al*, 1998.
	30 metastatic melanoma patients were injected with ALVAC virus encoding MAGE peptides	1partial response and 2 patients had disease stabilization. Evidence of CTL induction observed and correlated with clinical response	Van Baren *et al*, 2005.
Cellular Adaptive T cell Therapy	Myeloablative chemotherapy followed by autologous TIL transfer	Regression of metastases observed in 18 out of 35 patients (>50%)	Dudley ME *et al*, 2005.
	Melan-A specific T cell clones injected in 10 metastatic melanoma patients with IL-2 and IFN-α	1 complete response and 6 partial regression of metastasis or disease stabilisation. 'Epitope spreading' observed	Vignard *et al*, 2005.

(iii) Peptide Vaccines:- Any peptide presented by the MHC molecule on the surface of tumour cells is a potential target for cancer immunotherapy. In 1992, the first study to identify a nonamer peptide recognized by cytotoxic T lymphocytes in cancer patient was published (Traversari *et al*., 1992). Since then, several studies have identified immunogenic peptides presented by the tumour cells *in vivo* and recognized by the cytotoxic T lymphocytes in cancer patients (Romero *et al*., 2004). Peptide vaccines can be easily produced under GMP conditions, are relatively cheap and seldom responsible for serious side effects. These properties have made peptide vaccines the most attractive candidate for cancer immunotherapy in the past decade. A number of clinical trials have been conducted using peptide vaccines, where initial trials involved immunising patients with MHC class I restricted peptides alone; in some instances peptide specific CTLs were generated but this immunological response delivered little clinical responses if any (Boon *et al*., 2006; Lee *et al*., 1999). However, most of the other clinical trials have not generated encouraging results. In two recent independent clinical trials of stage IV melanoma

and CML patients, immunising with peptide vaccines containing epitopes from melanoma antigens (gp100, MART-1, tyrosinase and MAGE-3) and bcr-abl protein respectively, no objective clinical response in terms of tumour regression or remission induction was observed (Cathcart *et al*., 2004; Hersey *et al*., 2005). Experience from animal models showed that for an effective and lasting response leading to tumour regression, it was essential to activate CD4+ T helper cells. This has led to intensification in the efforts to identify MHC class II restricted peptides (Rojas *et al*., 2005; Zarour *et al*., 2000). Recent trials have used a combination of MHC class I and II peptides, but available evidence is limited and further investigation is required.

Limited clinical efficacy of the peptide vaccines can be attributed to a number of reasons including not being potent enough to break tolerance, immune escape, source of the MHC class II peptides (expressed by tumour cells) as well as the adjuvant and dose/schedule used for immunisation. Several approaches are currently been investigated to improve the potency of peptide vaccines. Modification of the anchor amino acids of the peptide can increase its binding affinity to MHC and TCR, inducing stronger immune response against tumour cells expressing native peptides. This approach has proved clinically beneficial in a significant clinical trial (Rosenberg *et al*., 1998). Peptide vaccines delivered through liposomes, nanobeads or in combination with CpG oligonucleotides have all shown an improved ability to generate high avidity CTLs; these approaches aim to deliver the peptide vaccines efficiently to the APC (Engler *et al*., 2004; Fifis *et al*., 2004).

(iv) Dendritic Cell Vaccines:- DC being the most important antigen presenting cells, have been extensively used in immunotherapeutic approaches where antigen has been directed to them or where DC themselves have been modified and injected back into the same patient. Although in most mouse models DC have been derived from the bone marrow, in human clinical trials, DC have mostly been generated from the CD34+ monocyte precursors from blood by culturing them *in vitro* in the presence of GM-CSF and IL-4 (Caux *et al*., 1996).

Over the years, researchers have devised various methods of manipulating DC for immunotherapeutic approaches (Banchereau & Palucka, 2005; Gilboa *et al*., 1998). Initial work involved pulsing DC with synthetic peptides from known antigens such as MART-1, tyrosinase or gp100 (Fong *et al*., 2001; Yu *et al*., 2001). However, the limitation of this strategy to specific MHC haplotypes led to either pulsing DC with antigens/tumour cell lysates or genetically engineering DC to express specific tumour antigens (Hsu *et al*., 1996; Lambert *et al*., 2001). These strategies preclude any knowledge of patient MHC type and are broadly applicable.

Another novel strategy has been to create hybrids of DC and tumour cells as these combine the whole antigen repertoire with the antigen stimulating capabilities of the DC (Rosenblatt *et al*., 2005). "Feeding" DC with tumour cell lysates or eluted peptides requires access to a relatively large quantity of the patient tumour, which is not always feasible. Hence, more recent work has focussed on transfecting DC with messenger RNA derived from the tumour cells (Heiser *et al*., 2000). Obtaining tumour tissues for nucleic acid isolation requires much less tissue along with advantage of being able to be combined with laser capture micro-dissection to specifically isolate tumour cells without surrounding normal cells. Modifying DC to secrete immuno-stimulatory cytokines such as IL-2, IL-12 or IFN-α along with the expression of tumour antigens can enhance the magnitude of the CTL responses in murine models (Huttner *et al*., 2005; Kuwashima *et al*., 2005). Several clinical trials have reported encouraging results by using allogeneic or autologous DC (Dallal & Lotze, 2000; Murphy *et al*., 1996).

Before DC based vaccination can be widely applicable in humans, several key points need to be addressed. DC consists of several different subsets and some of these can actually suppress the immune response instead of augmenting it. One study reported DC generated using IL-15 to be more potent stimulators of CTL response than IL-4 cultured DC (Mohamadzadeh *et al*., 2001). Hence, vaccination with the optimum DC subset in generating CTL responses will be crucial. Another recent study reported the presence of a novel interferon secreting killer DC (IKDC) subset in mice, which are capable of killing tumour cells as well as acting to present antigen; which property was predominantly dependent on activating factors (Chan *et al*., 2006). The existence of such a subset of DC in humans and whether they can be harnessed for immunotherapy remains to be proven. The maturation status of the cells and method of maturation is another important consideration (Dhodapkar *et al*., 2001). Most groups use a cocktail of pro-inflammatory cytokines IL-1β, TNF, IL-6 and Prostaglandin E2 for DC maturation. However, another group reported a combination of IL-1β and TNF with type I and II interferons to yield more potent DC for the induction of CTLs (Banchereau & Palucka, 2005). Among other considerations the major ones are the site of vaccination, dose, schedule and the pre-conditioning of the DC and injection site. A recent study generated engineered DC with drug inducible CD40 molecule, which is a potent means of promoting CTL generation and clearing established tumours in murine model (Hanks *et al*., 2005). To overcome the possible limitation of generating larger number of DC from patients, several groups are enduring to develop artificial APC (Kim *et al*., 2004).

(v) DNA Vaccines:- DNA vaccines consist of an antigen encoding gene on a bacterial plasmid backbone. Compared to peptide vaccines, which would be restricted to specific MHC haplotypes, DNA vaccines are unrestricted and can generate CTLs against multiple epitopes, being able to generate B cell as well as T cell immune responses (Pavlenko *et al*., 2004). Unlike viral vaccines, DNA vaccines are not influenced by the neutralizing antibodies against the viral vector. DNA vaccines can be administered in a number of ways such as intramuscular, intranasal, intramucosal or could be administered into the dermis by coating them onto gold particles and administered using a gene gun (Babiuk *et al*., 2003). Injected DNA can either be taken up by the somatic cells or the residing antigen presenting cells, depending on the site of immunisations. In the case of DNA transfecting somatic cells, APC pick up antigen by cross-priming and their role is vital in initiating the immune response. The route of immunisation with DNA vaccines seems to be important as several studies in animal models have showed that intramuscular immunisation preferentially generates a Th1 response, whereas immunising with the gene gun generates a Th2 response, although this method requires 100 times less antigen (Weiss *et al*., 2002).

DNA vaccination can be used in conjunction with other vaccination methods i.e. transfection of dendritic cells with plasmids encoding for tumour antigen or co-stimulatory/cytokine genes prior to injecting them. DNA vaccines, by virtue of intrinsic CpG motifs, provide the necessary danger signals leading to optimum activation of the APC. Recently, it has been shown that along with tumour antigen encoding plasmids, co-administration of plasmids encoding for dendritic cell chemotactic and growth factors enhances the antigen specific response in mouse models (Kim *et al*., 2000). DNA vaccines encoding multiple class I and II peptide epitopes have also been designed for HIV and malaria targeting either the MHC class I or II processing pathway by addition of specific signalling sequences (Velders *et al*., 2001). DNA vaccines by themselves are considered weakly antigenic; however when used in combination with viral vectors in a 'prime-boost' strategy they appear to be highly effective in generating antigen specific responses. Priming with plasmid vaccine followed by boosting with recombinant virus was shown to be highly effective compared to either of them alone (Amara *et al*., 2001).

(vi) Recombinant Viral and Bacterial Vaccines:- Viral and bacterial vectors are considered as the most promising approaches to stimulate potent immune response, especially to 'self antigens' by activating the APC due to their intrinsic high immunogenicity and the immune system's capacity to react to them. Retrovirus, poxvirus, alphavirus, adenovirus and herpes simplex

viruses have been investigated in several animal models and shown to be effective in promoting anti-tumour immunity (Bonnet *et al*, 2000). Viral vectors can be injected systemically or intra tumourally and used to modify tumour cells ex vivo prior to injection (Ali *et al*, 2002). Many viral vectors are disabled and safe to administer and ideally should infect dividing and non-dividing cells and have the capacity to accept large inserts of genetic information. Adenovirus has also been shown to be extremely potent in the generation of immune response in animals, however their efficacy in humans could be limited as about 85% of the population has antibodies against common serotypes. Recombinant alphaviruses are potentially very safe vectors that could be used as they need to be co-transfected with a helper plasmid for the generation of infective viral particles. Moreover they induce apoptosis upon infection which would facilitate cross-priming of the antigens. The immunotherapeutic potential of Disabled Infectious Single Cycle-Herpes simplex virus (DISC-HSV) in a murine tumour model has been extensively investigated (Ahmad *et al*., 2005). It was shown that injecting DISC/GM-CSF virus intratumorally, led to tumour rejection in up to 70% of the mice and combining this with other modalities increased its therapeutic benefit (Ali *et al*, 2002; Ali *et al*, 2004).

Recombinant bacterial vaccines from strains such as salmonella, BCG and Listeria monocytogenes are promising novel vectors for cancer immunotherapy since they can potentially be administered orally and are capable of infecting APCs directly. The dual phagolysosomal and cytoplasmic life cycle of listeria monocytogenes allows efficient processing of both MHC class II and class I antigens respectively (Gentschev *et al*., 2005; Weiskirch *et al*., 2001).

(vii) Novel vaccine delivery systems:- Limited clinical responses by most clinical trials till date have compelled researchers to look for novel delivery vehicles and strategies to enhance the delivery of vaccines in order to generate more potent immune responses. Liposomes have the ability to deliver "drugs" directly to the cytosol of the cells by fusion with the cell membrane and releasing the drug internally. This property has been exploited to deliver antigens to APC and liposomes containing tumour antigens have also been shown to generate antigen specific responses (Mandal & Lee, 2002). Liposomal vaccination has been shown to be clinically safe and effective in generating long lasting CD4+ and CD8+ T cell responses in animal models and in patients with advanced stage follicular lymphoma. Antigens, either coupled to beads made of iron, silica or latex, or trapped in poly-lactide-co-glycolide (PLG) microspheres, have been shown to generate CTLs and in some cases are able to provide tumour protection in animal models (Tartour *et al*., 2000). Exosomes have recently emerged as an alternative novel method

of immunisation. Exosomes are small membrane vesicles which are released from various cell types during fusion of multivesicular bodies with the plasma membrane (Cho *et al*., 2005), and carry both MHC class I and II antigens along with co-stimulatory molecules. These exosomes can be purified from various tumour cells and loaded with peptide ex vivo before injecting them into the patients. To overcome the reluctance of using viral vectors especially in immunocompromised cancer patients, virus like particles (VLP) have been designed, which consist of self-assembled proteins, derived from the viruses. These VLP are believed to be safer than conventional viruses and have similar potency. However, further research will be required to prove the clinical efficacy of these novel delivery vehicles.

(viii) Cellular Adoptive T cell Transfer:- One of the major limitations for cancer immunotherapy has been their inability to generate sufficient numbers of activated CTL. To overcome this, Rosenberg *et al* have pioneered cellular adoptive therapy, where tumour infiltrating lymphocytes (TIL) are isolated from autologous tumours, expanded and activated *in vitro* before being injected back into the patients. His group has recently shown that patients receiving myeloablative chemotherapy in the form of cyclophosphamide and fludarabine, followed by adoptive T cell transfer of autologous TIL, generated objective clinical response (>50% tumour reduction) in 18 out of 35 treated patients (Dudley *et al*., 2005; Rosenberg & Dudley, 2004). Regression of metastasis in these patients was observed in sites such as lung, brain, liver, lymph nodes and subcutaneous tissues (Dudley *et al*., 2005). Alternative novel strategy has been to clone tumour antigen specific T cell receptor into autologous T cells prior to injecting these engineered T cells back into patients. Local IL-2 support is essential for the activated T cells *in vivo*, however exogenous IL-2 administration to patients can have serious side affects. Hence, TILs engineered to contain the IL-2 gene are being developed, the local secretion of which would promote their own growth (Rosenberg & Dudley, 2004). In a recent clinical trial, adoptive transfer of Melan-A specific T cells in melanoma patients, not only provided clinical regression in some patients but it also led to expansion of T cells of higher avidity for other Melan-A epitopes, suggesting that some of the remarkable success of adoptive transfer can be attributed to the 'epitope spreading' phenomenon (Vignard *et al*, 2005).

1.5 Tumour Immune Escape

Despite the extraordinary success of several vaccination approaches in animal models, their application in humans have had limited clinical benefit. Current research is serving to unravel the complexities of the immune system and cancer, which is leading to discovery of novel

vaccination strategies to overcome the immune escape and tolerance mechanisms. One of the major reasons for this is that cancers in humans unlike animal models evolve over many years and undergo selection process to generate cells which are not only less immunogenic but are in fact immunosuppressive. Tumours can develop multiple escape mechanisms, even in the same individual. These mechanisms can be broadly classified as escape from detection, lack of susceptibility to killing and tumour induced immuno-suppression (Malmberg & Ljunggren, 2006; Muller *et al*., 2002).

(i) Escape from Immune Recognition Detection: - Immunosurveillance mechanisms detect any developing tumour and eliminate them via adaptive and innate immune cells. However, tumours develop mechanisms to evade these cells by various mechanisms such as loss of antigen, defects in the antigen processing, loss of presenting MHC molecules as well as up regulation of inhibitory receptors. Targeting a single antigen can lead to loss or down regulation of that antigen. Adoptive transfer of T cells specific for Mart-1 antigen led to selective down regulation of Mart-1 in the recurrent metastases while retaining the expression of other melanoma antigens gp100 and tyrosinase (Yee *et al*., 2002). Another important mechanism might be a mutation in the antigen which would affect the processing and presentation of the targeted epitope (Hoffmann *et al*., 2000). Similar cases have been documented with other forms of vaccination; hence it is crucial to target antigens which provide a growth advantage to the cancer cells making their loss difficult. However, this might be insufficient as antigen processing and presentation defects can occur independent of antigen loss. Various defects such as total loss of MHC class I, specific loss of HLA-haplotype locus, allele loss or mutations in proteins involved in antigen processing and transport (TAP) have been reported in a number of human tumours. HLA class I down regulation has been observed in about 16-50% of solid tumours including cancers of breast, lung, colon, cervix, prostate and melanoma (Chang *et al*., 2004; Rees *et al*., 1988). Mutations or defects in the production of β2 microglobulin chain results in total HLA loss as the class I chains are not assembled in the ER (Rosenberg *et al*., 2003). In the extensively investigated DISC-GMCSF/CT-26 tumour model, escape mechanisms were investigated in responder and non-responder mice on application of viral therapy following tumour inoculation. In this model, approximately 60% of mice with progressive tumour growth displayed MHC class I down-regulation (which was reversible on *in vitro* culture), whereas no change was observed in animals with regressive lesions. These experiments clearly demonstrate the importance of this mechanism in generating escape variants and tumour progression (Ahmad *et al*., 2004; Ali *et al*., 2002). However, total loss of HLA molecules would make these cells susceptible to NK cell

mediated killing in accordance with the 'missing self' hypothesis. To counteract this, cancer cells can selectively lose particular alleles which are targeted by immune cells and/or express minor HLA antigens which would inhibit the NK cells.

A numbers of human tumours are known to express stress related molecules like MICA and MICB which can be recognised by the NKG2D receptors on NK cells leading to their activation and tumour cell lysis. To avoid this NK mediated killing, tumour cells can shed MIC molecules, which not only avoids their recognition but also leads to deactivation of the NK cells. Soluble MIC molecules have been detected in the serum of cancer patients and their binding to NKG2D receptors causes their endocytosis and degradation, thus contributing to inefficient NK cell mediated immunosurveillance (Holdenrieder *et al*., 2006).

(ii) Lack of Susceptibility to Immune killing:- Another important mechanism of escape is decreased susceptibility to killing by the T and NK cells. T cells mediate tumour lysis mainly by granzyme/perforin pathway or death receptors Fas and TRAIL.

Granzyme B can be inhibited by serine protease inhibitor (PI-9) and is generally produced by activated T cells. In physiological conditions its role might be to prevent degranulation and destruction of dendritic cells by activated T cells. However, several human tumours also over express PI-9 rendering them resistance to granzyme B mediated cytotoxicity (Malmberg & Ljunggren, 2006). Cathepsin B is another such enzyme, which can inactivate perforin and is expressed by human tumours. Receptor mediated apoptosis can be overcome by tumour cells through expression of decoy receptors, soluble FasL or down regulation of death receptors. Death receptors Fas and TRAIL consist of cytoplasmic 'death domains', which are activated on binding of ligands triggering the downstream caspases and ultimately leading to apoptosis of tumour cells. Certain tumours can express decoy receptors (TRAIL-R3 and TRAIL-R4) which lack these death domains making them resistant to apoptosis on ligand binding. Another important molecule expressed in certain cases is the flice inhibitory protein (c-FLIP), which inhibits caspase 8 activation and apoptosis mediated by death receptors such as Fas and DR5 (Longley *et al*., 2006). Inhibiting the expression of c-FLIP was sufficient to sensitise human renal cell carcinoma cells *in vitro* (Brooks and Sayers, 2005). Over expression of other anti-apoptotic molecules such as survivin and bcl-2 has also been reported in certain cancer patients. However, tumour cells are also known to express FasL, which could bind to Fas molecules on the T cell surface leading to their apoptosis.

(iii) Tumour Mediated Immunosuppression:- Tumour cells can directly inhibit the immune response via secretion of immunosuppressive cytokines such as TGF-β and IL-10 and high levels of these cytokines have frequently been reported in cancer patients. Vascular endothelial growth factor (VEGF) secreted by many tumours not only helps in tumour neo-vascularisation but is also known to suppress transcription of NF-κB, thereby preventing DC maturation and activation (Oyama *et al*., 1998). Recently, in a very elegant model of sporadic immunogenic tumour model, Willimsky and Blankenstein showed that tumours do not escape immune detection by losing their intrinsic immunogenicity but they induce tolerance and expansion of non functional T cells (Willimsky & Blankenstein, 2005). In this model, immunised mice remained tumour free throughout life whereas all non-immunised mice developed progressive tumours, thereby suggesting that existing T cells in non immunised mice to be tolerant and unable to mediate tumour rejection. This suppression could be a result of tumour antigen presentation to the T cells without any co-stimulatory molecules, making them anergic (Abken *et al*., 2002). Another frequently employed mechanism by the tumour cells is the production of enzyme Indoleamine 2,3-di-oxygenase (Muller *et al*., 2005). IDO is ubiquitously expressed by normal tissues and catalyzes the rate limiting step in degradation of tryptophan. Over-expression of IDO at tumour sites depletes the essential amino acid tryptophan in the vicinity leading to growth arrest of T cells. Another possibility is that certain regulatory subsets of dendritic cells are also induced to produce this enzyme providing additional protection to the tumour cells (Munn *et al*., 2004).

One of the most important immunosuppressive mechanisms is thought to be the induction of TReg cells. TRegs account for 2-5% of the total CD4+ T cell population in humans. Conventional TRreg are identified by CD4+CD25+Foxp3+ phenotype although other variants are also known. Regulatory T cells consist of mainly two subtypes, natural and induced. Naturally occurring TReg cells are likely to be selected in the developing thymus and maintain peripheral tolerance to self-antigens. Organ specific antigens expressed ectopically in the thymus could lead to the generation of these suppressor cells (von Boehmer, 2005). These TRegs can protect tumour cells since most of the tumour antigens are in fact 'self-antigens'. Moreover, tumour cells can also induce TReg development for their survival. Initially, it was suggested that TReg act in an antigen independent manner, however, recent studies have identified antigen specific TRegs (Wang *et al*., 2004). One possibility is that tumour cells induce the development of these CD4+CD25+ cells from the CD4+ population of cells via immunosuppressive cytokines. Also, sub-immunogenic conditions during antigen presentation can lead to the generation of TReg (von Boehmer, 2005). The importance of this subset of T cells is underlined by the fact

that their deletion in combination with antigen specific immunotherapy has resulted in tumour regression of well-established poorly immunogenic tumours (Sutmuller *et al*, 2001). TReg cells have been shown to inhibit the proliferation of antigen specific CD4+ and CD8+ T cells in an IL-10 and TGF-β dependant manner (von Boehmer, 2005). TGF-β has been suggested to be involved in certain cases but most studies suggest this suppression to be cell contact dependant (von Boehmer, 2005). The precise mechanism of TReg mediated suppression still remains controversial especially *in vivo*. Other well documented suppressor cells are the NKT cells and the immature myeloid cells. It has been suggested that the NKT cells produce IL-13, which activates the immature myeloid cells to produce TGF-β (Terabe *et al*., 2003; Terabe *et al*., 2004). Fortunately, this suppression is reversible and these tolerant T cells can be rescued by culturing them in cytokines such as IL-15 (Teague *et al*., 2006).

Certain groups have also demonstrated shedding of minor HLA antigen HLA-G by tumour cells which induce apoptosis in antigen specific CD8+ T cells and NK cell inhibition (Chang *et al*., 2004).

1.6 MTA1 as a target for immunotherapy of cancer

In spite of the remarkable progress in medical diagnostics over the past few years, many cancer patients are still diagnosed in the terminal stages of the disease, at which point the conventional modalities of treatment are mainly palliative. Over the last 25 years, incidence of cancer has increased by 24% in the UK. This has fuelled an interest in the identification of antigens involved in the metastatic progression of the cancer and their potential application for therapy. Some of the genes associated with metastases are the WNT5A, WNT11, HMG-1(Y), MMP-2, MAP kinase phosphatase-1, WDNM1 (extracellular proteinase inhibitor), Trop 2 (tumor-associated calcium signal transducer-2), procollagen type IV alpha, secretory leukoprotease inhibitor, prenylated snare protein Ykt6, ceruloplasmin and chaperonin 10 (Kluger *et al*, 2004; Xie *et al*, 2004; Ebralidze *et al*., 1989; Dear *et al*, 1989; Basset *et al*, 1990). However, most of the antigens identified may not be applicable for therapy or indeed immunogenic. Hence, it would be very valuable to identify a metastasis associated gene which is immunogenic and could potentially be targeted by immunotherapy for late stage diseases or in a combination therapy at early stages to prevent metastatic occurrence. Metastasis Associated Tumour antigen 1 (MTA1) is such an antigen and was recently identified in our laboratory by applying SEREX technology and screening of prostate cancer cDNA libraries with autologous serum (Li G *et al*,

Unpublished), suggesting MTA1 to be immunogenic, at least for generation of CD4+ T cells as antibody production requires help from differentiated Th2 cells from CD4+ T cells.

MTA1 was originally identified using a differential cDNA library screening using rat mammary adenocarcinoma cells (Pencil *et al*, 1993; Toh *et al*, 1994). MTA1 gene encodes an 82 kDa protein. Analysis of the gene sequence of MTA1 revealed that it has a proline rich region (SH3 binding motif), putative zinc finger DNA binding motif and a leucine zipper motif. Moreover, the human MTA1 protein is also rich in SPXX motifs, which are usually expressed in gene regulatory and DNA binding proteins. Also, it contains three nuclear localization signals (Nicolson *et al*, 2003; Nawa *et al*, 2000). This evidence suggests that MTA1 might be localized in the nucleus and act to repress transcription. Indeed, through indirect immunofluorescence it was found that MTA1 was mainly localized in the nucleus (Nawa *et al*, 2000).

Histone proteins help in the organization of the DNA into nucleosomes, which are regular repeating structures in the chromatin and the acetylation status of the histone proteins effects gene expression by altering the transcription of the genes (Marks *et al*, 2001). Recently, it was shown that MTA1 might be a part of the nucleosome remodeling (NuRD) and histone deacetylase complex (HDAC) (Toh *et al*, 2000). Moreover, expression level of MTA1 correlated inversely with the acetylation status of histone H4 in invasive oesophageal carcinomas, which correlated positively with the prognosis of the patients. Hence, it has been suggested that strategies inhibiting MTA1 function could prove to be a novel approach to treat certain cancers (Toh *et al*, 2004).

Screening of various tumours and cell lines such as breast, esophageal, colorectal, gastric and pancreatic cancer, has shown MTA1 to be over expressed in metastatic cells as compared to the primary tumour (Tang *et al*, 2003; Sasaki *et al*, 2002; Toh *et al*, 1999; Nicolson *et al*, 2003). It has also been shown that expression of MTA1 enhances the cellular motility and invasive potential of the cancer cells (Hofer *et al*, 2004; Mahoney *et al*, 2002). MTA1 expression is also detectable in various normal cells, although at very low levels as compared to cancer cells. Moreover, cells expressing higher levels of MTA1 have a faster growth rate suggesting that it might be involved in cellular proliferation (Nicolson *et al*, 2003). Furthermore, experimental inhibition of MTA1 protein expression using antisense phosphorothioate oligonucleotides resulted in growth inhibition of human breast cancer cells (Nawa *et al*, 2000). Thus, MTA1 might be essential for the growth and/or invasive potential of cancer cells and hence targeting

MTA1 might not allow cancer cells to generate antigen loss variants. Considering the expression pattern and the diversity of MTA1 expression in different tumours, MTA1 might potentially be an ideal target for immunotherapy. However, it is also expressed in normal tissues at lower levels and it might be considerably difficult to generate an immune response against and that too at the risk of auto-immunity. But, it has been shown in the past that immune response can be generated against self antigens like p53 and survivin without severe adverse effects (Murakami *et al*, 2004; Reker *et al*, 2004; Vierboom *et al*, 1997). However, till date, none of the published studies have investigated the immunotherapeutic potential of MTA1.

Human MTA1 is 85% and 96% similar to murine MTA1 at the genetic and amino acid level respectively (Nawa *et al*, 2000). Moreover, its function and distribution have also been shown to be quite similar, which could allow development of murine model to explore immunotherapeutic potential of MTA1.

1.7 Aims and Objectives of the study

This study proposes to investigate the immunotherapeutic potential of MTA1 in a murine model as a pre-requisite for using human MTA1 as a target for immunotherapy in patients. Firstly, MTA1 will be validated as a target for therapy by confirming its over expression in various human cancers, compared to its expression in murine tissues and cell lines. Subsequent to its validation, this study will attempt to generate immune response against MTA1 in murine model and identify peptides epitopes for immunotherapy and immune-monitoring. Considering the 'self-antigen' status of MTA1, potent vaccination strategies such as DNA and viral vaccines (syngeneic and xenogeneic) along with depletion of Tregs might be required and will also be evaluated.

2.1 Materials

2.1.1 Reagents

Culture Media	Company
DMEM	Bio Whittaker, Europe
1640 RPMI	Bio Whittaker, Europe

Supplements added to Culture Media	Company
Foetal Calf Serum (FCS)	Bio Whittaker, Europe
2-mercaptoethanol	Bio Whittaker, Europe
Penicillin/Streptomycin	Bio Whittaker, Europe
HEPES buffer	Bio Whittaker, Europe
Fungizone	Bio Whittaker, Europe
Geniticin (G418)	Bio Whittaker, Europe

Other Reagents	Company
Trypsin	Gibco, UK
Versene	Gibco, UK
Heparin	Sigma, UK
DNAase	Sigma, UK
Collagenase	Calbiochem, UK
Trypan Blue	Sigma, UK
Lipopolysaccharide	Sigma, UK

Molecular Grade Chemicals	Company
Molecular Grade Water	Sigma, UK
Absolut Ethanol	BDH, UK
Isopropanol	Sigma, UK
RNA Stat 60	AMS Biotechnology, UK
Chloroform	Sigma Aldrich
Agarose	Bioline
Tryptone	Oxoid
Yeast	Oxoid
Bacteriological Agar	Oxoid
Sodium Chloride	Sigma
Kanamycin	Sigma
Tetracyclin	Sigma
Ampicillin	Sigma
Phenol-Chloroform IsoAmyl Alcohol	Sigma
Absolute Ethanol	BDH
α-Chymotrypsin	Sigma Aldrich
Aprotinin	Sigma Aldrich
BSA	Sigma
Sucrose	BDH Lab Supplies

PBS tablets pH 7.2-7.4	OXOID
Acetic Acid	Fisher Scientific Ltd
Tween 20	Promega
Sodium azide	Sigma
Trypan Blue	Sigma
Ethidium Bromide	Sigma
Sodium Chloride	Fisher Scientific Ltd
Sodium Hydroxide	Fisher Scientific Ltd
Tris	Fisher Scientific Ltd

Other Reagents	**Company**
EMLA Anaesthetic Cream	Astra Zeneca, UK
Micro Scint 0	Packard
Tritiated Thymidine	Amersham
Chromium 51	Amersham
Incomplete Freunds adjuvant (IFA)	Gibco

2.1.2 Media, Buffers and Solutions

Prepared as indicated

T cell Media

Ingredients	**Quantity**
Complete RPMI	500 ml
10% FCS (by volume)	50 ml
Glutamine	5 ml
20 mM HEPES	10 ml
50 µM 2 Mercaptoethanol	500 µl
50U/ml Penicillin/Streptomycin	5 ml
0.25 µg/ml Fungizone	500 µl

BM-DC media

Ingredients	**Quantity**
Complete RPMI	500 ml
10% FCS (by volume)	25 ml
Glutamine	5 ml
20 mM HEPES	10 ml
50 µM 2 Mercaptoethanol	500 µl
50U/ml Penicillin/Streptomycin	5 ml
0.25 µg/ml Fungizone	500 µl

PBS-BSA wash for FACS

Ingredients	**Quantity**
PBS tablets	10/litre

BSA	0.1% (1g/litre)
Sodium Azide	0.02% (0.2 g/litre)

RIP Buffer

Reagent	**gm/500 ml**	**mM**
Sodium Chloride	4.38	150
Tris	3.027	50
EDTA, anhydrous	0.931	5

Western Blot Lysis Buffer

Ingredients	**Quantity**
RIP Buffer	5 ml
Igepal	50 µl
Deoxycholate acid	25 mg
10% SDS	50 µl
500 mM Benzamidine	10 µl
100 mM PMSF	5 µl
200 mM Sodium Valrpoate	25 µl
1 M Sodium Fluoride	5 µl

Other Buffers

Buffer	**Composition**
PBS	1 tablet dissolved in 100 ml distilled water
PBA	PBS 0.1% (w/v) BSA 0.02% (w/v) Sodium Azide
TBS	10mM Tris 150nM NaCl pH 7.4
1 x TAE Freshly prepared from 10x TAE	40 mM Tris Acetate 1 mM EDTA
Glutaraldehyde Solution	0.1 M Sodium phosphate, pH 7.0 1mM MgCl2 0.25% Glutaraldehyde
X-Gal Solution	0.2% X-Gal 1mM MgCl2 150mM NaCl 3.3mM K4Fe(CN)6 3.3mM K3Fe(CN)6

60mM Na2HPO4
40mM NaH2PO4

2.1.3 RT-PCR Enzymes, Restriction Enzymes and Reagents

Reagent	Company
M-MLV-RT	Promega
Oligo dT Primers	Promega
RNasin Inhibitor	Promega
Taq Polymerase	Bioline
T4 Ligase Enzyme	Promega
EcoRI Restriciton Enzyme	Promega
BamHI Restriciton Enzyme	Promega
XbaI Restriciton Enzyme	Promega
AgeI Restriciton Enzyme	Promega
SpeI Restriciton Enzyme	Promega
ApaI Restriciton Enzyme	Promega
Pfu Polymerase	Promega
Phusion Taq polymerase	Finnzyme
TOPO-TA vector	Invitrogen
TOPO-Blunt Vector	Invitrogen
pcDNA3 plasmid	Invitrogen
SYBR Green Master Mix	Biorad
dNTPs	Bioline
DNA ladder (1Kb plus)	Invitrogen
10X Reaction Buffer	Promega
Magnesium Chloride	Promega

2.1.4 AntiBodies and Kits

Cell line/Antibody	Source/Manufacturer
Goat anti-mouse FITC	Sigma
HB54 (HLA-A2.1)	Hybridoma
Anti-MTA1 antibody	Santa Cruz Biotechnology,USA
Rabbit Anti Goat-HRP antibody	DAKO
CD80	Cambridge Biosciences
CD40	Hybridoma FGK-45
CD25-FITC	Serotec
CD11c	Hybridoma
MHC II IA/IE	BD Pharmingen
Goat Anti-Mouse MTA1	Santa Cruz Biotech
Mouse Anti-FLAG	SIGMA
Mouse Anti-FLAG FITC	SIGMA
Rabbit anti-goat IgG – HRP	DAKO

Streptavidin – HRP	Zymed, USA
Goat Anti-Mouse – HRP	DAKO
Anti-Rat FITC	Serotec
Anti-hamster FITC	Serotec
Anti-Goat FITC	Sigma
Mouse CD4: Dynabeads Mouse CD4	Dynal, Europe
Mouse CD8: Dynabeads Mouse CD8	Dynal, Europe
Mouse CD4: Detach A beads CD4	Dynal, Europe
Mouse IFN-γ ELISA kit	R&D Systems, UK
Mouse IL-4 ELISA kit	R&D Systems, UK
Anti-Human HLA-DR (L243)	BD Pharmingen

2.1.5 Laboratory Plastic ware, glass ware and sharps

Item	**Company**
T25 and T75 tissue culture flasks	Sarstedt, UK
50 ml screw top tubes	Sarstedt, UK
10 ml and 5 ml pipettes	Sarstedt, UK
20 ml Universal tubes	Sterilin UK
Centrifuge Tubes (15ml)	Sarstedt, UK
Bijou tubes (7 ml)	Sterlin, SLS, UK
FACS tubes	Elkay, UK
10 ml syringes	Becton Dickenson
BD Microlance 3 needles	Becton Dickenson
24 well and 6 well flat bottom culture dishes	Sarstedt, UK
96 well round bottom plates	Sarstedt, UK
Pasteur pipettes	Sarstedt, UK
1.5 ml eppendorf tubes	Sarstedt, UK
0.5 ml eppendorf tubes	Sarstedt, UK
1.2 ml Cryovials	TPP, UK
Pipette tips < 1ml	Sarstedt, UK
96 well ELISA plates	Costar, UK
Petri dishes	Sterilin UK
25 ml Pipettes	Sarstedt, UK
10 ml Pipettes	Sarstedt, UK
5 ml Pipettes	Sarstedt, UK
Haemocytometer	Weber
96 well plate harvester filters	Perkin Elmer
Scalpels	Swann Morton Ltd.
PCR Tubes	Micronic Systems
0.2µm Filters	Sartorius, UK
Realtime PCR tubes	Strategene, Germany
0.5 – 10µl tips	Sarstedt, UK
20 – 200µl tips	Sarstedt, UK
200 – 1000µl tips	Sarstedt, UK

2.1.6 Electrical Equipment

Equipment	Manufacturer
Refrigerated centrifuge	Mistral 1000, MSE
Flow Cytometer	Beckman Coulter
Clenz	Beckman Coulter
Isoton	Beckman Coulter
Liquid Nitrogen Freezer	Forma Scientific
-80°C Freezer	Ultima II, Revco
Class II safety cabinets	Walker
37°C incubator	Forma Scientific
96 well plate harvester	Packard
Light microscope	Olympus
96 well plate reader	Tecan
Top count scintillation counter	Packard
Drying Cabinet	Scientific Laboratory Supplies Ltd
PCR Thermal Cycler	Hybaid, Germany
Water Baths	Grant Instruments
Real Time PCR Thermal Cycler	Bio-rad
Microscope	Nikon
Power Packs	Bio-rad
Electrophoresis gel tanks	Bio-rad
Microwave	Matsui
UV Spectrophotometer	Sanyo
Transilluminator	Ultra Violet Products
Whirlimixer	Scientific Industries

2. 2Methods

2.2.1 Determination of MTA1 Expression by Conventional RT-PCR

2.2.1.1 Cell Lines and Tissues

Various cell lines used in this study are described below in table 2.

Table2.1:- Cell Lines and their descriptions

Name	Description	Media	Source
CT-26	Murine colon carcinoma	DMEM+10% FCS	Prof Ian Hart (St Thomas Hospital)
CL-25	Murine colon carcinoma expressing β-galactosidase	DMEM+10% FCS+G418	Prof Ian Hart (St Thomas Hospital)
B16	Murine melanoma	RPMI 1640+2 mM L-glutamine	Richard Vile (Hammersmith Hospital)
A20	Murine B cell lymphoma	RPMI 1640+2 mM L-glutamine	ATCC
RENCA	Murine Renal cell carcinoma	RPMI 1640+2 mM L-glutamine	Dr. Robert Wiltrout (National Cancer Institute, Bethesda, MD)
EL4	Lymphoid cells	RPMI 1640+2 mM L-glutamine	Prof Ian Hart (St Thomas Hospital)
CMT 93	Murine rectal carcinoma	RPMI 1640+2 mM L-glutamine	Richard Vile (Hammersmith Hospital)
RMA/S	Lymphoblastoid	RPMI 1640+2 mM L-glutamine+10% FCS	Dr. Colin Brooks (University of Newcastle)
RMA/S-A2	Transgenic lymphoblastoid	RPMI 1640+2 mM L-glutamine+10% FCS+G418	Dr. F Lemonnier (Institut Pasteur, Paris)
EL4-HHDII	Transgenic Lymphoid cells expressing HHDII molecule	RPMI 1640+2 mM L-glutamine+10% FCS	Dr. F Lemonnier (Institut Pasteur, Paris)
T2	Human Lymphoblastoid	RPMI 1640+2 mM L-glutamine +G41810% FCS	Dr. F Lemonnier (Institut Pasteur, Paris)
BHK-21	Syrian hamster kidney	DMEM+10% FCS	ATCC
293	Human Embryo Kidney	DMEM+10% FCS	ICRF
K562	Chronic Myeloid Leukaemia	RPMI 1640+2 mM L-glutamine+10% FCS	ATCC

Murine tissues were harvested from naïve Balb/c mice and immediately snap frozen in liquid nitrogen and stored at -80˚ C until RNA was isolated from them.

2.2.1.2 RNA extraction and cDNA synthesis

Total RNA was isolated from the cell lines and the tissues using RNA STAT-60 (AMS Biotechnology, UK) following manufacturer's instructions. Briefly, the tissues were grounded to a powder in liquid nitrogen and 1 ml of RNA-STAT60 added to them and stored at room temperature for 5 minutes. 0.2ml of Chloroform was added and the homogenate shaken vigorously for 60 seconds and left at room temperature for 3 minutes. Samples were then centrifuged at 14,000 rpm for 10 minutes. The aqueous phase was transferred to a fresh eppendorf and 0.5 ml of isopropanol was added to them. Samples were incubated at room temperature for 8 minutes followed by centrifugation at 14,000 rpm for 15 minutes. Supernatant was discarded and the pellet washed with 75% ethanol. RNA pellet was then dried and resuspended in molecular grade water and the concentration and purity of the RNA was measured on UV spectrophotometer.

RNA was then reverse transcribed into cDNA as follows. 2µg of RNA was taken in an eppendorf along with 0.5µg of oligo d(T_{15}) primer. Tube was heated at 70˚C for 5 minutes and then placed on ice. Following mix was then added to the tube

5 µl of 5x Reaction Buffer

1 µl of dNTPs (12.5 mM)

25 units rRNasin Ribonuclease Inhibitor

200 units of M-MLV Reverse Transcriptase.

Nuclease free water was then added to make the final volume to 25 µl. Contents of the tube were gently mixed and heated at 39.2˚C for 80 minutes followed by cooling on ice and heating at 95˚C for minutes and then storing them at -20˚C.

2.2.1.3 PCR amplification

PCR was performed on a DNA Thermal cycler (Thermo Hybaid, USA). Primers were supplied by Sigma Genosys (UK). All the tissues and cell line samples were pre-screened for house keeping gene mGAPDH, which yielded a 400 bp product.

For mMTA1 screening, primers used were 5'-GCGAGAGCTGTTACACCACA and 3'-ACTGCTGAGCACACTGGATG, which yielded a 508 bp product. For amplification by PCR, 1µl of cDNA was supplemented with 5 µl of 10x PCR buffer, 0.8 µl each of 10mM dNTP, 20 pM each of primer solutions, 1.25 unit of thermostable Taq polymerase, 1.5 mM $MgCl_2$ (Bioline), and water to a final volume of 50 µl.

PCR for MTA1 was initiated by a melting step at 95˚ C lasting for 5 minutes, followed by 34 cycles of denaturation at 95˚ C for 1 min, annealing at 55˚ C for 1 minute and extension at 72˚ C

for 1 minute. It was followed by a final extension step at 72˚ C for 5 minutes. All the primers used for the study are listed below in table 5.

Table 2.2:- Primers used for PCR, Cloning and sequencing of murine and human MTA1

Conventional PCR Primers (5' > 3')	
mGAPDH Forward	ACTCCACTCACGGCAAATTC
mGAPDH Reverse	CCTTCCACAATGCCAAAGTT
mMTA1-F	GCGAGAGCTGTTACACCACA
mMTA1-R	ACTGCTGAGCACACTGGATG
hGAPDH-F	ACCACCAACTGCTTAGCACC
hGAPDH-R	CCATCCACAGTCTTCTGGGT
hMTA1-F	CGCTCAAGTCCTACCTGGAG
hMTA1-R	TGGTACCGGTTTCCTACTCG
Primers for Murine MTA1 cloning	
mMTA1-F	ACCATGGCCGCCAACATGTACAGG
mMTA1-R	CGCCTAGTCCTCAATAACAATGGGCTC
mMTA1 Seq -1	AAAACTGGAGACCAAGGTGTG
mMTA1 Seq -2	GAGAGCTGTTACACCACACA
Primers for Human MTA1 cloning	
HMTA1-F	ACCATGGCCGCCAACATGTA
HMTA1-R	GGCCCCTAGTCCTCGATGACGATGGGCTCG

PCR products were visualized using a 1.5% (wt/vol) agarose gel containing 1 μg/ml of ethidium bromide (BDH Laboratories, UK).

2.2.2 Determination of Human and mouse MTA1 expression by Real-time RT-PCR

2.2.2.1 Human Cancer and normal tissues

Samples of Breast, Gastric and Colon carcinoma mRNA, along with patient matched normal tissue mRNA, were kindly provided by Dr. Aija Line (Latvia). Mouse cell lines and tissues are described above.

2.2.2.2 cDNA Synthesis from mRNA

Reverse Transcription of the mRNA was performed as described earlier with exception of using Random primers instead of Oligo dT.

2.2.2.3 Real-Time Quantitative PCR

For preparing the standard curve, total RNA from a normal testis sample (T7) was serially diluted. Primers for various genes (mMTA1, mGAPDH, m18S, hMTA1, hGAPDH and h18S) were designed with the assistance of the Primer Vs program accessible from the following website:

(http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi.)

All primers were designed to generate PCR products of approximately 100 bp in size to optimize the RT-Q-PCR.

Table 2.3 Forward and Reverse primer sequences of primers used for RT-Q-PCR

Gene	Primer Sequences (5' > 3')
mGAPDH-F	CCACCCAGAAGACTGTGGAT
mGAPDH-R	TTCAGCTCTGGGATGACCTT
mMTA1-F	CTCCTGCTCAATGGGAAGTC
mMTA1-R	CTTCGGTGGCCATGTAAAAT
m18SR-F	GTAACCCGTTGAACCCCATT
m18SR-R	CCATCCAATCGGTAGTAGCG
MTA-1 TW F	CGCTCAAGTCCTACCTGGAG
MTA-1 TW R	TGGTACCGGTTTCCTACTCG
GAPDH TW F	ACCACCAACTGCTTAGCACC
GAPDH TW R	CCATCCACAGTCTTCTGGGT
H18S F	CAACTTTCGATGGTAGTCG
H18S R	CCTTCCTTGGATGTGGTA

RT-Q-PCR was performed using Biorad real time Thermocycler (Biorad) using SYBR green fluorescent dye. Thermocycling for each reaction was done in a final volume of 12.5 µl containing 0.5µl of template, 6.5 µl of SYBR green master mix (Invitrogen, UK) containing Hot Start Taq DNA polymerase, and pre-optimised amounts of gene-specific forward and reverse primers. This was then made up to 12.5 µl with double distilled water. In each experiment, at least 4 no template controls were included to rule out any contamination and also to indicate the degree of amplification due to primer dimers. The cycling conditions for each gene were;

Human MTA1

50 ºC for 2 mins
95 ºC for 8:30 mins
95 ºC for 30 secs
58 ºC for 1 min
72 ºC for 30 secs
} 40 cycles
95 ºC for 1 min
58 ºC -10 secs (70 cyc for dissociation curve)

Human 18S ribosomal RNA

50 ºC for 2 mins
95 ºC for 8:30 mins
95 ºC for 30 secs
54 ºC for 1 min
72 ºC for 30 secs
} 40 cycles
95 ºC for 1 min
54 ºC -10 secs (85 cyc for dissociation curve)

Human GAPDH

50 ºC for 2 mins
95 ºC for 8:30 mins
95 ºC for 30 secs
}

Murine GAPDH

95 ºC for 3 mins
58 ºC for 20 secs
72 ºC for 30 secs
} 40 cycles

58 ºC for 30 min 72 ºC for 30 secs 95 ºC for 1 min 58 ºC -10 secs (70 cyc for dissociation curve)	45 cycles	95 ºC for 1 min 58 ºC for 1 min

Murine MTA1		**Murine 18S ribosomal RNA**	
95 ºC for 3 mins		95 ºC for 3 mins	
58 ºC for 20 secs 72 ºC for 30 secs	40 cycles	58 ºC for 20 secs 72 ºC for 30 secs	40 cycles
95 ºC for 1 min		95 ºC for 1 min	
58 ºC for 1 min		58 ºC for 1 min	

2.2.3 Determination of MTA1 protein expression

2.2.3.1 Cell Lines

Murine cell lines have been described in section 2.1.1. HaCaT cell line was a generous gift from Dr Hong Wan (Cancer Research, UK), and was cultured in DMEM+10% FCS. K562 cells were obtained from EUCAPS and were grown in RPMI 1640 +10% FCS, whereas MDA-MD-435 cells were obtained from Queens Medical Centre (Nottingham) and were cultured in DMEM+10% FCS.

2.2.3.2 Cell Lysate Preparation

Cells were harvested and washed twice in ice cold PBS at 400 rpm for 3 min at 4°C. Cell pellet was resuspended in 500µl of lysis buffer (150mM NaCl, 50mM Tris-Base pH 8.0, 5mM EDTA, 1% v/v IGEPAL CA-630, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, 1mM benzamidine, 0.1mM PMSF, 1mM sodium ortho-vanadate, 1mM sodium azide) and the tubes were agitated for 30 min at 4°C. The tubes were then allowed to stand on ice for one hour followed by centrifugation at 14000 rpm at 4°C for 30 mins and supernatant transferred to a fresh eppendorf. For proteins from tissues, various tissues were harvested from naïve balb/c mice and tissues were homogenized using a homogenizer in lysis buffer and the above process repeated. The samples were stored at –20°C until analysis by protein assay and SDS-PAGE.

2.2.3.3 Protein assay

Protein concentration was determined in the lysate preparation by performing a protein assay as described by the manufacturer protocol (Biorad). The standard was made of BSA diluted in lysis buffer in serial dilutions. Briefly, to 5 µl of the samples and standards 25 µl of Reagent A was added. To each well, 200µl of reagent B was then added. Each sample was run in duplicate. The reaction was left to develop for 30 min and the plate was read at 750nm on a Spectrafluor (Tecan).

2.2.3.4 SDS-PAGE and transfer

1x reducing buffer was added to all the samples and boiled for 5 min at 95°C to denature proteins, before being loaded on the polyacrylamide gel. As a standard, BSA was run with the samples. The gel was run at 90V through the 4% stacking gel (15% (v/v) acrylamide /bis, 25% 0.5M Tris HCL pH6.8, 60% dH_20 plus 0.1% (v/v) TEMED and 1% (v/v) 10% ammonium persulfate) and 120V through the 10% resolving gel (33.3% (v/v) acrylamide/bis, 25% 1.5M Tris HCl pH 8.8, 41.7% dH_20 plus 0.1% (v/v) 10% ammonium persulfate). Proteins were then transferred at 13V onto nitrocellulose membrane for 40 mins using a semi-dry transfer system (Biorad) according to manufacturer instructions.

2.2.3.5 Western Blotting

Membranes were stained with Ponceau S, and the standard lane was cut from the rest of the membrane. The membrane was blocked overnight in 5% milk-TBS-T at 4°C under constant agitation. The primary antibody (anti-human/mouse MTA1 antibody, Santacruz Biotechnology, USA) was then added at 1:1000 dilution in 5% milk-TBS-T and incubated for 2 hrs at room temperature. After washing the membrane 4 times for 15 mins in TBS-T at room temperature, the secondary antibody (HRP conjugated) was added to the membrane at a 1: 1000 dilution in 5% milk-TBS-T and incubated for 2 hour at room temperature. Membrane was washed 4 times for 15 mins at room temperature in TBS-T, and revealed using ECL chemioluminescence kit (Amersham). Hyperfilm ECL (Amersham) films were used to detect the luminescence.

2.2.4 Cloning of Mouse MTA1 and Human MTA1

Briefly, full length mouse MTA1 (Pubmed accession no NM_054081) was amplified from naïve mouse testis, using forward primer 5'-ACCATGGCCGCCAACATGTACAGG-3' (141-161 bases) and reverse primer 5'-CGCCTAGTCCTCAATAACAATGGGCTC-3' (2214-2240

bases). PCR was performed for 40 cycles and was initiated by a melting step at 95˚ C lasting for 5 minutes, followed by 40 cycles of denaturation at 95˚ C for 1 min, annealing at 68˚ C for 1 min and extension at 72˚ C for 4 min. It was followed by a final extension step at 72˚ C for 5 mins. For amplification by PCR, 1µl of cDNA was supplemented with 5 µl of 10x PCR buffer, 0.8 µl each of 10mM dNTP, 20 pM each of primer solutions, 1 unit of pfu polymerase (Promega) and water to a final volume of 50 µl. Full size MTA1 cDNA band of approximately 2.1 kb was visualized on 1% Agarose gel, band extracted and cloned into TOPO-Blunt vector following manufacturer's instructions. Cloned plasmid was isolated from multiple colonies and sequenced to confirm that there were no mutations (MWG-Biotech). For directional cloning, mMTA1 was digested using HindIII and XbaI double digest from TOPO-Blunt and inserted into pcDNA3 mammalian expression vector (Invitrogen).

For human MTA1 cloning (Pubmed accession no NM_004689) (Forward bases 188-201 and Reverse bases 2312-2337), PCR reaction was set up with Phusion Taq Polymerase (Finnzyme) and using the primers described earlier. PCR conditions were as follows:- 98˚C for 2 minutes, 35 cycles of 98˚C for 20 seconds and 72˚C for 90 seconds followed by final extension step of 72˚C for 5 minutes. PCR product was run on gel and was band extracted followed by cloning into the TOPO-Blunt vector (Invitrogen) as Phusion Taq polymerase generates blunt end products. Human MTA1 sequence was confirmed by sequencing and was subcloned into pcDNA3 using BamHI and XbaI enzyme sites.

2.2.5 Determination of Immunogenicity of MTA1 peptides

2.2.5.1 Animals

Balb/c and C57bl/6J mice were either bred in house or purchased from Harlan (Oxon, U.K.) and HHD II mice were a generous gift from Dr. F Lemonnier (Institut Pasteur, Paris). All animals were maintained in accordance with the Home Office Codes of Practice for the housing and care of animals.

2.2.5.2 Peptides

Murine MTA1 protein sequence was screened, using a web based algorithm (SYFPEITHI), for peptides potentially binding to H-2Kd and H-Ld molecules (Balb/c) or H-2Db and H-Kb molecules (C57bl/6). Human MTA1 protein sequence was screened for peptides binding to HLA-A2.1 molecules (HHD II) mice. Various peptides (Table 2) were chosen based on their binding score and were synthesized (Alta Biosciences, Birmingham, UK).

Table 2.4:- Peptides selected using SYFPEITHI to test for cytotoxicity

Mouse Strain	Peptide No	Sequence	MHC Motif	Binding Score
Balb/c	**MTA1 622**	KSYPTKVRLI	H2-Kd	27
	MTA1 168	RYQADITDL	H2-Kd & H2-Ld	28 & 22
	MTA1 436	RPGPNRNNM	H2-Ld	23
	MTA1 298	KYGKDFTDI	H2-Kd	26
	MTA1 148	VYDPQQKTL	H2-Kd	25
	MTA1 551	SSSSVLSSL	H2-Kd & H2-Ld	20 & 20
	MTA1 208	QFLVVARSV	H2-Kd	24
C57Bl/6	**MTA1 699**	PAPVNDEPI	H2-Db	29
	MTA1 496	YMPINSAAI	H2-Db	25
	MTA1 12	NSSSNPYLI	H2-Db	24
HHD II	**MTA1 22**	YLIRRIEEL	HLA-A2.1	30
	MTA1 57	ALADKHATL	HLA-A2.1	30
	MTA1 109	FLSRQLESL	HLA-A2.1	27

2.2.5.3 T2 binding assay

T2 cells were harvested, counted and re-suspended at 4 x 10^6 cells per ml and were plated at 1.6 x 10^6 cells/well in 40 µl volume in a 96 well plate. Peptides were added to these cells at 100µg/ml, 10µg/ml and 1µg/ml in a 10µl volume and incubated overnight at 37˚ C. Cell were harvested the next day and stained with primary antibody for HLA-A2 (HB54 hybridoma supernatant), washed and stained with goat anti-mouse secondary. Cells were then washed and analyzed using a Flow Cytometer for the mean fluorescence intensity.

2.2.5.4 Coating of gold particles by DNA

DNA was coated onto 1.0 Micron gold particles (Biorad, Hemel Hempstead, Hertfordshire, UK) using manufacturers' instruction and administered by Helios Gene Gun (Biorad). Breifly, 200 µl of spermidine was added to 16.6 µg of gold followed by sonication. 200 µl of 1M calcium chloride was added to DNA-Spermidine solution followed by incubation at room temperature for 10 minutes. Tubes were spun at 13,000 rpm for 1 min and gold particles re-suspended in dry ethanol. After repeating the above step 2 more times, particles were resuspended in 0025mg/ml of PVP in dry ethanol. During these steps, tubing was dried using nitrogen for 15-20 minutes using nitrogen gas. Re-suspended gold particles were loaded into the dry tubing using a syringe and the tube replaced on the roller/dryer (Biorad). Following incubation for 30 mins, ethanol was gently removed using the syringe and the tube was rotated on the roller along with nitrogen gas

being passed through it for 5 mins. Bullets were then cut using guillotine and stored at 4ºC until used for immunisation.

2.2.5.5 Immunisations

All mice were immunized with 100µg of the CTL peptide along with 140µg of the helper peptide in 1:1 dilution in Incomplete Freunds Adjuvant (IFA) in a total volume of 100µl. The injection was given at the base of the tail. Mice were boosted with the same dose after one week. Helper peptide for the Balb/c mice was ISQ derived from ovalbumin and Hep B helper peptide was used for C57Bl/6 and HHD mice.

In certain experiments, mice were immunised with gold particles coated with human or mouse DNA, using gene gun (Biorad). Three rounds of immunisations were undertaken at one week's interval.

2.2.5.6 LPS Blasts

Spleens were harvested from naïve mice and single cell suspensions prepared. LPS blasts were set up in a T75 flask by culturing 60 x 10^6 spleen cells, in the presence of 1mg LPS and 7µg/ml of Dextran Sulphate. After 48 hours, the cells from the LPS blast flask were harvested, washed, resuspended in 5 ml of T cell media and irradiated. These LPS blasts were washed again and pulsed with the peptide for 1 hour at 37˚ C. After washing these cells were used for *in vitro* re-stimulation of the splenocytes harvested from immunized mice.

2.2.5.7 Harvesting Splenocytes from immunised mice and in-vitro re-stimulation

One week after boosting, spleens were harvested from the immunized mice and single cell suspensions were prepared in sterile conditions. Cells were counted, resuspended and plated in a 24 well plate at 2.5 x 10^6 cells/500µl. 5 x 10^5 /500µl irradiated and peptide pulsed LPS blasts were added to the splenocytes to make a final volume of 1ml in each well of 24 well plate. Supernatants were collected usually on day 3 and 5 for cytokine testing.

2.2.5.8 Cytotoxicity Assay

On day 5 of in-vitro stimulation, splenocytes were harvested, washed twice in serum free media, resuspended in CTL media, counted and used as effector cells. Target cells were harvested by trypsination (only CT-26), washed and labeled with chromium-51. A standard 4 h Cr-release assay was performed and the percentage specific cytotoxicity was determined using the following equation: %specific cytotoxicity = (experimental release-spontaneous release)/(maximum release-spontaneous release) x 100.

2.2.6 Proliferation assays

2.2.6.1 Mice, Peptides and Immunisations

FVB/N-DR1 and C57BL/6-DR4 colonies were bred at The Nottingham Trent University animal house. FVB/N-DR1 animals were received from Dr. Altman and C57BL/6-DR4 mice were purchased from Taconic. FVB/N-DR1 F2 mating positive animals were maintained inbred by ensuring they have a common F0 ancestor. Animals had already been PCR genotyped to verify that they were HLA-DR1 and HLA-DR4 positive.

Peptides were selected using web based algorithms SYFPEITHI and PAPROC.

Two peptides, which were predicted to bind to HLA-DR1 and HLA-DR4 molecules with high affinity were chosen and purchased from Alta Biosciences.

Table 2.5 HLA-DR1/DR4 restricted peptides derived from MTA1 used in proliferation assays

Peptide Region	Sequence	HLA-DR1*0101 binding score	HLA-DR4*0401 binding score
MTA1 497	**R** N P **Y** L **P** I N **S** A A I K A E	30	28
MTA1 550	**P** D P **V** K **S** V S **S** V L S S L T	29	26

Each animal was immunised with 100µg of the peptide, diluted in PBS and emulsified in 1:1 dilution with incomplete Freunds adjuvant (IFA) (Sigma). 100µl of this peptide emulsion was administered at the base of each animal's tail. Two rounds of immunisation with the same peptide were undertaken at seven-day intervals. For investigating the natural processing of peptides, in certain experiments mice were immunised with gold particles coated with human or mouse DNA, using gene gun (Biorad). Three rounds of immunisations were undertaken at one week's interval.

2.2.6.2 BM-DC generation for proliferation assay

BM-DC were generated as described by Inaba and coworkers with modifications (Inaba *et al*, 1992). Briefly, hind limbs of naïve mice were harvested and all muscle was removed using scalpel and tweezers. After cutting the ends of the bone, bone-marrow was flushed and the cells collected, centrifuged, re-suspended in 1ml BM-DC media and plated at 1x 10^6 cells per well/ml with 100ng/ml of mGM-CSF (X63 supernatant). On day 2 and day 4, non-adherent cells were washed out by gently removing 700µl of media from each well, and 750µl of fresh media containing GM-CSF added in its place. On day 7, BM-DC were replated with the 10µg/ml peptide of interest or control peptide for 4-6 hours. LPS was then added at 1µg/ml to induce

complete maturation. The cells were then incubated overnight at 37° C, 5% CO_2. The following day, BM-DC were washed in T cell media, re-suspended in 1ml of T cell media and pulsed with 10µg/ml of peptide for 4-6 hours at 37° C, 5% CO_2. These cells were plated at 5 x 10^3 per well in a round bottom 96 well plate. BM-DC were always used at a 1 DC to 10 splenocyte ratio in proliferation assay.

2.2.6.3 Splenocyte preparation and re-stimulation with peptide *in vitro*.

Spleens of immunised animals were harvested and the cells flushed out with T cell media. Cells were collected and placed on ice while the spleen was being digested. The remaining tissue (spleen) was digested using an enzyme cocktail (0.1U/ml DNAase (Sigma) + 1.6 mg/ml collagenase (Sigma)) for 1 hour at 37° C. Single cell suspension was prepared, counted and cells were plated in 24 well plates at 2.5 x 10^6 and 3.5 x 10^6 cells per well in 1 ml of T cell media for HLA-DR1 and HLA-DR4 mice respectively. 10 µg/ml of the peptide was added to each well. On day 6, cells were used at 5 x 10^4 cells per well as responders for the proliferation assay.

2.2.6.4 Murine CD8+ T cell depletion

Depletions were done on day 6, using CD8 specific dynabeads (Dynabeads Mouse CD8, Dynal) and following the manufacturer instructions. CD8 cells attached to the beads were depleted using a magnet. The remaining cells were collected, washed once with T cell media and subsequently used for the proliferation assay or plated in a 48 well plate (Rojas *et al.,* 2005). Protocol for CD8+ depletion has been optimised in our laboratory and the remaining cells were shown to consist negligible number of CD8+ T cells.

2.2.6.5 Proliferation assay for murine CD4+ T-cells

The cells collected after T cell depletion, were re-suspended in 4 ml of T cell media and counted. These cells were then plated in 96 well round bottom plate at cell density of 5 x 10^4 cells per well. Peptide pulsed syngeneic BM-DC were used as antigen presenting cells in all the experiments. Responder cells were co-cultured with BM-DC either pulsed with the relevant peptide, an irrelevant peptide or no peptide in some experiments. Pulsed BM-DC were added to the wells at a density of 5 x 10^3 cells per well. To ascertain the MHC restriction of the response, MHC blocking antibody (L243 anti-HLA-DR, see appendix) was added to the relevant wells. As control a matched isotype antibody was also used in these experiments. Each culture was performed in triplicates or quadruplicates for approximately 60 hours. Tritiated thymidine (Amersham) was added at a final concentration of 0.037MBq/ml 16 to 18 hours prior harvesting.

Cells were harvested using a 96-well harvester (Packard) onto a 96-well UniFilters GF/C plate (Packard) and the plate was left to dry for 1 hour in a drying cabinet. 40 µl of scintillation fluid (Microscint 0, Packard) was added to each of the filter wells. Filters were counted on a Top-Count scintillation counter (Packard).

2.2.6.6 Second week proliferation and cytokine assessment of murine T cells.

For HLA-DR4 mice, day 6 CD8+ depleted splenocytes were also cultured at 0.5-1 x 10^6/well in 48 well plate for a further 7 days and proliferation was repeated as described in 3.8. 4-6 x 10^5 splenocytes were plated with 4-6 x 10^4 peptide pulsed BM-DC in 48 well plates for assessing cytokine production.

2.2.6.7 CD25+ Depletion and DNA immunisations for *in vivo* experiments

Plasmid DNA vaccination was performed using helium propelled gene gun (Biorad) with 1µm gold particles coated with pcDNA3-mMTA1/hMTA1. Three rounds of immunisations were undertaken at a week's interval. Where indicated, mice were immunised with 100-150µg/kg of cyclophosphamide (CPM) four days before the vaccination. Confirmation of CD25+ depletion was done by positively selecting CD4+ T cells, using CD4+ T cell isolation kit (Miltenyi), and staining for CD25+ cells (Serotec).

2.2.7 Generation and optimisation of Semliki Forest Virus (SFV) particles

2.2.7.1 Sub-cloning of mMTA1 in pSMART2b vector

All plasmids for the Semliki Forest Virus generation were kindly provided by Dr. Rod Bremner (University of Toronto and Toronto Western Research Institute). Following the cloning of mMTA1 in TOPO-Blunt, to sub-clone it into pSMART2b vector, TOPO-Blunt /mMTA1 was double digested with BamHI and NsiI. The digested products were run on the gel and the band corresponding to mMTA1 (2.1Kb) was excised and the DNA extracted and ligated in pSMART2b vector after digesting the vector with the same enzymes. Insert was confirmed by sequencing as well as by transfecting RENCA cell line with pSMART2b/mMTA1 followed by RT-PCR.

2.2.7.2 Generation of virus particles

Generation of SFV virus particles was optimized for SFV-βgal. For production of infective viral particles, pSCAβ and pSCAhelper plasmids were co-transfected in 293 cells at different molar ratios. Different methods of transfection were investigated (Electroporation, Calcium phosphate

and Lipofectamine). Calcium phosphate transfection was observed to be the most efficient method of transfection and combined with 1:3 molar ratio of pSCAβ: pSCAhelper gave the highest titer of virus and this combination was used in most of the experiments.

Calcium phosphate transfection was performed using the ProFection® Mammalian Transfection System—Calcium Phosphate (Promega) and following the manufacturer's instructions. Briefly, 293 cells were plated at 5-8x10^5 cells/well/3 ml of media in a 6 well plate, a day before transfection. On the day of transfection, cells were washed once and media was replaced 2-3 hrs before transfection. DNA and HBS solutions were prepared in two separate sterile 1.5 ml eppendorfs. In one tube, 5-12 μg of DNA was diluted in water and 37 μl of 2M $CaCl_2$ to make the final volume of 300 μl. This DNA mixture was added to 2xHBS solution in another sterile tube with constant vortexing. The DNA-HBS-$CaCl_2$ mix was incubated for 30 minutes and added to the cells drop wise. After incubating the plate for 16-18 hours at 37ºC and 5% CO_2, media was removed from the transfected cells, cells washed once with PBS and fresh media added to the cells. After further 48 hour incubation, mediated containing the lysed cells and virus was harvested following a freeze thaw cycle, spun at 2000 rpm for 10 minutes at 4ºC. After centrifugation, the supernatant containing the virus was transferred into a fresh tube and stored at -20ºC until use. Viral particles generated in the way were inactive and prior to use, they were activated by adding 1/20^{th} of total volume of 10mg/ml of α-chymotrypsin to cleave the p62 glycoprotein into E2 and E3 proteins. After 45 minute incubation at room temperature, 10mg/ml solution of aprotinin was added to 1/15^{th} of total volume to stop the protease activity.

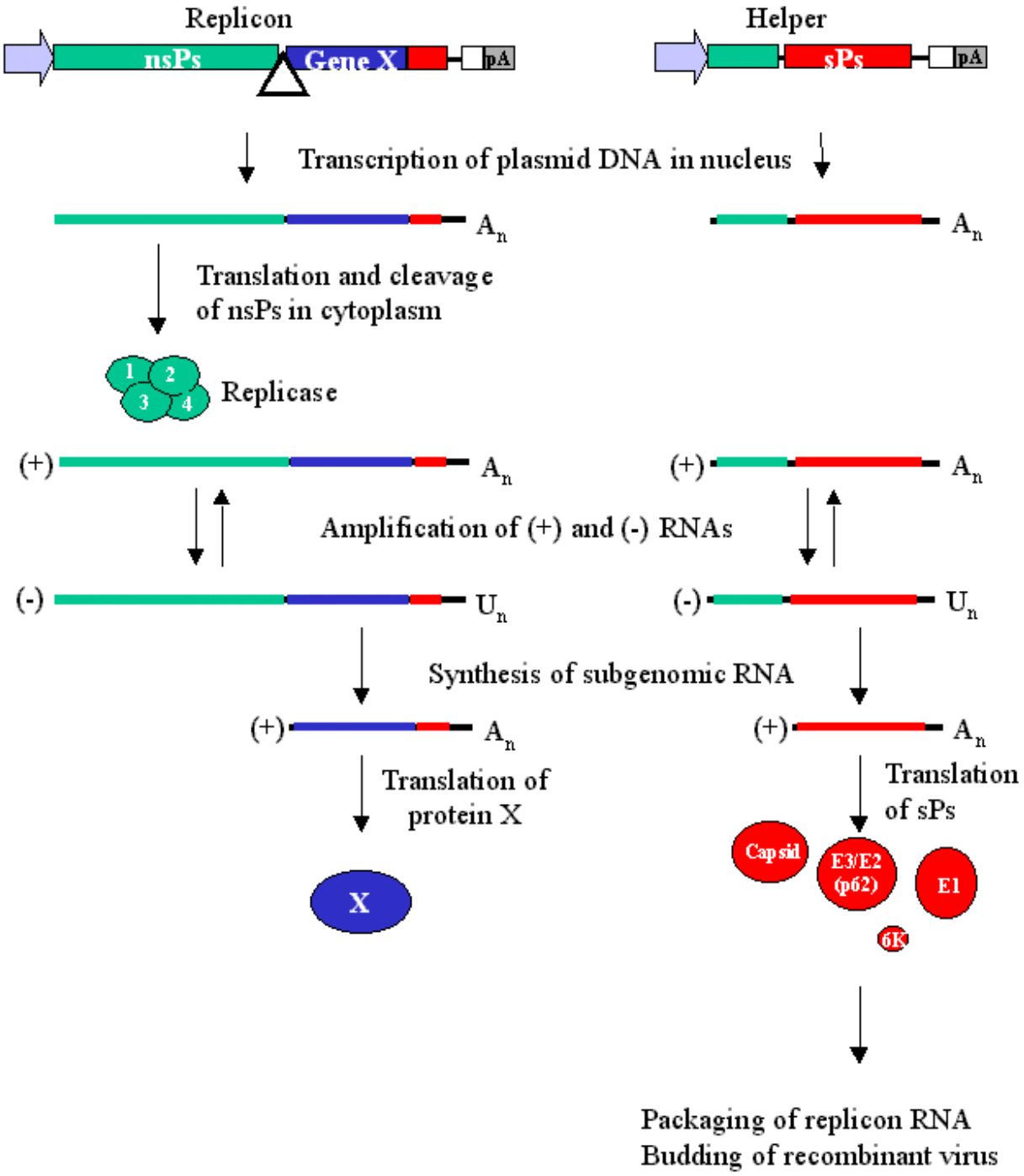


Figure 2.1 Schematic Representation of SFV expression vector generation

2.2.7.3 Titration of Virus: - To calculate the titer of the virus, BHK-21 cells were infected with different volumes of activated virus for 45 minutes at 37ºC. After incubation, cells were washed once with PBS and incubated for 18-24 hrs after addition of fresh media. To visualise the infected cells by SFV-βgal, X-gal assay was performed on them. Briefly, cells were washed twice with PBS and fixed with glutaraldehyde for 15 minutes at 37ºC. Cells were washed with PBS twice after fixing followed by addition of 1 ml of Xgal solution per well. Colour was

allowed to develop for 2-3 hours and blue cells counted. Titer of virus was calculated taking into consideration the number of infected and uninfected cells as well as the surface area of a well in 6 well plates and finally adjusting for the virus dilution used for cell infection.

For titration of SFV/mMTA1 virus, after infection of BHK-21 cells as above, infected cells were visualised by immunohistochemistry. 18-24 hours post-infection, cells were fixed with -20ºC methanol for 1 min followed by addition of mouse anti-FLAG primary antibody (Sigma, UK). Following incubation at room temperature for 1 hour, cells were washed twice and secondary antibody (Goat anti-mouse HRP, DAKO) was added. After further 30 minutes incubation at room temperature, cells were washed and stained with DAB solution (DAKO). Colour was allowed to develop and infected cells (dark brown stained) were counted to determine the titer.

2.2.7.4 Direct Immuno-fluorescence assay

24 hours prior to transfection, $1x10^5$ BHK cells/well were plated in 8 chamber slides. Cells were transfected with pSMART2b-mMTA1 using Lipofectamine and media replaced after 6 hours. 24-36 hour after transfection, media was removed and cells fixed with -20ºC methanol for 1 min at room temperature. Anti-FLAG-FITC antibody was added at 1:1000 dilution in PBS for 1 hour at room temp, followed by washing with PBS 3 times. Slide was dried at 37ºC and mounted with fluorescent mounting liquid and images were acquired using a confocal microscope (Leica).

2.2.7.5 BM-DC infection with SFV-Bgal

BM-DC were generated as discussed before. On day 7, DC were harvested and replated at $5x10^5$ cells/500µl/well in a 24 well plate. SFV-Bgal virus were activated and $1x10^5$ viral particles were added per well. After one hour, 500µl of BM-DC media was added and cells incubated for 18-24 hours before harvesting them. Cells were cyto-spun onto a slide and stained using X-gal assay.

2.2.7.6 FACS for BM-DC phenotype analysis

DC were harvested for FACS analysis. 2-5 x 10^5 of cells were used per tube. Cells were washed twice in PBS+0.1% BSA+0.02% NaN_3 and incubated on ice for 30 minutes with primary antibody. Rat anti-mouse CD80, Macrophage/Monocyte marker (F4/80), DEC205, I-A (murine class II) and CD40, mouse anti-human HLA-DR and hamster anti-mouse CD11c monoclonal antibodies were used in these experiments (see appendix for details). Appropriate isotype controls were used in each experiment. Following incubation with the primary antibody, cells

were washed twice in PBS BSA and incubated for 30 minutes on ice with FITC coupled goat anti-rat IgG, goat anti-mouse IgG or goat anti-hamster IgG as secondary antibodies as appropriate. Finally the cells were washed in PBS+0.1% BSA+0.02% and resuspended in 300µl of sheath fluid and FACS analysis was performed.

2.2.7.7 Viral immunisation and *in vivo* challenge experiments

Mice were immunised twice with $1x10^6$ SFV virus particles at 7 day interval as previously described. After 1 week of last immunization, mice were challenged with $8x10^4$ CT26 cells for Balb/c or $4x10^5$ EL4-HHD cells (HHD II) mice. Animals were monitored twice a week for tumour development and size and were sacrificed when the tumour reached a size of $100mm^2$ according to the Home Office guidelines.

2.2.7.8 Statistical Analysis

Data are expressed as mean +/-SD and represent one of at least 3 separate experiments, unless stated otherwise. For proliferation and cytotoxicity assays, statistical analysis was performed using Student's T test (unpaired). For RT-Q-PCR and in vivo tumour progression assays, analysis was performed using Prism 3.03 software (*=$p<0.05$, **=$p<0.01$, ***=$p<0.005$).

Chapter 3:- VALIDATION OF MTA1 AS A POTENTIAL ANTIGEN FOR I MMUNOTHERAPY IN A MURINE MODEL

3.1 Introduction

Vaccines have been used for several infectious diseases and are responsible for the eradication of small pox and decreasing the incidences of diseases such as polio, mumps and rubella. Traditional vaccines for infectious diseases consist of live attenuated pathogens, and due to their success early cancer vaccines used whole tumour cell vaccines, which in mouse tumour models proved to be effective in preventing tumour, however they were not potent enough for tumour therapy. Since the identification of the first human tumour antigen (MAGE1) in 1991, considerable efforts have been made to search for novel tumour antigens, leading to identification of hundreds of potential targets for therapy. Antigen specific vaccination targeting MAGE, tyrosinase, carcino-embryonic antigen (CEA) and NY-ESO, amongst others have proved very successful in prevention and therapy of cancers in animal models (Ye *et al*., 2004; Muders *et al*., 2003). However, significant therapeutic benefits have eluded immunologists in human clinical trials. One of the reasons for their failure may have been due to the choice of antigens targeted.

Cancers with viral aetiology have been targeted with vaccines against known viral antigens and these are proving to be successful in clinical trials (Harper *et al*, 2006). Viral antigens are highly immunogenic and the immune system has no previous experience, thereby eliminating the problem of tolerance. Moreover, highly restricted expression of these antigens in virus infected, normal or cancer cells precludes autoimmunity. However, most human cancers are the result of environmental and genetic factors rather than viral. Although tumour specific unique non-viral antigens are widely considered as good targets, their identification in each individual patient can be time-consuming and expensive, thereby not feasible for wider application. Thus, shared tumour antigens seem to be the most practical therapeutic targets for cancer immunotherapy as they are widely expressed in a variety of tumours. Ideally tumour antigens should not be expressed in normal cells. However, cancer cells arise from their normal counterparts and therefore share similar antigens. Several studies have demonstrated that as long as tumour antigens are not highly expressed in normal tissues, cancer vaccines generated auto-immune response is negligible or highly restricted (Schreiber *et al*., 1999; Trefzer *et al*., 2000). Some of the commonly targeted ubiquitously expressed antigens are MUC-1, p53 and HER-2/neu. MUC-1 has been shown to be over-expressed in breast and pancreatic cancers, in addition to epithelial

cells, fibroblasts and B-cells. CTL recognising MUC-1 peptides have been generated and shown to specifically recognise breast cancer cells (Grosso *et al*, 2004). Similarly several studies have demonstrated CTL mediated lysis of p53 over-expressing tumour cells but not cells expressing normal levels of p53 (Murakami *et al*, 2004). The Her-2/neu proto-oncogene encodes a tyrosine kinase, widely over-expressed in breast and ovarian cancers and recognised by the immune system. Several target peptides have been identified from Her-2/neu, but more importantly, one study showed a commonly shared epitope between breast, colon, lung and renal cancers (Scardino *et al*, 2001). This underlines the utility of targeting a shared antigen. Another important characteristic of tumour antigen has to be its role in oncogenic transformation of cells. Antigen specific targeting of non-essential tumour antigens would eventually lead to down-regulation of such antigens allowing cancer cells to escape (Yee *et al*., 2002). Hence, a rational vaccine strategy should target antigens involved in the maintenance of the cancer phenotype and having a non-redundant role in oncogenesis.

Although technical advances in surgery and medicine have enabled us to surgically remove most primary tumours and treat residual disease, mortality rates due to cancers remains largely unaffected due to inability to target metastatic cells. Immunotherapeutic intervention to target metastatic cells seems to be an appropriate strategy, especially in combination with other therapies. This has led to a surge in interest for identification of metastatic related genes in cancer cells. Metastasis is a complex series of events starting from detachment of neoplastic cells from primary tumour, invasion of surrounding tissues and penetration of blood and lymphatic vessels, adhesion to endothelial cells of distant organs, extravasation, colonisation and angiogenesis. During these events, tumours cells also have to evade the immune system. Several genes are involved in controlling these events and their identification can not only provide a clue to this complex process, but also provide targets for therapy. Search for these genes have led to identification of several potential candidates in breast cancer such as mts-1, nm23, WDNM-2 and stromelysin-3; which are potentially involved in metastases of breast cancer (Ebralidze *et al*., 1989; Steeg *et al*, 1998; Dear *et al*, 1989; Basset *et al*, 1990).

Amongst several genes involved in metastasis, MTA1 was first identified by Toh *et al* (1994), using rat mammary adenocarcinoma metastatic system. Using derivatives of a single 13762NF tumour cell line with different metastatic potential, differential hybridisation screening identified 10 genes, one of which was MTA1 (Toh *et al*., 1994). They also identified the human and mouse homologues of rat MTA1 gene and demonstrated human MTA1 expression in several human

breast cancer cells. Furthermore, MTA1 expression correlated with the metastatic potential of the human breast cancer cell lines; ratio of MTA1 expression in non-metastatic to invasive to metastatic cell lines was 1:2:4 (Toh *et al*., 1995). Rat MTA1 gene expression was also observed in several normal rat organs, indicating its essential function in normal organs as well as cancer cells. Contrary to its low level expression in normal tissues, MTA1 is highly expressed in testis. Spermatogenesis is a highly complex yet controlled process of proliferation, mitosis, meiosis and differentiation, and is likely to be controlled by a number of genes. Furthermore, spermatozoa are highly motile, contain specific proteolytic enzymes and adhesion molecules; properties which are generally associated with metastatic cells (Alberts *et al*, 1989). Recent study showed a direct interaction of MTA1 and endophilin 3 in the cytoplasm by using a yeast two-hybrid system, suggesting that MTA1 might also be involved in the regulation of endocytosis mediated by endophilin 3 (Aramaki *et al*., 2005).

In 1998, MTA1 was shown to be a part of the novel Nucleosome Remodelling and Histone Deacetylase complex (HDAC) (Xue *et al*, 1998). In eukaryotes, genes are packed in chromatin and nucleosomes are the repeating units of chromatin. Nucleosomes consist of 146 base pairs of DNA wrapped around the core histone octamer, which are highly conserved proteins throughout evolution. This formation of genes makes them inaccessible to transcription factors. Acetylation of histone changes its conformation and makes DNA more accessible for transcription. This process of histone acetylation and deacetylation is controlled by multi-subunit chromatin remodelling complexes, histone acetyltransferases (HAT) and histone deacetylases (HDAC) (Marks *et al*, 2001). HDAC are primarily involved in making chromatin compact by removal of charge-neutralising acetyl groups. Transcription repression has been associated with cellular transformation in a number of studies. It has been proposed that HDAC may repress transcription of number of genes involved in cell-cycle arrest, differentiation and apoptotic cell death (Marks *et al*., 2001). Indeed, a number of compounds with HDAC inhibitory activity have shown great promise in inhibition of tumour growth *in vivo* and *in vitro* (Marks PA *et al*, 2000). Several of these compounds are already in clinical trials, suggesting the importance of targeting these complexes. Moreover, HDAC are also known to interact with transcription factors. Being a sub-unit of HDAC, suggested that MTA1 has a role in transcription repression and gene regulation, and seems to have an essential role in transformation. MTA1 contains zinc finger and leucine zipper domains, generally observed in transcription factors, indicating that it can also function as a transcription factor. Thus, MTA1 is likely to have an essential role in the transformation process and could prove a valuable asset to target metastatic cells. Other members of the MTA1

family have since been identified, three members (MTA1, MTA2, MTA3) and six isoforms (MTA1, MTA2, MTA3, MTA1s, MTA1-ZG29p, MTA3L). MTA2 and MTA3 are also sub-units of the NuRD complex, whereas MTA1s was shown to interact with oestrogen receptor α in the cytoplasm, sequestering it and making breast cancer cells unresponsive to hormone therapy (Kumar *et al*., 2002).

Just being essential for transformation does not make MTA1 a target for immunotherapy and it needs to be carefully evaluated as a potential target. Other key parameters to be examined regarding MTA1 are, its expression level in various cancers and at different stages of cancer, the effect of its inhibition and over-expression in cancer and normal cells, respectively, and its immunogenicity. The initial identification of MTA1 in breast cancer led researchers to suggest its role in breast cancer progression. Indeed, a recent study showed that transgenic mice with increased MTA1 levels had extensive branching of the mammary glands and increased proliferation of the ductal and alveolar epithelial cells (Bagheri-Yarmand *et al*., 2004). Interestingly, 30% of these mice developed focal hyperplastic nodules and approximately 7% exhibited mammary tumours in 18 months. Studies have also revealed MTA1 over-expression in a number of different cancers such as gastrointestinal, ovarian, oesophageal, prostate and lung (Tang *et al*, 2003; Sasaki *et al*, 2002; Toh *et al*, 1999; Nicolson *et al*, 2003). In most of these studies, MTA1 over-expression was correlated with tumour progression, invasion and metastasis. To validate MTA1 as a potential target for immunotherapy, this aspect of the study was designed to investigate its expression in various human tumours compared with patient matched normal tissues. MTA1 mRNA levels were determined in gastric, colon, breast and prostate cancers. Furthermore, being 94% identical at protein level to the mouse MTA1, we validated a mouse model for investigating human MTA1 as an immunotherapeutic target. Expression of MTA1 was therefore determined in various murine tumour cell lines and normal mouse tissues using conventional RT-PCR. These results were confirmed and expression levels quantified using real time PCR. Rarely, mRNA levels of a gene do not correlate with its protein level and hence we compared MTA1 protein expression in murine tumour cell lines and normal tissues by western blotting. A recent study suggested MTA1 to be predominantly localised to the nucleus along with low levels in cytoplasm, contradicting previously published studies where MTA1 was found to be only localised in the nucleus. This also led us to investigate murine MTA1 localisation in cancer cells.

3.2 Results

3.2.1 Expression of human MTA1 in human tumour tissues by RT-Q-PCR

Previously published studies have showed over-expression of MTA1 in human gastric, colon, breast and prostate cancers, amongst others. To confirm these studies and for validation of MTA1 as a target for immunotherapy, MTA1 expression was investigated in colon, breast, gastric and prostate cancer samples and patient-matched normal tissues, using Real time quantitative PCR (RT-Q-PCR). Briefly, 2μg of RNA was reverse transcribed to generate cDNA using random primers from the samples. A maximum quantity was used for samples with very low concentration of RNA. RT-Q-PCR was performed for 40-45 cycles and the relative quantity of MTA1 was estimated by dividing the starting quantity of MTA1 by that of house keeping genes. It is noteworthy that two different housekeeping genes were estimated, GAPDH and ribosomal 18SRNA as suggested by several previously published studies (Bustin, 2000). Wide variations were observed in GAPDH values whereas 18SRNA values were the most consistent and were therefore used for all future analyses. Indeed, it has been documented that GAPDH mRNA levels are not constant as previously believed and should not be used for normalising PCR data as it can be widely affected by factors such as hypoxia, oxidative stress, pregnancy, during cell cycle and might even be up-regulated in cancers (Bustin, 2000). However, GAPDH is still widely used as a housekeeping gene, especially for conventional PCR.

As seen in figure 3.1A, the relative expression of MTA1 in colon carcinoma samples is higher than the normal tissues, although the difference does not reach statistical significance. This might be due to the low number of samples available (n=10) and the fact that some cancer samples did not show an increase in the MTA1 expression. Indeed, individual MTA1 expression levels in each samples showed a clear increase compared to the normal tissues, with 6 out of 9 samples having an increased MTA1 levels, out of which 2 (Co21 and Co22) show a dramatic increase (>10 times) (fig 3.1B). Interestingly, patient Co22 also had lymph node involvement and previous studies in different cancers have related MTA1 levels to lymph node involvement and metastasis. Moreover, all three patients with lymph node involvement showed higher MTA1 levels in their primary tumours. It is noteworthy that none of the patients had distant metastases, which are likely to have the highest MTA1 expression.

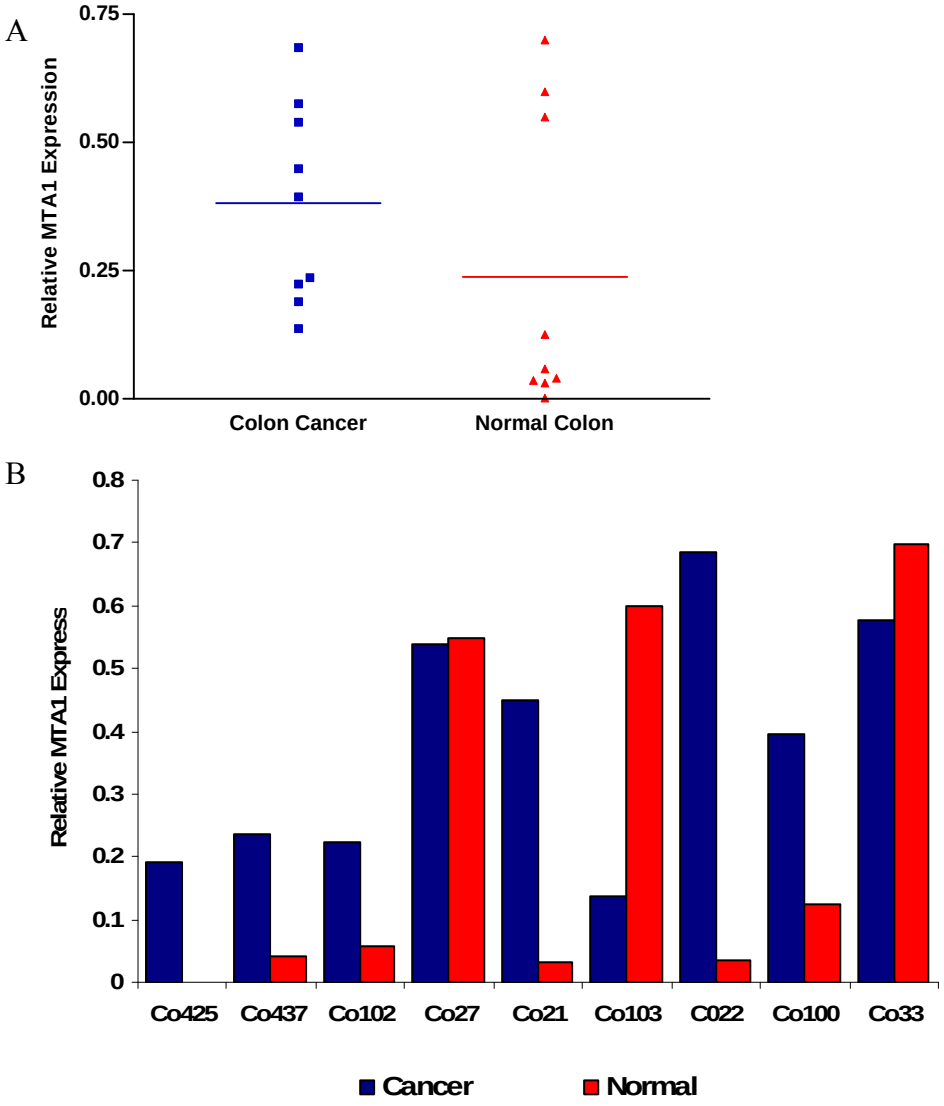


Figure 3.1:- Expression of MTA1 in colon carcinoma and patient matched normal colon tissue *(A)Graph analysing nine tumour and normal tissues from patients (B) Individual patient variation of MTA1 expression*

Table 3.1 Colon Carcinoma sample information and their relative MTA1 expression

Sample Code	TNM Stage	Differentiation	Localisation	Relative MTA1 Expression
Co425T	1.0.0	Moderate	Rectum/Sigmoid	0.19
Co425N				0.00
Co437T	3.1.0	Low	Sigmoid	0.24
Co437N				0.04
Co102T	3.0.0	Moderate	Rectum	0.22
Co102N				0.06
Co27T	4.0.0	Moderate	Sigmoid	0.54
Co27N				0.55
Co21T	3.1.0	Moderate	Ascendens	0.45
Co21N				0.03
Co103T	3.0.0	Moderate	Rectum/Sigmoid	0.14
Co103N				0.60
Co30T	3.1.0	Moderate	Caecum	-
Co30N				0.09
C022T	3.0.0	Moderate	Rectum	0.68
Co22N				0.03
Co100T	3.1.0	Moderate	Rectum/Sigmoid	0.39
Co100N				0.12
Co33T	3.0.0	Well	Sigmoid	0.57
Co33N				0.69

Similar to the results obtained with colon cancer samples, over-expression of MTA1 was also observed in gastric cancer samples. Relative expression of MTA1 in gastric cancer samples were significantly higher than the normal tissues ($p=0.0074$) (fig3.2A). Moreover, individual variation of MTA1 expression in cancer showed dramatic increase of MTA1 levels in sample Ga418 (>10 times) (fig3.2B). Overall, a significant increase was observed in 5 out of 10 samples. However, MTA1 expression level could not be correlated with the lymph node involvement or tumour progression in this study.

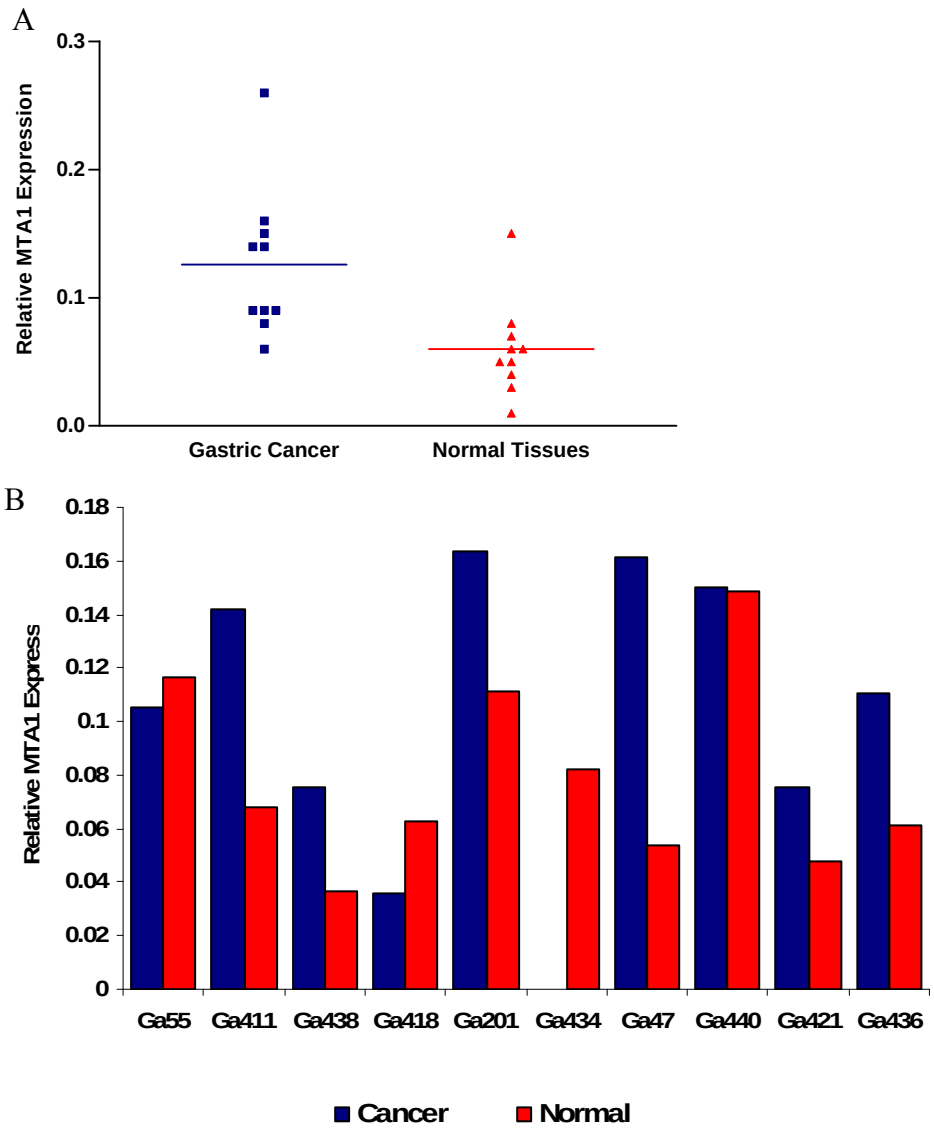


Figure 3.2:- Expression of MTA1 in gastric carcinoma and patient matched normal gastric tissue*(A) Graph analysing nine tumour and normal tissues from patients (B) Individual patient variation of MTA1 expression*

Table 3.2 Gastric Carcinoma sample information and their relative MTA1 expression

Sample Code	TNM Stage	Differentiation	Lauren's Classification	Relative MTA1 Expression
Ga55T	4.1.0	GIII	Diffuse	0.08
Ga55N				0.06
Ga411T	3.0.0	GIV	Intestinal	0.26
Ga411N				0.06
Ga438T	2.0.0	GII	Intestinal	0.09
Ga438N				0.07
Ga418T	3.0.0	GIII	Diffuse	0.14
Ga418N				0.01
Ga201T	NA	Low	NA	0.09
Ga201N				0.04
Ga434T	2.0.0	GII	Intestinal	0.15
Ga434N				0.15
Ga47T	NA	GIII	Intestinal	0.14
Ga47N				0.05
Ga440T	3.1.0	GIII	Diffuse	0.16
Ga440N				0.03
Ga421T	3.0.0	GIII	Diffuse	0.08
Ga421N				0.06
Ga436T	2.0.0	GI	Intestinal	0.26
Ga436N				0.06

To verify previously published studies of MTA1 over-expression in breast cancer samples, MTA1 expression was compared in breast cancer as well as the normal breast tissues. As expected, MTA1 expression was increased in breast cancer samples as compared to normal breast tissues, however, this did not reach the level of statistical significance. One of the reasons for that might be that several normal tissue samples had very low and/or poor quality RNA and could not be amplified for either the house keeping gene or MTA1 and had to be excluded from final analyses. Moreover, increased numbers of samples might be required to conclusively suggest MTA1 over-expression in breast cancer tissues. Individual patient variation could only be compared in four samples where two of them had significant increase in MTA1 levels in cancer tissue compared to normal tissue. Patient Br12 had more than 10 times increase in MTA1 levels and it correlated with her tumour size, although information regarding the node involvement or metastasis was not available for this patient. Amongst the samples investigated, patient Br11 had the highest degree of node involvement but this could not be correlated with MTA1 expression.

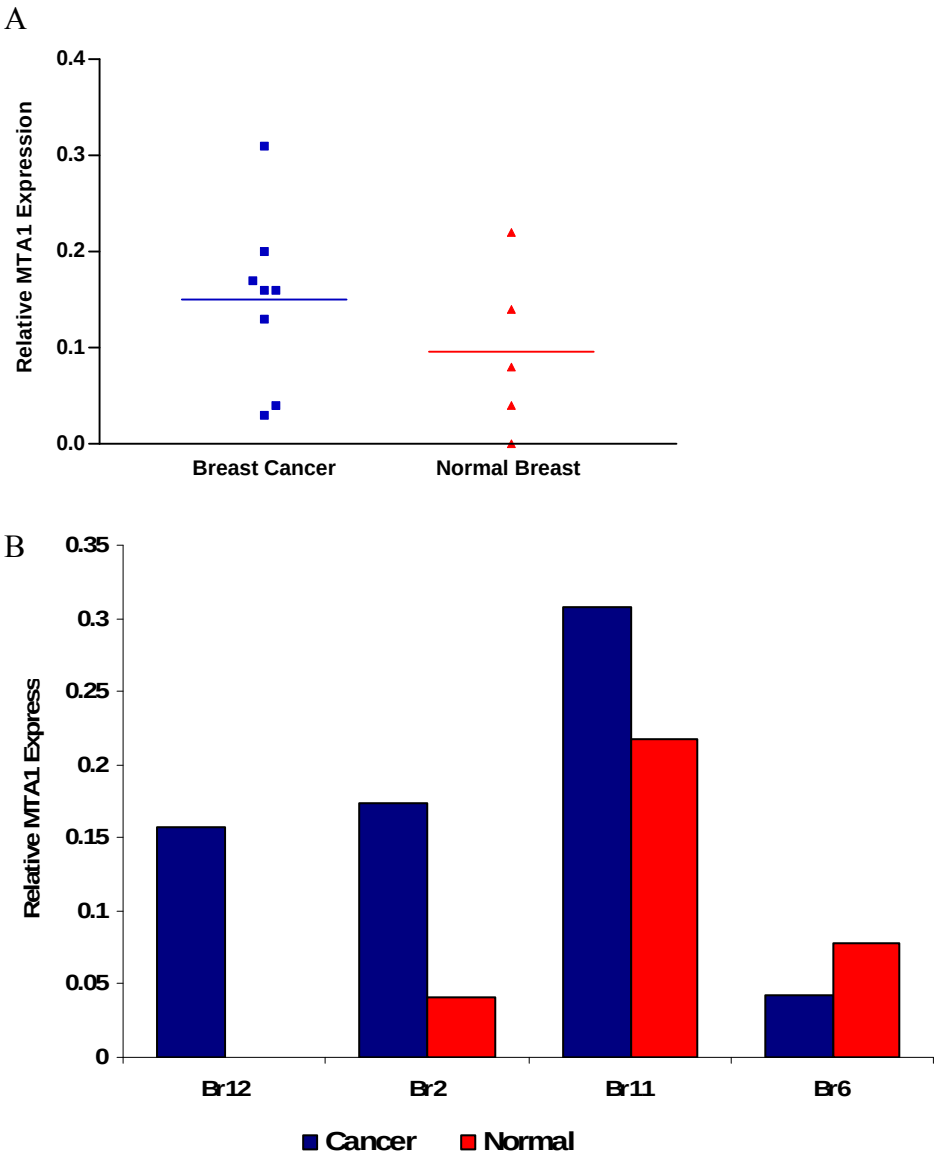


Figure 3.3:- Expression of MTA1 in breast carcinoma and patient matched normal breast tissue*(A) Graph analysing nine tumour and normal tissues from patients (B) Individual patient variation of MTA1 expression*

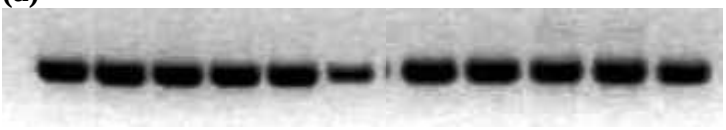
In order to validate a mouse model for MTA1 immunotherapy, we investigated the expression of mouse MTA1 in normal mouse tissues and tumour cell lines. Several normal tissues (brain, kidney, lung, heart, testis, liver, thymus and spleen) were obtained from naïve mouse and immediately snap frozen in liquid nitrogen. Frozen tissues were crushed using mortar and pestle and mRNA was isolated from them using RNA-STAT60 (AMS Biotechnology, UK) and reverse transcribed using random primers. Expression of MTA1 was initially determined using conventional RT-PCR. All the samples contained non-degraded RNA as shown by normal expression of the house keeping gene mGAPDH (fig3.4a). MTA1 was found to be over-expressed in all the tumour cell lines compared to the normal tissues except testis (fig 3.5).

Table 3.3 Breast Carcinoma information and their relative MTA1 express

Sample Code	TNM Stage	Differentiation	Histology	Relative MTA1 Expression
Br1T	1.0.0	Moderate	Infiltrative	-
Br1N				-
Br2T	3.1.0	Low	Infiltrative signet	0.17
Br2N				0.04
Br6T	2.1.0	Moderate	Infiltrative	0.04
Br6N				0.08
Br7T	2.1.0	Moderate	Infiltrative	0.13
Br7N				-
Br8T	3.1.0	Moderate	Infiltrative, medul	0.16
Br8N				
Br9T	-	Fibroma	NA	0.03
Br9N				-
Br10T	1.0.0	Moderate	Infiltrative	0.2
Br10N				-
Br11T	2.3.0	Moderate	Infiltrative	0.31
Br11N				0.22
Br12T	4.-.-	NA	Infiltrative	0.16
Br12N				0.00
Br13T	1.0.0	Moderate	Infiltrative	-
Br13N				0.14

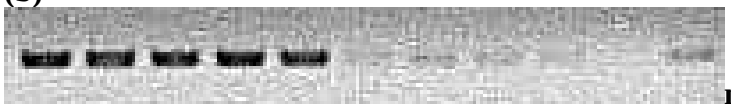
3.2.2 Expression of murine MTA1 in mouse cancer cell lines and normal tissues

(a)



mGAPDH

(b)



mMTA1

Figure 3.4 Agarose gel electrophoresis of mGAPDH and mMTA1 following RT- PCR *Lanes:-1, CT26; 2, A20; 3, RENCA; 4, CMT 93; 5, B16; 6, Brain; 7, Liver; 8, Lung; 9, Muscle; 10, Spleen; 11, Kidney*
Following completion of RT-PCR, 20 µl of the final products were run on the 1.5% agarose gel. (a) Primers for mGAPDH were designed to amplify a 300 bp product. Bands were seen at the expected size, confirming that the band is mGAPDH. GAPDH PCR was performed for 24 cycles.
(b) Primers for mMTA1 were designed to amplify a 508 bp product, which is the size at which the bands were seen confirming the expression of mMTA1. mMTA1 PCR was performed for 30 cycles. It can be clearly seen that the mMTA1 is highly expressed in the tumour cell lines compared to normal tissues.

Although genomic DNA contamination could not be ruled out in the samples, the primers used were located on different exons and hence MTA1 amplification from genomic DNA would have generated a much larger band size. The level of MTA1 expression in the testis was similar to the tumour tissues. In comparison, all the normal tissues expressed MTA1 at very low levels. It is

noteworthy, that mMTA1 transcripts were also detected in muscle tissues, which contradicts previously published results, where no MTA1 expression was detected (Simpson *et al*, 2001). However, in those experiments, MTA1 expression was determined by *in-situ* hybridization, which lacks the sensitivity of RT-PCR.

Expression of MTA1 was not limited to a particular strain of mice as tumour cells of different backgrounds expressed high levels of MTA1. Higher Expression of MTA1 in tumour cells and testis (data not shown) suggests that it might be important for the proliferation of cells.

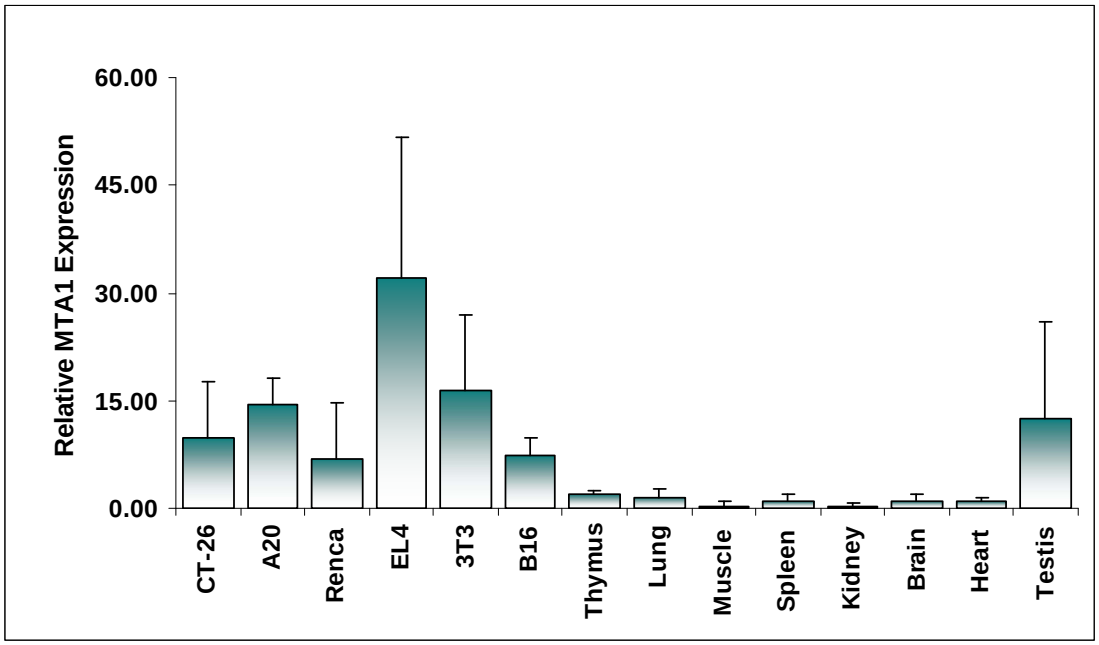


Figure 3.5:- Real time PCR analysis of mouse MTA1 in mouse tumour cell lines and normal tissues (n=3)

To confirm the data obtained from conventional PCR, murine MTA1 expression was determined and compared in tumour cell lines and normal mouse tissues using quantitative real time PCR. As for the human tumour tissues, mMTA1 was amplified for 40-45 cycles and its relative expression was determined by dividing the standard quantity of mMTA1 by that of house keeping gene 18S ribosomal RNA. As seen from figure 3.5, RT-Q-PCR confirmed the conventional PCR results with high relative expression of MTA1 in all cell lines tested compared to all the normal tissues, except testis. Importantly, low levels of MTA1 could be detected in the thymus by RT-Q-PCR suggesting its potential expression in adult thymus, which could lead to central tolerance and deletion of MTA1 specific T cells.

3.2.3 Expression of MTA1 protein in mouse cancer cell lines and normal tissues

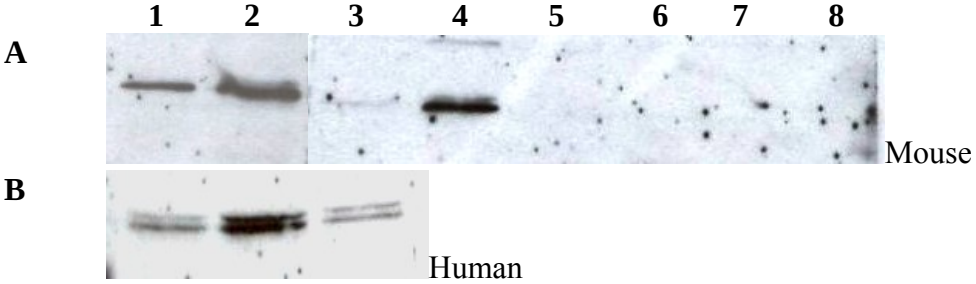


Figure 3.6 Western Blot of murine tumour cell lines, normal tissues and human tumour cell lines.
30µg of protein was loaded in each lane and western blot performed by probing with anti human MTA1 antibody cross reactive with murine mta1.
A:- from 1-8, CT26, A20, RENCA, EL4-HHD, Liver, Lung, Brain, Kidney
B:- from 1-3, HaCaT, K562, MDA-MB-435.

Although murine MTA1 transcripts were detected at high levels in all the cell lines tested, it can be argued that the level of actual protein might be affected by post translational modifications and that RNA levels do not necessarily reflect levels of protein in the cell (Gygi *et al*., 1999). Indeed, MTA1 contains several phosphorylation sites which could influence its protein expression and function (Toh *et al*., 1997). Hence, to confirm the protein expression of MTA1 in cell lines and tissues, protein lysates were prepared from various cell lines as well as tissues by homogenisation. Protein concentration was determined in all the samples and western blotting was performed with equal quantities of proteins (30µg) and blots probed with MTA1 antibody. As seen from figure 3.6, an approximately 80KDa size immunoreactive band corresponding to MTA1 was observed in most of the samples. High level of MTA1 protein expression was observed in CT26, A20 and EL4-HHD cell lines, whereas, RENCA cells expressed the lowest levels of MTA1 protein. Several groups have previously reported MTA1 expression at lower level in normal tissues (Nicolson *et al*, 2003, Simpson *et al*, 2001). Interestingly, although MTA1 expression was detected at lower levels in tissues by RT-PCR, western blot was unable to detect protein expression in normal tissue lysates. This might be due to protein expression in tissues being lower than the detection threshold of the technique rather than non expression of MTA1. Furthermore, variable expression of MTA1 was also observed in the human tumour cell lines tested, with HaCaT and MDA-MB-435 cells expressing it at lower levels, and higher levels being expressed in CML cell line K562. Interestingly, in the human cell lines, two distinct bands could be seen, which could either be other MTA family members or a splice variant of MTA1. However, this needs to be further investigated.

Thus, MTA1 protein is preferentially expressed at higher levels in tumour cells compared to normal tissues. This suggests that MTA1 could be exploited as a target for immunotherapy

where the immune response would be predominantly directed against tumour cells with less likelihood of serious autoimmune reactions.

3.2.4 Localisation of mouse MTA1

Most studies have suggested that MTA1 is localised in the nucleus. However, two recent studies have contradicted this and showed MTA1 to have some limited cytoplasmic localisation as well (Yaguchi *et al*., 2005; Aramaki *et al*., 2005). To investigate the localisation of MTA1, murine MTA1 was cloned into the pSMART2b vector (see chapter 4). BHK cells were transfected with pSMART2b/mMTA1 vector using Lipofectamine and MTA1 localisation observed by immunofluorescence after staining for the FLAG tag. Mouse MTA1 was observed to be localised completely in the nucleus as seen in figure 3.7.

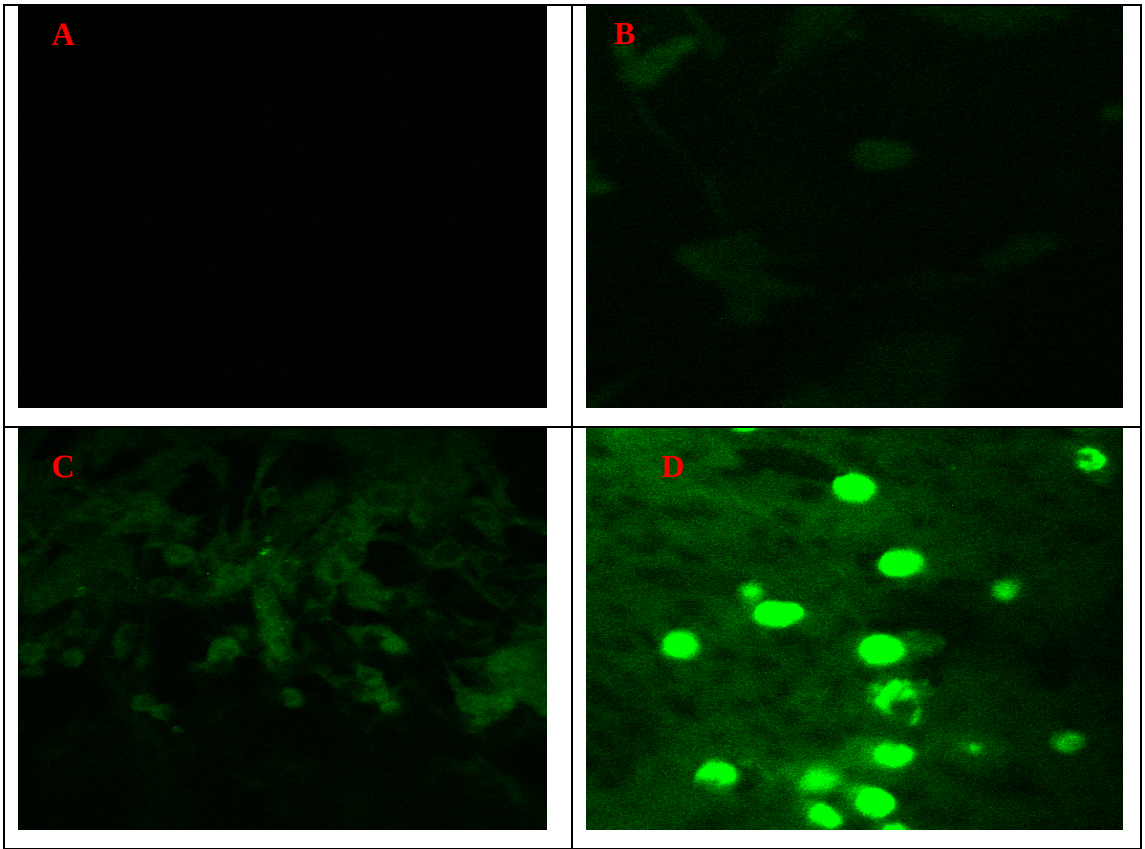


Figure 3.7 Immunofluorescence assay for localisation of MTA1 in BHK cells after transfection with pSMART2b/mMTA1 *A- no antibody, B-Isotype control antibody, C-Non transfected BHK and FLAG antibody, D-Transfected and FLAG antibody*

3.3 Discussion

Metastasis involves a complex series of events which are controlled by multiple genes and some of these genes are likely to be good targets for therapy. In view of the overwhelming failure of chemotherapy and radiotherapy regimes to control late stage metastatic disease, it becomes all the more vital to devise strategies targeting metastatic cells. MTA1 is one of the several antigens proposed to play a role in metastatic process and is a likely candidate for immunotherapy.

Studies in the past few years have showed MTA1 to be over-expressed in various human tumours such as pancreatic, prostate, lung, oesophageal, gastrointestinal and breast cancers (Hofer *et al*., 2004a; Hofer *et al*., 2004b; Sasaki *et al*, 2002; Toh *et al*, 1999; Toh *et al*., 1997; Toh *et al*., 1995). In order to validate MTA1 as a target for therapy we confirmed the expression analysis of MTA1 in various human cancer tissues obtained from patients and compared it to the normal tissues. MTA1 expression levels were normalised to two different house keeping genes (GAPDH and rRNA), but due to non-reliability of GAPDH levels only 18SRNA levels were taken into account for normalisation. It has been recently reported in the literature that GAPDH is an un-reliable house keeping gene for normalisation in RT-PCR due to its wide variability in response to different factors and should not be the gene of choice for this purpose (Bustin, 2002). MTA1 was found to be over-expressed in both gastric and colorectal carcinomas (tumour/normal ratio>2), in 6 out of 9 colorectal cancers and in 5 out of 10 gastric cancers. However, mean expression level analysis were only statistically significant for gastric cancer. In individual colorectal cancers, MTA1 expression was correlated with the tumour size and lymph node involvement. This is slightly higher than the previously published study, where approximately 40% of colorectal and gastric cancers over-expressed MTA1 compared to the paired normal colon or gastric epithelium respectively; which might be due to different techniques used in both studies, as Toh *et al* used semi quantitative PCR (Toh *et al*., 1997). However, in their study, clinical-pathological correlation of colorectal cancer showed that MTA1 over expressing tumours exhibited significantly deeper wall invasion and a higher rate of metastasis to lymph nodes and were likely to be at a more advanced Duke's stage. Although, our results suggest a correlation between MTA1 expression and lymph node involvement, analysis of a larger set of samples is required to allow statistical comparison. Previous study had also established a link between MTA1 expression and serosal invasion, metastasis and vascular involvement for gastric cancers (Toh *et al*., 1997). Unfortunately, no such correlation was observed for gastric cancers in the present study. One of the main reasons for this could be that the RNA samples used in this

study were derived from primary tumours of patients who were still in a relatively early stage of the disease. The highest level of MTA1 expression is likely to be present in the metastatic cells, which none of the patients had and hence no correlation could be established between MTA1 level and metastases. A similar pattern was observed in breast cancer samples. However, several normal breast tissue samples had either low or poor quality RNA and could not be amplified. For the individual samples evaluated, MTA1 levels could not be correlated to tumour stage. Interestingly, in a recently published study of 263 breast cancer patients, MTA1 levels did not correlate with tumour grade but with the microvessel density of the tumours (Jang *et al*, 2006). This suggests that MTA1 might also be involved in tumour angiogenesis and is a potential target for anti-angiogenic therapy as well.

It is noticeable that in most cases, an increase in MTA1 expression in tumour tissue is only 3-4 fold compared to normal tissue. This is in agreement with other studies showing an increase of various metastasis related genes by 2-4 fold in human tumours (Dear *et al*., 1989). Another essential aspect to consider during analysis of tumour antigens is the source of RNA as well as the technique used for obtaining the tissue. All the tissues used in this study were obtained from radical surgery and this usually results in tissue consisting of different cell types. RNA obtained from such tissues can be diluted out as a result of the presence of heterogeneous cell populations. The recent emergence of laser capture micro-dissection (LCM) has provided a crucial breakthrough for specifically isolating foci of tumour cells from a heterogeneous tumour population. This is achieved by selecting individual areas or cells and directing a brief laser pulse at the area to isolate them (Bustin, 2002). Indeed, it was recently observed that when compared to benign prostate tissue, MTA1 levels were not significantly elevated in prostate cancer samples obtained by radical prostatectomy. However, when similar study was performed with prostate cancer samples isolated using LCM, a significant difference was observed in MTA1 expression (Walton T, unpublished).

Analysis of the expression data of MTA1 revealed that MTA1 was over-expressed in most cancers of different histological types. However, the question still remains whether MTA1 expression increases as a result of the metastatic process or plays an essential role in the process? Firstly, low level expression in most normal tissues and high levels in testis suggests that it does indeed have a role in proliferation and migration of cells. Several recent studies have provided evidence supporting this hypothesis. Nawa *et al* showed that treatment of a human breast adenocarcinoma cell line, over-expressing MTA1, with anti-sense phosphorothioate

oligonucleotides inhibited its cell growth to 22% of mock treated cells, whereas similar treatment of cells expressing MTA1 at normal levels did not have any effect on their growth (Nawa *et al*., 2000). Complementing this study, Mahoney *et al* demonstrated multiple effects of MTA1 over-expression in human immortalised keratinocytes (HaCaT cells) (Mahoney *et al*., 2002). Over expression of MTA1 in HaCaT cells led to increased migration and invasion of immortalised keratinocytes, allowing them to grow in an anchorage independent manner as well as contributed to their expression of anti-apoptotic Bcl-2 family member Bcl-xl. More importantly, this was also confirmed in an *in vivo* mouse model of MTA1 dysregulation leading to development of mammary tumours (Bagheri-Yarmand *et al.*, 2004).

MTA1 was recently cloned in our laboratory using SEREX suggesting its immunogenicity, at least for the CD4+ T cells. Human MTA1 is 94% identical to mouse MTA1 at protein level and this led us to investigate MTA1 as a target for immunotherapy in a murine model. Most of the studies to date have concentrated on MTA1 expression in human tumours and tissues. It was necessary to determine the relative expression of mouse MTA1 in tumour cell lines and normal tissues to validate using MTA1 as a target in the mouse model. Hence, initially, the expression of mMTA1 was determined; using RT-PCR, in murine cancer cell lines of different background and expression compared with that in normal murine tissues. MTA1 was found to be highly over-expressed in all the tumour cell lines tested, whereas the expression in normal tissues was quite low compared to them. This result is in agreement with previous studies performed in humans and rats, where MTA1 expression in tumours was nearly two to four times that of normal tissues (Nicolson *et al*, 2003). However, previous studies failed to demonstrate expression of MTA1 in muscle cells, but in this study MTA1 appears to be expressed in muscle cells although at much lower levels (Simpson *et al*, 2001). The reason for this discrepancy may be that Simpson *et al* (2001) used Northern blotting, which lacks the sensitivity of RT-PCR. Furthermore, for relative quantification of MTA1 in tumour cells and normal mouse tissues, RT-Q-PCR was performed, which not only confirmed the results of conventional PCR but also showed MTA1 levels to be several fold higher in tumour cell lines compared to normal mouse tissues. However, the mouse tumour cell lines used were mostly derived decades ago and might have undergone additional genotype and phenotype changes during *in vitro* cultures, which would explain differences observed in human vs. mouse tissues (4 fold vs. 10 fold increase). Furthermore, *in vivo* expression of MTA1 (or other tumour antigens) could also be affected by the immune system or other regulatory mechanisms.

Hence, any immune response against MTA1 could also potentially lead to an autoimmune destruction of the organs expressing MTA1, although high level of protein expression is generally required in cells, for peptide-MHC complexes to be expressed on the surface in sufficient quantities to be recognised by CTLs (Stevanovic and Schild, 1999). This could help the CTLs generated against MTA1 antigen to distinguish normal tissues from tumour cells and thereby causing minimal auto immune reaction, if any.

Furthermore, western blotting was performed to confirm and compare the protein expression of MTA1 in murine tissues and tumour cell lines and also to eliminate any possibility of post-translational modifications altering the MTA1 protein expression, which was observed in all the cell lines tested although RENCA seemed to express it the least amount of MTA1 compared to other cells. Moreover, no MTA1 protein expression was observed in normal tissues by western blotting, which is likely to be due to lower sensitivity of western blotting compared to RT-PCR. However, this again confirms that MTA1 is expressed at very low levels in normal tissues, which could limit the auto immune response.

Lastly, localisation of MTA1 was investigated using immuno-fluorescence and as expected showed nuclear localisation of MTA1. Two recent studies have contradicted previously published results and suggested that MTA1 might have limited nuclear localisation. Yaguchi *et al* identified several splice variants of MTA1 in mice and showed differential localisation of these variants (Yaguchi *et al*., 2005). Using a yeast two-hybrid system Aramaki *et al* showed that mMTA1 interacts with endophilin 3 in the cytoplasm and suggested its possible involvement in endophilin 3 mediated endocytosis (Arakami *et al*., 2005). Thus the results here contradict these studies and are in agreement with most other studies proving nuclear localisation of MTA1. Nuclear localisation does not sequester antigens from the immune system as revealed by a number of auto-immune diseases where antibodies are generated to nuclear antigens (Nakken *et al*., 2003). However, CD4+ T cells reactive to nuclear antigens undergo rigorous tolerance or deletion mechanisms (Nakken *et al*., 2003).

Thus, the present study confirms that MTA1 has most of the characteristics of an ideal tumour antigen such as low level expression in normal tissues, important role in oncogenic transformation of cells as well as being a late stage disease antigen. Moreover, high similarity in protein sequence and function between mouse and human MTA1 makes it a convenient antigen to investigate its immunogenic potential in murine model, with direct application for human disease. However, being a self-antigen, tolerance to MTA1 is likely to be a key factor in generating immune response with the added risk of stimulating an auto-immune reaction.

Chapter 4:- Construction of Plasmid and Viral Vectors encoding MTA1

4.1 Introduction

In the past few years, gene based immunisation strategies have evolved dramatically for infectious diseases and cancer. Contrary to peptide vaccination, 'gene immunisation' can be applied to the whole population, without the need for identifying naturally processed peptides for different MHC haplotypes. This makes it widely applicable to general population. Another advantage of this method is its ease of production to good manufacturing practice (GMP) standards and cost of production. Apart from these, they can be delivered by multiple routes, are naturally immunogenic and activate multiple arms of the immune system (Pavlenko *et al*., 2004). Gene-based strategies can mainly be divided into two, 'naked' plasmid based as well as viral/bacterial vector vaccination. Moreover, apart from their direct administration as a vaccine, they can also be used to modify tumour cells and APC *in vitro* before injecting them in order to make them more immunogenic or increasing their antigen presenting ability, respectively.

Wolff and colleagues first noticed the *in vivo* protein expression on injection of plasmid encoding for the protein, paving the way for a revolution in vaccination strategies (Wolff *et al*, 1990). They observed that *in vivo* injection of DNA or RNA expression vectors encoding genes can lead to long term detectable protein expression (up to 2 months) in the muscles injected. Plasmid vaccination consists of a bacteria derived plasmid backbone encoding the gene of interest. Three essential components of a plasmid vector are expression cassette consisting of a promoter and a polyadenylation signal, antigen encoding gene sequence as well as an origin of replication and selection marker for propagation of plasmid in bacteria. Recent completion of the human genome project has provided immunologists with sequence information of virtually all genes in the human body and utilising molecular biology tools, it is now possible to clone these genes into expression plasmids with relative ease. DNA vaccines can be delivered intra-muscularly, through skin via gene gun, administered orally through mucosal route, intra-peritoneal and intra-dermal injections.

Different immunisation routes might have different immunological outcomes. The ability of 'naked' DNA vaccines to generate and polarise the immune response can be due to several pathways. Firstly, resident APC in the muscle may take up the plasmid directly to produce the protein, process it and present it to generate the immune response. Secondly, muscle cells might uptake the DNA but are inefficient antigen presenters. Release of proteins from the transfected muscle cells undergoing apoptosis and their uptake by professional APC can also generate immune response, termed 'cross-priming'. Lastly, skin resident APC (langerhans cells) can be

directly transfected on intradermally administered DNA via gene gun (Condon *et al*., 1996). Thus different pathways are likely to be involved in DNA based immunisation although the final response might be the result of a combination of above three mechanisms.

Table 4.1:- Advantages and Disadvantages of DNA vaccines

Advantages	Disadvantages
Cost-Effective	Prior knowledge of antigen sequence
Easily Manufactured to GMP standards	Might require additional adjuvants
Safe	Codon optimisation might be necessary
Easy to administer	Different routes of vaccination might generate response
Several routes of vaccination	
Low doses required	
Primers multiple arms of immune response	
Antigens can be linked to several co-stimulatory molecules or cytokines	
Innate adjuvant properties	

Several studies have provided contradicting results regarding polarisation of the immune response by gene gun and intra-muscular immunisation. Bombardment of skin with DNA coated gold particles uses 100-1000 times less DNA than other routes of vaccination. Saline–DNA immunisation either intramuscularly or intradermally leads to the generation of Th1 response and production of IgG2a antibodies in mice, whereas gene gun immunisation has been reported to generate a Th2 response and IgG1 antibodies. This polarisation of the response is independent of the dose of DNA and is likely to be influenced by the different cell types transfected with the injected plasmids (Torres *et al*., 1997; Felquate *et al*., 1997). Contradicting these studies, gene gun mediated DNA immunisation has been shown to provide protective and therapeutic anti-tumour immunity mediated by CTLs (Condon *et al*., 1996; Bowne *et al*., 1999; Gold *et al*., 2003). In these studies, vaccinating mice with as little as 1 μg of DNA through gene gun was enough to generate anti-tumour immune response.

Recent years have also witnessed an unprecedented interest in use of viral vectors as alternative and more potent vectors for immunotherapy. Immunising with viral vectors can mimic natural viral infection, thereby generating more potent immune response and efficiently penetrate most cell types, which makes them particularly useful for *in vitro* or *in vivo* application. However,

there is also a wide spread concern regarding viral vectors for immunotherapy as there is a possibility of progressive viral infection, especially in immuno-compromised patients. This is evident by a number of different viral vectors being developed with high bio-safety levels and negligible chances of *in vivo* generation of infectious recombinant virus. New generation viral vectors have genes, essential for replication, deleted by genetic manipulation to make them attenuated. Other viruses such as avipox virus derived vectors take advantage of natural host restriction as they are unable to multiply in human cells. Another important characteristic for a viral vector is their low intrinsic immunogenicity, since antibody response to viral proteins can limit their efficacy as a therapeutic agent. Viral vectors commonly used for therapy include the following:-

Table 4.2 Characteristics of Different viral vectors (Adapted from Bonnet et al., 2000)

Vector	Biology	Pre-existing immunity in human	Duration of Gene Expression	Safety
Retroviruses	Diploid RNA strand	No	Good	Risk of insertional mutagenesis
Vaccinia viruses	Double stranded DNA replicative	Yes	Transient	Well documented safety
Avipox viruses	Double-strand DNA non replicative in mammalian cells	Yes	Transient	Very good
Adenovirus	Double-stranded DNA (linear)	Yes (highly immunogenic)	Transient	Good
Adeno-associated viral vector	Single-strand DNA	Yes	Transient	Risk of insertional mutagenesis
Herpes simplex virus	Double-stranded DNA (linear)	Yes	Transient	Neurovirulence,
Alphavirus (SFV, EEV)	RNA viruses with replicon	No	Good	Safe in animals, not fully characterised in humans

SFV=Semliki Forest Virus, EEV=Equine Encephalitis Virus

Adenovirus

Adenoviruses are medium sized non enveloped double-stranded DNA viruses. There are more than 50 serotypes of adenovirus known of which serotype 2 and 5 have mostly been used for

vector development (Jooss & Chirmule., 2003). They infect the cells using a viral fibre knob to bind to coxsackievirus receptor (CAR) and other co-receptors. Adenovirus replicate inside the nucleus of infected cell without integrating into their genome. They are associated with a number of infections in humans such as upper respiratory tract infections, conjunctivitis and other infections in immuno-compromised hosts. Consequently, they are capable of infecting a wide range of cell types. Their main advantages are high level of protein expression and relative ease of high titre generation. They are capable of generating cell mediated and humoral immune response in immunised animals. First generation adenovirus derived vectors had deletion in genes necessary for replication, which were followed with second generation vectors having deletions in other genes responsible for blocking host immune response. However, more than 85% of the population has neutralising antibodies to adenovirus, precluding their use for human *in vivo* immunotherapy (Bonnet *et al*., 2000). Although antibodies to adenovirus have made their use limited, novel recombinant vectors, high protein expression, ability to infect dividing and non-dividing cell and use of uncommon adenovirus serotypes might still make them useful for immunotherapy

Adeno Associated virus (AAV) vectors

AAV are single stranded DNA virus of parvovirinae family of 4.7 kb genome. Eight different serotypes of AAV have been identified and wild types AAV1-6 have not been associated with any known human disease. AAV-2 has been the most extensively studied serotype for immunotherapy. AAV vectors can stably integrate into the human genome on chromosome 19 (Jooss & Chirmule., 2003). They can infect dividing and non-dividing cells and their greatest advantage is their long term gene expression compared to other vectors (Bonnet *et al*., 2000). Recombinant AAV vectors need helper virus to replicate and this can also limit their production at high titres without a stable helper cell line. Moreover, they have a limited insert capacity (4.5 Kb) which might preclude their use in cancer vaccination, since vector coding for multiple genes (antigens along with co-stimulatory/cytokine genes) is likely to be more efficacious compared to one encoding only the antigen.

Poxvirus Vectors

Poxviruses are large, enveloped double stranded DNA virus and are the only virus with intra-cytoplasmic replicating capability. They can be generated to high viral titres using primary chicken embryo fibroblasts and their major advantage is their ability to accept large inserts (up to 30 Kb). This would allow simultaneous insertion of multiple genes and wider application.

Among several poxviruses investigated, modified vaccinia Ankara strain (MVA) has shown high bio-safety profile and is highly efficacious as demonstrated in the small pox eradication programme (Bonnet *et al*., 2000). NYVAC stain was derived from the original Copenhagen strain by deletion of 18 open reading frames. Other poxviruses such as canarypox and fowlpox virus are potentially safer due to their inability to multiply in human cells. Poxvirus vectors have proved their efficacy in immunotherapy of cancers in animal studies (Jourdier *et al*, 2003).

Herpes Simplex Viral vectors

HSV are large, enveloped, double stranded DNA containing virus with a 152 kb genome. HSV viral genome encodes for 73 distinct genes, of which 38 are believed to be redundant and can be replaced with foreign genes. They are able to infect most types of dividing cell and do not integrate into the host genome. Moreover, several variants of HSV have been constructed with modifications to ensure that replication competent virus is not generated *in vivo*. DISC-HSV was derived by deleting the essential glycoprotein (gH) gene from HSV virus allowing the replication competent viruses to be formed only in competent cell line having the gH gene (Rees *et al*., 2002). This provides DISC-HSV with an added level of safety. HSV vectors have good safety profile and have proven highly efficacious in animal models, where injection of DISC-HSV/GM-CSF intra-tumorally led to tumour regression in 60% of mice with well established tumours. Being cytolytic, they are also likely to induce cross-priming (Ali *et al*., 2002). Moreover, previous exposure to HSV and antibody presence to it, was unable to decrease therapeutic efficacy of DISC-HSV.

Retroviruses

Retroviruses are diploid positive-strand RNA viruses. They are likely to integrate into the genome of the cell and hence carry a chance of insertional mutagenesis. However, retroviruses only infect dividing cells and are inefficient at infecting APC, which could limit its use for human *in vivo* application. It is particularly useful for *in vitro* modification of tumour cells due to stable integration and long term protein expression (Hodge & Schlom, 1999). Lentiviral vectors derived from the HIV-1 have generated a lot of interest recently for clinical gene therapy, due to their capability of infecting non-dividing cells as well. Replication incompetent vectors have recently been designed which uses either helper genes or packaging cell lines for virus generation and are considered safer for human application (Buchschacher & Wong-Staal, 2000). However, HIV derived vector systems still cause serious concerns for human therapy due to a possibility of wild-type virus generation *in vivo*.

Alpha virus

Commonly used alpha viruses are Sindbis virus, Semliki forest virus (SFV) and Venezuelan Equine Encephalitis virus and have proved very efficient gene delivery vehicles. Alphaviruses have a single stranded RNA genome of approximately 12 kb and surrounded by an icosahedral capsid protein shell. They have the ability to infect different cell types, generate high level of protein and are cytolytic in nature, leading to cell lysis 48-72 hour post-infection.

Semliki Forest Virus as choice of vector for immunotherapy

SFV is a 65-70 nm spherical virus with a single strand RNA. Viral genome is surrounded by a nucleocapsid, which is formed by 240 copies of the capsid protein arranged to form an icosahedral shell. Nucleocapsid is surrounded by a lipid bilayer derived from host cell membrane into which glycoprotein spikes formed by proteins E1, E2 and E3 are inserted. SFV mRNA consists of two open reading frames (ORF), the first of which codes for the four non structural proteins and the second ORF codes for capsid and envelope proteins. SFV infects cells via binding of its envelope protein to different cell surface receptors (figure 4.1). Upon SFV infection, host cell protein synthesis shuts down in favour of virus encoded protein production (Riezebos-Brilman *et al*., 2006).

Low infection efficiency, limited host range, safety and complexity of the system are some of the hurdles in viral vector based gene therapy. Recombinant SFV vectors for immunotherapy have generated considerable interest due to their ability to overcome these challenges. Because of genetic manipulation, recombinant SFV are inactive and have to be activated with α-chymotrypsin before use, which makes it particularly safe for handling (Berglund *et al*., 1993). Also, they are only capable of a single round of infection; high level protein expression followed by apoptosis facilitating cross-priming and priming adaptive and humoral immune system also make them a popular choice. Due to dsRNA intermediates generated upon infection, they are potent in activating dendritic cells through TLR3 (Riezebos-Brilman *et al*., 2006). Moreover, humans do not have any antibodies to SFV, which allows them to be administered without decrease in efficacy due to neutralising antibodies.

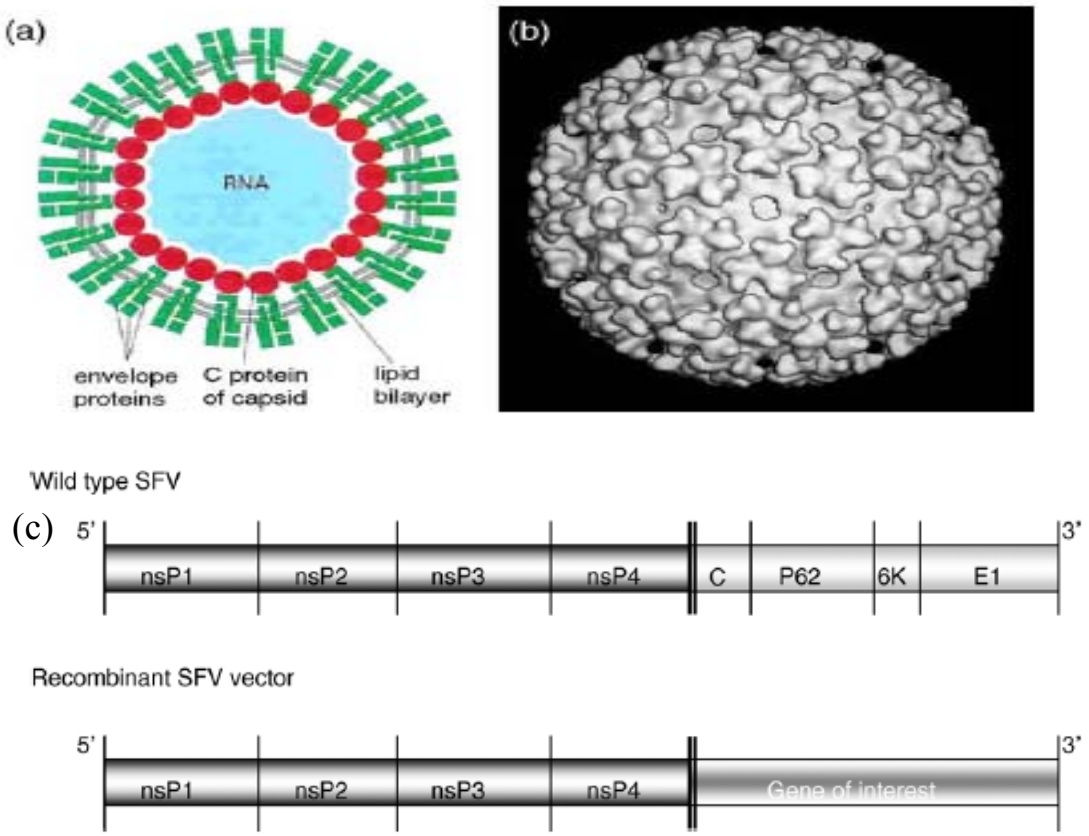


Figure 4.1:- SFV structure *(a) Schematic structure of SFV (b) Cryo-Electronmicroscopy image reconstruction (c) Schematic structure of genome of SFV and its recombinant SFV vector (taken from Riezebos-Brilman et al., 2006). nsP= non-structural proteins*

Original SFV expression vector system employed a plasmid with SP6 RNA polymerase promoter and the structural protein coding region replaced with gene of interest (Liljestrom & Garoff., 1991). This was complemented with a helper RNA encoding for the viral structural proteins. Thus, upon co-transfection of both helper and gene coding RNA, recombinant virus particles are generated. Moreover, mutation in the p62 protein gene generates viruses which are inactive and have to be proteolytically treated with α-chymotrypsin. These two safeguard mechanisms make it unlikely for replication competent virus would be generated upon transfection or *in vivo* application for therapy. However, RNA based SFV expression system still require the generation of capped RNA transcripts *in vitro* and specialised handling conditions. These obstacles were overcome by DiCiommo and Bremner, who constructed a DNA based expression system by replacing the SP6 promoter with RNA polymerase II dependent cytomegalovirus immediate early (CMV IE) enhancer/promoter to drive transcription *in vivo* (DiCiommo & Bremner, 1998). Both helper and replicon plasmids were designed and conditions were optimised for co-transfection to generate high levels of virus titre. Co-transfection of both plasmids into BHK cells generated upto 20-30 pg/cell of virus encoded foreign protein

(DiCiommo & Bremner., 1998). This improved SFV expression system was later enhanced further by expanding the multiple cloning site as well as addition of FLAG and HIS10 epitope and affinity tags (DiCiommo *et al*., 2004).

The SFV vector system has been evaluated in several animal models for generation or preventive and therapeutic immune response to cancer (Daemen *et al*., 2000; Daemen *et al*., 2003; Daemen *et al*., 2004; Ni *et al*., 2004; Riezebos-Brilman *et al*., 2005). Moreover, a recent study also demonstrated the ability of SFV vector to generate an immune response in an immune-tolerant mouse model, demonstrating its usefulness in breaking tolerance (Riezebos-Brilman *et al*., 2005). Thus, ease of manipulation, good efficacy for therapeutic purposes as well as ability to overcome tolerance led us to investigate SFV expression system as a means for breaking tolerance to MTA1.

In order to investigate application of plasmid DNA vaccination for generating immune response to MTA1, this study describes the construction of a plasmid expression vector coding mMTA1. Also, human MTA1 was cloned into the expression vector for investigating xenogeneic immunisation. Furthermore, both mMTA1 and hMTA1 were cloned into SFV vectors and virus generation optimised. This was followed by confirmation of protein production by the various vectors generated.

4.2 Results

4.2.1 Cloning of mouse and human MTA1 into mammalian expression vector pcDNA3

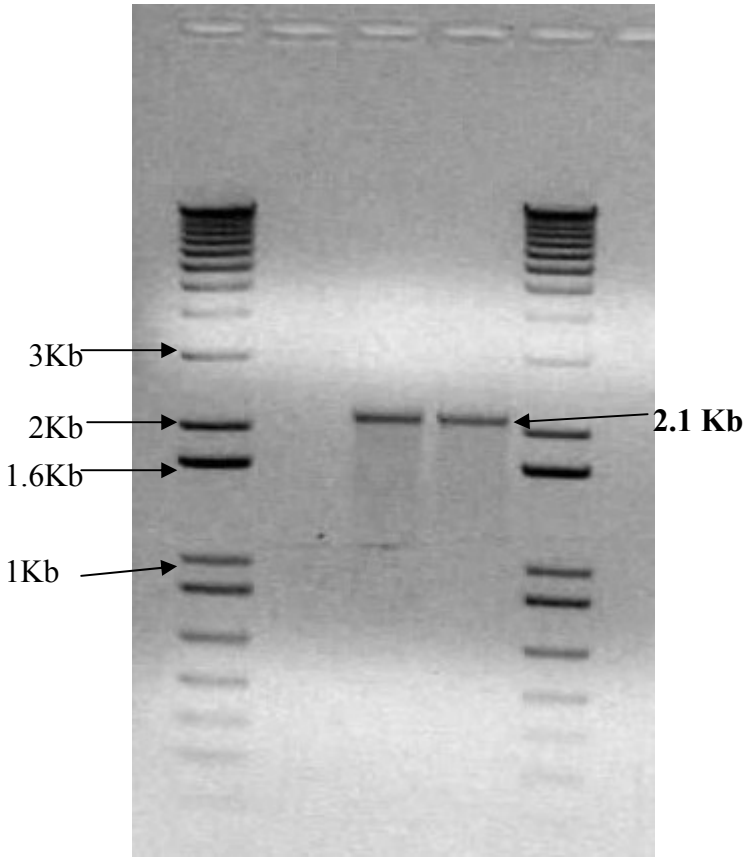


Figure 4.2 Agarose gel electrophoresis of full length mMTA1 following RT- PCR from mouse testis for 40 cycles

In order to immunise mice with full length plasmid as well as viral vaccination, the full open reading sequence of MTA1 was cloned into pcDNA3. Full length mouse MTA1(mMTA1) was first amplified from mouse testis using RT-PCR for 40 cycles, using a high fidelity Taq polymerase (Phusion, Finnzymes, UK). It is noteworthy, that mouse testis was used for generating amplified MTA and not a tumour cell line which might have mutations in the MTA1 gene, although no documented mutations have been reported in MTA1 till date. After running the amplified mMTA1 on an agarose gel, a band corresponding to 2.1Kb (mMTA1) was clearly visible and was extracted (Fig 4.2). Since Phusion Taq polymerase generates blunt ended products, full length mMTA1 cDNA was cloned into TOPO-Blunt vector (Invitrogen), using manufacturer's instructions.

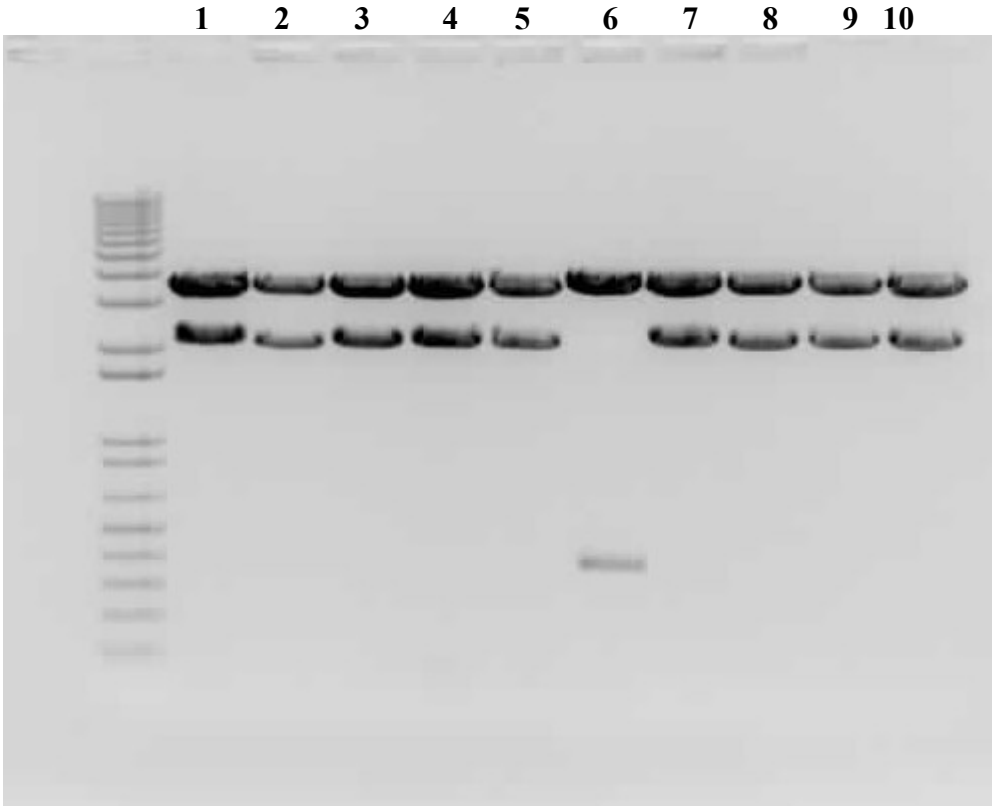


Figure 4.3 Agarose gel electrophoresis after double digestion of TOPO-Blunt clones with insertion.

Briefly, blunt ended mMTA1 was incubated with 1µl of TOPO-Blunt vector and 1µl of salt solution for 5 minutes. TOPO vectors take advantage of the enzyme DNA topoisomerase I, whose function is to nick and re-ligate DNA fragments. Five minute ligation was enough for insertion of MTA1, without a need for addition of DNA ligase enzyme. Following the ligation, XL1B bacteria were transformed with TOPO-Blunt/mMTA1 and plated on LB agar with kanamycin for selection of clones containing the plasmid. After overnight incubation at 37˚C, 10 colonies were picked and grown in LB broth with 50µg/ml of kanamycin overnight. Plasmids were isolated from the selected clones. To confirm the presence of mMTA1 insert in TOPO-Blunt, EcoRI restriction digest was performed on the plasmids, since TOPO-Blunt vectors have EcoRI restriction sites at both ends of the insert (see appendix). As seen in figure 4.3 band corresponding to 2.1kb could be detected in 9 out of 10 clones picked.

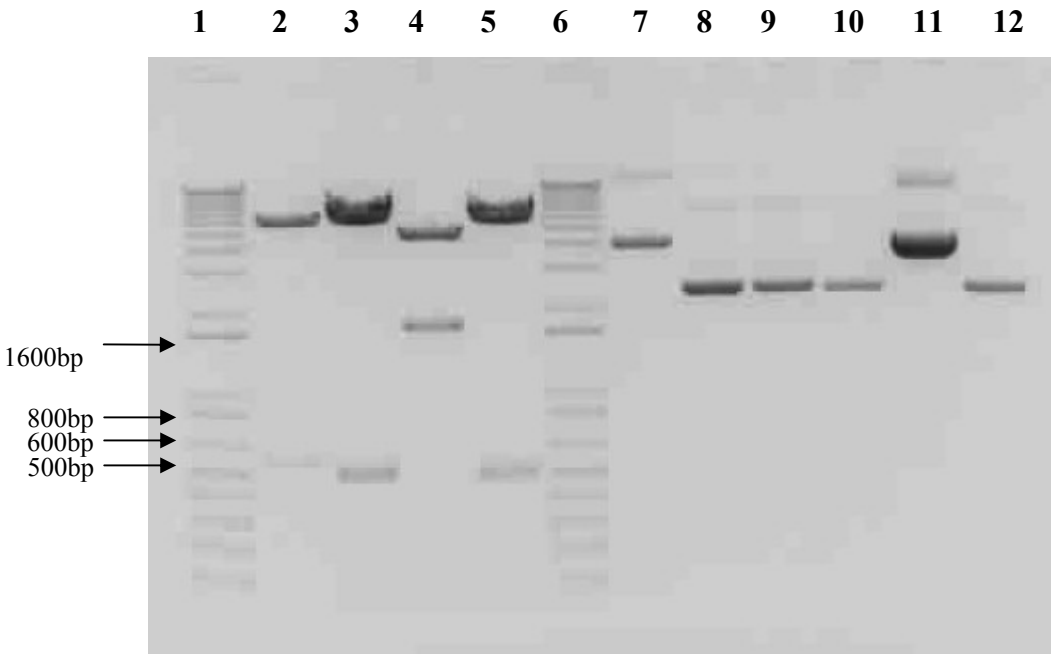


Figure 4.4 Agarose gel electrophoresis of TOPO-Blunt/mMTA1 clones after BamHI-AgeI double digest (lanes 1 & 6-1kb plus ladder, 2-5 &7-12 TOPO-Blunt/mMTA1 clones)

Furthermore, blunt ended mMTA1 could insert in either forward or reverse orientation into TOPO-Blunt vector. Clones with mMTA1 in correct orientation could be either identified by sequencing or restriction digestion of mMTA1 with one enzyme cutting inside mMTA1 and other on the vector to generate specific sized bands. Indeed, Age I restriction enzyme cuts mMTA1-TOPO construct approximately 500bp upstream of the start codon. Hence, if mMTA1 was in the correct orientation then BamHI-AgeI double digestion would generate a band of approximately 500bp on agar gel electrophoresis, and a band of approximately 1500bp would indicate it in the reverse orientation. As seen in figure 4.4, 3 clones had mMTA1 in the correct orientation. One of them was sequenced to confirm that there were no mismatches.

After three attempts, full length mMTA1 clone was obtained with 100% identical sequence to published mMTA1 sequence. Finally, to sub-clone mMTA1 into pcDNA3, both TOPO-Blunt/mMTA1 (clone 5) and pcDNA3 were double digested with HindIII and XbaI restriction enzymes and run on Agarose gel (Figure 4.5). Digested corresponding bands of pcDNA3 and mMTA1 were gel extracted and ligated overnight at 4˚C with T4 DNA ligase enzyme, followed by plating on LB agar with ampicillin and selection of clones. Clones were confirmed to contain mMTA1 by restriction digestion (data not shown).

Similarly, human MTA1 was cloned from human testis into pcDNA3 after confirming 100% sequence homology to the published sequence (data not shown).

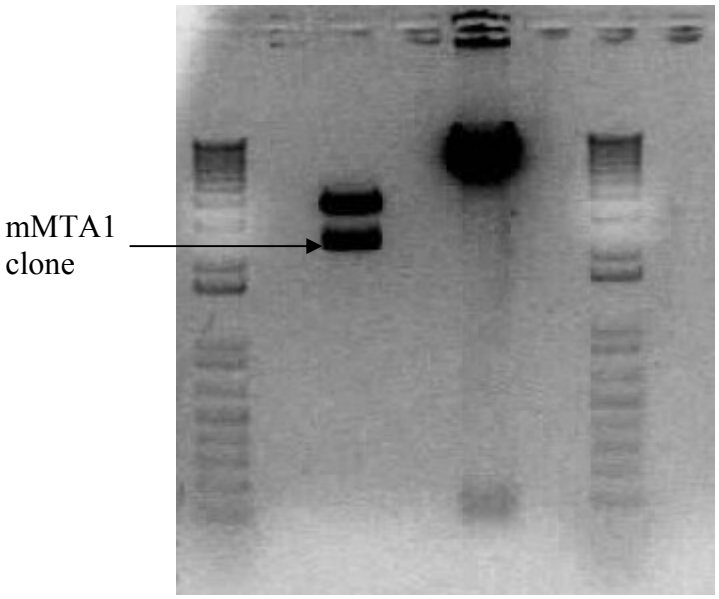


Figure 4.5 HindIII-XbaI double digest of TOPO-Blunt/mMTA1 and pcDNA3 (from left to right lanes 1-ladder, 3- TOPO-Blunt/mMTA1, 5-pcDNA3, 7-ladder)

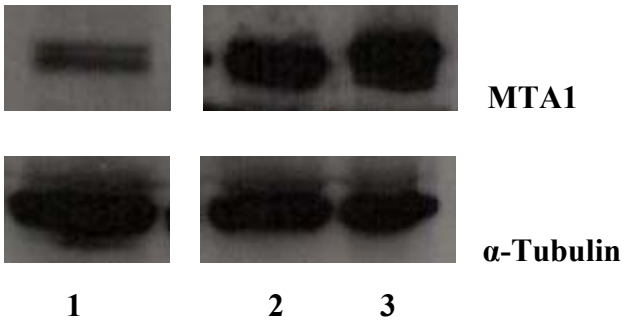


Figure 4.6 Western-blot of 293 cells48 hours after transfection with pcDNA3/mMTA1 or pcDNA expression vectors and α-tubulin western blot as a control *(Lane 1-control, 2-mouse MTA1, 3-human l*

To confirm mouse and human MTA1 protein expression by pcDNA3 vectors, human embryonic kidney (HEK 293) cells were transfected with pcDNA3 encoding for either mouse or human MTA1 using lipofectamine 2000 reagent and following manufacturer's instructions (Invitrogen) (see methods). 24-48 hours after transfection, lysates were prepared from the transfected cells, protein assay was performed and 20μg of protein of transfected and non-transfected control samples were loaded on 10%SDS-PAGE gels. Western blot was performed by transferring the gel proteins onto a nitrocellulose membrane and probed with either MTA1 antibody or α-tubulin antibody as a control. High levels of MTA1 protein expression was detected in cells transfected with expression vectors encoding the human or mouse MTA1, compared to control cell lysates (Figure 4.6). It is noteworthy that the MTA1 signal was observed in control cells (no transfection), which is in agreement with our previous results showing variable MTA1

expression in most tumour cell lines. To confirm that the increased expression of MTA1 observed in transfected cells was not due to overall increase in the total protein loaded (although equal amounts of protein were loaded), blots were also probed with α-tubulin antibody as a control protein. No significant changes in α-tubulin levels were observed. These results were also confirmed by RT-PCR (data not shown).

4.2.2 Cloning of mouse MTA1 into pSMART2b vector for SFV vector generation

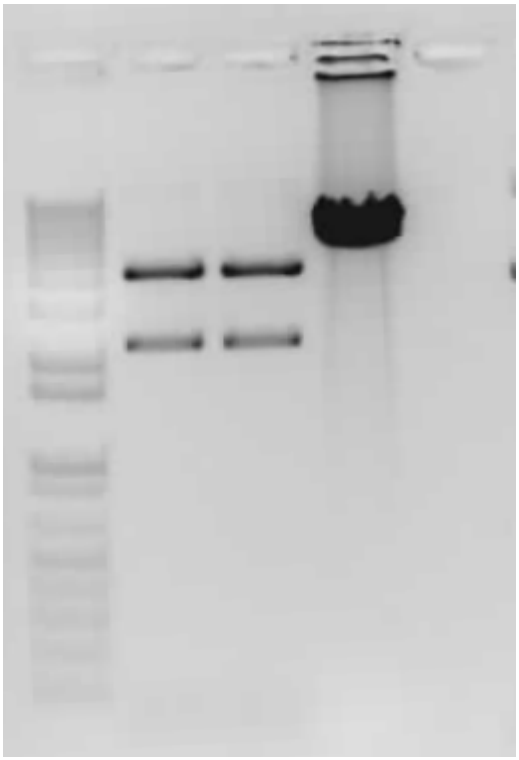


Figure 4.7 Double digest of TOPO-Blunt/mMTA1 and pSMART2b vector with BamHI and NsiI (Lanes 1-ladder, 2&3-TOPO-Blunt/mMTA1, 4-pSMART2b)

Plasmids for SFV vector generation were obtained from Dr. Rod Bremner (Canada). In order to generate SFV/MTA1 vectors, human and mouse MTA1 sequences had to be cloned into either pSMART2a or pSMART2b vectors. Both these vectors are essentially similar except that the gene of interest, when cloned into them, will be in different reading frames. It is essential for the gene of interest to be in correct reading frame with the upstream capsid-flag tag for the protein to be produced using these vectors. From the sequencing data of TOPO-Blunt/mMTA1 and TOPO-Blunt/hMTA1, it was determined that open reading frame of MTA1, when sub-cloned, would be in frame in pSMART2b vector and not in pSMART2a. To sub-clone MTA1 sequence from TOPO-Blunt vectors, TOPO-Blunt/mMTA1 was double digested with BamHI and NsiI restriction enzymes, along with pSMART2b vector (Figure 4.7). Digested vector (pSMART2b)

and MTA1 band were extracted and ligated overnight as described before. Clones of vector from transformed E.Coli XL1B cells were isolated and checked for insertion of mMTA1 by repeating the BamHI-NsiI digestion (Figure 4.8). As seen below, 3 clones with mMTA1 insertions were identified and were further used for confirmation of protein production after transfection. Similar procedure was repeated for SFV/hMTA1 vector generation (data not shown).

Figure 4.8 BamHI-NsiI double digest of pSMART2b/mMTA1 (lanes 1-ladder, 2-6 pSMART2b/ mMTA1 clones

To confirm production of MTA1 protein by pSMART2b-mMTA1/hMTA1 vectors, BHK cells were transfected with the vectors using Lipofectamine and protein production was confirmed by western blotting 48 hours after transfection. α-tubulin protein expression levels were used as controls. As seen in figure 4.9, high levels of MTA1 expression was observed in cell lines transfected with pSMART2b-mMTA1/hMTA1 vectors, confirming the in frame insertion of MTA1 sequences in pSMART2b vector. To confirm that MTA1 protein could not be produced by pSMART2a vector, mMTA1 was sub-cloned into the pSMART2a vector and protein production investigated after transfection as above. No protein production could be detected in this case (data not shown).

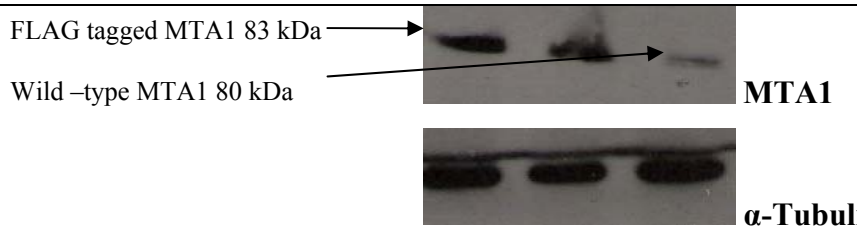


Figure 4.9 Western-blot of BHK cells 48 after transfection with pSMART2b/mMTA1 or hMTA1 *(lanes 1-pSMART2b/MMTA1, 2-pSMART2b/hMTA1, 3- Control non transfected)*

4.3 Optimisation of SFV/βgal generation

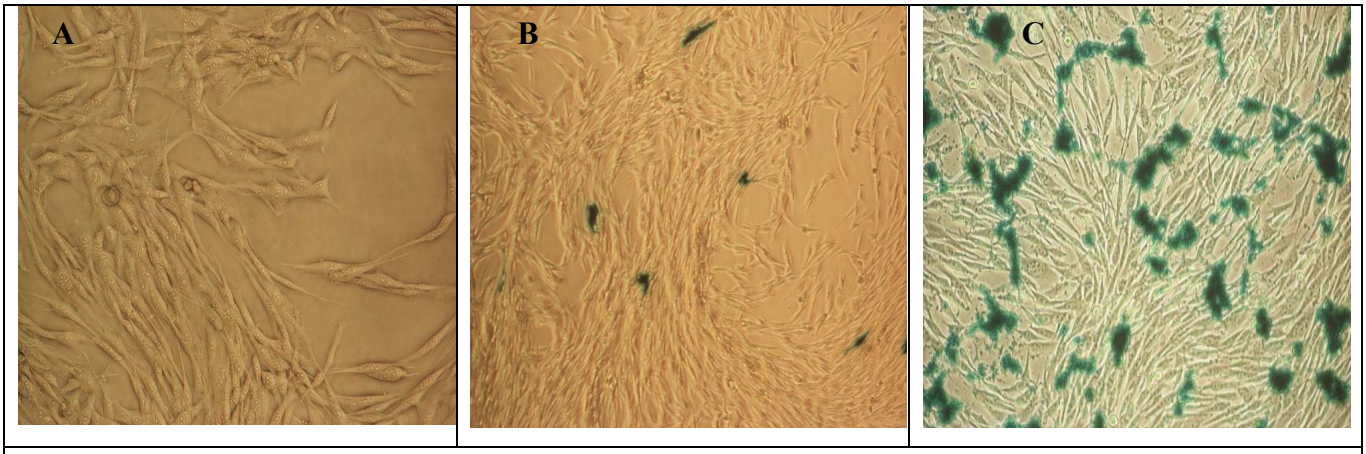


Figure 4.10 Xgal assay BHK cells 24 hours after infection with SFV-βgal vector generated using original protocol and modified protocol *A=non infected cells, B=Cells infected with virus generated without freeze/ thaw, C=virus generated after modified freeze thaw protocol*

SFV vector generation requires co-transfection of the helper plasmid as well as the replicon plasmid (containing gene of interest). Factors, such as method of transfection and molar ratios of the two plasmids are known to affect the titre of virus generated. Lipofectamine and calcium phosphate (Promega, UK) mediated transfection methods were first investigated for virus production via transfection of HEK 293 cells and results confirmed that $CaPO_4$ transfection method was better (data not shown). Previously, it had been suggested that 24-48 hour post-transfection of 293 cells with both plasmids, virus can be harvested from the supernatants of the cells (DiCommio & Bremner, 1998). However, virus titre obtained through this method was very low. We hypothesised that most of the virus was still inside the cell and cell lysis by freeze/thawing might increase the titre of virus obtained. To test this, supernatants of the transfected cells were collected before and after freeze/thaw, and virus harvested from them. Following activation, equal quantities of supernatant was used to infect BHK cells and β-galactosidase protein expression was determined by performing an X-gal assay. A significant increase in virus titre was observed from the supernatant of cells after the freeze thaw cycles

($1.34x10^5$ pfu/ml vs $2x10^6$ pfu/ml) (Figure 4.10). Hence, this modified protocol was then used for virus harvesting. Moreover, different molar ratios (1:1, 1:2, 1:3) of pSCaβ and helper plasmids were transfected using $CaPO_4$ and its effect on virus titer examined. No significant difference was observed by using different molar ratios of the plasmids; however a molar ratio of 1:3 generated slightly better titer and it was decided to use this ratio for all future experiments (data not shown).

4.2.3 Dendritic cell infection by SFV/B-gal

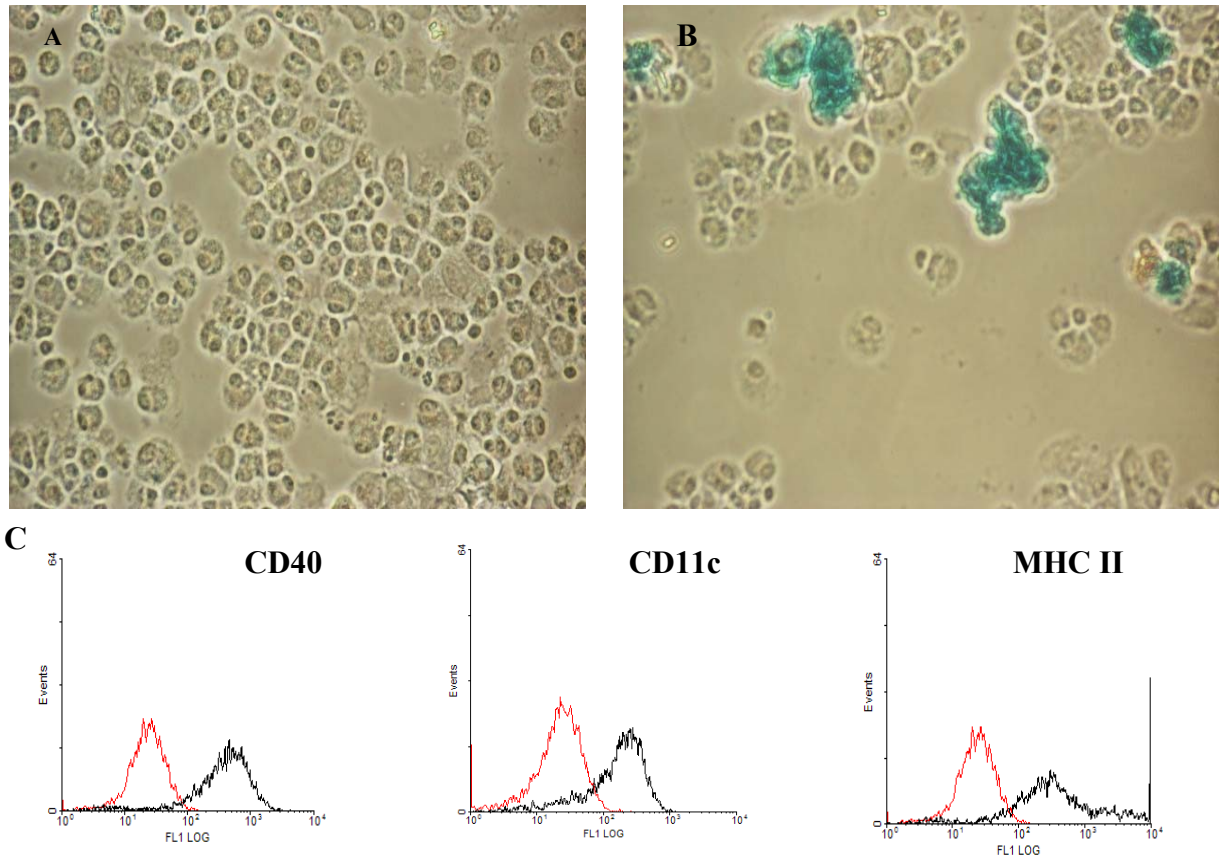


Figure 4.11 Xgal assay 24 hour post-infection of dendritic cells with SFV/β-gal. *A-Control non infected DC, B-Infected DC, C-FACS analysis for selected dendritic cell markers*

Recent studies have suggested that SFV vectors are inefficient in infecting DC and most of the immunological response to SFV immunisation was due to cross-priming, where the infected cells undergo apoptosis and antigens released from them are engulfed by APCs, processed and presented (Huckriede *et al.*, 2004). To confirm these findings, dendritic cells were generated from bone marrow of balb/c mice and infected with SFV-βgal for 1 hour at 37ºC. 24-48 hours post-infection, X-gal assay was performed on them to verify the expression of β-galactosidase

enzyme. As seen in figure 4.11, although SFV infected only about 15-20% of the DC, it was still approximately 100 times more than that observed by Huckriede *et al* (0.14%)-(Huckriede *et al*., 2004). These results were confirmed in two independent experiments and were independent of strain of mice, since DC derived from C57BL/6 background mice showed similar susceptibility to infection (data not shown). Moreover, maturation status of DC had no significant affect on their ability to be infected (data not shown).

4.2.4 Determination of MTA1 expression after infection of BHK cells with SFV-mMTA1

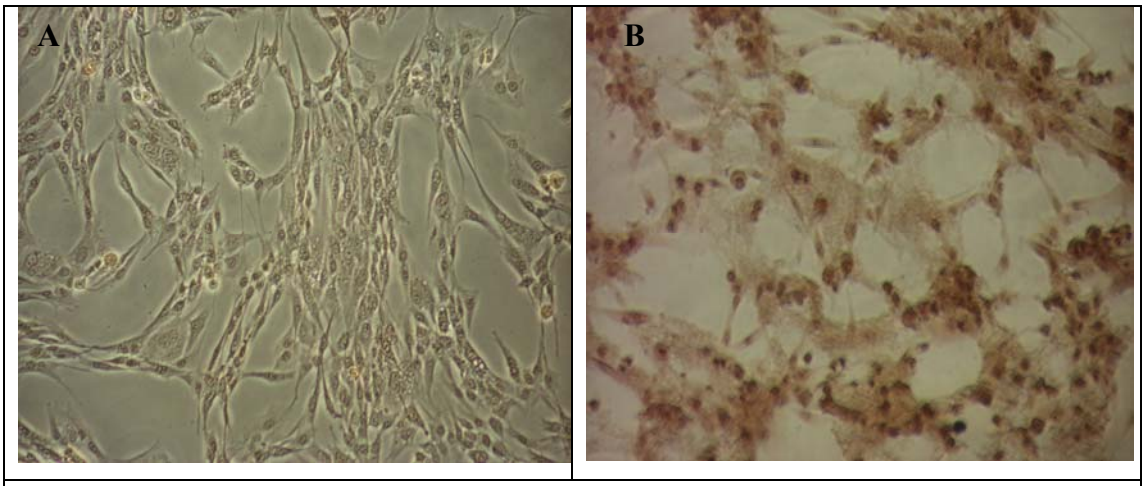


Figure 4.12 Immunohistochemisty staining of BHK cells 48 hour post infection of SFV/mMTA1 (A-Non infected cells, B-SFV/mMTA1 infection)

Finally, to confirm MTA1 protein production in cells infected with SFV-MTA1 vectors, BHK cells were infected with $1x10^6$ pfu/ml of the activated virus for 1 hour and generation of FLAG-tagged MTA1 protein was visualised using immunohistochemistry. Infected BHK cells were stained with FLAG M2 (Sigma, UK) primary antibody followed by anti-mouse secondary antibody conjugated to HRP. DAB solution was added after washing three times to visualise brown staining of the infected cells. As seen in figure 4.12, negligible background staining was observed in control cells, whereas dark brown staining was observed in infected BHK cells indicating FLAG tagged MTA1 production. Moreover, immunohistochemistry revealed MTA1 to be localised in the nucleus, which agrees with our immuno-fluorescence assay.

4.3 Discussion

Nucleic acid vaccines can be delivered in a variety of forms such as plasmid DNA or RNA based, through modification of cells *in vitro* and viral/bacterial vectors. Choice of vector is dictated by its purpose and application. Nucleic acid vaccines offer the flexibility and specificity of generating potent immune response against multiple antigens and in certain cases are also able to polarise that response. Vaccination with whole protein antigens is expensive and time consuming to generate. Moreover, injected proteins are likely to be degraded within a few days, whereas nucleic acid vaccination can direct protein expression *in vivo* for few months or longer due to integration in the host genome.

4.3.1 Generation of mammalian expression vector encoding MTA1

Various vaccination methods have been shown to generate immune prevention and rejection responses, such as peptide, DNA, viral etc. Although reverse immunology approaches and transgenic mice application have made the identification of immunogenic and naturally processed peptides from tumour antigens relatively quick, it can still be very difficult, especially for self antigens. Most of T cells specific for high affinity epitopes from self-antigens are known to be either deleted or tolerised via central and peripheral tolerance mechanisms. Hence, identification of peptides from such antigens might require testing hundreds of peptides with medium binding affinity to particular MHC molecule, which is time consuming and expensive. Nucleic acid vaccination overcomes this limitation as it allows vaccination with whole gene or part of it and is applicable to the whole population. Various co-stimulatory molecule or cytokine genes can be included in these vaccines; moreover, antigens can be directed to specific antigen processing compartments by including signal sequences (Liu *et al*., 2004; Stevenson *et al*., 2004). As an alternate strategy to generate immune response against MTA1 derived peptides, mouse MTA1 was cloned into a mammalian expression vector. Moreover, recent studies by Houghton *et al*, have proved xenogeneic immunisation to be an extremely useful tool in breaking tolerance to several self-antigens (Bowne *et al*., 1999; Gold *et al*., 2003; Naftzger *et al*., 1996). Hence, to investigate xenogeneic immunisation, the human MTA1 gene was also cloned in an expression vector. Having assembled and constructed the expression vector, it is essential to confirm that it performs as expected before moving into *in vivo* studies. This was confirmed by transfecting cells with these expression vectors and analysing the mRNA expression of MTA1 by RT-PCR (data not shown). However, mRNA level of a gene does not always reflect its protein expression, which needs to be confirmed independently. Human embryonic kidney 293 cells were transfected with pcDNA3-mMTA1/hMTA1 plasmids using lipofectamine reagent and

MTA1 protein expression was determined after 48 hours by western blotting. High levels of MTA1 expression was observed in transfected cells compared to control non-transfected cells, establishing the protein expression capability of the generated expression vectors.

4.3.2 SFV vector generation and optimisation

Numbers of viral and bacterial vectors have been used in recent years for modification of cells *in vitro* as well as for *in vivo* application in animals and humans. Viruses and bacteria are naturally immunogenic and efficient at infecting different cell types which make them potent weapons for immunotherapy. Adenovirus and pox virus vectors are the most widely used viruses to date but they have their limitations as well. One of the most important considerations in choosing a vector is the possibility of generating replication competent vectors *in vivo*, which could be potentially lethal in immuno-suppressed cancer patients.

Alpha viruses (Semliki Forest Virus, Sindbis virus, Venezuelan Equine Encephalitis virus) are generating considerable interest for immunotherapy approaches. Necessity to simultaneously transfect with two different plasmids (helper and replicon) for virus production, limits the chances of generating replication competent virus *in vivo*. As an added bio-safety level, virus produced is inactive and need activation by cleavage of p62 protein. These mechanisms make SFV as a safe option for human immunotherapy. Original RNA based SFV vector system was modified and improved by DiCiommo and Bremner by constructing a DNA based system, which obviates the need for *in vitro* transcription and makes manipulation easier. However, DNA based expression system does have an adverse effect on the titre of virus generated. Co-transfection of DNA plasmids produced titres of approximately 1-2x10^6 pfu/ml, whereas by transfecting *in vitro* transcribed RNA generated 10-100 times higher titre (DiCommio & Bremner., 1998; Liljestrom & Garoff., 1991).

Recombinant SFV (rSFV) particle generation was optimised for various parameters known to affect it. Co-transfecting β-gal reporter plasmid along with helper plasmid allowed easy optimisation of virus production and titre. Firstly, different methods of transfection were compared (Calcium phosphate, Lipofectamine, GeneJuice) and the calcium phosphate method was determined to be cheapest and most reliable method for HEK 293 cells, as previously suggested (Bremner *et al*., 1998). Thereafter, different molar ratios of helper to replicon plasmid were co-transfected in order to determine the best concentrations. Although different ratios did not have significant effect on virus titre production, molar ratio of 1:3 was slightly better and was used for virus generation. Higher concentrations of helper plasmid would mean that most of the cells transfected with replicon plasmid would also be transfected with helper plasmid. Lastly,

virus harvested from cell supernatant of co-transfected cells was of low titre and previous experience in our laboratory suggested that most of the virus was retained inside the cells and lysing them was likely to enhance the titre. SFV is a cytolytic virus and replicates inside the infected cell for 48-72 hours before causing cell apoptosis. Indeed, a single cycle of freeze-thaw significantly increased the titre of the virus. SFV virus was also found to be efficient at infecting different cell lines (human and mouse) (data not shown). Finally, to generate recombinant SFV (rSFV) particles encoding mMTA1 and hMTA1, these genes were first cloned into pSMART2b vector. Protein expression from these vectors was confirmed by transfecting cells followed by determination of MTA1 protein expression 48 hours later by western blotting. Moreover, rSFV-MTA1 particles were generated and after activation they were used for infecting BHK cells *in vitro,* leading to high level of MTA1 production, thus confirming viral activity and efficacy.

4.3.3 SFV vector can infect dendritic cells

CD8+ T cell priming is an essential requirement in most cases for tumour rejection. This CTL generation is related to the ability of vectors to infect DC directly or indirectly. Rationale for vaccine design for cancer requires knowledge of antigen presentation following vaccination. Following SFV vaccination *in vivo*, there are two likely scenarios. DC can either become infected directly, producing virus encoded proteins, degrading them and presenting them on MHC class I molecules or they may uptake antigens indirectly after lysis of other infected cells and present them on MHC class I molecules through cross-priming. Several studies have produced contradictory results regarding DC infection by SFV. Immunisation with DC pulsed with SFV encoding tumour antigens or cytokines has shown to be efficient in generating CTLs and tumour protection/rejection, which suggests that SFV can efficiently infect DC (Yamanaka *et al*., 2003; Yamanaka *et al*., 2002). However, these findings have been contradicted by Huckriede *et al* (2004), who revealed inefficient infection of DC by SFV *in vitro* (0.14% DC infection at MOI of 1000). Furthermore, they hypothesised that CTL priming *in vivo* by SFV immunisation was due to cross-priming. In order to prove this, they generated SFV particles with different abilities to produce stable influenza nucleoprotein (NP) and suggested that rapid intracellular degradation of NP would not produce enough protein for cross priming, whereas stable high level expression of NP may improve cross-priming. Indeed, ubiquitin tagged NP (rapidly degradable form) produced by SFV was inefficient in generating CTLs, whilst SFV producing high levels of NP generated more potent immune response as measured by cytotoxicity assay and tetramer analysis (Huckriede *et al*., 2004). Interestingly, SFV producing

NP at normal levels also generated good CTL responses, suggesting that cross-priming might not be the only mechanism for CTL priming on SFV immunisation.

To resolve this discrepancy, we investigated the *in vitro* efficiency of SFV to infect Balb/c mice bone marrow derived DC. Modified protocol of Inaba *et al* (1992) was used to generate BM-DC from mice hind limbs and more than 80% cells expressed DC markers (CD11c, MHC class II and CD40)(fig 4.11c). In our hands, up to 20% of DC were infected with SFV/B-gal as visualised with B-gal assay (MOI of 10), compared to maximum of 0.15% by Huckeriede *et al* (Huckriede *et al*., 2004). This difference cannot be attributed to titre of virus used for infection as even at MOI of 1000, no significant change in DC infection by SFV was observed by Huckriede. Moreover, infectivity was independent of DC maturation status as well as strain of mice (data not shown). Different capabilities of DC infection by SFV in these studies could be due to different methods of SFV production used (RNA based vs DNA based) or the method of DC generation in these studies. It is noteworthy, that our method of BM-DC generation is very similar to that described by Huckriede *et al*, and is unlikely to cause this incongruity. Most of the viral vectors used are efficient at DC infection and can also cause maturation of the DC. Although, SFV was reasonably efficient at infecting DC, it did not seem to affect their maturation (data not shown).

In conclusion, expression vectors for human and mouse MTA1, as well as the SFV vector for both were constructed and investigated for their ability to produce protein *in vitro* following transfection. These vectors need further *in vivo* investigation for their ability to generate immune response and tumour prevention/rejection.

Chapter 5 Investigating potential immunogenic and naturally processed peptides from MTA1

5.1 Introduction

Tumour vaccination employs tumour antigens in order to generate an immune response for rejection of cancerous cells. In general, antigens shed by tumour cells are taken up by the APC, processed and are presented on their surface bound to MHC class I and class II antigens (fig 5.1). These MHC-peptide complexes are recognised by the CD8+ and CD4+ T cells and this interaction along with other co-stimulatory molecules trigger the immune system to react against cancer cells presenting the same peptides (at least in case of CD8+ T cells).

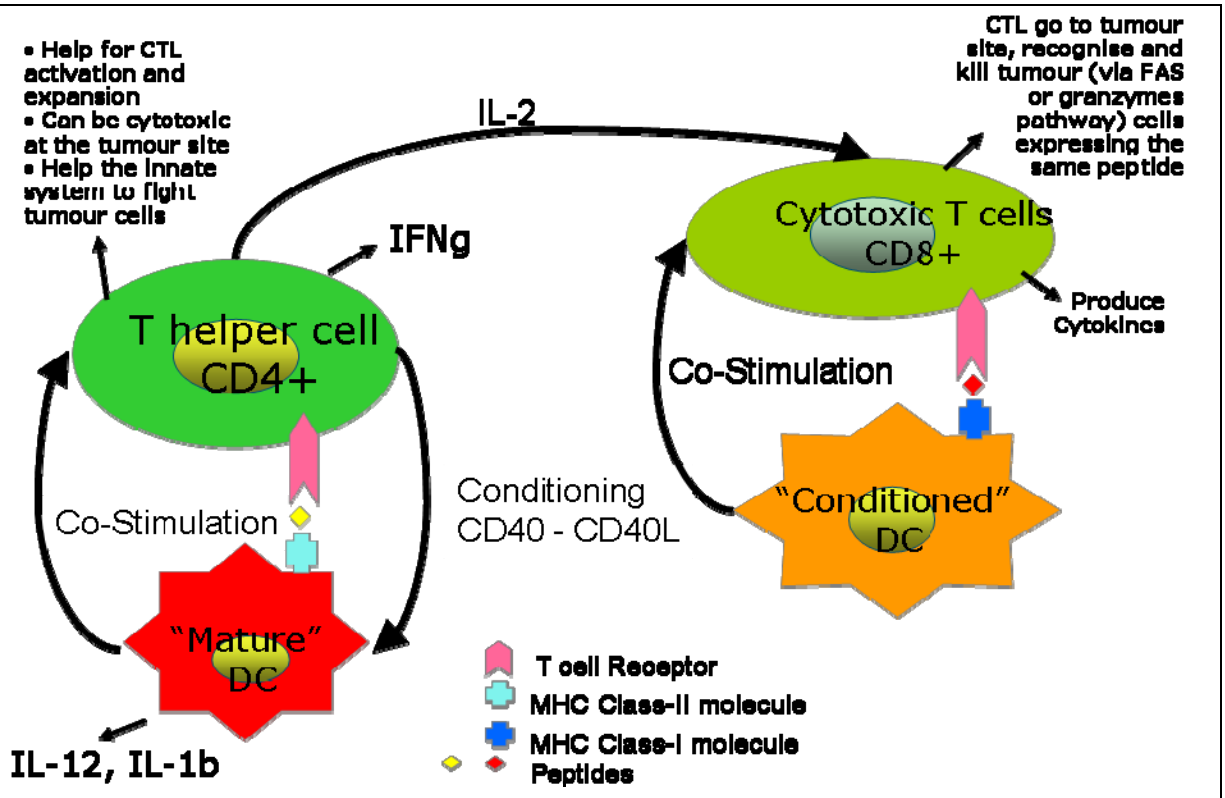


Figure 5.1:- Schematic diagram of interaction of DC with CD4+ and CD8+ T cells to initiate an immune response.

Identification of MHC class I and class II epitopes that can be processed and presented *in vivo* to prime T cells holds tremendous promise for several reasons. Firstly, immunisation with a CTL epitope is able to mediate tumour regression in several animal models and generate peptide specific CTLs in human cancer patients. Wild type or modified peptides (to increase MHC binding and immunogenicity) can either be delivered on their own with an adjuvant or pulsed on

DC before administration. Also, peptide vaccination is generally considered better than whole protein administration as immune system is likely to be tolerised to dominant portions of the protein but not all the peptides (Disis *et al*., 1996). Being convenient chemical entities, they are cheap, safe and easy to synthesise, and their administration is unlikely to generate serious ethical considerations, unlike viral vectors. Another important application of peptide discovery is their use for making of MHC class I tetramers, which have started to revolutionised immune monitoring of cancer patients before and after vaccination. Lastly, peptide identification can lead to sequence information of TCR with the ability to bind to it. Autologous T cells transfected with TCR for known naturally processed epitopes before adoptive transfer can remarkably increase the persistence of T cells and clinical benefits gained from it (Morgan *et al*., 2006). Thus, identification of these peptide epitopes holds immense promise for the field of immunotherapy and has led to intense efforts in the last few years for their discovery.

5.1.1 Approaches for epitope identification

Since the identification of the first human tumour antigen and CTL epitope, MAGE-1, rapid strides have been made for development of novel techniques for CTL epitope identification. Two most commonly used approaches are "direct immunology" and "reverse immunology" (Figure 5.2). Direct approaches begins with documented T cell reaction against tumour cells and refines to identify the peptide, whereas the reverse approach predicts T cell epitope and then investigates its immunogenicity.

1. ***cDNA expression cloning :-*** Screening of the genomic DNA library derived from melanoma cells using the autologous melanoma reactive CD8+ T cells led to the identification of the first tumour antigen MAGE-1 by Boon and colleagues in 1991 (van der Bruggen *et al*., 1991). In this approach, DNA or cDNA libraries isolated from tumour cells are transfected into cells expressing the relevant MHC molecule and the resulting cells are then screened for their ability to generate an immune response from the tumour infiltrating T cells by means of either target cell lysis and/or cytokine release. Further experiments using truncating regions of MAGE-1 gene, resulted in the identification of a small region of DNA encoding the peptide recognised by the CTL, thus leading to the discovery of the first nine amino acid peptide target (Traversari *et al*., 1992). Substantial improvements have since been made to this approach by constructing better expression libraries (Smith *et al*., 2001). However, one of the major limitations of this

approach is the isolation and generation of CD8+ T cell clones from the tumour infiltrating lymphocytes, which can be immensely difficult.

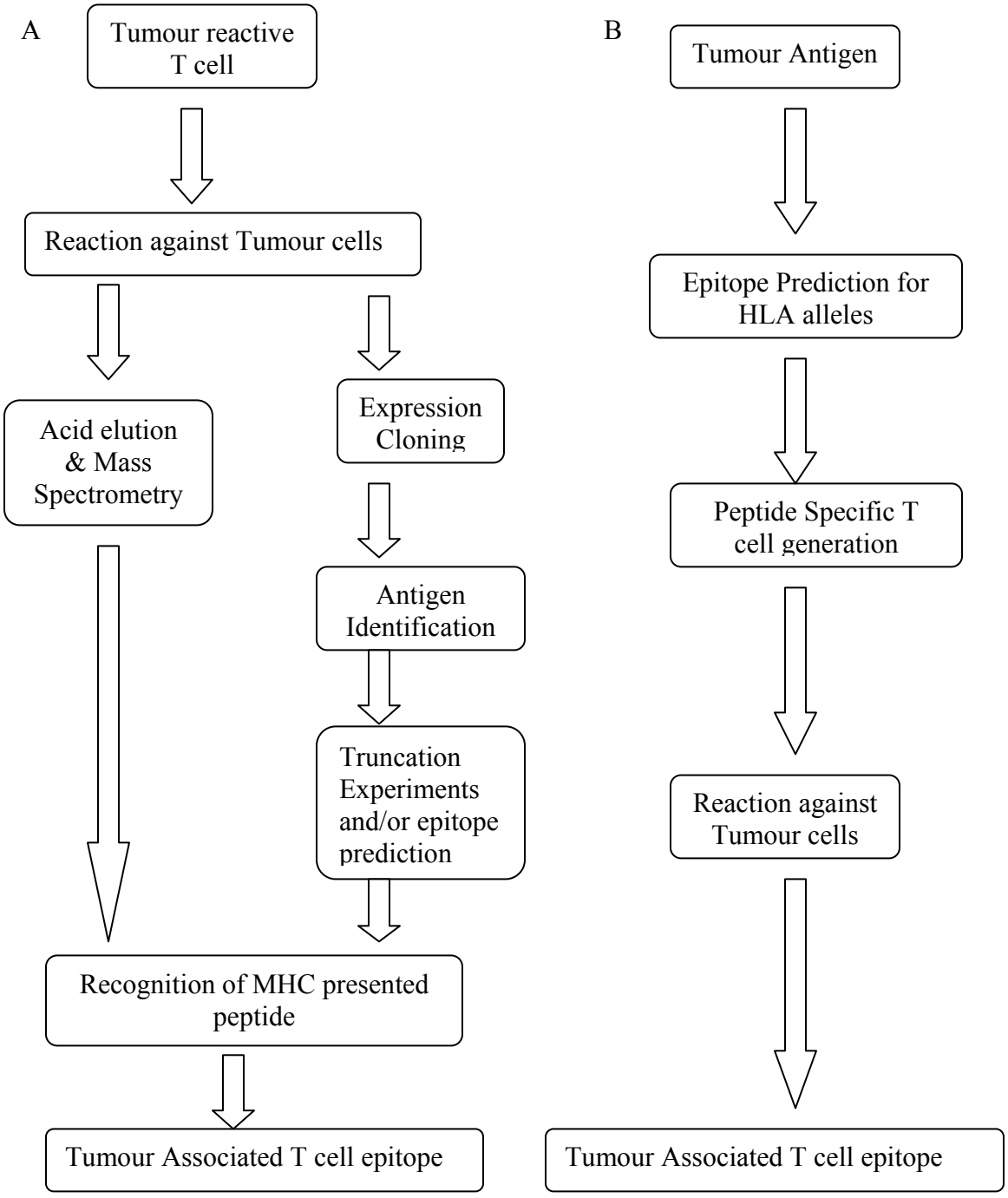


Figure 5.2 Direct and Reverse Immunology approaches for epitope identification (Adapted from Stevanovic, 2002)

2. ***Biochemical Approach:***- Peptides presented by the MHC class I epitopes can be directly identified by elution of the MHC class I-peptide complex from the surface of the tumour cells

and then analysing them by mass-spectrometry. Storkus *et al* pioneered this approach when they first eluted an MHC class I bound peptide from influenza A infected cells, by treating them with citrate phosphate buffer at pH3.3. Treating cells with this buffer led to the elution of the cell surface MHC bound peptide, which were subsequently fractionated by reverse-phase high performance liquid chromatography (RP-HPLC) (Storkus *et al*., 1993). Although, this approach was applied to the identification of a viral peptide, several tumour associated peptides have since been discovered via this method (Cox *et al*., 1994; Clark *et al*., 2001; Bonner *et al*., 2002). The advantage of this technique is the fact that it can directly identify naturally processed peptides, which can serve as part of peptide vaccines. Moreover, this approach is able to identify post-translationally modified peptides. However, it requires large number of tumour cells to be generated for elution. Although MHC class I peptide elution has been reliably demonstrated by mild acid elution, identification of MHC class II peptides by this method has proved elusive owing to the variability of the length of the MHC class II peptides (Halder *et al*., 1997). Also, MHC class II molecules are fairly stable at pH 3.3 and can only be eluted at much lower pH, which is generally toxic to the cells (Danielle Barry, personal communication). Major limiting factor for this characterization is the requirement for highly sensitive equipment to identify low number of tumour specific peptides from thousands of irrelevant normal peptides. Moreover, identification of peptides presented on the surface of tumour cells does not guarantee its immunogenicity and they still need to be assessed *in vitro* assays.

3. ***Reverse Immunology:*** - Knowledge gained from previously identified peptides through other approaches is used to predict peptides which are most likely to bind to particular HLA alleles. Use of this technique for peptide identification is known as 'reverse immunology' method. Protein sequence of the antigen of interest can be screened for potential immunogenic peptides using a number of algorithms available on the World Wide Web. 'SYFPEITHI' and BIMAS are the most commonly used algorithms for this purpose. In these algorithms, peptides are given a score according to their binding prediction to the various HLA molecules. This prediction score is based on presence of specific amino acids at the anchor positions of the peptide. For example, for HLA-A201 molecule, positions 2 and 9 are the anchor residues and presence of certain residues (aliphatic amino acids leucine, isoleucine, valine or methionine) at these positions would enable this peptide to bind this particular HLA molecule with strong affinity. Following

prediction, the peptides are synthesised and their immunogenicity tested, using either *in vitro* models with human PBMC or transgenic mouse models. T cells are generated from PBMC of cancer patients/normal healthy donors or from transgenic mice (carrying particular HLA allele) after immunisation with the peptides. The final step in the approach is the testing the recognition of human tumour cells (expressing the antigen) by T cells, raised against these peptides. The drawback of this method is that the protein sequence of the antigen has to be known and immunogenicity of the peptide does not guarantee its natural processing. However, improvements have been made to the earlier versions of the prediction algorithms to account for the natural processing of the peptide. Several peptides, for MHC class I and class II molecules have been identified using this approach till date (Rojas *et al*., 2005; Touloukian *et al*., 2000; Zarour *et al*., 2000). The advent of MHC-transgenic mouse models has boosted efforts in epitope identification through 'reverse immunology' as they can save a considerable amount of time and resources, and have several advantages over cell cultures. Apart from efficient exploration for immunogenic peptides, natural processing can also be easily looked at in these transgenic mice by allowing *in vivo* cells to process genes/proteins and present peptides. Interestingly, natural processing of the transgenic mouse models and humans have shown to be similar as several peptides identified using transgenic mice have shown to be naturally processed by the tumour cells in humans (Rojas *et al*., 2005; Theobald *et al*., 1995; Theobald *et al*., 1997). However, it is noteworthy that transgenic mice do not necessarily process antigens as humans (Street *et al*., 2002). Also, transgenic mice are only available for the most common HLA alleles. Another variant of this approach is using the predicted peptide to calibrate a capillary-chromatography-mass spectrometric system and by comparing these results with the ones obtained after acid-elution, naturally processed peptides can be identified. This approach has been termed as 'predict, calibrate and detect' method, although it does not provide information regarding the immunogenicity of the peptide (Pascolo *et al*., 2001).

Apart from the anchor residues in the peptide sequence, other amino acids also have an important role in HLA binding (Udaka *et al*., 1995). To overcome these limitations, recently more powerful tools such as multilayered artificial neural networks (ANN) and HMM have been developed. These tools have been reported to be more accurate in their predictions (approximately 80% sensitivity and specificity) (Viatte *et al*., 2006). A recent study used ANN to predict peptides

from known tumour antigens and further investigated peptides, most of which would not be predicted by motif based algorithms. Of the 7 peptides predicted, 6 generated good CTL responses and T cells specific for 4 of these peptides could be detected in melanoma patients (Bredenbeck *et al*., 2005). Interestingly, most of these peptides were not good HLA ligands, suggesting that in relying on popular motif based algorithms several immunodominant peptides may be neglected. This study also underlines the importance of MHC-peptide complex affinity for TCR instead of simply reliant on HLA binding to peptide. A recent study highlighted the limitations of this approach by directly identifying a peptide produced from gp100 antigen by splicing of the protein; such peptides would never be predicted by any algorithms as having immunotherapeutic potential (Vigneron *et al*., 2004).

In spite of the progress achieved in the field of 'reverse immunology' for the prediction of MHC class I peptides, its application for MHC class II peptides has lagged behind. Apart from the ignored role of CD4+ T cells in anti-tumour response, MHC class II structure related difficulties have also hindered progress in this area. MHC class II peptides can vary between 12-22 amino acids in length due to an open peptide binding groove, with most peptides being 13 to 16 amino acids long. These peptides have no definitive amino or carboxy terminal making it extremely difficult to predict high binding peptides with higher accuracy (Touloukian *et al*., 2000). Although, several algorithms do provide MHC class II predictions, their sensitivity and specificity is lower than that of class I epitopes.

5.1.2 Aims of the Chapter

This study aims to predict immunogenic peptides from MTA1 using web based algorithms and testing them using syngeneic and transgenic mouse models. HHD II (HLA-A0201) transgenic mice would be utilised for class I peptides identification. Although the main focus would be on generation of CTL responses, MHC class II peptides would also be investigated using HLA-DR0101 and HLA-DR0401 transgenic mice would be used for MHC class II peptides. Following determination of immunogenicity, natural processing of the peptides will be further analysed.

5.2 Results

5.2.1 Identification of HLA-A0201 restricted peptides from MTA1 using HHD II transgenic mice

5.2.1.1 T2 Binding assay

Human MTA1 protein sequence was screened for potential binding peptides to HLA-A2 using web based algorithm 'SYFPEITHI'. Three peptides with highest binding affinity were selected and synthesized. These were named MTA1 22, 57 and 109. However the prediction of the algorithm does not necessarily mean that the peptide is a good binder to the HLA-A2 molecule. Hence, before testing the immunogenicity of these peptides in transgenic mouse model, their HLA-A201 binding affinity was tested in a T2 binding assay. T2 cells are human lymphoblastoid cells and express HLA-A2 molecules. However, they are TAP deficient, which is essential in transporting the peptides to the ER where they will bind to HLA molecules, hence T2 cells express empty MHC molecules on their surface, which are rapidly internalized unless stabilised by externally added peptide. T2 cells were incubated with different concentrations of the peptide for 12 hours and the stability of the HLA-A201 molecule was determined using FACS, after washing off the excess peptide.

Binding of the peptide to the empty HLA-A2 molecules on the surface of the T2 cells stabilises it or else the HLA-A2 molecules are regularly internalized and degraded in the cell before being replaced by other empty HLA molecules. Thus, increased mean fluorescence intensity in the FACS analysis provides an idea of the binding affinity of the peptide i.e. a higher binding peptide will have higher mean fluorescence intensity than a weak binder. From the mean fluorescence intensity, the fluorescence ratio (FR) was calculated as follows:-

$$FR = \frac{\text{Mean channel fluorescence of test peptides}}{\text{Mean Channel fluorescence of DMSO}}$$

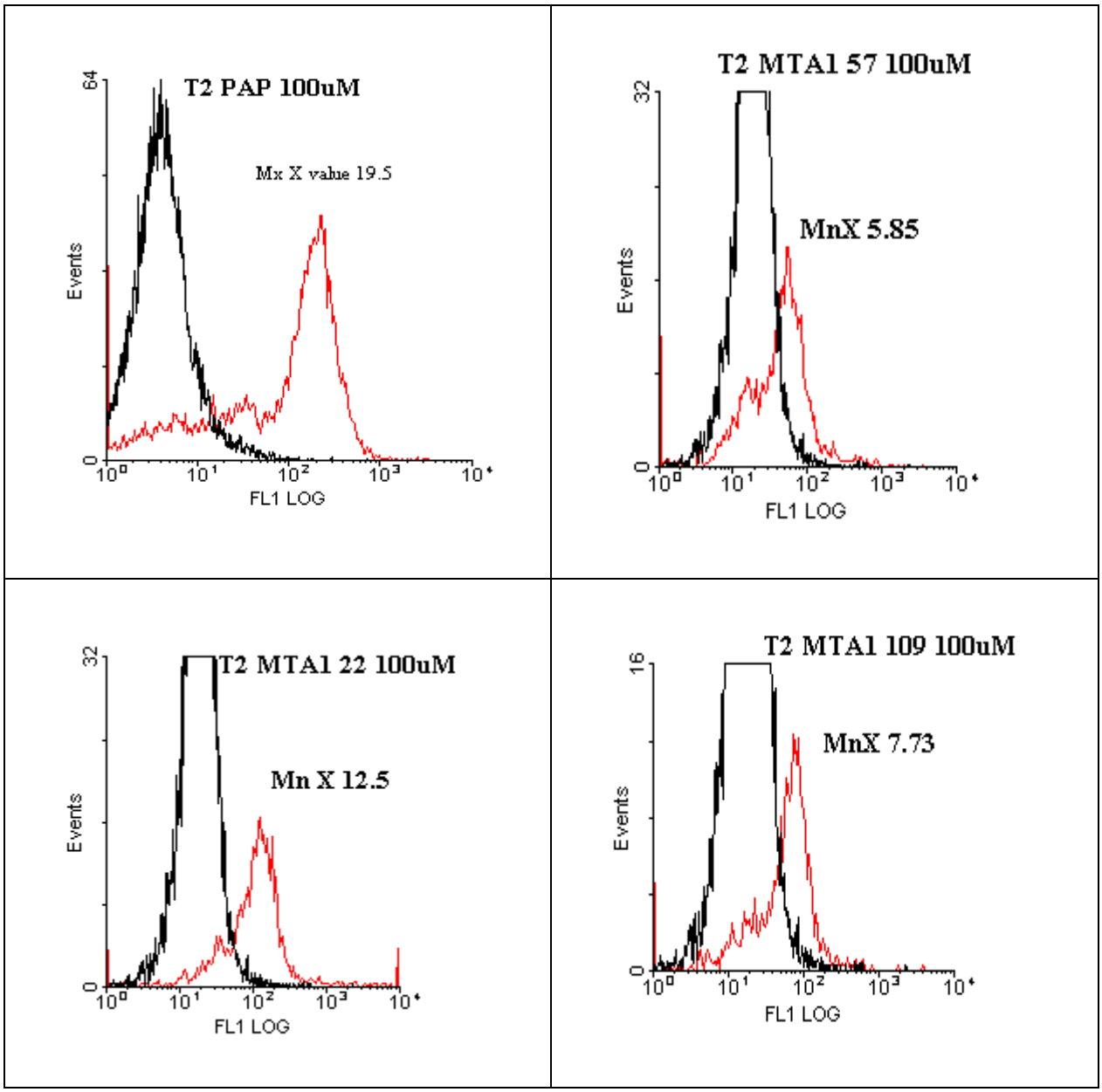


Figure 5.3: - FACS analysis of the T2 binding assay for human MTA1 peptides for HLA-A201 molecules*.T2 cells which are TAP deficient and express empty HLA-A201 molecules, were incubated with different concentration of peptides overnight. Excess peptide was washed off and the cells were stained for the HLA-A201 using primary antibody (HB54) and secondary antibody (goat-anti mouse FITC) to determine the stability of the HLA-A201 molecules on the surface of T2 cells. Results shown are for 100µg/ml concentration of peptide.*

The fluorescence intensity was compared with DMSO as all the peptides were suspended in DMSO. Any peptide with FR<1 was considered as a non binder, 1<FR<1.5 was considered as a weak binder and FR>1.5 was taken as an indicator of a strong binder. Even though, the predictive binding scores for both MTA1 22 and MTA1 57 were the same, on T2 binding assay

MTA1 22 was observed to bind to HLA-A2 more strongly and hence stabilized more HLA-A2 molecules on T2 cells as suggested by the fluorescence ratio (figure 5.3). FR for MTA1 22 was 3.5 when used at 100µg/ml and it seemed to be a very strong binder, whereas for MTA1 57 and MTA1 109 it was 1.67 and 2.2 respectively at the same concentrations (figure 5.4 & table 5.1). Interestingly, although the predictive binding score for MTA1 109 is lower than MTA1 57, its FR is higher, suggesting that predictive binding score might give an idea of good binders but they are not necessarily accurate. Moreover, better binder to HLA molecules, do not necessarily guarantee immunogenicity and these results would have to be correlated with generation of an immune response when injected in the HHD II mice, which are transgenic for HLA-A2 molecules.

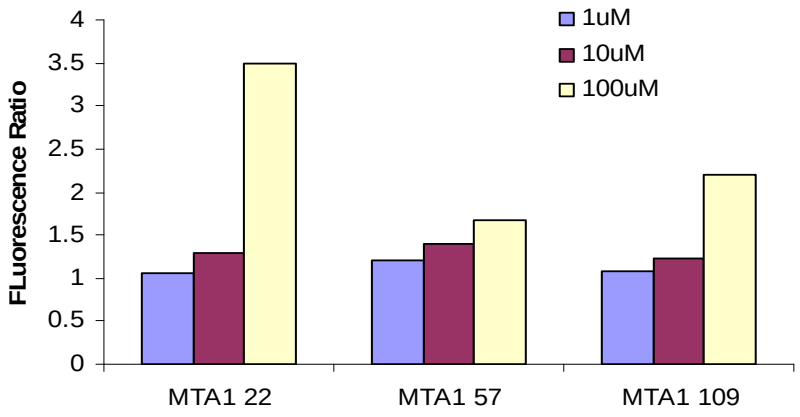


Figure 5.4 Fluorescence Ratio (FR) of human MTA1 peptides at various concentrations in T2 binding assay. Experiment was repeated twice with similar results.

Table 5.1:- Results of T2 binding assay of human MTA1 peptides

Protein	**Peptide Region**	**Predicted**	**Fluorescence Ratio**		
		Score	**1µM**	**10µM**	**100µM**
PAP	135-143	24	-	-	5.58
hMTA1	22-31	30	1.06	1.3	3.5
hMTA1	57-65	30	1.2	1.39	1.67
hMTA1	109-117	27	1.08	1.23	2.2

PAP:-Prostate Acid Phosphatase was used as a positive control

5.2.1.2 Investigating immunogenicity of peptides predicted from human MTA1 sequence for HLA-A0201 (MTA1 22, 57 and 109)

HHD II transgenic mice were immunised once with 100µg of the MHC class I peptides in 1:1 emulsion with IFA as described in methods section. Murine MHC class II peptide derived from hepatitis B was used as a helper peptide for each immunisation. Seven days after immunisation, splenocytes were harvested and re-stimulated *in vitro* with LPS blasts loaded with relevant peptide for 5 days. On day 6, the immunogenicity of the peptides was determined by evaluating the T cells generated against these peptides in a standard 4 hour chromium release assay. RMAS/A2 cells, relevant and irrelevant peptide pulsed were used as targets to determine the specificity of the response (Figure 5.5). Peptide specific killing of pulsed RMAS/A2 cells was detected for peptides MTA1 22 and MTA1 57. Minimal lysis of RMAS/A2 cells pulsed with irrelevant peptides was observed confirming the specificity of the immune response. Moreover, this lysis was completely blocked by HLA-A2 antibody, further consolidating the fact that T cells generated were capable of killing in an HLA-A2 restricted manner (data not shown). T cells could not be generated against MTA1 peptide 109 and hence was deemed non-immunogenic and no further experiments were carried out with this peptide (data not shown).

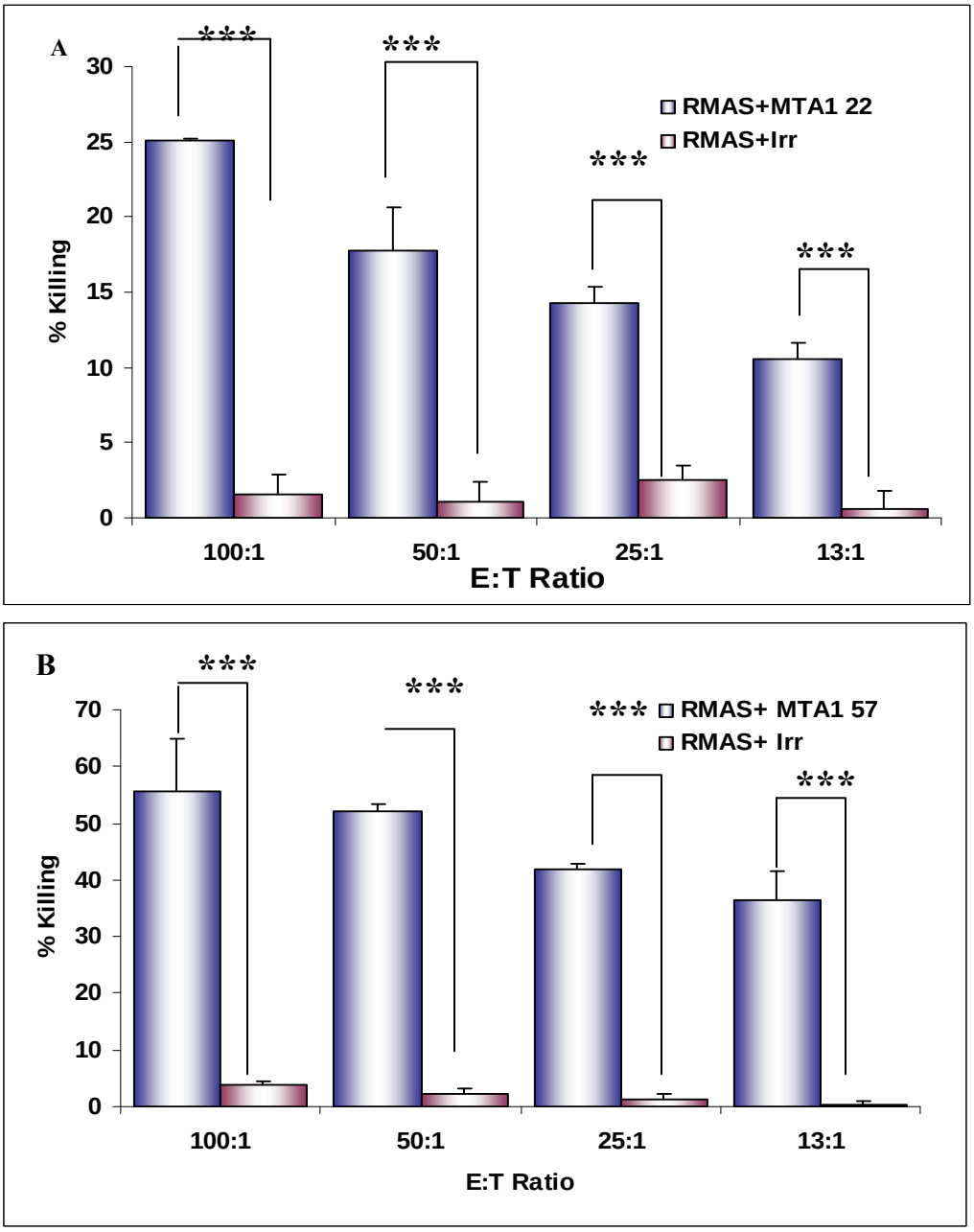


Figure 5.5 Cytotoxicity assay using T cells generated from HHD II mice immunised with hMTA1 peptides 22(A)(n=4) and 57(B) (n=4)

*5.2.1.3 Determination of natural processing of immunogenic pepti*des

In order to investigate the natural processing of MTA1 peptides, we decided to use the gene gun mediated full length MTA1 DNA vaccination followed by *in vitro* evaluation of splenocytes from the immunised mice for specific peptides. To validate this strategy, p53 cDNA was used as a positive control. Mice were immunized 3 times (7 days interval) with a p53 encoding plasmid

using a gene gun. A week after the last immunisation, splenocytes were harvested and re-stimulated with a previously published naturally processed p53 149 peptide (Theobald *et al*., 1995). Following five days of *in vitro* re-stimulation, T cells generated against this peptide were evaluated in a cytotoxicity assay against target cells pulsed with p53 149 peptide or an irrelevant peptide. As seen in figure 5.6, peptide specific killing was observed only for EL4-HHDII cells pulsed with the p53 149 peptide, whereas no killing of irrelevant targets was observed. As a control for this experiment, splenocytes harvested from naïve mouse were re-stimulated similarly and evaluated for killing. No peptide specific killing could be generated from naïve mice (data not shown). Thus, *in vitro* re-stimulation is not enough to generate peptide specific CTLs against p53 peptide 149. This suggests that in mice immunised with p53 cDNA, p53 protein was produced and processed, presenting p53 149 peptide *in vivo* to prime specific T cells against it, which were then expanded *in vitro* with the use of LPS blasts.

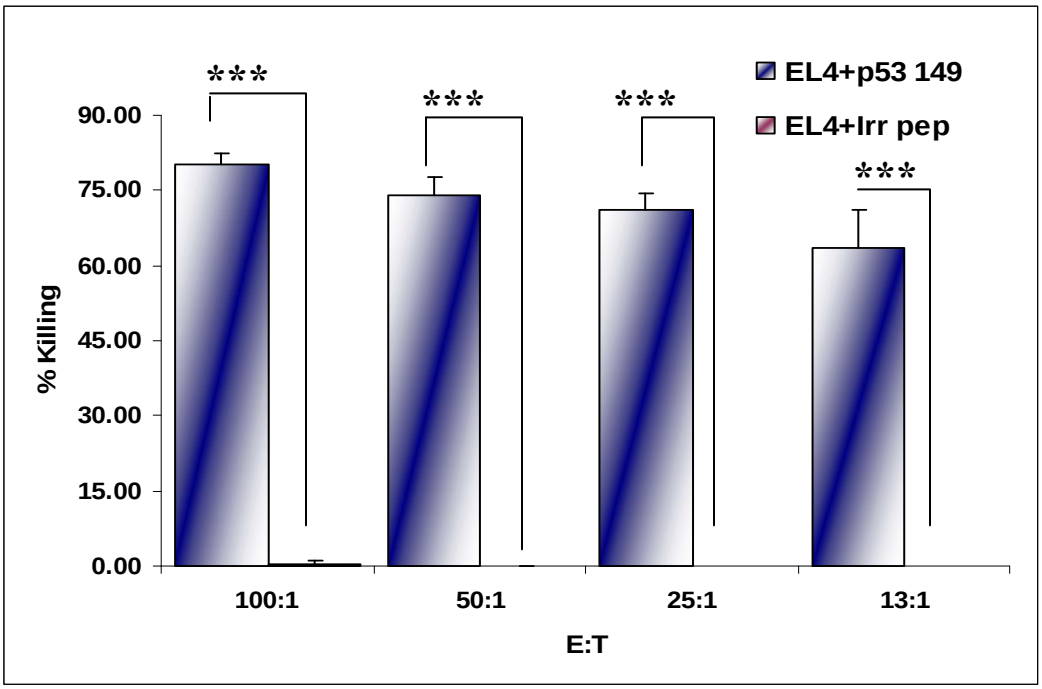


Figure 5.6:- Cytotoxicity assay using T cells generated from HHD II mice immunised with plasmid encoding p53 followed by a 5 day in vitro re-stimulation with p53 149 peptide*. Result shown is a representative of 2 independent experiments (n=4)(***=p<0.0001)*

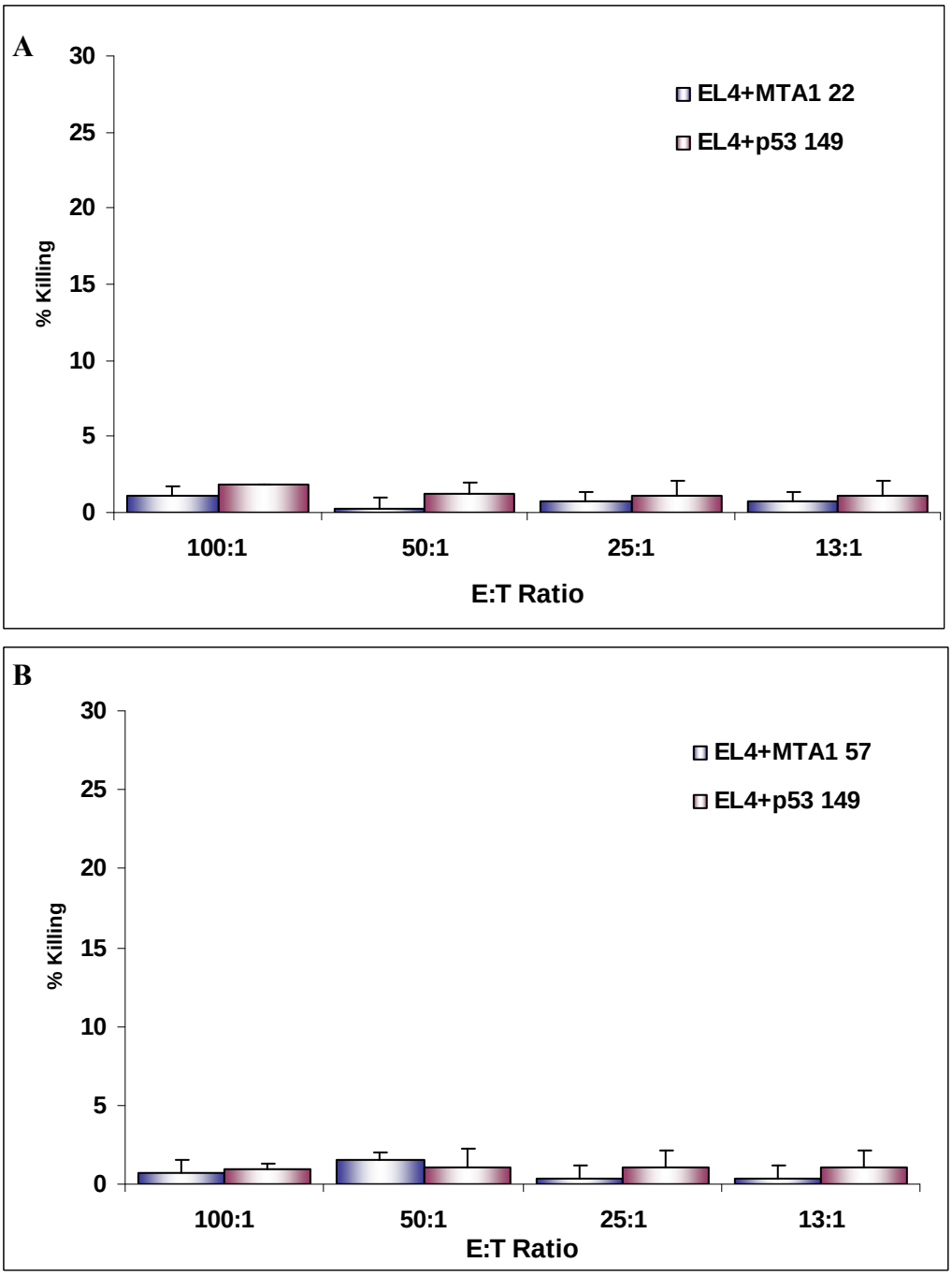


Figure5.7:- Cytotoxicity assay against EL4/HHD II cells pulsed with MTA1 peptides after 3 immunisations with MTA1 DNA and in vitro restimulation with MTA1 22(A) and 57(B) peptides. *As a control EL4/HHDII cells pulsed with p53 149 peptide were used. Results shown are representative of two independent experiments (n=4).*

Natural processing of MTA1 peptides 22 and 57 was investigated after immunising with MTA1 plasmid using gene gun. However, no peptide specific killing was observed in these experiments, suggesting that both immunogenic peptides identified for MTA1 were not naturally processed (Figure 5.7). Other strategies were also used to confirm this finding. T cells generated against

these peptides after peptide immunization (as before) were evaluated for the killing of human HLA-A2+/MTA1+ tumour cell line as well as for EL4/HHD cell line, transfected with human MTA1 (to increase the expression of MTA1) (data not shown). Later experiment, also confirmed that the non killing of EL4/HHD cells was not due to low surface expression of the processed peptide, which were below the activation threshold level for mediated CTL lysis.

5.2.2 Identification of MHC class I peptides from murine MTA1 peptides in syngeneic Balb/c mice

5.2.2.1 Optimisation of Immunisation protocol for balb/c mice

Unlike for the HHD II mice, a single immunisation with peptide emulsified in IFA was not sufficient to generate peptide specific CTL. In order to optimise this method, the TPH peptide (derived from β-galactosidase) was used as a control. Various parameters were evaluated to determine the best protocol for optimisation and *in vitro* re-stimulation (Figure 5.7). Firstly, a single immunisation regime was compared to immunise and boost regime, followed by LPS blast mediated *in vitro* re-stimulation (fig 5.8 A & B). It was observed that boosting after seven days of immunisation was more potent in generating peptide specific CTLs as observed from the cytotoxicity assays. Potent *in vitro* re-stimulation may cause antigen induced cell death of the peptide specific T cells. To investigate this possibility, two different *in vitro* re-stimulation methods were compared. LPS blast mediated re-stimulation generated a more potent peptide specific T cell response, as observed by higher killing in cytotoxicity assay (25% vs 5%), although specific killing was observed with both methods (fig 5.8 C & D). Adjuvants are also likely to affect the potency of the immune response generated. CpG as an immune modulator has been highly successful in a number of animal models and is also currently being used in human clinical trials. Hence, CpG as an adjuvant was compared to IFA (fig 5.8 E & F). In balb/c mice, immunising with peptide and CpG was unable to generate any significant CTL response. Whether this is related specifically to the strain of mice, is not known and needs further investigation, as CpG was found to be a very potent adjuvant in C57BL/6 mice (data not shown). Thus, for all future immunisations in balb/c mice, two rounds of immunisations seven days apart, followed by LPS blast mediated re-stimulation was considered the optimum protocol.

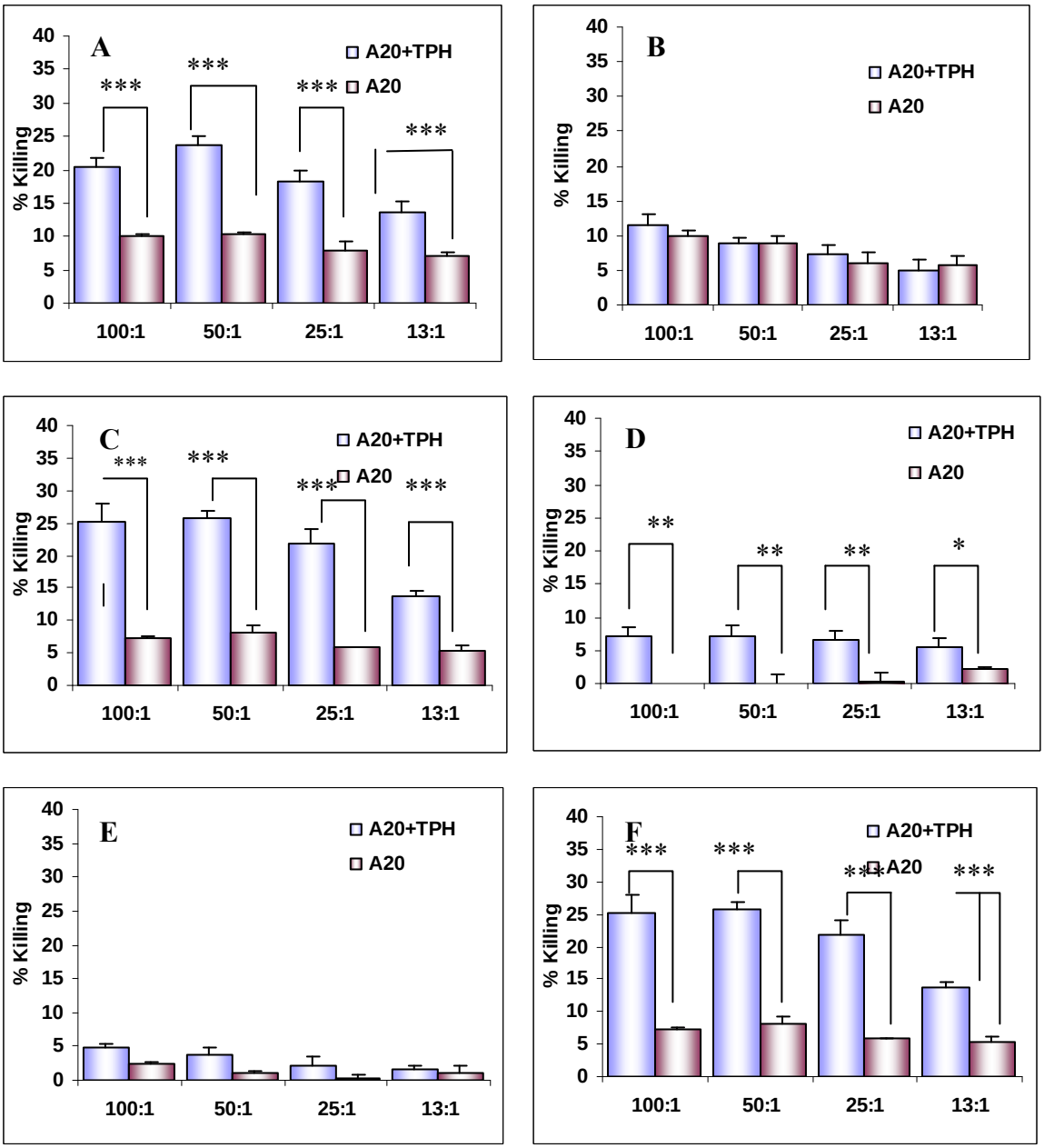


Figure 5.8:- Optimisation of immunisation protocol in Balb/c mice with TPH peptide. *Cytotoxicity assay against A20 cells pulsed with TPH or non pulsed after immunisation I Balb/c mice with TPH Imm and boost (B) Immunised once (C) In vitro restimulation with blasts (D) restimulation with peptide (E) CpG as an adjuvant (F) IFA as adjuvant. Results shown are representative of two independent experiments. (n=6).*

5.2.2.2 Identification of immunogenic peptides from MTA1 in syngeneic Balb/c mice

Protein sequence of murine MTA1 antigen was screened for potentially immunogenic peptides using a web based algorithm "SYFPEITHI". Seven peptides were selected based on their binding score to H2-Kd or H2-Ld MHC motifs for Balb/c mice. Three of the peptides were able to generate CTL (figure 5.9). Peptides which generated CTL response in more than 50% of mice tested and the specific killing was at least twice than the non-specific were considered as immunogenic. MTA1 622, 208 and 168 were immunogenic as they generated CTL which demonstrated moderate killing when used against A20 cell line pulsed with the relevant peptide. The killing was specific for the relevant peptides as no killing was observed against irrelevant peptide pulsed targets. The A20 cell line expresses high levels of murine MTA1 antigen but were not killed by the CTLs generated against either MTA1 622, 168 or 208, when unpulsed A20 cell line were presented as targets to them. These results suggest that none of the above immunogenic peptides were naturally processed. MTA1 peptide 298 generated a positive CTL response in only 1 out of 3 mice and was considered as only weakly immunogenic (figure 5.10D).

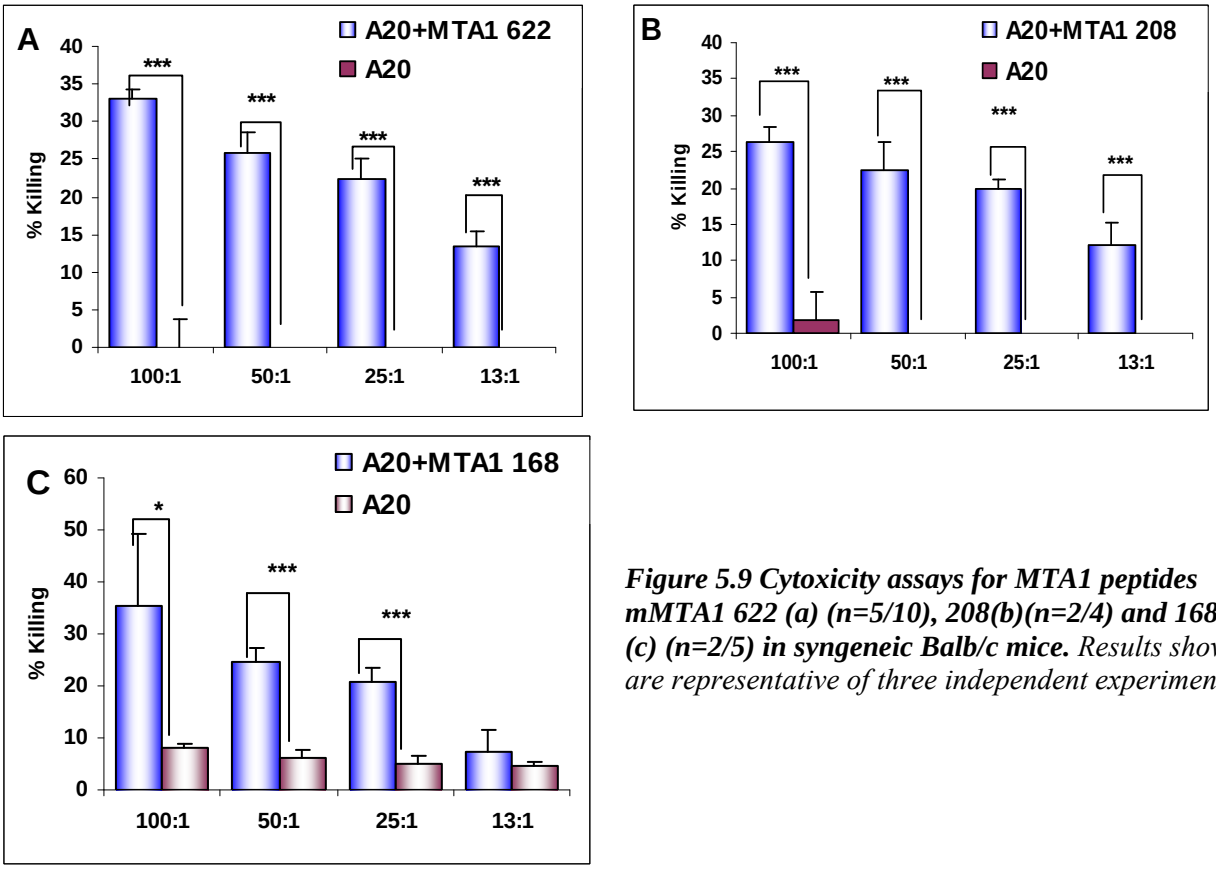


Figure 5.9 Cytoxicity assays for MTA1 peptides mMTA1 622 (a) (n=5/10), 208(b)(n=2/4) and 168 (c) (n=2/5) in syngeneic Balb/c mice. *Results shown are representative of three independent experiments.*

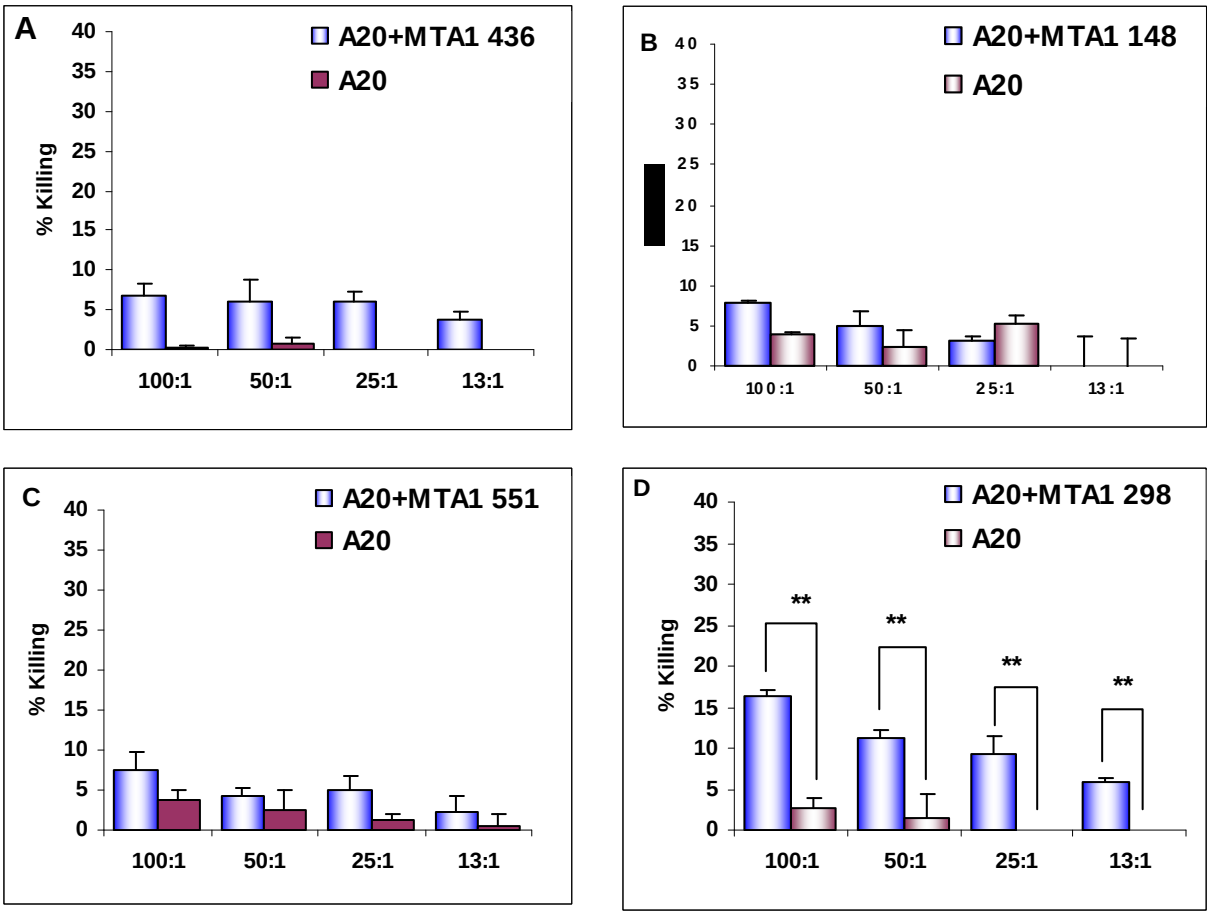


Figure 5.10 Cytotoxicity assays for murine MTA1 peptides 436 (A), 148 (B), 551 (C) and 298 (D)
Results shown are representative of three independent experiments (n=4).

Other murine MTA1 peptides (436, 148 and 551) were also tested in cytoxicity assays but none of them were immunogenic (figure 5.10). The inability of the peptide specific CTLs to lyse A20 cells (naturally over-expressing MTA1) was not cell line dependant as peptide specific CTLs were also found to be unable to lyse CT-26 and Renca cells (data not shown). To confirm these findings, immunogenic and non immunogenic peptides were all tested for their ability to generate peptide specific CTL after DNA immunisation of mice using gene gun as explained before. None of the peptides were able to generate CTLs (data not shown).

This data confirms that most of the peptides evaluated were not immunogenic and the ones that were immunogenic were not naturally processed. It is widely established that tolerance mechanisms ensure the deletion or tolerisation of high affinity CTLs for 'self antigens'. It is

noteworthy, that the peptides used in this study were chosen to represent both high and medium binding affinity epitopes.

5.2.3 Identification of immunogenic HLA-DR restricted peptides using HLA-DR0101 and HLA-DR0401 transgenic mice

MTA1 was recently identified in our lab as a SEREX antigen, which indicates that it is capable of generating an antibody response, which in turn is dependent on the CD4+ T helper cells (Li *et al*, 2004). Hence, we also investigated the immunogenicity and naturally processing of MHC class II peptides (specifically for HLA-DR4 and HLA-DR1) from MTA1 antigen. Two peptides, MTA1 497 and 550 were selected based on their predicted binding affinity to HLA-DR4 and HLA-DR1 molecules specifically. Mice were immunised twice with the peptides, following which splenocytes were harvested and re-stimulated for 6 days *in vitro* with the peptide. These cells were co-cultured with peptide pulsed syngeneic BM-DC in order to detect peptide specific proliferation. As seen from figure 5.11, both peptides induced specific proliferation in HLA-DR4 mice, where as no response was obtained in HLA-DR1 mice. However, MTA1 497 induced response in 1out of 3 mice (Figure 5.11D), whereas MTA1 550 proved to be highly immunogenic as it generated proliferation of CD4+ T cells in 3 out of 3 mice tested (Figure 5.11C). For both these peptides specific proliferation could be blocked by HLA-DR antibody (L243) and not by control antibody, confirming HLA-DR restricted proliferation. These results were also confirmed by ELISA for secretion of IFN-γ and/or IL-5 in supernatants of splenocytes re-stimulated with peptides *in vitro*.

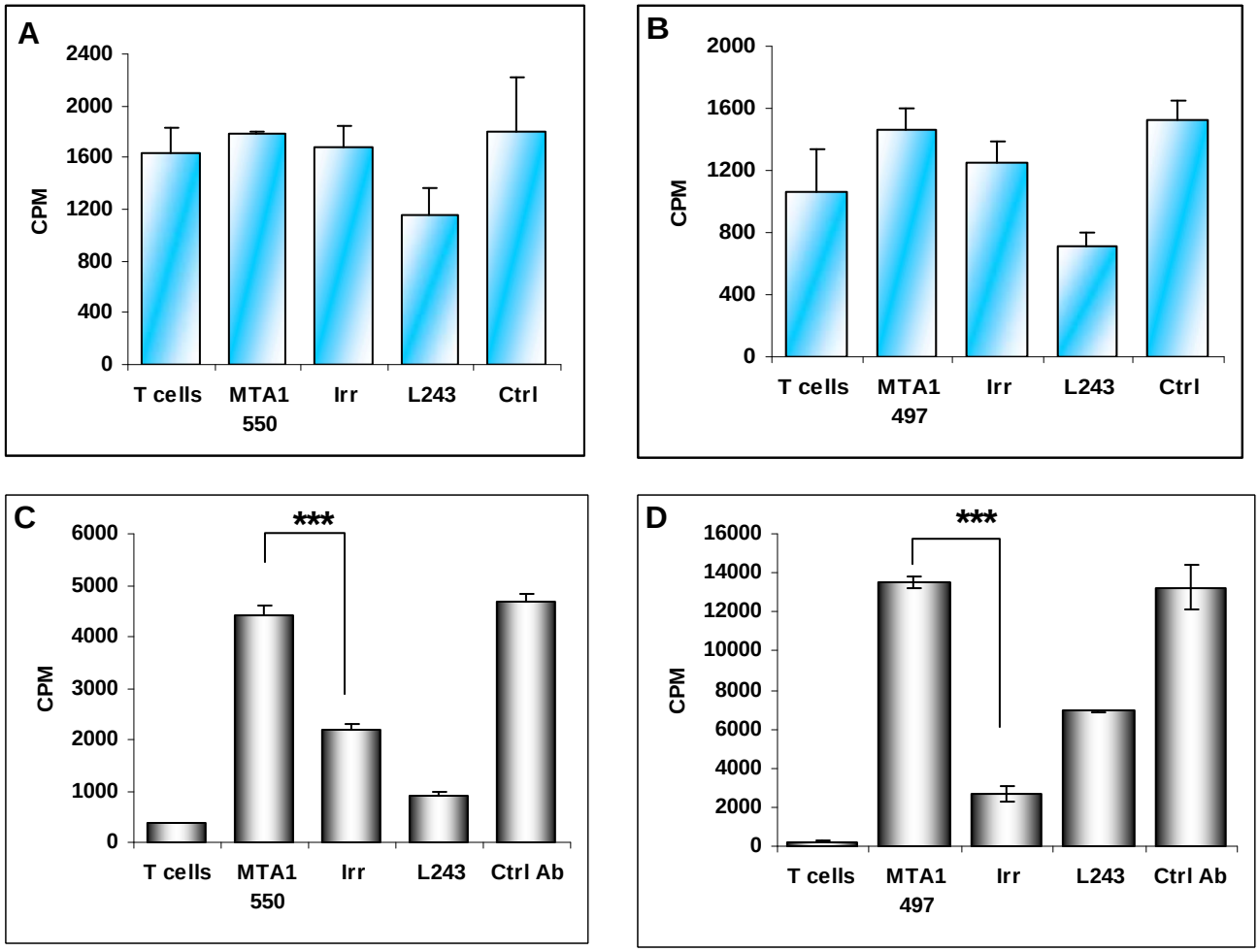


Figure 5.11:- Proliferation results of MTA1 497 (A) and 550 (B) peptides in HLA-DR1 (A and B) & HLA-DR4 (C and D) transgenic mice. *Mice were immunized with 100µg of peptide in 1:1 dilution with IFA and boosted one week later. Splenocytes were harvested a week later and restimulated in vitro with the peptide for 7 days and then tested for proliferation by incubating them with syngeneic BM-DC pulsed with peptide(n=4).*

Next, the natural processing of MTA1 497 and 550 peptides in HLA-DR0401 peptides was investigated. To achieve this, HLA-DR4 transgenic mice were immunised 3 times with pcDNA3/hMTA1 using gene gun, followed by re-stimulation of splenocytes from these mice with peptides *in vitro*. After 5 days of re-stimulation, proliferation was performed using DC pulsed with relevant peptides or an irrelevant (Flu) peptide. It was expected that if any of the peptides were processed and presented *in vivo* during immunisations, DC would prime CD4+ T cells against them and a week of *in vitro* re-stimulation would expand them further, allowing their proliferation, when presented with the same peptide by DC. No peptide specific proliferation was observed for both these peptides, suggesting that none of them were naturally

processed (figure 5.12). To confirm these results, natural processing was also investigated using a recently described method (Rojas *et al*., 2005). Following two immunisations with peptides, splenocytes were harvested, re-stimulated with peptides and their proliferation investigated using DC as APC pulsed with lysate of cells demonstrating either high expression or very low expression of MTA1. As a control, splenocytes harvested from mice immunised with Flu peptide were used. Results obtained by this method confirmed previously obtained results suggesting that MTA1 497 and 550 were not naturally processed (data not shown).

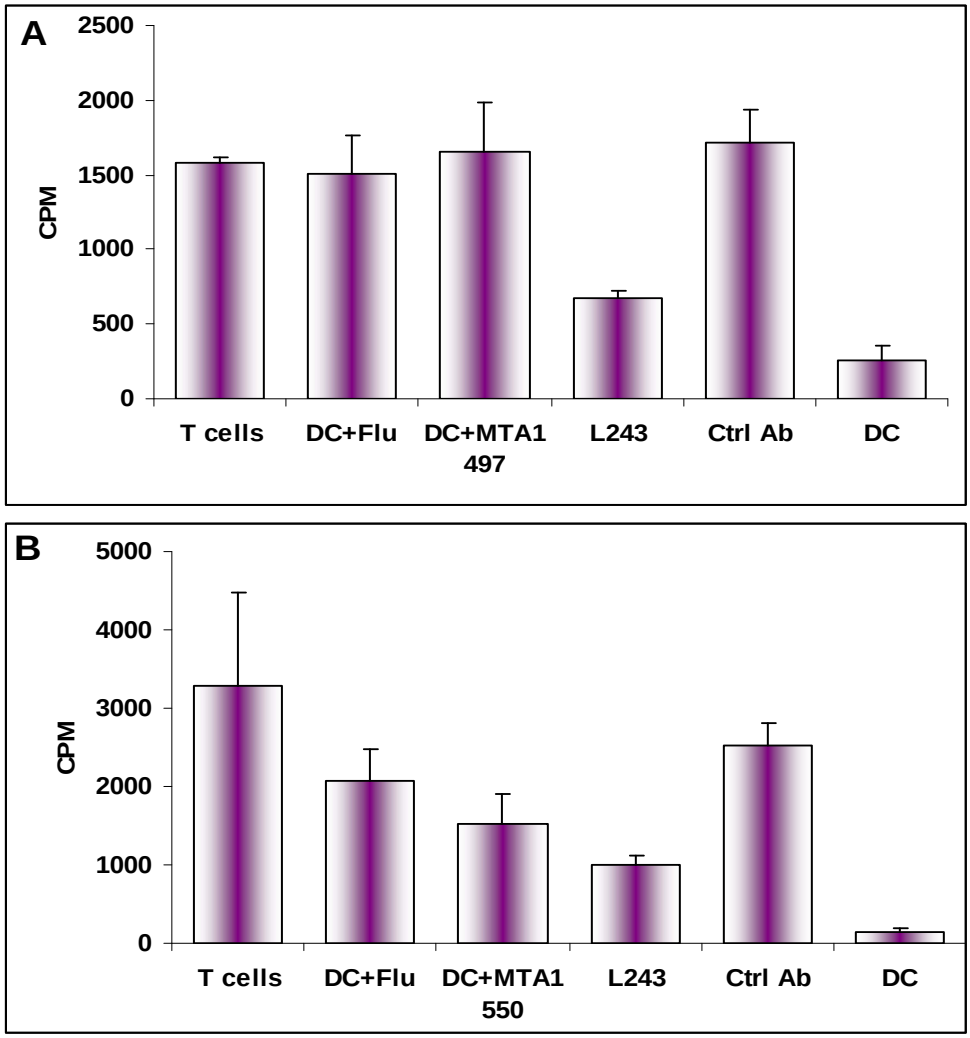


Figure 5.12:- Proliferation assay to evaluate natural processing of MTA1 487 (A) and MTA1 550 (B) peptides. *HLA-DR4 transgenic mice were immunised 3 times with pcDNA3/MTA1 using a gene gun. Splenocytes were harvested from these mice and after 5 days of in vitro re-stimulation with peptides, proliferation assay was performed using DC as APC to present relevant peptides of control (Flu) peptide. L243 and isotype control antibodies were used to confirm restriction of response. Result shown is representative of 2 independent experiments (n=6).*

Finally we sought to determine whether it is possible to generate an immune response to the full MTA1 sequence following DNA immunisation. Moreover, several studies indicate the tendency of immune response to be skewed towards Th2, hence we immunised HLA-DR4 transgenic mice with pcDNA3/hMTA1 intramuscularly and repeated the proliferation assay using DC transfected with hMTA1 or βgal as a control. No specific proliferation was observed in this case and immunogenicity of MTA1 could not be determined (Figure 5.13). Similar study was also performed for class I response in a cytotoxicity assay, where antigen (MTA1) specific killing was not observed.

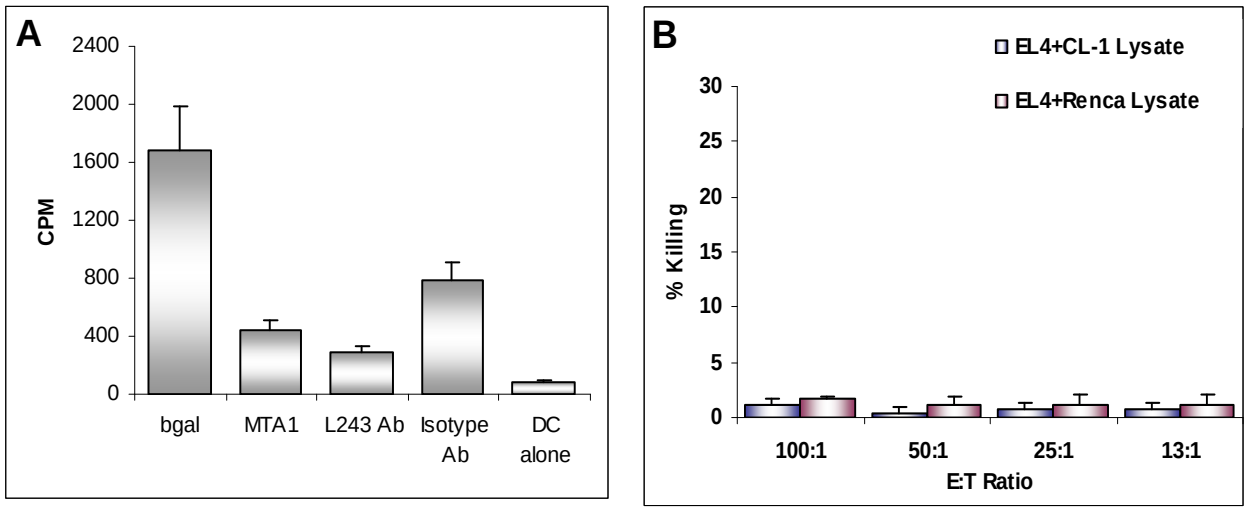


Figure 5.13:- Proliferation (A) and cytotoxicity assay (B) after intramuscular immunisation of HLA-DR4 and HLA-0201 transgenic mice with pcDNA3/hMTA1. *DC transfected with MTA1 were used as APC in proliferation assay. In the cytotoxicity assay, EL4-HHD II cells pulsed with lysate of cells either over-expressing MTA1 (CL-1) or low MTA1 expressing MTA1 (Renca) were used as targets (n=4).*

Table 5.2 summarises the peptides investigated in this study for their immunogenicity. These results suggest that either MTA1 is not immunogenic owing to deletion of most antigen specific T cells or the immunisation protocols used in this study have been unable to break tolerance to MTA1 and more potent strategies need to be designed.

Table 5.2:-Summary of the peptides tested and their immunogenicity

Mice Strain	Peptides	Immunogenicity	No of Mice tested	Cytokines IFNγ/IL-5
Balb/c	MTA1 622	Yes	5/10	2
	MTA1 168	Yes	2/5	1
	MTA1 436	No	0/4	0
	MTA1 298	No	1/3	0
	MTA1 148	No	0/3	0
	MTA1 551	No	0/4	0
	MTA1 208	Yes	2/4	0
C57BL/6	MTA1 699	Yes	4/4	2
	MTA1 496	Yes	4/4	2
	MTA1 12	No	0/2	0
HHD II	MTA1 22	Yes	4/4	ND
	MTA1 57	Yes	4/4	2
	MTA1 109	No	0/4	0
HLA-DR0101	MTA1 497	No	0/4	ND
	MTA1 550	No	0/4	ND
HLA-DR0401	MTA1 497	Yes	3/4	ND
	MTA1 550	Yes	2/4	ND

ND=Not done

5.3 Discussion

5.3.1 Human MTA1 derived class I peptides

Several studies in animal models have shown the efficacy of peptide specific CD8+ T cells to provide protection against tumour challenge or completely eradicate established tumours. One of the most convenient methods of immunization has been the use of peptides with an adjuvant; this obviates the need to generate any vectors, are well tolerated and perhaps the safest mode of vaccination. Immunisation of Her/2-neu transgenic mice with a Her-2/neu CTL epitope was shown to be effective in protection as well as therapeutic experiments in these animals (Gritzapis *et al*., 2006). A major factor limiting the success of immunotherapy *in vivo* is the inadequate number of T cell generated. Recent clinical trials have shown incredible success in patients treated with adoptively transferred T cells. These T cells are generally isolated from the TIL and expanded *in vitro*; peptides presented by DC or artificial APC *in vitro* can be used efficiently for this purpose. Adoptive T cell therapy is also limited by the number of T cells generated for *in vivo* injections. In another recent trial, genetically modified T cells expressing a specific TCR were used to immunise metastatic melanoma patients. Two months post-infusion, more than 10% of peripheral circulating lymphocytes consisted of transferred T cells, leading to objective clinical regression in some patients (Morgan *et al*., 2006). This TCR cloning and patient monitoring is only possible if the identity of peptide targeted is known. Moreover, clinical trials conducted with dendritic cells pulsed with peptide have also shown promising results. In a recently conducted trial of 20 metastatic renal cell carcinoma patients, DC pulsed with MUC-1 peptide induced objective clinical regression of metastasis in 3 patients and stabilised the cancer in 4 others (Wierecky *et al*., 2006). Thus peptide identification from known tumour antigens forms an essential component of immunotherapy for several cancers.

Limited success in human clinical trials of the peptide vaccines has been ascribed to the limited range of epitopes used as well as targeting peptides expressed by most common alleles only. Effective peptide vaccine for cancer would have to target multiple epitopes from several antigens and presented by different MHC alleles, to minimise the chances of immune escape through antigen or MHC down regulation. Considering the benefits of peptide identification, we sought to identify CTL epitopes from MTA1 using the ‘reverse immunology’ approach in transgenic and syngeneic mouse models. MTA1 sequence was screened using computer-based algorithm

'SYFPEITHI' to identify potentially immunogenic peptides. It was hypothesized that T cells repertoire to MTA1 would be limited in syngeneic balb/c mice, whereas HHD II transgenic mice are less likely to be tolerant to hMTA1. Minor differences in the protein sequence can have a major impact on immune response in case of 'self antigens'. Hence, we decided to investigate immunogenic CTL peptides from mMTA1 in balb/c mice and hMTA1 in HHDII mice. Peptides were chosen for both syngeneic balb/c mice as well as transgenic HHD II mice. Of the 3 peptides selected for evaluation in HHD II mice, 2 were immunogenic as demonstrated by development of peptide specific CTL in cytotoxicity assay. However, CTL generated against these peptides were neither capable of killing murine EL4-HHD II cells (expressing MTA1) nor human tumour cells MCF-7 (HLA-A0201+, MTA1+). It is noteworthy, that CTL generated from transgenic HHD II mice have been shown to efficiently lyse human tumour cells in an MHC restricted manner (Gritzapis *et al*., 2004; Gritzapis *et al*., 2006). These results were confirmed by investigating *in vivo* presentation of these peptides following gene gun immunisation. Moreover, inability to generate immune response in HHD II mice was not dependant on method of immunisation as even intra-muscular immunisation produced the same results (data not shown).

The Immunisation protocol had to be optimised when using balb/c mice as single immunisation, as in HHD II mice, this was not potent enough. This could be due to strain differences in the mice as HHD II mice have been generated from the C57 background. Also, binding affinity of peptide is also likely to play a major role in this. Following optimisation, seven MHC class I epitopes, including high and medium binding affinity peptides, were evaluated for immunogenicity. Three of these were immunogenic but not naturally processed, since CTL generated against them could only lyse peptide pulsed target cells but not unpulsed cells naturally over-expressing mMTA1. To confirm these results, peptides were also used to re-stimulate splenocytes from pcDNA3/mMTA1 gene immunised mice and tested in a cytotoxicity assay (data not shown). This suggested that MTA1 immunisation was unable to prime CTLs to these peptides *in vivo* following gene immunisation. Of course, the possibility that the immunisation strategy was not potent enough, cannot be neglected.

5.3.2 Identification of MHC class II peptides and role of CD4+ T helper epitopes in immunotherapy

Due to the overwhelming evidence of CD8+ T cells being directly responsible for killing of tumour cells *in vitro* and *in vivo*, CD4+ T cells had been neglected during the early years of cancer immunotherapy. The result of this was that the phenomenal success of peptide vaccines in animal models was not translated in human clinical trials (Chen *et al*., 2004; Lee *et al*., 1999). Different animal models have produced contradictory results regarding CD4+ T cell role in anti-tumour immune response. It is hypothesised that pathogenic infections produce strong 'danger signals', leading to up-regulation of CD40 molecules on DC thereby bypassing CD4+ T cell help. This phenomenon has also been observed in non-pathogenic tumour models, which contradicts the above theory. CD4+ T cell help might be dependent on the MHC class I epitope affinity. Franco *et al* showed that peptides with high affinity for their MHC class I molecules do not require CD4+ T cell help [31]. Generation of CTL is influenced by the period of MHC restricted peptide display on APC, TCR-MHC binding affinity/duration and whether or not CD4+ T cell mediated help is necessary. Considering that most MHC class I epitopes for over-expressed self antigens are likely to be of low-medium binding affinity, CD4+ T cell help would prove critical for their generation. Gradually, CD4+ T cells have taken the centre stage due to their central role in orchestrating multiple arms of the immune system. Not only is T cell help required for optimal effector and memory CTL response (Ali SA, 2000), but in certain animal models CD4+ T cells have been shown to mediate tumour regression via an IFN-γ mediated mechanism (Egilmez *et al*., 2002).

Although, most studies seem to suggest that CD4+ T cells do not need to recognise peptides on the surface of tumour cells in an MHC restricted manner for inducing cell lysis, several others have shown that it is likely in some cases. Independent studies have shown CD4+ T cells to directly mediate tumour cell lysis through TRAIL, FasL and granzyme-perforin dependent pathways, traditionally employed by CTL (Echchakir *et al*., 2000; Schattner *et al*., 1996; Thomas & Hersey., 1998a; Thomas & Hersey., 1998b). Another debate has been the source of the CD4+ T cell epitope, where some researchers suggest to use an irrelevant (derived from foreign antigen i.e. tetanus toxoid and hepatitis B), whereas others have argued the importance of using helper epitope from the same antigen as CTL epitope in a peptide vaccine (Slingluff *et al*., 2001).

MTA1 was recently identified in our laboratory using SEREX technology, which relies on high titre IgG antibodies in cancer patient's serum towards cancer antigens. This antibody production is supported by T helper 2 cells, implying the presence of CD4+ T cells against MTA1. In order to investigate this, we evaluated two peptides derived from human MTA1 in HLA-DR1 and HLA-DR4 transgenic mice. MTA1 497 and 550 peptides were shown to be immunogenic in HLA-DR4 but not in HLA-DR1 transgenic mice. However, both of the peptides were found not to be naturally processed as neither DNA immunisation primed CD4+ T cells *in vivo* nor the cells generated through peptide immunisation proliferated in response to DC presenting processed MTA1 gene or cell lysates containing high level of MTA1. Although, it is generally assumed that CD4+ T cells are less likely to be tolerised against 'self-antigens', recent studies have suggested that nuclearly localized antigens are not averse to mechanisms of central and peripheral tolerance (Nakken *et al*., 2003). Considering the overwhelming number of epitopes in the medium binding affinity group, it was deemed impractical and costly to screen large number of peptides to identify naturally processed peptides, although we believe that there is a likelihood of it existing.

Prediction of naturally processed CD4+ T cell epitopes is much more difficult compared to CTL epitope due to larger peptide size and variability of amino acid number. Surface elution of MHC class II peptides, although possible, is technically more difficult (Peakman *et al*., 1999). Recent emergence of ANN and HMM based methods might make this prediction easier and more accurate. Another recently evolving strategy has been the use of overlapping peptides spanning the whole protein sequence of an antigen. PBMC from cancer patients can then be tested against these peptides for release of cytokines or killing, providing evidence of T cell existence against some peptides *in vivo* (Vittae *et al*., 2006). However, constructing an overlapping peptide library is still quite expensive and there might be other issues relating to the purity of individual peptide pools.

5.3.3 Tolerance and its affect on T cell repertoire

For years it has been believed that the T cell repertoire is purged of the self antigen reactive high affinity T cells in order to protect the host from auto-immune reactions**.** This theory has recently been questioned by several reports suggesting that in several cases high affinity self-reactive T cells can escape thymic deletion and escape into the periphery. This could either be due to non

expression of self antigen in the thymus, inefficient processing of antigen in thymus or due to insufficient density of peptide-MHC levels in thymus to reach the threshold for deletion of thymocytes (Houghton & Guevara-Patino, 2004). Also, cryptic epitopes, which are not processed efficiently by cells, are likely to escape from inducing deletion (Gross *et al*., 2004). Thus, high affinity T cells for self antigens can also be possibly found in the peripheral T cell pool. Moreover, TCR have a very high level of cross reactivity. Theoretical calculations estimate that a single TCR can potentially react to $1x10^6$ different peptide antigens (Mason, 1998). These properties of T cells offer cancer immunologists a window of opportunity to reactivate these T cells for mounting an attack against cancers expressing self-antigens.

Apart from central deletion mechanisms, T cells encountering self antigens in the periphery are anergised and require much higher levels of antigens or mutated antigens to be re-activated (Kawahata *et al*., 2002). MTA1, being expressed at low levels in the thymus and other normal tissues, is likely to be affected by both these mechanisms. Whether the thymus plays a role in deletion of T cells against MTA1 is still unknown and would be relying on presentation of MTA1 derived peptides in the thymus to developing thymocytes. However it is likely that peripheral tolerance mechanisms do have an affect on T cells reactive against MTA1. Clearly, more potent strategies are needed to overcome anergy and generate immune response to MTA1. Modification of wild type peptides in order to increase their binding affinity to MHC or TCR has been shown to be effective in several studies. In a transgenic mouse model expressing human TCR for gp100 peptide, Yu *et al* showed that inability of wild type peptide to generate immune response was due to fast dissociation rate of peptide and that modification of the peptide increased the stability of peptide-MHC molecules, leading to activation of transgenic T cells (Yu *et al*., 2004). Similarly, another study demonstrated modified p53 peptides to be more immunogenic, CTLs generated against it to be efficient in recognizing wild type peptide as well as being able to lyse tumour cells expressing p53 gene (Petersen *et al*, 2001). However, the challenge lies in being able to identify peptides likely to benefit from modification as medium to low binding group of peptides from a given tumour antigen can be extremely large. To overcome this limitation, Alan Houghton's group has shown xenogeneic immunisation, which exploits natural variation in sequences of mouse and human homologue genes, to be highly effective in breaking tolerance against tumour associated self-antigens (Gold *et al*., 2003).

Cytokines such as IL-15 have also been shown to rescue tolerant CD8+ T cells in a transgenic mouse model. In another TCR transgenic model, T cells against self-antigen expressed in liver are tolerised but can be rescued to proliferate and react against antigen by IL-15 cytokine, and these T cells were able to protect mice against leukaemia. This study confirms that high affinity T cells are not necessarily deleted and given the right method, can be rescued for tumour immunotherapy (Teague *et al*, 2006).

5.3.4 Strategies for enhancing the potency of peptide vaccines

Major criticism against peptide vaccines has been their lack of immunogenicity or potency. Injected peptides *in vivo* can be degraded by proteases, leading to rapid clearance. These limitations can be overcome by using potent adjuvant and correct immunisation strategies. Combining peptides with oil based adjuvant would lead to depot formation of peptide with slow release, thereby avoiding rapid clearance through proteases. Also, GM-CSF as an adjuvant can be beneficial due to its ability to recruit professional APC to the site of injection. Other adjuvants used with peptide vaccines with high efficacy are IL-2, IL-12, CpG oligonucleotides and interferons (Buteau *et al*., 2002). Peptide based vaccination can also be benefited by targeting them to specific compartments of APC by injecting them in a DNA form with signalling sequences. Also, linking peptides to nanobeads, liposomes or linker sequences are more efficacious in targeted delivery of peptides (Engler *et al*., 2004; Fifis *et al*., 2004).

In conclusion, reverse immunology approaches were unsuccessful in identification of naturally processed peptides from MTA1, for MHC class I and class II peptides. Inability to identify these peptides could either be due to non-existence of such peptide specific CTLs (due to deletion in thymus) or more likely due to inaccurate prediction and ineffective vaccination methods to overcome tolerance for re-activation of anergised T cells. Strategies such as xenogeneic DNA or viral vectors might be able to overcome this limitation and will be investigated in future studies for MTA1 immunotherapy.

Chapter 6:- Evaluation of MTA1 as an immunotherapeutic target in an *in vivo* model using DNA based vaccine

6.1 Introduction

DNA based vaccines have revolutionized the field of immunotherapy in several ways. In-spite of various advantages of peptide vaccines, lack of knowledge of immunogenic tumour specific peptide epitopes, which can be targeted by therapy, is likely to be a major limitation. Moreover, as seen in this study, identification of naturally processed peptides from tumour shared 'self-antigens' (which form the bulk of tumour antigens), can be quite difficult. Deletion of high avidity T cells against self antigens ensures that only T cells with low to medium binding affinity are allowed to survive in the periphery and they too could be under the control of peripheral tolerance mechanisms. Prediction of peptides using web based algorithms predicts literally hundreds of peptides with medium to low binding affinity for a given antigen, and screening all of them is impractical and expensive, especially for individual HLA alleles. DNA based vaccine not only obviates this need for individual peptide identification, but can also generate antibody and CTL response simultaneously, which could mediate tumour protection or regression.

DNA based vaccination methods have demonstrated good efficacy in mediating tumour rejection in animal models. Gene gun mediated immunisation of HLA-A0201 mice with plasmid DNA encoding for immuno-dominant epitopes from HPV16 and HPV17 were able to protect mice from a lethal challenge of tumour cells expressing these antigens. However, DNA immunisation was less effective at treating established tumours compared to viral vaccine in the same study (Eiben *et al*., 2002). Interestingly, antigens used in that study were highly immunogenic as they are derived from a virus (HPV). In another study, intramuscular immunisation of murine tyrosinase related protein -1 (mTrp-1), enabled mice to be protected from a lethal challenge of poorly immunogenic B16 melanoma cells (Bronte *et al*., 2000). In the same study, the vaccination of mice with a recombinant vaccinia virus encoding mTrp-1 led to tumour regression of well established B16 tumours. It is noteworthy, that mTrp-1 is a 'self-antigen' expressed in normal skin; although previous studies had revealed that regression of melanoma coincided with vitiligo, in this study no autoimmune reactions were observed. More importantly, DNA vaccines have shown to be safe in several human clinical trials, although lack of objective clinical response has also been their feature.

Several strategies have recently been devised to increase the immunogenicity and potency of 'naked' DNA vaccines. For instance, linking the DNA encoded protein to ubiquitin, targets it to MHC class I processing pathway and is found to be more effective in CTL generation (Velders *et al*., 2001). Similarly, gene can be targeted to the endosomal/lysosomal compartment by linking it to lysosome associated membrane protein (LAMP-1) for efficient processing and presentation to CD4+ T cells (Ji *et al*., 1999). Addition of co-stimulatory or cytokine genes in the plasmid enhances the potency and effectiveness of the vaccine. Although several cytokine genes have been combined with DNA vaccination, GM-CSF is one of the most commonly used cytokine as an adjuvant. Co-administration of plasmids encoding GM-CSF and gp100, generated potent immune response for mediating tumour protection and regression, and enabled to do so at very low levels of gp100 plasmid (Rakhmilevich *et al*., 2001). GM-CSF is a strong chemoattractant for the dendritic cells and this strategy essentially uses this property to target administered antigens to professional APCs. Tumour cells manage to anergise T cells by expressing the antigens without any co-stimulatory molecules on their surface. To overcome this limitation, DNA vaccines co-administered with co-stimulatory molecules has been attempted and are shown to be more effective in generating CTL response capable of mediating protection in mice (Corr *et al*., 1997). Other advances facilitating antigen delivery to APC such as *in vivo* electroporation, mucosal jet injection as well as encapsulation of DNA into microparticles/liposomes has further enhanced the efficacy of DNA vaccines (Liu *et al*., 2004).

One of the criticisms of DNA cancer vaccines has been their lack of immunogenicity and inability to break tolerance against 'self antigens'. In recent years, Houghton and colleagues have endeavoured to overcome this by pioneering the use of xenogeneic (vaccinating with a homologous gene from another species) immunisation (Bowne *et al*., 1999; Gold *et al*., 2003). Modification of amino acids in specific locations on a peptide can increase its binding to MHC or TCR, thereby overcoming tolerance to native peptide specific low avidity T cells. But this strategy still needs to identify these low immunogenic naturally processed peptides. Xenogeneic immunisation uses the same strategy but has additional advantages of DNA vaccine. Several tumour associated 'self-antigens' are highly conserved in humans and mice, probably owing to their essential role in cell survival. Minor differences in amino acid sequences of such conserved proteins can be used i.e. mouse genes to immunise humans or vice versa, for bypassing immune

tolerance to self antigens. This is achieved not only by the generation of modified peptides with increased affinity for MHC or TCR but may also be due to altered processing and presentation of cryptic epitopes. Immune response, leading to tumour protection and/or regression, against several self antigens such as gp75, trp-2 and gp100 has been generated using this approach (Weber *et al*., 1998; Gregor *et al*., 2004; Bowne *et al*., 1999). Interestingly, the immune response generated against self-antigens was shown to be mediated by different immune cells, depending on the antigen. When Trp-1 antigen was targeted, the immune response was mediated by NK cells and B cells, whereas CD4+ and CD8+ T cells were responsible for mediating tumour rejection in case of Trp-2 (Bowne *et al*., 1999). Furthermore, xenogeneic immunisation can be used in combination with other adjuvants such as anti-CTLA4 antibody to enhance their efficacy and overcome other peripheral tolerance mechanisms like regulatory T cells (Tregs) (Gregor *et al*., 2004).

Apart from the recessive tolerance mechanisms, the immune response to tumour antigens is also controlled by dominant regulatory mechanisms, mainly suppressive T cells. Although several subpopulations of suppressive cells have been discovered (NKT cells, GR1+CD11c+ myeloid cells), perhaps the most important cells are the regulatory T cells (Tregs). Recent years have seen remarkable interest in the role of these cells and the importance of Tregs in controlling the immune system is underlined by the fact that their depletion in animal models leads to severe auto-immune diseases; and their adoptive transfer causes their abrogation. Tregs have been divided into two subgroups, naturally occurring and induced. Tregs can be induced to develop from CD4+ T cells depending on the antigen stimulation conditions (Chakraborty *et al*., 1999) and have the phenotype CD4+CD25+; however in the recent years it has emerged that most T cells can express CD25 upon activation. FOXP3 (Foxp3 in mice), a member of the forkhead/winged helix member of transcription factors, is now regarded as the marker of choice for detecting these Tregs, which are defined by the phenotype CD4+CD25+Foxp3. Forced expression of Foxp3 can convert normal CD4+ T cells into having a suppressive phenotype and has been shown to be critical for their development. Apart from these, other surface molecules such as glucocorticoid induced tumour necrosis factor receptor (GITR) and CTLA-4 are frequently expressed by the Tregs (Sakaguchi, 2004).

Numerous studies have shown the immuno-suppressive effects of Tregs on CD4+ and CD8+ proliferation as well as the cytolytic capability of CTLs (Sakaguchi, 2004). Different models have demonstrated multiple mechanisms used by CD4+CD25+ Tregs for suppression of immune response. Cytokines like IL-10 and TGF-β may be responsible for Treg mediated suppression in some cases, whereas in others cell-cell contact was critical. Contact dependant inhibition of T cells may be mediated by CTLA-4 and/or GITR, however, their role is still being investigated (Read *et al*., 2006). Treg mediated modulation of the APC activity (down-regulation of co-stimulatory molecules) has been deemed responsible for this. Interestingly, *in vitro* studies have revealed that antigen specific stimulation of Tregs was necessary for their suppressive action, although once activated, suppression was non-specific. However, if Tregs suppress other T cells in a non-specific manner then how does the adaptive immune system manage to respond to pathogens? One possibility is that potent stimulation of CD4+ and CD8+ T cells might be able to overcome Treg mediated immuno-suppression. Indeed, a recent study suggested that activated CD8+ T cell mediated release of IFN-γ was able to overcome Treg mediated immuno-suppression and led to development of antigen specific CD4+ T cells instead (Nishikawa *et al*., 2005). By establishing a clone of CD4+ TIL, a recent study managed to identify Tregs with specificity for the LAGE-1 antigen, re-enforcing the belief in existence of antigen specific Tregs in the periphery (Wang *et al*., 2004).

It has become evident that the thymus produces Tregs as a separate subpopulation of T cells. High level of antigen expression in the thymus during development of thymocytes seems to be responsible for their development from antigen specific T cells (Sakaguchi, 2004). Several studies have also suggested that Tregs and CD4+ T cells, with identical antigen specificity, can co-exist in the periphery, and Tregs are approximately 10-100 times more sensitive to the antigen compared to effector CD4+ T cells. It has been hypothesized that Tregs exist in the periphery and are constantly stimulated by the APC presenting self-antigens in a non-inflammatory context, thereby controlling the actions of potential auto-reactive T cells.

Frequency of Tregs is increased in peripheral blood, TIL and tumour draining lymph nodes of cancer patients. Moreover, in a recent study of ovarian cancer, Treg accumulation at tumour sites correlated with poor prognosis of patients (Curiel *et al*., 2004). In the same study, secretion of chemokine CCL22 by tumour cells and macrophages was shown to be responsible for Treg

accumulation in tumours, underlining their importance in immuno-evasion by cancer cells. Complementing the human studies, depletion of Tregs along with antigen specific vaccination has been shown to induce tumour regression in several animal models (Shimizu *et al*., 1999; Onizuka *et al*., 1999). Different groups have sought several strategies for Treg depletion.

Several studies have used anti CD25 antibody for depletion of Tregs as CD25 is highly over-expressed on them (Shimizu *et al*., 1999; Onizuka *et al*., 1999). Although useful in mouse models, it is unlikely to be used in humans as activated T cells also seem to express CD25 and this antibody is likely to deplete them as well. Glucocorticoid-induced tumour necrosis factor receptor family related protein (GITR) and CTLA4 are responsible for cell-cell contact dependant inhibition of effector T cells. Hence, anti GITR and anti-CTLA4 antibodies have also been recently employed, as they block the inhibitory effects of GITR and CTLA4 molecules, making immunotherapy more efficacious (Yamaguchi & Sakaguchi., 2006). Moreover, these antibodies can be used in combination for enhanced effect (Ko *et al*., 2005). Denileukin diftitox or Ontak (IL-2 diptheria toxin fusion protein) is currently being used in patients of non-Hodgkin's lymphoma and cutaneous T cell lymphoma (Baecher-Allan & Anderson., 2006). CD25 molecules form the α-chain of IL-2 receptor and Ontak might lead to depletion of Tregs, since they express highest levels of CD25. Ontak binding to cells introduces diphtheria toxin in CD25+ cells, causing their lysis. Pharmacological agents have also demonstrated some efficacy in Treg inhibition. Low dose cyclophosphamide selectively induces apoptosis in Tregs and has shown to enhance vaccination efficacy in mouse models (Ghiringhelli *et al*., 2004). Similarly, in patients receiving fludarabine based chemotherapy for chronic lymphocytic leukaemia, a decreased frequency of Tregs was observed. Confirming this study, Beyer *et al*, demonstrated enhanced sensitivity of CD4+CD25+ T cells to fludarabine then CD4+CD25- cells (Beyer *et al*., 2005).

Considering the inability to break tolerance and identify naturally processed peptides by simple peptide vaccines, it was decided to investigate other potent methods of vaccination such as DNA vaccination, xenogeneic vaccination, viral vaccines as well as combining them with Treg depletion.

6.2 Results

6.2.1 Evaluation of syngeneic DNA immunisation for MTA1

Full length murine MTA1 was amplified from mouse testis and cloned into TOPO-Blunt shuttle vector. Sequencing was performed to exclude possibility of any possible mismatches during amplification, following which mMTA1 was cloned into pcDNA3 mammalian expression vector (invitrogen) (see chapter 4). Mouse MTA1 protein expression from pcDNA3-mMTA1 was confirmed by transfecting a cell line followed by western blotting assay 24-48 hours later from cell lysate of transfected and non-transfected cell line. Gene gun immunisation has been used recently with good efficacy in several murine tumour models. Hence, we coated 1 micron gold particles (Biorad) with the MTA1 expressing construct pcDNA3-mMTA1 (preparation and characterisation of construct is described in chapter 4). These DNA coated gold particles were then administered intradermally three times at seven day interval to the shaved abdomen of balb/c mice using a helium gene gun (Biorad). A week after final immunisation, mice were challenged with $8x10^4$ CT-26 cells subcutaneously and monitored twice a week until the tumour size reached $1mm^2$, when mice were euthanized as per the home office schedule. No significant difference in either tumour growth rate or survival benefit in immunised mice was observed compared to the control gene immunised or naïve mice (n=5) (fig 6.1). Although, a slight increase in survival was observed in 1 out of 5 mMTA1 immunised mice, the difference was not significant and was inconclusive.

Considering the possible development of tolerance to MTA1 in syngeneic Balb/c mice, xenogeneic immunisation was evaluated as an option to overcome tolerance. After cloning hMTA1 into pcDNA3 and confirming protein expression *in vitro* using this vector (see chapter 4), gold bullets coated with pcDNA3-hMTA1 were prepared as before and were used to immunise mice. As controls, mice immunised with pcDNA3-mMTA1, empty pcDNA3 and naïve mice were also challenged with CT-26 cells. Unfortunately, no significant survival benefit was observed in these experiments, suggesting that even xenogeneic immunisation was unable to generate immune response to MTA1 (fig 6.2). Similar experiments were also performed in HHD II transgenic mice and challenged with EL4-HHD II cells (which express MTA1 at high levels) with identical results. However, EL4 HHD II cells were inconsistent in tumour growth in HHD II mice and hence the results of *in vivo* experiments from them were not considered and all further

experiments were performed in balb/c mice. It has been observed in our laboratory that immune activation using gene gun vaccination in HHD II mice might be antigen dependent i.e. immunising with p53 gene was highly effective, whereas it was inefficient for another tumour antigen (HAGE) (McArdle et al, unpublished observations).

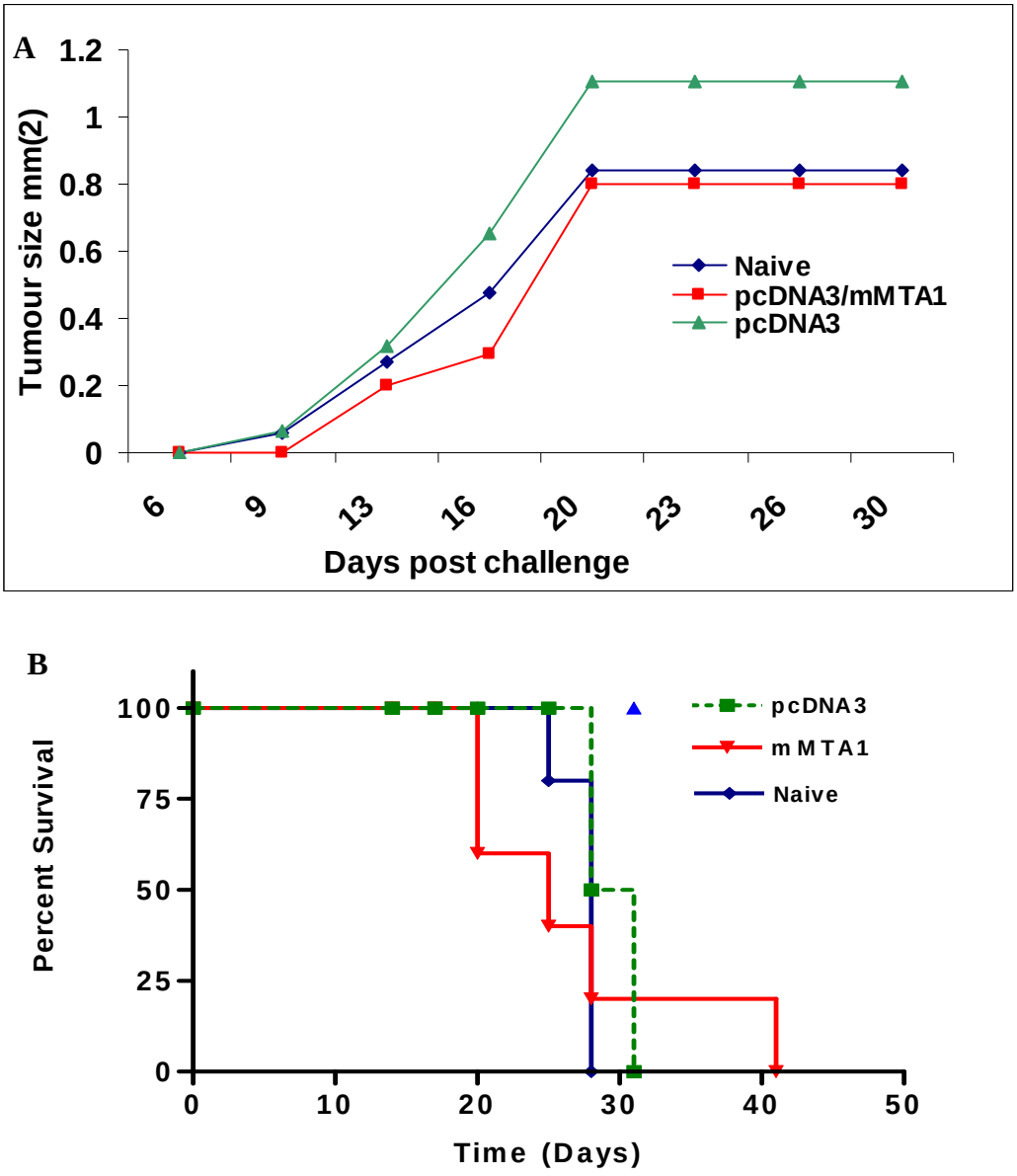


Figure 6.1:- Tumour Challenge experiment following gene gun immunisation in balb/c mice ***Balb/c mice. (A) Tumour growth rate (B) Survival Curve.*** *Mice were immunised 3 times with gold particles coated with pcDNA3-mMTA1 at 7 day intervals, followed by challenge with CT-26 cells. No significant survival benefit was observed in vaccinated mice compared to controls. Results shown are representative of two independent experiments with 5 mice per group.*

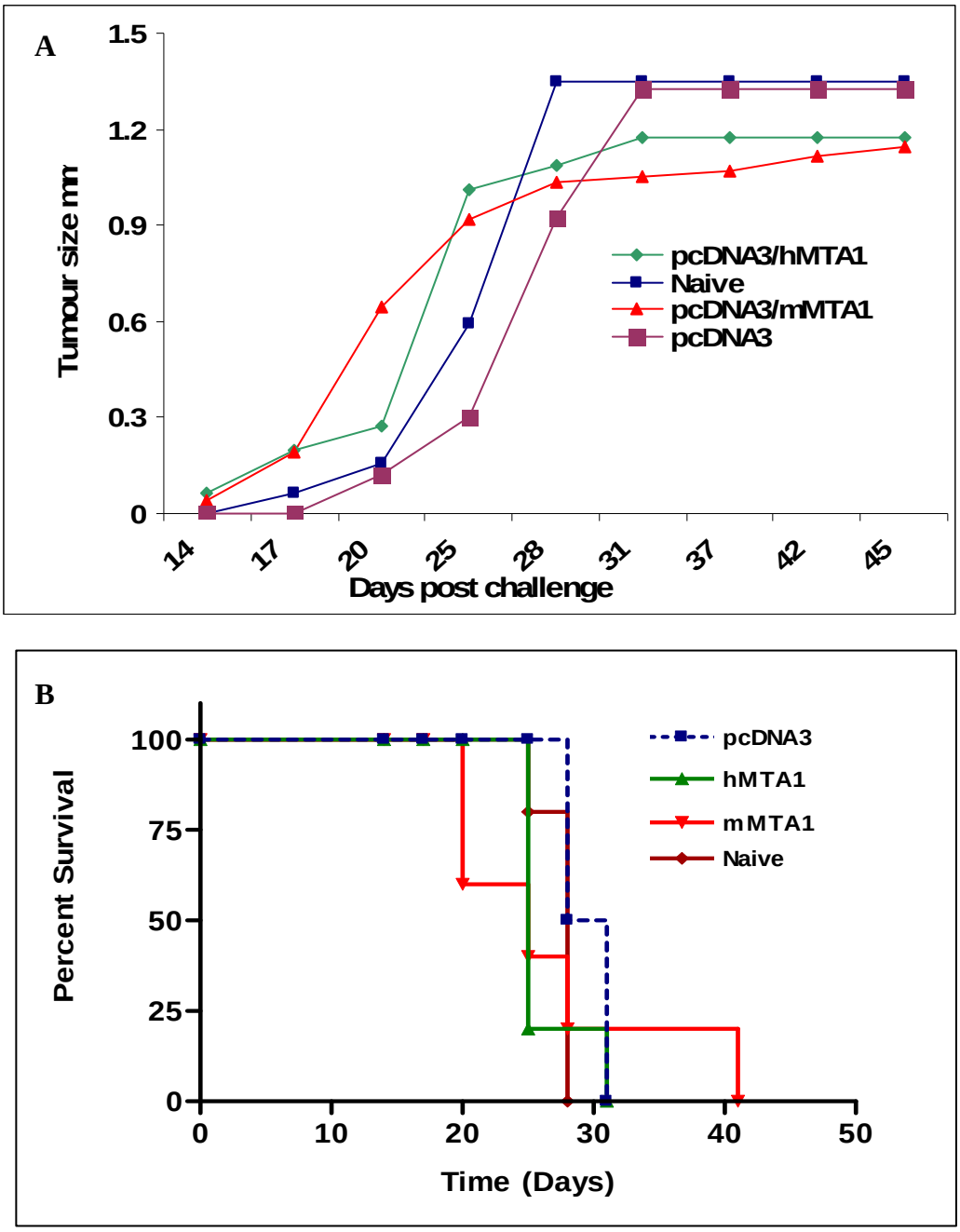


Figure 6.2:- Tumour Challenge experiment following syngeneic and xenogeneic MTA1 gene gun immunisation in balb/c mice. (A) Tumour growth rate (B) Survival Curve. *Mice were immunised 3 times with gold particles coated with pcDNA3-mMTA1 or pcDNA3-hMTA1 at 7 day intervals, followed by challenge with CT-26 cells. No significant survival benefit was observed in vaccinated mice compared to controls. Results shown are representative of two independent experiments with 5 mice per group.*

Several studies have suggested that gene gun immunisation is more likely to polarise the immune response to Th2 type, and this response is not inefficacious in generating anti-tumour immunity due to lack of CTL generation. To exclude this possibility, we immunised balb/c mice IM twice at seven day interval with 100µg of pcDNA-mMTA1/hMTA1 genes or control genes along with 100µg CpG as an adjuvant. Seven days after the final immunisation, mice were challenged with CT-26 cells as before. The results obtained were similar to those obtained using gene gun vaccination, where no significant suppression of tumour growth or survival advantage was observed in vaccinated mice compared to non-vaccinated mice (fig 6.3). Approximately 20 days after tumour challenge, all animals had to be sacrificed as their tumours reached $1cm^2$. It is noteworthy that even xenogeneic intramuscular immunisation was unable to generate any response at all.

Thus, plasmid DNA immunisation on its own was unsuccessful in generating any immune response to MTA1 in this model.

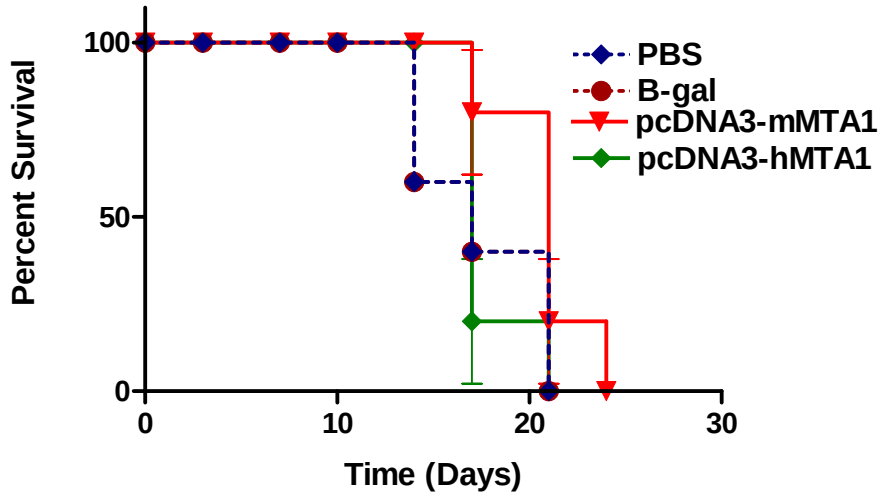


Figure 6.3:- Intramuscular syngeneic and xenogeneic MTA1 immunisation followed by tumour challenge and monitoring for tumour growth (A) and percent survival (B). *Result shown is a representative of two independent experiments.*

6.2.2 Combination of cyclophosphamide and gene gun immunisation

Regulatory T cells (Tregs) have been shown to suppress immune response to self antigens and to tumours. It was hypothesized that peripheral regulatory T cells might be involved in suppressing

the immune response to MTA1. To test this possibility, it was decided to deplete the Tregs and combine it with MTA1 vaccination. Anti CD25 (PC-61) antibody binds to IL-2α receptor and inhibits their proliferation, and has been used in various studies for this purpose. However, although purification of antiCD25 antibody from the PC-61 hybridoma cell line was attempted, insufficient amounts of antibody were obtained. As an alternative, it was decided to use low dose cyclophosphamide for Treg depletion. Naïve balb/c mice were injected twice with either 100µg/kg or 150µg/kg, followed by isolation of CD4+ T cells from the spleens four days later and stained for CD25+ cells. As shown in figure 6.4, compared to naïve mice, the number of CD4+CD25+ T cells had decreased by almost a third following cyclophosphamide administration (7% vs 2.1%). Noticeably, increasing the dose of cyclophosphamide from 100µg/kg to 150µg/kg did not make a significant difference (2.1 vs 2.7%). Cyclophosphamide at higher doses can be immunosuppressive and might lead to depletion of CD4+CD25- T cells as well; hence 100mg/kg was used as the optimum dose of cyclophosphamide for Treg depletion. Moreover, our results were supported by other studies, showing 100mg/kg cyclophosphamide as being efficacious in Treg depletion (North *et al*., 1982; Ercolini *et al*., 2005). It is however important to remember that, Treg depletion is only temporary and these repopulate the periphery within 7-9 days.

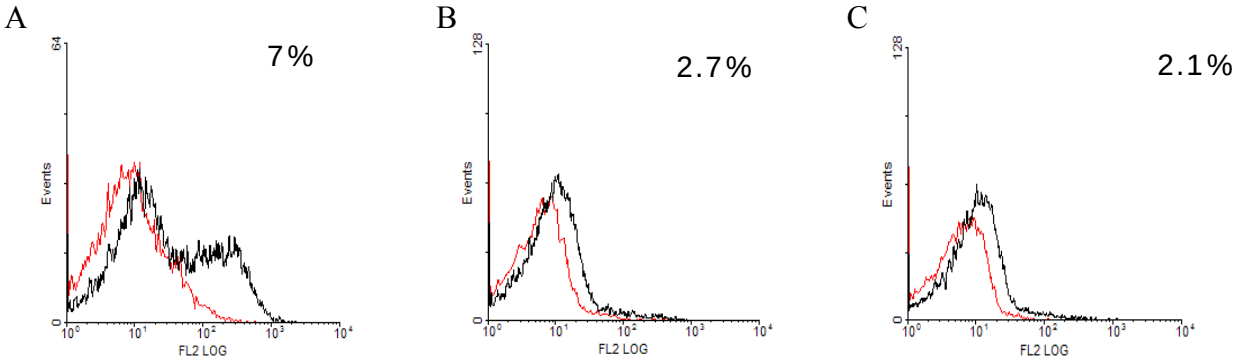


Figure 6.4:- FACS staining for CD25+ cells after isolation of CD4+ T cells from naïve and cyclophosphamide administered mice. *CD4+ T cells were isolated from naïve (A) or mice injected with 100mg/kg(B) or 150mg/kg(C) of cyclophosphamide twice at 5 day intervals and stained for CD25+ cells.*

To combine Treg depletion with MTA1 specific vaccination, we injected mice with 100µg/kg of cyclophosphamide and immunised four days later with pcDNA3-mMTA1/hMTA1 using a gene gun. Following three immunizations at three days interval, mice were challenged with CT-26

cells and monitored for tumour development and survival. Treg depletion using cyclophosphamide combined with MTA1 specific vaccination was not able to produce any significant survival benefit or delay in tumour growth in these mice compared to control animals (fig 6.5). However, the possibility of immunisation protocol (e.g. timing) not being optimum could not be ruled out.

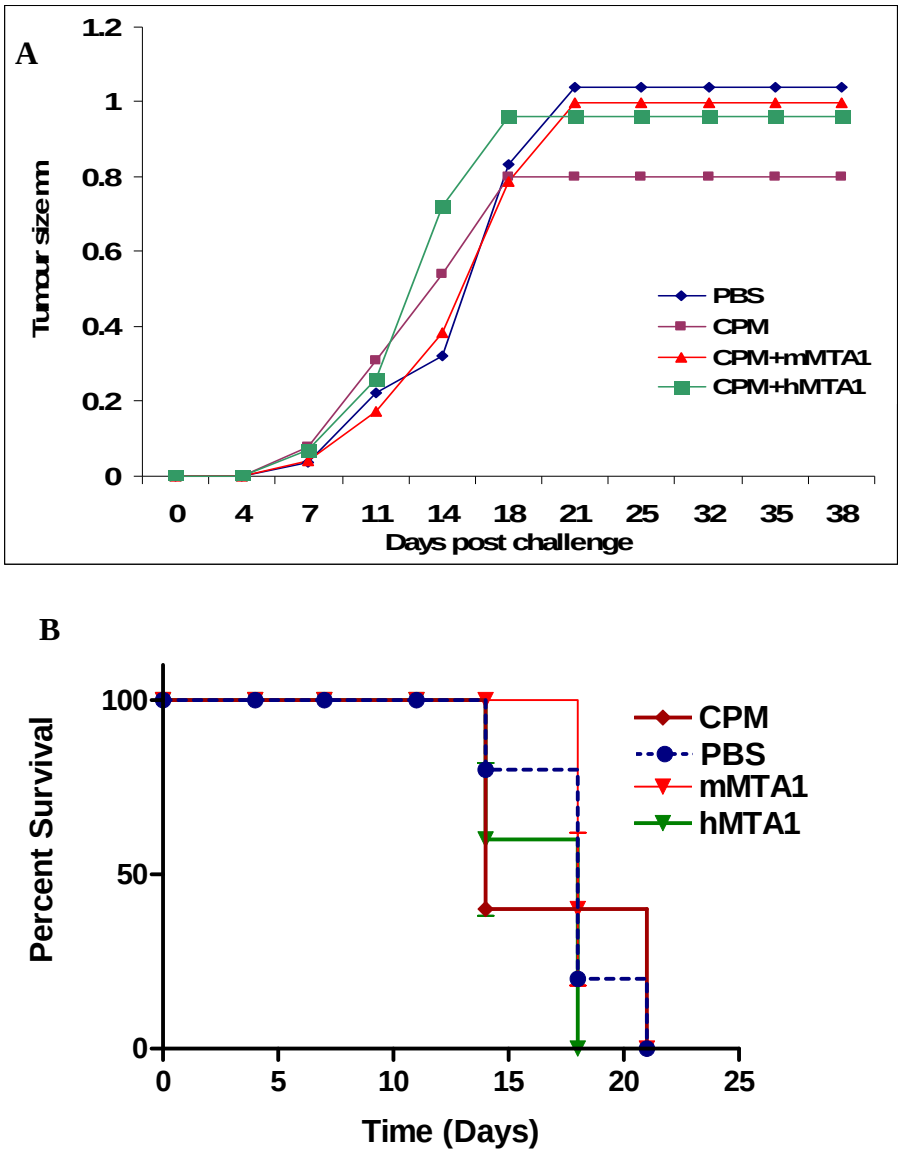


Figure 6.5:- Combination of cyclophosphamide (CPM) and genegun immunisation. *Balb/c mice were immunised thrice with CPM 4 days before gene gun immunisations of pcDNA3-mMTA1/hMTA1, followed by CT-26 challenge 7 days after last vaccination and monitored for tumour growth rate (A) and survival (B). Result shown is a representative of 2 independent experiments with 5 mice per group.*

6.2.3 SFV as a vector for immunotherapy using a 'non-self' antigen

Following the failure of DNA vaccination to generate an immune response to MTA1, it was next decided to investigate viral vaccination. To demonstrate the ability of SFV vector to generate antigen specific immune response *in vivo*, naïve balb/c mice were immunised with SFV-βgal twice at 3 days interval ($1x10^6$ pfu). A week after the booster immunisation, splenocytes were isolated from the immunised mice and re-stimulated *in vitro* for 5 days with immunodominant TPH peptide from beta-galactosidase (bgal) followed by evaluating the specificity of CTLs generated in a cytotoxicity assay using either CT-26 or CL-25 tumour cells which is a CT-26 derived clone transfected with the bgal gene). TPH peptide specific cell lysis was observed with 40% of CL-25 cells lysed by T cells generated from SFV-βgal immunised mice, whereas negligible lysis of CT-26 cells was observed, suggesting that immune response generated was b-gal specific (fig 6.6). More importantly, negligible lysis of CL-25 cells was observed when TPH specific T cells were generated from naïve mice or control SFV immunised mice (data not shown). These results suggest that β-gal protein was efficiently produced *in vivo* upon SFV-βgal immunisation and TPH peptide was naturally processed and presented to prime naïve TPH specific CTLs.

Next, SFV was compared with other viral vectors (Adenovirus-βgal and DISC-βgal) in terms of generating TPH specific CTLs. Following two immunisations of naïve balb/c mice with either of SFV-βgal, Adeno-βgal or DISC-βgal, splenocytes were stimulated with TPH peptide for 5 days *in vitro* as before evaluating them in a cytotoxicity assay. As seen in figure 6.7, TPH specific CTLs generated from all three groups of mice were able to specifically lyse CL-25 cells. Non-specific killing in terms of lysis of CT-26 cells was less than 5% (data not shown). Although, all three vectors were able to generate CTL responses against TPH, at $1x10^6$ pfu, SFV-βgal proved to be most potent and generated approximately 60% lysis of CL-25 cells (fig 6.7). Surprisingly, Adenovirus was least effective in these experiments generating a maximum of 10% killing. Previously published experiments using SFV have demonstrated good efficacy when using $1x10^6$ pfu particles in generating antigen specific immune response (Ni *et al*., 2004). Moreover, attempts to further concentrate SFV virus by sucrose gradient method were unsuccessful. Thus it was decided to use this titre for immunisations in the following *in vivo* experiments.

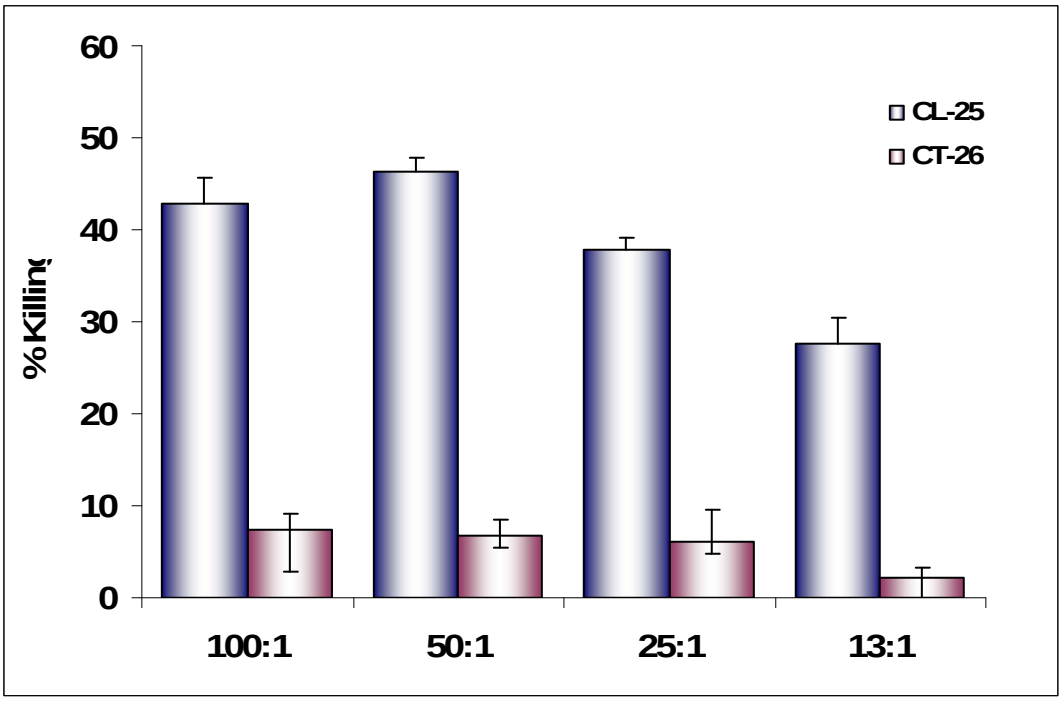


Figure 6.6:- Cytotoxicity assay using CTLs generated from SFV-βgal immunised mice. *Balb/c mice were immunised twice with $1x10^6$ pfu SFV-βgal at 4 day interval. Splenocytes were harvested seven days after last immunisation, re-stimulated in vitro for 5 days with immunodominant TPH peptide (from beta galactosidase protein), followed by testing the CTLs generated in a standard 4 hour chromium release assay against CT-26 or CL-25 cells.*

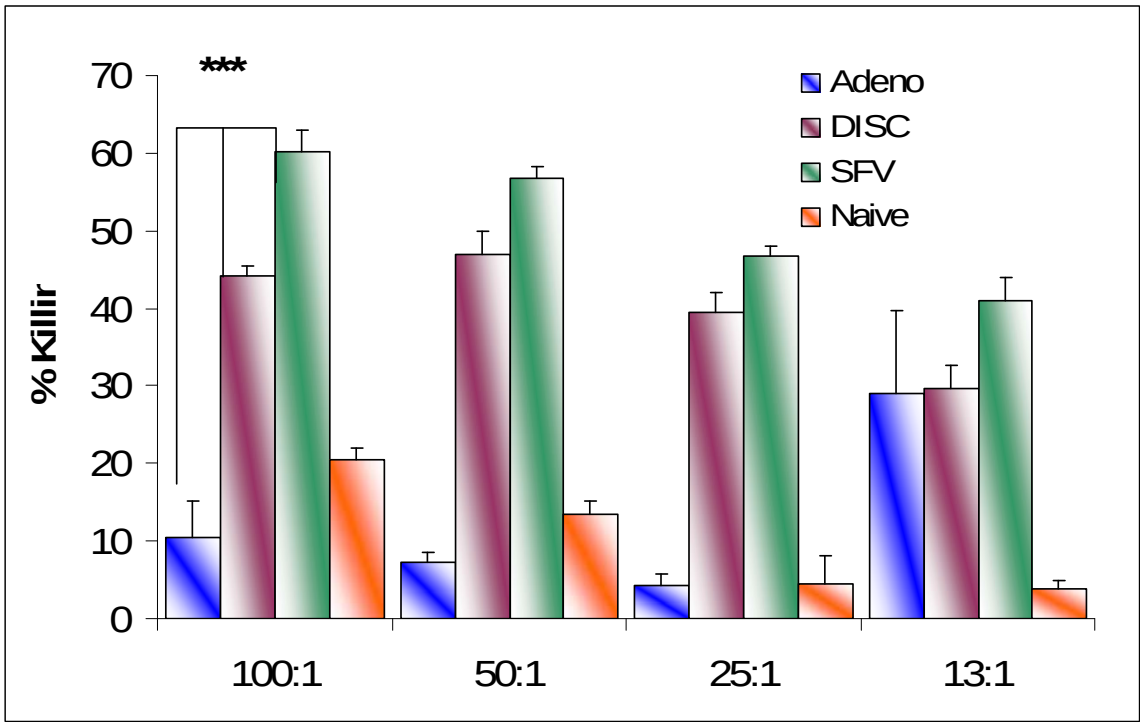


Figure 6.7:- Comparision of SFV-βgal with Adeno-βgal and DISC-βgal vectors at 1x106 pfu for their ability to generate Bgal specific CTLs. *Balb/c mice were immunised with 1x10^6 pfu of either of the vectors twice, followed by in vitro restimulation of splenocytes from these mice with TPH peptide and were evaluated in cytotoxicity assay against CL-25 cells. ($p<0.001$)Statistical analysis done using student's t test.*

Furthermore, to determine whether this *in vitro* cytotoxicity translated into anti-tumour immune response *in vivo*, balb/c mice were immunised twice with 1x10^6 pfu SFV-βgal particles and after seven days of last boost, mice were challenged with either CL-25 or CT-26 cells subcutaneously. These animals were then monitored for tumour development and survival. A significant delay in tumour progression was observed in all the mice immunised with SFV-βgal and challenged with CL-25 cells. More importantly, this translated into significantly improved survival of these mice ($p=0.0007$) and upto 50% of the animals were completely protected from tumour challenge (Fig 6.8A). Control mice (PBS injected) developed rapidly progressing tumours and had to be killed. This study also assessed SFV-βgal in a therapeutic study. In this experiment, balb/c mice with well established palpable (2-3 mm^2) tumours were immunised with 1x10^6 pfu SFV-βgal intra-peritoneally and monitored for tumour growth rate. As seen in figure 6.8B, mice vaccinated with SFV-βgal displayed slow tumour progression and survived significantly longer, although all mice eventually succumbed to the tumours. In comparison, both control groups of mice (injected

with PBS or SFV-βgal immunisation in mice having CT-26 tumours) developed progressive tumours. Considering the fact that no effect of SFV-βgal immunisation was observed in mice harbouring CT-26 tumours, it was presumed that the immune response developed was antigen specific (βgal) and the delayed tumour growth was not a result of a non specific activation of immune system due to the virus administration.

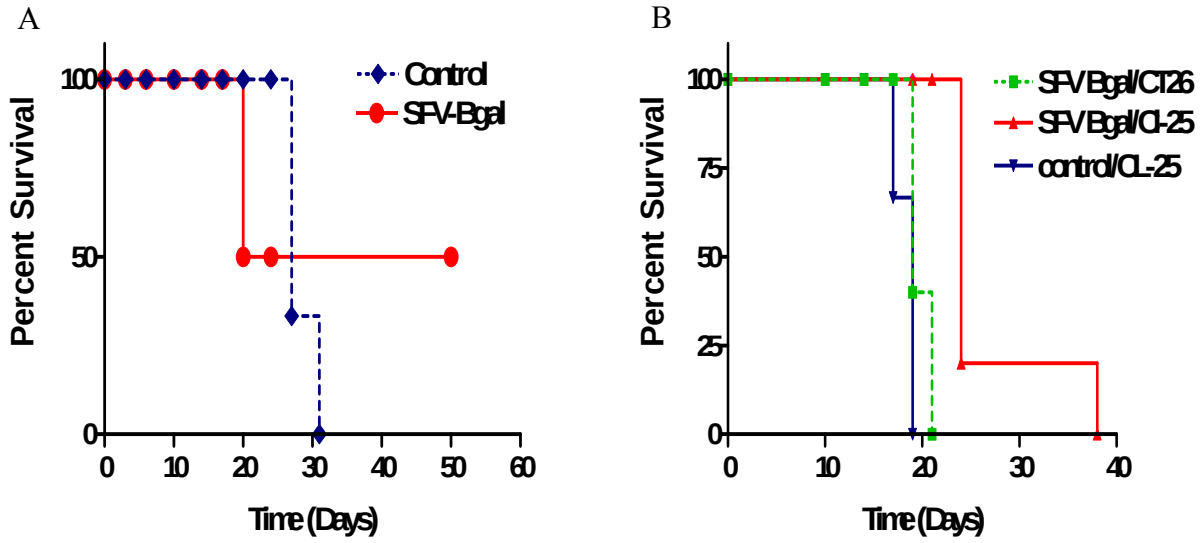


Figure 6.8:- Tumour protection (A) and therapy (B) studies using SFV-βgal. (A) *Balb/c mice were immunised twice with 1x10^6 pfu of SFV-βgal followed by CL-25 tumour challenge seven days later. Animals were monitored for tumour growth rate and survival benefit. (B) Ten days after injecting 8x10^4 CL-25 cells, mice were immunised twice with 1x10^6 pfu of SFV-βgal and monitored for tumour growth. Results shown are a representative of two independent experiments with 5 mice per group.*

6.2.4 SFV vector for immunotherapy targeting MTA1

Having established SFV as a reasonably efficient vector for anti-tumour immune response, it was next decided to investigate SFV vaccination for MTA1. Murine and human MTA1 were cloned into pSMART2b vectors as described previously (chapter 4). Co-transfection of pSMART2b-mMTA1/hMTA1 along with a helper plasmid in 293 cells was used to generate SFV-mMTA1 and SFV/hMTA1 particles. 1x10^6 pfu SFV-mMTA1/hMTA1 particles were used to immunise naïve balb/c mice twice, followed by challenge with CT-26 tumour cells seven days later. Delayed growth of CT-26 tumours was observed in mice immunised with either SFV-mMTA1 or SFV-hMTA1. A statistically significant survival benefit was observed in SFV-hMTA1

immunised mice compared to control (p=0.022) (fig 6.9); although SFV-mMTA1 immunised animals also showed delayed tumour growth and survived longer, it was not statistically significant (p=0.055). Further experiments need to be carried out to improve on this result by using higher titre of the virus and/or combining it with other immuno-modulatory agents, but lack of time did not permit it.

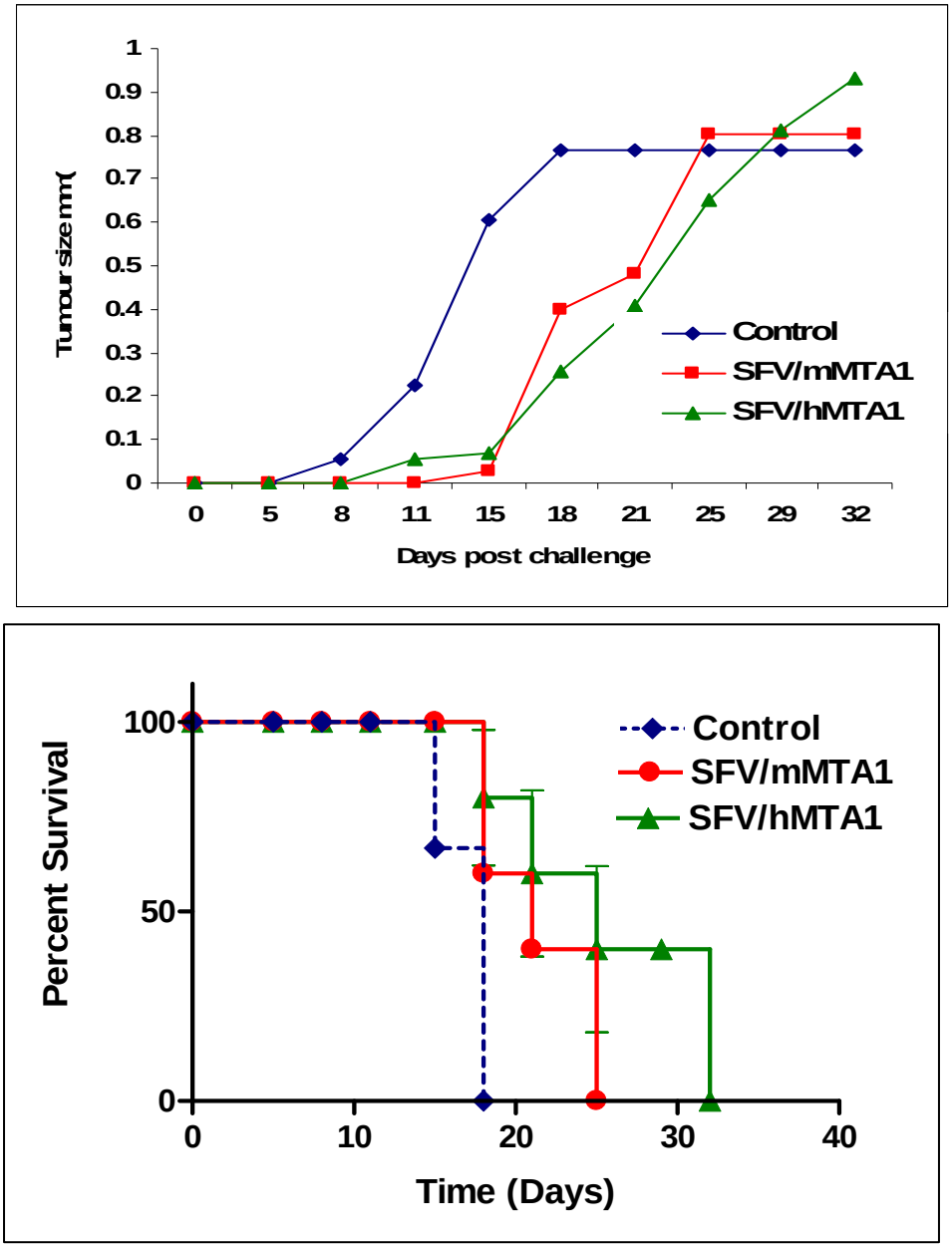


Figure 6.9:- Delayed CL-25 tumour development following SFV-mMTA1/hMTA1 vaccination. *Balb/c mice were immunised twice with 1x10^6 pfu of SFV-mMTA1/hMTA1 followed by CT-26 tumour challenge seven days later. Animals were monitored for tumour growth rate (A) and survival benefit (B). Results shown are representative of two independent experiments with 5 mice per group.*

6.3 Discussion

Previous attempts to identify naturally processed epitopes from MTA1 antigen were unsuccessful and hence we decided to target the full length MTA1 gene for therapy. This would obviate the need to identify peptides for therapy, although without known epitopes, monitoring an immune response to MTA1 could be difficult.

6.3.1 Syngeneic plasmid DNA vaccination

In this study, firstly syngeneic balb/c mice were immunised with gold particle coated with pcDNA3-mMTA1, using a helium gene gun, followed by challenge of tumour cells over-expressing MTA1. The inability to protect mice in this study was not surprising, considering that several groups have questioned the limited potency of DNA vaccines, especially when using gene gun. It was also difficult to generate anti-tumour response in mice whilst targeting beta-galactosidase antigen using this method (data not shown). Although, gene gun immunisation has been successfully used for mediating anti-tumour immune response, most studies have targeted foreign antigens, which are highly immunogenic (Curcio *et al*., 2003). Whether this method is potent enough to break tolerance to self-antigens is not clear. Indeed, a similar study using gp75 antigen demonstrated the inability of different strategies to overcome tolerance in a syngeneic model, whereas xenogeneic gp75 (insect or human) was potent enough to generate CTL response in mouse model (Naftzger *et al*., 1996). In that study, syngeneic gp75 vaccine as a cell based, purified protein, peptide and in combination with different cytokines was unable to generate any antibody or CTL response to mouse gp75. Moreover, different routes of vaccination (intraperitoneal, sub-cutaneous and intradermal) were also unable to break tolerance to gp75, whereas gp75 produced in insect cells or human gp75 vaccination was able to generate auto-antibodies capable of mediating tumour rejection. Interestingly, no CTL responses were detected in any of those experiments; leading the authors to hypothesise that gp75 might be processed exclusively through MHC class II pathway (Naftzger *et al*., 1996). Another interesting study clearly demonstrated the differences of gene gun mediated vaccination in non-tolerant and tolerant animal models. In wild-type balb/c mice, intra-dermal immunisation of rat-her2/neu plasmid using gene gun was able to provide complete protection in immunised mice and this immune response was dependent on CD4+ and CD8+ T cells, among other cells. However, the

same experimental therapy was unable to provide any protection in Her2 tolerant Balb-neuT mice (Curcio *et al*., 2003; Cavallo *et al*., 2006).

6.3.2 Xenogeneic plasmid DNA vaccination

Subsequently, this study assessed xenogeneic immunisation as a means to overcome tolerance as suggested by several recent studies. This strategy of vaccinating syngeneic balb/c mice with human MTA1 gene was also unsuccessful in mediating anti-tumour immune response as observed by no protection to tumour challenge in the mice immunised with hMTA1 gene. Furthermore, to exclude the possibility that the mode of vaccination may have been responsible for the inefficient priming *in vivo*, we repeated the same experiment but immunised the mice intramuscularly with 100μg of syngeneic and xenogeneic plasmids with CpG as adjuvant. Intramuscular immunisation did not enable vaccinated mice to reject CT-26 tumour cells over expressing MTA1 either. Xenogeneic immunisations have been successfully used by Alan Houghton's group to overcome tolerance against several self-antigens and mediate tumour protection/rejection (Gregor *et al*, 2005). However, there are key differences in their studies and this study targeting MTA1. Antigens targeted by Houghton and colleagues have mainly been melanoma differentiation antigens (gp100, gp75, tyrosinase and trp-2), whereas MTA1 is a ubiquitously expressed self antigen, which may result in more profound tolerance to MTA1 than tissue antigens. Notably, realtime PCR results in this study suggested MTA1 mRNA to be also expressed in the thymus at low levels (fig 3.5). It would be interesting to determine whether the protein expression of MTA1 in the thymus could lead to central tolerance and deletion of MTA1 specific T cells. Indeed, a similar strategy targeting prostate specific membrane antigen (PSMA) resulted in generation of auto-antibodies but no CTL response (Gregor *et al*., 2005). Thus, success of this strategy could be vitally dependent on the antigen targeted and its expression pattern. Also, antigen similarity between mouse and human homologues of these differentiation antigens is between 65-85% (gp100-77% identical, trp-2-83% identical), compared to 94% similarity between human and mouse MTA1 (Zhai *et al*., 1997; Bowne *et al*., 1999). It is possible that protein difference within a certain range might be crucial to be able to break tolerance i.e. difference of 23% between human and mouse gp100 could be one of the major factors enabling xenogeneic vaccination to break tolerance, whereas a 6% difference between

MTA1 sequences is not sufficient to modify the processing and binding of CTL epitopes from mMTA1. Other possibility for inefficiency of xenogeneic immunisation in this study could be attributed to the codon bias. Most amino acids can be coded by different genetic codes (for eg:- lysine can be coded by AAA or AAG) and different species tend to have a certain bias for these genetic codes, especially related to the GC content. This affects the stability of the mRNA and the translation efficiency through tRNA. It is known that codon bias exists in certain species affecting the translation efficiency of xenogeneic genes in them. Intramuscular plasmid DNA vaccination encoding an epitope from listeria monocytogenes was unable to generate any CTL response when the wild type sequence was used for vaccination, whereas when the codons were optimised for the mouse system, the translation efficiency of the epitope *in vivo* increased remarkably. Moreover, improved translation efficiency due to codon bias correction translated into epitope specific CTL generation and partial protection of mice from listeria infection (Uchijima *et al*., 1998).

6.3.3 Combination of Cyclophosphamide (CPM) and plasmid vaccination

Finally, to enhance DNA vaccine potency, either depletion of Tregs or inhibiting their suppressive action has been shown to enhance anti-tumour immune response against self antigens. CTLA-4 molecule expressed by Tregs binds to B71/B72 molecules on APC surface and inhibits their antigen presentation efficiency. CTLA-4 antibody can inhibit this interaction, thereby increasing vaccination efficiency. Anti CTLA-4 antibody in combination with xenogeneic DNA vaccination was able to generate CTL and anti-tumour response to self antigens (Gregor *et al*., 2004). Similarly, anti-GITR antibody in combination with xenogeneic DNA vaccine enhanced CTL response to self antigen (Cohen *et al*., 2006). Other studies have shown that depletion of Tregs can be equally effective in generating anti-tumour immune response (Turk *et al*., 2004; Onizuka *et al*., 1999). Low dose cyclophosphamide has been shown to be toxic to Tregs by inducing apoptosis in them and this led us to investigate a combination of low dose CPM with DNA vaccine (Ghiringhelli *et al*., 2004). Four days after CPM administration Treg population decreased to approximately 2% from 7%, which is in agreement with previous studies. In this study, balb/c mice were immunised with 100μg/kg of CPM followed four days later by DNA vaccine three times at seven days interval and challenged with

CT-26 cells. This strategy, depletion of Tregs along with DNA vaccination, was unable to provide significant protection in mice, which developed progressive tumours. Although, this study failed to generate MTA1 specific immune response, role of Tregs in suppression of MTA1 specific CTLs is still inconclusive and further work needs to be done to optimise Treg depletion either with CPM or PC-61 (anti IL-2α) antibody. Also, other methods for suppression of Treg function (anti GITR and anti CTLA-4) alone or in combination with DNA or viral vectors for MTA1 might need to be investigated.

The success of DNA vaccination is crucially reliant on the efficient translation of injected plasmid, duration of expression and the cells targeted by immunisation. In hindsight, inefficient translation of gene-gun and intramuscular DNA vaccinations cannot be ruled out as a cause for inefficient immune response in these studies. Several recent studies have endeavoured to increase DNA efficiency protocols by injection of plasmid intramuscularly followed a few seconds later with electric pulses, which remarkably enhanced vaccination efficacy in animal models. Vaccination by electroporation increases cell permeabilisation at injection site, thereby facilitating plasmid entry into cells. Also, infiltrating DC and other inflammatory cells due to the electric field, could further enhance the immunogenicity of plasmid coded antigen (Cavallo *et al*., 2006). Based on evidence from several studies described above, significant improvements can be undertaken to vaccination protocol used for MTA1 in this study, before MTA1 can be ruled out as an immunotherapeutic target.

6.3.4 SFV vector as an immunotherapeutic agent

Finally, it was hypothesised that delivering MTA1 in a highly immunogenic setting could trigger a potent immune reaction and would be able to overcome peripheral tolerance mechanisms, which might influence anti-MTA1 immune response. The SFV vector was chosen due to several reasons; relative ease of manipulation, safety profile, ability to prime cell-mediated and humoral immune responses, high level protein expression and negligible chance of integration in host cell genome (Daemen *et al*., 2000). In this part of the study, SFV potency to generate an immune response to foreign antigens was first evaluated. SFV-βgal particles were generated and tested for their capability to generate immune response to an immunodominant TPH peptide from β-galactosidase antigen. CTLs generated in balb/c mice by SFV-βgal immunisation were capable

to specifically killing CL-25 cells, which have been stably transfected to express βgal antigen. At a similar dose, SFV was more potent than adenovirus and DISC-HSV in terms of generating higher cytotoxicity against relevant target cells. However, these experiments were only performed at one concentration ($1x10^6$ pfu), and higher dose of adenovirus and DISC-HSV might be equivalent or better than SFV. It is noteworthy, that several studies have used doses of up to $1x10^8$ pfu for the above viral vectors for anti-tumour immune responses.

SFV vectors have been evaluated in a number of animal models for their efficacy in generating CTL responses and anti-tumour immune response. Immunisation of naïve C57BL/6 mice with SFV vector coding for E6 and E7 proteins (human papilloma virus proteins) provided protection from tumour cells expressing these proteins in 40% of animals (Daemen *et al*, 2000). In that study, injection of as few as 10^4 virus particles was able to generate CTL response against E6 and E7 protein expressing cell line, whereas injecting 10^6 particles led to significantly delayed tumour growth and complete protection in 10% mice. A subsequent study from the same group demonstrated regression of well established (500 mm^3) tumours in the same cervical cancer mouse model (Daemen *et al*., 2004). Although all routes of immunisation were capable of generating CTLs in this model, the authors observed intravenous injections to be the most effective. In another study, intra-peritoneal injection of 10^6 SFV viral particles expressing P1A gene was sufficient to protect 80% of animals from challenge of tumour cells expressing P1A antigen (Ni *et al*., 2004). Moreover, SFV vaccination was also able to generate therapeutic immune response in 50% of mice in the same tumour model.

To confirm these results and investigate whether βgal specific CTL induction would translate into protective and therapeutic anti-tumour response *in vivo*, we used CL-25 cells as a tumour model. Intra-peritoneal vaccination of naïve balb/c mice with $1x10^6$ pfu SFV-βgal delayed tumour growth in all mice and protected upto 50% of the mice from tumour development. Moreover, SFV-βgal administered to mice with well established tumours, significantly delayed tumour growth but was noticeably less efficient compared to prophylactic experiments. This is not surprising since in therapeutic experiments, the immune system is likely to be manipulated and/or suppressed due to the presence of tumour cells and immuno-suppressive factors secreted by it. Although, no tumour regression was observed in therapeutic experiments, it is likely to be

due to low dose of the virus injected and increasing the virus dose for vaccination would have to be evaluated in future therapeutic experiments.

6.3.5 SFV-MTA1 mediated delay in CT-26 tumour progression

Having established the efficacy of SFV particles in protecting mice from tumour development, the SFV-MTA1 vector was tested for the ability to protect mice from MTA1 over-expressing tumour. Initial experiments have demonstrated a significant delay in tumour growth in mice vaccinated with SFV-hMTA1. Although, delayed tumour growth was observed on vaccination with SFV-mMTA1, it did not reach the level of statistical significance. This delay in tumour progression in vaccinated animals was reproducible. It should be emphasised that none of the vaccinated animals survived the tumour challenge, indicating that the immune response was relatively weak. Experiments need to be conducted using higher titre of the virus and its combination with other therapeutic interventions such as Treg depletion. It is noteworthy that the immunisation protocol and dose of immunisation used in this study were similar to a recently published study and was shown to be highly effective in preventive and therapeutic experiments (Ni *et al*., 2004). However, the antigen targeted in that study, P1A, a cancer testis antigen with less likelihood of being able to generate immune tolerance. Lack of time did not permit to perform these experiments but are necessary to conclusively appraise the therapeutic potential of MTA1.

These studies suggest a strong regulatory component against MTA1 making the generation of strong immune response difficult. But it would be reasonable to assume that given the optimal protocol and strategy of immunisation, immune response can be generated against most antigens, albeit at the risk of potential autoimmunity developing. Although, MTA1 boasts of having most of the characteristics for an ideal tumour antigen, its expression in several normal tissues is likely to play a major role in suppressing the immune response to it. It would be crucial to firstly evaluate MTA1 protein expression in thymus during development. Central tolerance may affect MTA1 CTL repertoire and may depend on this thymic expression. However, since MTA1 was recognised and cloned by SEREX in our laboratory, it suggests that central tolerance might not be very stringent for CD4+ T cells. In the study identifying MTA1 by SEREX, approximately 10-15% of the prostate cancer patients produced antibody response to MTA1, although the

number of serum samples analysed was small (n=13). More samples from cancer patients need to be analysed, including other cancers to confirm the immunogenicity of MTA1, not only in prostate but other cancers as well. Another potential hurdle in generating immune response to MTA1 and other self-antigens is that they are expressed in normal tissues in the absence of co-stimulatory molecules which would rapidly tolerised any CTLs generated, leading to decreased protective and/or therapeutic efficacy of vaccination. It has been showed that CD4+CD25+ cells can be generated from naïve CD4+ T cells *in vitro* depending on antigen stimulation protocol. Thus, it is likely that chronic presentation of MTA1 in a non-immunogenic context by normal tissues may generate MTA1 specific Tregs and immunising with MTA1 coding vectors would actually activate these cells, thereby suppressing the immune system and making clinical progress worse. Indeed, Nishikawa *et al* (2003) have recently shown that DNA immunisation using SEREX defined self-antigens (similar as MTA1) leads to activation of antigen specific Tregs and enhanced tumour growth in immunised animals compared to control irrelevant gene vaccinated mice. Interestingly, this phenomenon could be reversed by IFN-γ either produced by CD8+ T cells or externally administered (Nishikawa *et al*., 2005a; Nishikawa *et al*., 2005b). From the above study, it can be hypothesised that administration of SEREX defined self-antigens in a highly immunogenic context (viral vectors) could cause high level IFN-γ secretion and reversal of Treg generation. Whether MTA1 immunisation would also generate Tregs is not known and needs to be evaluated.

Thus, caution needs to be exercised in selecting antigens for immunotherapy of cancer. In spite of the fact that MTA1 has showed limited potential, as an immunotherapeutic target, it seems reasonable to propose that other metastasis associated antigens should be looked at in future, if immunotherapy needs to be applied to late stage cancer patients.

Chapter 7:- Discussion

Cancer is an intractable disease of varied spectrum and stages and can be defined as "uncontrolled cellular proliferation which at its advanced stages spreads to distant organs of the body" and is self sufficient for their growth. Normal cell growth and division, which is controlled by several key genes called oncogenes and tumour suppressor genes, is deregulated owing to genetic events affecting these genes. According to the immunosurveillance theory, these transformed cells are constantly eliminated from the body. However, as suggested by Robert Schreiber, a state of equilibrium is reached at some point where the rate of elimination of cancer cells is balanced by their repopulation (Dunn *et al*, 2004). During this phase the cancer cells are also edited by the immune mechanisms selecting for more aggressive and less immunogenic tumour cells (Escape). Taking advantage of the fact the T cells can recognise tumour antigens and eliminate cells expressing them, strategies to activate T cells against such antigens has gained unprecedented interest over the past few years. But caution must be exercised whilst selecting the target antigen as an immune reaction against non-essential antigen might actively select for cells which down-regulate specific antigen and thereby hasten the immuno-editing and escape stage. This compelled us to investigate the criteria defining the ideal tumour antigen for immunotherapy.

7.1 Tumour Antigens and Immunotherapy

Over the past one and a half decades, since the identification of the first tumour antigen (MAGE-1), immunotherapy has rapidly evolved as the most promising strategy to selectively target cancer cells. Moreover, being mediated by our own immune system it is likely to be devoid of the distressing and sometimes life threatening side effects of chemotherapy and radiotherapy. However, initial enthusiasm generated from the phenomenal success of immunotherapy in animal models suffered a set back because of limited clinical benefit in humans. Whilst investigating immunotherapy for potential clinical application in human patients, two key parameters have to be considered the tumour antigens targeted and the relevant animal model used to investigate it.

A number of characteristics should be used to define an ideal tumour antigen and pursuing them in animal and human studies. Although a number of groups are advocating personalised vaccines based on individual tumour antigens expressed, this is likely to be quite expensive and impractical, especially in the developing world. Hence, antigens widely over-expressed in tumours are the most suitable targets. For example, bcr-abl is over-expressed in majority of CML patients and has been extensively investigated for immunotherapeutic purpose (Clark *et al*, 2001). Antigens like bcr-abl and viral antigens are very specific for certain tumours but similar antigens for most other cancers have not been identified till date. Cancers are derived from normal human tissues and most of them over-express certain essential genes required for growth and proliferation. Such antigens are shared between several different tumour types and are the most practical targets as they could be applicable to the majority of population. Although, targeting over-expressed self-antigens can generate auto-immune response as seen in melanoma clinical trials, several other studies have demonstrated no such reactions (Yagi *et al*., 2006). Expression of such genes in normal tissues is unlikely to generate the threshold of MHC-peptide complexes capable of activating T cell responses. Another important criterion is the role of the antigen in transformation of cells. Targeting a non-essential antigen by therapy could eventually lead to de-selection of tumour cells expressing such antigens, thereby immuno-editing and selecting of less immunogenic and escape variants from tumours. Thus, antigens essential for maintaining the oncogenic phenotype are most promising targets. However, another strategy could be to target several non-essential proteins, which would also make generation of escape variants less plausible.

Finally, it is widely agreed that immunotherapy is likely to be more successful if used in combination with the conventional modalities of therapies (Lake & Robinson, 2005). Removal of the primary tumours by surgery and targeting the metastasis by immunotherapy is likely to be highly effective, since removal of the bulk of tumour not only leaves less cancer cells to be dealt by the immune cells but also eliminates most of the immuno-suppressive factors released by the cancers. Thus, late stage antigens involved in establishing the metastatic phenotype of tumour cells seem to be ideal candidates for immunotherapy.

7.2 MTA1 as a potential candidate for cancer

Initially identified in 1994 by Toh *et al*, several studies have since demonstrated over-expression of MTA1 in human cancers such as lung, hepatocellular, breast, pancreatic, oesophageal, colorectal, gastric, ovarian, laryngeal and prostate cancer (Tang *et al.*, 2003; Sasaki *et al.*, 2002; Toh *et al.*, 1999; Nicolson *et al.*, 2003; Moon *et al.*, 2004; Nawa *et al.*, 2000). Independently, MTA1 was identified using SEREX expression cloning by Li *et al*, demonstrating the presence of antibody response in cancer patients against MTA1 (Li *et al*, submitted; Assudani *et al*, 2005). Approximately 40-50% of the cancers over-expresses MTA1 most of which have been correlated with metastatic and angiogenesis, and seems like a good target as it would be applicable for majority of the population. Moreover, most of the above studies were conducted using primary tumours and it is reasonable to assume that MTA1 expression would be even higher in the metastatic cells. Moreover, being highly conserved in different species, MTA1 is likely to be essential for the growth and survival of tumour cells and indeed normal cells. MTA1 is a part of HDAC complex and is involved in transcription repression (Toh *et al.*, 2000). However, its structure suggests that it may also have a role as a transcription factor. Contradicting most previous studies suggesting MTA1 to be localised in nucleus, a recent study showed that it can interact with endophilin 3 in the cytoplasm of brain cells. Several splice variants of MTA1 were identified in recently mice and it might be interesting to explore whether different splice variants of MTA1 are responsible for its varied functions (Yaguchi *et al.*, 2005).

Three independent studies provided evidence regarding non-redundancy of MTA1 for growth and metastatic potential of tumour cells. Over-expression of MTA1 in immortalised keratinocytes cell line (HaCaT) led to increased invasive and migratory potential of these cells along with increased resistance to apoptosis by up-regulation anti-apoptotic molecule Bcl-xl (Mahoney *et al*., 2002). These results were also confirmed in a pancreatic cancer model as well (Hofer *et al*., 2004). Complementing these studies, down-regulation of MTA1 had the opposite effects. Down-regulation of MTA1 expression using antisense phosphorothioate oligonucleotides resulted in growth inhibition of human breast cancer cells whereas siRNA mediated MTA1 inhibition led to decreased malignant phenotype in human oesophageal carcinoma cells (Nawa *et al*., 2000; Qian *et al*., 2005). All these studies provide strong evidence supporting MTA1's role in late stage cancer progression. However, the mechanism used and the target genes for MTA1 over-expression were not known until recently. MTA1 expression was significantly correlated

with intratumoral microvessel density (MVD) in human breast cancer samples and suggested that amongst other function, one of the major role of MTA1 was facilitating angiogenesis which is essential for cancer cells to metastasise in the first place and for progressive growth at distant organs (Jang *et al*., 2006). Indeed, a recent study showed MTA1 to be responsible for increased stabilisation of hypoxia inducible factor-1 alpha (HIF-1α), thereby up-regulating vascular endothelial growth factor (VEGF) and enhancing angiogenesis (Moon *et al*., 2006). In breast cancer cells specifically, breast cancer-amplified sequence 3 (BCAS3) was shown to be a target for MTA1, which is responsible for increased tamoxifen resistance in them (Gururaj *et al*., 2006). Considering the expression data and role of MTA1 in cancers, it can be hypothesised that not only can MTA1 be targeted in late stage metastatic cancer cells of different backgrounds but it can also be used in combination with traditional therapies to improve therapeutic efficacy.

Finally, immunogenicity of an antigen would be the deciding factor in endorsing it with target of immunotherapeutic potential. Not all tumour antigens are immunogenic and it would especially be so for over-expressed self antigens such as MTA1. Immune cells are educated during development to ignore self-antigens; however the number of auto-immune diseases indicates that this process is not water tight and in specific priming conditions, these immune cells can be activated to react against self antigens. Although MTA1 is an over-expressed self antigen, its identification by SEREX in our laboratory (Li *et al*., submitted; Assudani *et al*, 2005) provides some evidence that MTA1 has been recognised by the CD4+ T cells, which in turn stimulated B cells to produce antibodies against it in cancer patients. It was hypothesised that, CD8+ as well as CD4+ T cells may exist in patients, whose tumours over-express MTA1. Combined together these studies strongly suggested that should MTA1 be immunogenic, it would represent the ideal immunotherapeutic target against metastatic cancer and therefore MTA1 immunogenicity was investigated.

7.3 Animal models for cancer immunotherapy

Animal models have been used extensively in the past to model human diseases including cancer. However, recently reliance on animal models has been seriously questioned due to lack of significant clinical benefits of therapies, which showed remarkable benefit in them. Nonetheless, animal models have provided great insights into human disease leading to

discovery of number of drugs and medical interventions. Although it can be argued that human physiological systems is quite different to that of mice, it is more likely that poor animal models used in several studies are responsible for the failure of clinical trials in humans.

Most immunotherapeutic models of cancer use mice as a choice of animal due to obvious reasons; short life providing quick answers, relatively inexpensive, easy to handle, ease of genetic manipulation and similar physiological systems as humans. A number of therapeutic strategies have been investigated on transplantable tumours in mice. These tumours grow quite rapidly and are injected at a site (sub-cutaneous) which is not their usual anatomic location. Moreover, antigens used in the studies are mostly foreign and highly immunogenic (ovalbumin is the most commonly used antigen), whereas most tumour antigens are likely to be self in origin and less immunogenic (Dullaers *et al*., 2006). Other models employ mice producing T cells with transgenic TCR for a specific epitope, which is a highly artificial system as antigen specific T cells are quite rare in normal circumstances (Marzo *et al*., 1999). Hence, selection of an animal model to investigate a novel therapeutic antigen or any other intervention can be most critical in attaining results which can be translated in to humans. Thus it was necessary to first confirm expression levels of MTA1, not only in human cancers but also in murine cancers and tissues.

7.4 MTA1 expression in human cancers

MTA1 over-expression was confirmed in several tumours of different histological origin such as gastric, colorectal, breast and prostate cancer (data not shown). Levels of MTA1 in cancer samples were comparable to those previously published. Approximately 40-60% of cancer samples were shown to be over-expressing MTA1 and the rise was generally 2-4 folds. Interestingly, other studies have demonstrated similar rise for other metastasis antigens as well. This suggests that, although the levels of such metastasis associated genes do not increase a great deal, but it is enough to support the migration and metastatic ability of tumour cells. The numbers of samples examined in this study were small but some correlation between tumour size and node involvement was observed for colorectal cancers but not gastric cancers. A similar increase in MTA1 level was observed in breast cancer samples, although several samples could not be evaluated accurately due to very low quantities of mRNA available from them. MTA1 has been extensively investigated in breast cancer and has been correlated to angiogenesis. Interestingly, MTA1s a splice variant of MTA1 has been implicated in binding to oestrogen

receptor α (ERα) in the cytoplasm of breast cancer cells (Kumar *et al*., 2002). Oestrogen receptor expression is considered as a good prognostic marker for breast cancers and is down-regulated due to binding of MTA1s to ERα. Thus, by inhibiting MTA1s, ERα expression can be restored in the nucleus and thereby making breast cancer cells responsive to hormonal therapy. This gives an additional strategy to exploit this group of antigens in breast cancer patients. MTA1 over-expression has also been correlated with prostate cancer progression (Hofer *et al*., 2004). MTA1 expression in prostate cancer samples was also studied and only 2 out of 10 patients demonstrated significantly elevated MTA1 levels compared to benign prostate tissue (data not shown), but could not be correlated with disease progression in these cases. Larger numbers of samples need to be examined to draw accurate conclusions, preferably from metastatic samples. However, metastatic samples are quite difficult to obtain. LCM samples should be used in future studies to accurately evaluate MTA1 expression in cancerous cell without the surrounding stroma or normal cells. Moreover, similar to breast cancer MTA1 expression should also be correlated with angiogenesis in other cancers. A recent study observed a paradoxical relationship between MTA1 and p53 tumour suppressor gene (Qian *et al*, 2005). Inhibition of MTA1 by RNAi also decreased p53 protein levels, although cell proliferation also decreased. Being a tumour suppressor gene, increase in p53 has generally been associated with inhibition of cell proliferation and would be expected to correlate with decreased MTA1 levels. This observation also needs to be confirmed to explain the complex biological role of MTA1 in cancer cells.

7.5 Validation of MTA1 in a mouse model

As discussed previously, animal models can provide a wealth of information if correctly chosen. MTA1 is a highly conserved molecule, being 84% and 96% similar at the DNA and protein levels respectively between mouse and human. Also, its expression pattern, localisation and functions seem to be identical. Interestingly, several splice variants of MTA1 have been recently identified in mice, most of which are yet to be discovered in humans. One of the splice variants of mouse MTA1 is similar to the MTA1s antigen identified in human breast cancers. Considering these factors, it was hypothesised that results obtained from murine studies targeting MTA1 could be directly applicable to humans as well. To verify MTA1 expression at mRNA and protein levels in murine tissues and cancer cells, this study evaluated its expression using conventional PCR, real-time PCR and western blotting. Both conventional and real-time PCR

confirmed that MTA1 was highly over-expressed in all the tumour cells examined compared to low level expression in most normal tissues except testis. Interestingly, spermatogenesis is a highly active process requiring high motility and proteolytic enzymes, and these properties are also shared by metastatic cells.

These results were further confirmed at the protein level of MTA1 as mRNA and protein levels, do not necessarily always correlate. However, in this study western blotting (WB) confirmed the results of PCR and high level of MTA1 protein was observed in all cell lines compared to normal tissues, where no protein expression could be detected. Previous studies have demonstrated low level protein expression of MTA1 using immuno-histochemistry in normal tissues but in this study, perhaps the levels of MTA1 were below the detection limit of WB. Increasing the quantity of protein loaded by per lane for WB might have enabled MTA1 protein detection. This could be highly significant since threshold levels of MHC-peptide expression is required on the cell surface for recognition and mediation of CTL activity and very low protein levels of MTA1 may not allow that threshold to be reached, thereby preventing auto-immune response of CTLs generated against MTA1 by vaccination (Stevanovic & Schild, 1999).

These results justify investigating MTA1 as a target for immunotherapy against cancer in a mouse model, with a potential to being directly relevant to future human studies.

7.6 Peptide vaccines and MTA1

Identification of epitopes presented on the surface of tumour cells in an MHC restricted manner can be recognised by CTL leading to tumour lysis. Theoretically, CTLs generated against any such single epitope would be able to mediate tumour rejection. Indeed, a minority of patients demonstrate significant clinical responses in trials of peptide vaccines (Rosenberg *et al*., 1998; Rosenberg *et al*., 2004). Identification of such peptides can prove to be considerably challenging but recent technological advances and availability of transgenic mice expressing human HLA molecules have expedited this procedure. Several algorithms are available on the World Wide Web, which allows prediction of peptides that could bind with higher affinity to a particular MHC allele. These peptides can then be synthesised and tested *in vitro* using human PBMC from donors or *in vivo* in transgenic mice. This study used the widely used software SYFPEITHI to predict peptides from MTA1 protein likely to be immunogenic. These peptides were then tested in transgenic mice for immunogenicity and natural processing. Three out of seven peptides

evaluated in syngeneic balb/c mice were found to be immunogenic. However, CTLs generated against these peptides were unable to kill tumour cells naturally over-expressing MTA1 at high levels, suggesting that these peptides were not naturally processed and presented by tumour cells. Similarly naturally processed peptides could not be identified from human MTA1 in HLA-A2 transgenic mice.

Inability to identify naturally processed peptides using the reverse immunology approach can be due to several reasons. Firstly, it is now widely believed that high affinity CTLs for self-antigens are likely to be deleted in the thymus and CTLs allowed to leave the thymus would be of medium to low binding affinity. Activating these CTLs would require highly immunogenic strategy and peptide vaccine on their own is not potent enough to do that. Thus, peptides identified as non-immunogenic in this study were perhaps less immunogenic and naturally processed, but the vaccination protocol used was not able to enhance this response. It should however be noted, that different adjuvants were attempted for vaccination, such as IFA, CpG and IFA/CpG combination with similar results (data not shown). It is more likely that the peptides tested in this study were indeed not naturally processed. Hundreds of peptides can be classified as medium/low binding epitopes according to their predicted binding score. But evaluating each of them is both expensive and time-consuming. Although prediction softwares have improved in recent years to take natural processing into account this is still not very accurate. Moreover, as established by a recent study, alternate splicing by the proteasome can produce a completely novel peptide from some protein (Vigneron *et al*., 2004). These peptides would be missed by currently available algorithms, but are nonetheless potential therapeutic targets. A more direct approach would be to elute peptides from the surface of tumour cells over-expressing MTA1 and mass spectrometry mediated identification of MTA1 derived peptides. However this technique is time consuming, laborious, expensive and requires huge numbers of cells. Moreover, eluted and identified peptide still does not guarantee their adequate immunogenicity for *in vivo* killing of tumour cells.

Since MTA1 is a self antigen, it is likely to be processed in the thymus by MHC class I processing pathway causing central tolerance of CD8+ T cells. CD4+ T cells may escape from this mechanism, since; MTA1 identification by SEREX suggests the existence of CD4+ T cells against it. Using this argument two class II human MTA1 derived peptides were evaluated in

HLA-DR4/DR1 transgenic mouse models. Both peptides were non-immunogenic in HLA-DR1 mice but were highly immunogenic in HLA-DR4 transgenic mice. However, neither of these peptides were naturally processed as tested using two different methods (gene gun & lysate, see chapter 5). It is now known that intracellular proteins can also be presented in MHC class II restricted context, which if true for MTA1, would affect CD4+ T cell repertoire as well (Bogen *et al*., 1990). More peptides need to be investigated for MHC class II to draw conclusions regarding mechanism(s) underlying the existence of T helper cells against MTA1.

7.7 DNA vaccine and MTA1

DNA based vaccine strategies obviate the need to identify specific epitopes from antigens and can be used to target whole antigens which might generate immune response against multiple epitopes, irrespective of HLA type of the person. To test this, mouse MTA1 cDNA sequence was cloned into a mammalian expression vector, which was then used to immunise mice using either the gene gun or intramuscular method. Immunised mice were not only tested to reactivity against specific peptides but also for their ability to be protected from CT-26 tumour challenge, naturally over-expressing MTA1. Immunisation with mouse MTA1 was neither able to generate response to any of the peptides previously tested nor provide any protection from fatal tumour challenge. The inability of mMTA1 DNA vaccine to generate immune response was not surprising, as similar studies targeting self antigens using plasmid vaccine have demonstrated very limited potential, even with different methods of vaccination (Naftzger *et al*., 1996; Curcio *et al*., 2003). It was hypothesised that syngeneic DNA immunisation was not potent enough to be able to break tolerance to MTA1 and considering several recent studies, xenogeneic vaccine was employed as the next strategy (Bowne *et al*., 1999; Gold *et al*., 2003; Naftzger *et al*., 1996). Immunisation of mice with human MTA1 was also unable to provide syngeneic balb/c mice with protection from CT-26 tumour cell challenge. Furthermore, it is widely acknowledged that Tregs are responsible for suppression of self-antigen specific immune response and their depletion helps to overcome this. This led us to try a combination therapy regime consisting of Treg depletion using low dose cyclophosphamide and DNA vaccination. Although, this combination regime failed to provide significant benefit in immunised animals, further experiments are required with other Treg depletion methods to draw definitive conclusions.

In spite of a number of advantages of DNA vaccines, this study underlines the problems associated with it as well as additional factors that need to be considered. Immune response to DNA based vaccines is crucially dependent on antigen delivery and persistence, targeting the right cells, adjuvants as well as ensuring the appropriate processing depending on the response desired. Although, the presence of unmethylated CpG islands in the plasmid DNA are known to be good adjuvants in certain studies, their lack of potency is paradoxically blamed as the causative factor for failure of DNA vaccines, which might also be the case in this study. To overcome this, strategies such as co-administration of cytokine genes, co-stimulatory molecules, xenogeneic vaccine, using gene gun to target skin resident langerhans cells as well as including signalling molecules to target antigen to either MHC class I and class II pathway, have all been successfully applied in different studies. Moreover, accounting for codon bias also resulted in immune response generation to previously non-immunogenic native antigen. Consensus also needs to be reached regarding the optimum vaccination strategy for DNA vaccines. Several studies advocate intramuscular route as method of choice whereas other propose gene gun mediated plasmid delivery. Th2 polarisation of the immune response by gene gun mediated vaccination could be a potential problem for cancer immunotherapy and this needs to be properly addressed in future studies. Moreover, the intramuscular route is generally accompanied by administration of cardiotoxin (muscle degenerating drug) in animal studies, to increase inflammatory cell influx at vaccination site leading to superior antigen presentation. Whether this strategy would be feasible in humans is still not clear. The failure of DNA based vaccines in human clinical trials could be attributed, in part, to poor planning based on conflicting results obtained in animal studies and improper optimisation of vaccination strategies.

Understanding of these factors would be all the more important, for use of ‘self-antigens’ as targets for immunotherapy, since most studies have used viral or foreign antigens, and the response to these antigens may not apply to ubiquitously expressed ‘self-antigens’.

7.8 Viral Vaccine and MTA1

The aforementioned high vaccination efficacy as well as potent immunogenicity of viral vector compelled investigation into this method of vaccination. Several vectors, viral and bacterial, have been used in the recent years for cancer immunotherapy. These pathogens are naturally capable of infecting a majority of cell types, using the cellular machinery for their propagation

leading to high levels of viral antigen production by the cells. These properties have made these vectors an attractive choice for cancer immunologists. Vectors such as adenovirus, canarypox virus, fowlpox virus and herpes simplex virus have been extensively used for this purpose. Although most of these vectors have been highly successful in animal models, their application in humans raises certain health and safety, and ethical concerns due to their chance of integration into human genome as well as opportunist progressive infection in terminally ill immuno-suppressed patients. This has dictated researchers to come up with novel vectors, which are highly safe without losing their potency.

Semliki forest virus is one of the new generation vectors with multiple bio-safety levels for safe application in humans. Genes coding for the virus have been split into two separate vectors, one coding for antigens and the other coding for the structural proteins needed for viral particles assembly. In order to generate infectious viral particles, both plasmids have to be co-transfected in the same cell. This makes the possibility of *in vivo* recombinant viral particle generation negligible. Moreover, the virus generated is inactive and requires activation by α-chymotrypsin before use. The original SFV particle system was RNA based and required *in vitro* transcription to generate mRNA from plasmids followed by their transfection into virus producing cells (Atkins *et al*., 1996). Bremner and colleagues (1998) modified this delivery vehicle and constructed a DNA based system, where plasmids could be directly transfected into packaging cells (DiCiommo *et al*., 1998). This DNA based SFV vector was evaluated for its capability to generate antigen specific immune response. In this study, SFV-Bgal showed good potency in generating antigen specific CTL response in immunised mice. At similar titres, SFV was slightly better than DISC-HSV and Adenovirus in generating CTLs against immuno-dominant TPH peptide from b-gal. Moreover, cytotoxic activity also translated into protective and therapeutic effect, since mice immunised with SFV-Bgal were protected from lethal tumour challenge of tumour cells expressing B-gal protein and vaccination of mice with established tumours delayed their growth. These results confirmed the efficacy of SFV as a potential efficient vector for cancer immunotherapy as also seen in other tumour models.

From this perspective, we evaluated whether tolerance to MTA1 could be overcome using a viral vector such as SFV, to enable protective immune response against tumour cells over-expressing MTA1. Preliminary studies suggested that SFV-hMTA1/mMTA1 mediated vaccination did

indeed delay the growth of tumour in these mice compared to control mice. Although, all the mice eventually succumbed to their tumours, significant longer survival and delayed growth was observed in vaccinated animals. However, further experiments are required to confirm these findings as well as increase the protection observed by combining SFV with other strategies such as DNA prime- viral boost, Treg depletion, increase in SFV titre and different routes of vaccination (i.v.). Higher titres and intravenous vaccination have been more effective in generating anti-tumour immune response and will have to be investigated in future studies.

Construction of DNA based SFV expression systems has come at a cost of low titre generation and further study will have to be undertaken to generate higher titres. One of the ways this could be achieved is by establishing a packaging cell line, which stably expresses the helper plasmid. Transfecting of replicon plasmid (antigen coding) into these cells would be sufficient to produce viral particles. Another novel strategy would be the use of a recently developed listeria monocytogenes (LM) vector. LM are facultative intracellular bacterium adapted to live in the cytosol and can be taken up by the APC, thereby directing antigen to both MHC class I and II pathways (Singh & Paterson., 2006).

7.9 Is there a future for MTA1 in cancer therapy?

Although, MTA1 has shown limited potential as an immunotherapeutic target in this study, much more work needs to be done in order to confirm its applicability. Its expression in late stage tumours needs to be further characterised and its exact role needs to be delineated. A recent study suggested MTA1 could be required in the initial stages of metastasis (invasion) but is not necessary for later stages (Hofer *et al*., 2006). Thus, if immunogenic, vaccination against MTA1 could be used prophylactically in early stage cancer patients to prevent cancer spread and in late stage patients to eliminate metastasis, in combination with surgery to remove primary tumours. Moreover, MTA1 expression determination in primary tumours, metastatic cells or in the lymph nodes might also be useful as a diagnostic or prognostic marker for cancer patients. In a recent study by Nishikawa *et al* (2005), immunisation with SEREX defined self antigens were found to induce generation of Tregs instead of CTL or CD4+ T cells (Nishikawa *et al*., 2005). MTA1 belongs to a similar category of antigens and can be viewed in a similar manner. It can be hypothesised that immunisation with such an antigen can be used for immuno-suppression in

auto-immune diseases. Moreover, in the above study, a combination of SEREX defined self antigen with a CTL epitope enhanced the potency of the immune response to the MHC class I epitopes. Thus, IFN-γ produced from the CTL (against MHC class I peptide) was able to reverse the generation of Treg into CD4+ T cells against SEREX antigens. It can therefore be envisioned that MTA1 may be applicable for immunisation along with other antigens to enhance the immune response against it.

Other members of the MTA1 family have not been extensively investigated and although MTA2 and MTA3 seem to have similar functions, they could also be potential targets. MTA2, MTA3 and MTA1s have been implicated in progression of breast cancers but their role in other cancers hasn't been investigated till date. Considering the splice variants of MTA1 identified in murine studies, more variants of MTA1 or its family members may exist and need to be identified.

7.10 Self-Antigens and Immunotherapy

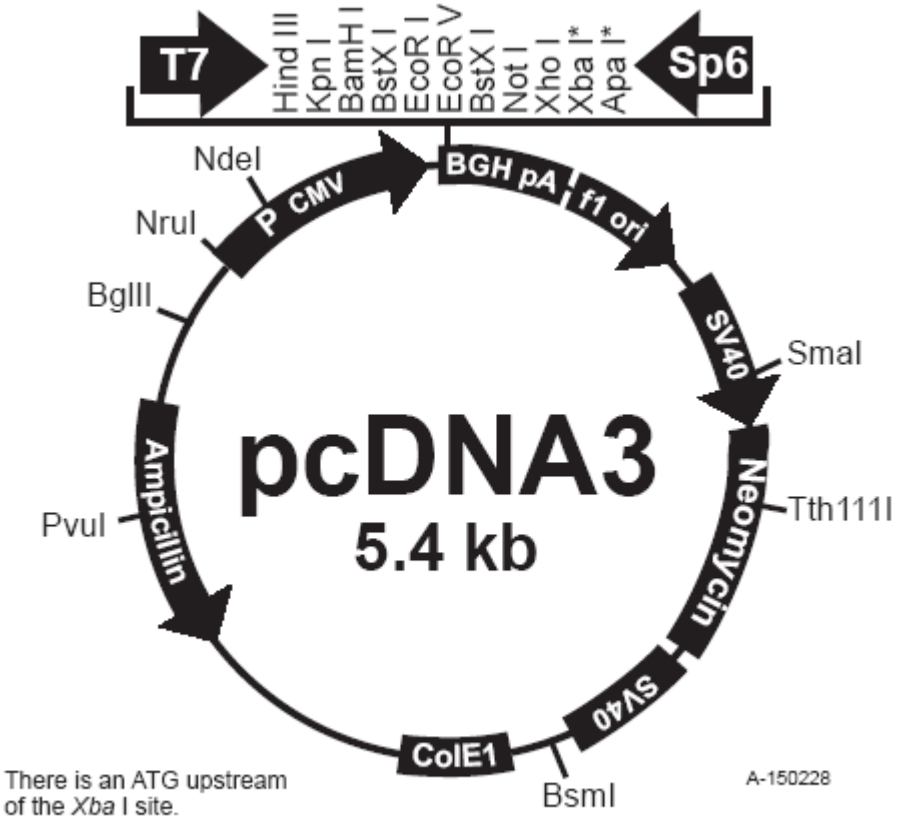
Self antigens remain the largest group of tumour antigens identified till date and are rightly being investigated as immunotherapeutic targets by several groups. Caution needs to be exercised whilst targeting such antigens as auto-immunity is a likely side-effect of strong immune response against them. The pros and cons will have to be weighed up for cancer therapy and auto-immune side effects that occur following immunisation. Scientists now acknowledge the need to develop novel vaccination strategies and vectors for targeting this group of antigens, due to their wide spread application. Immune responses have been successfully generated against several 'self-antigens' in animal models and in humans, but further refinements will be needed to translate them into objective clinical responses in cancer patients. According to this author, along with the antigens, an effective tumour vaccine might have to also include cytokines, chemokines, potent adjuvant, combined with an effective strategy for prior depletion of immuno-suppressive cells such as Tregs, NKT cells and Gr1+ myeloid cells.

Clearly, a long road lies ahead for cancer immunologists but, remarkable progress has been achieved in the past few years and with continued technological advances, cancer patients will be identified at a much earlier stage (owing, for example to proteomic biomarker profiling), giving immunotherapy the best chance to succeed. Advocates of personalised cancer vaccines argue the

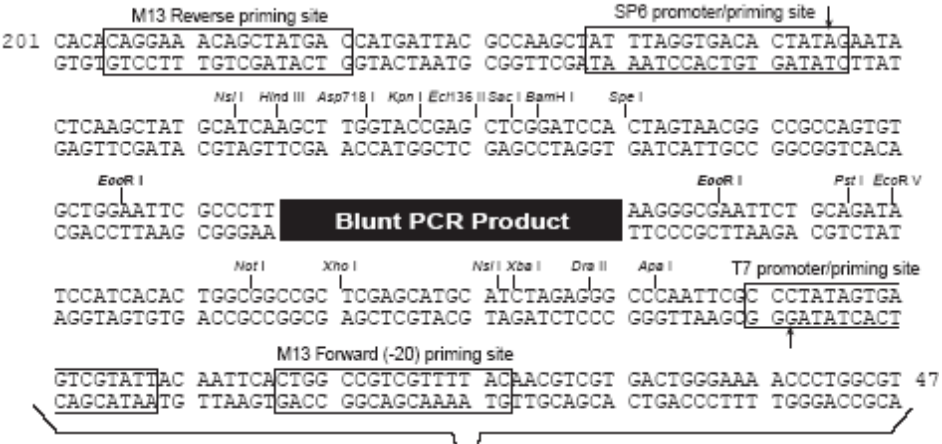
unique nature of each individual cancer. The availability of cheaper gene and protein array chips in the future might allow us to specifically identify antigens over-expressed by individual cancer allowing tailored vaccines to be applied in such patients.

Appendix

Plasmid map of pcDNA3



Plasmid map of TOPO-Blunt Vector



Comments for pCR®-Blunt II-TOPO®
3519 nucleotides

lac promoter/operator region: bases 95-216
M13 Reverse priming site: bases 205-221
LacZ-alpha ORF: bases 217-576
SP6 promoter priming site: bases 239-256
Multiple Cloning Site: bases 269-399
TOPO®-Cloning site: bases 336-337
T7 promoter priming site: bases 406-425
M13 (-20) Forward priming site: bases 433-448
Fusion joint: bases 577-585
ccdB lethal gene ORF: bases 586-888
kan gene: bases 1099-2031
  kan promoter: bases 1099-1236
  Kanamycin resistance gene ORF: bases 1237-2031
Zeocin resistance ORF: bases 2238-2612
pUC origin: bases 2724-3397

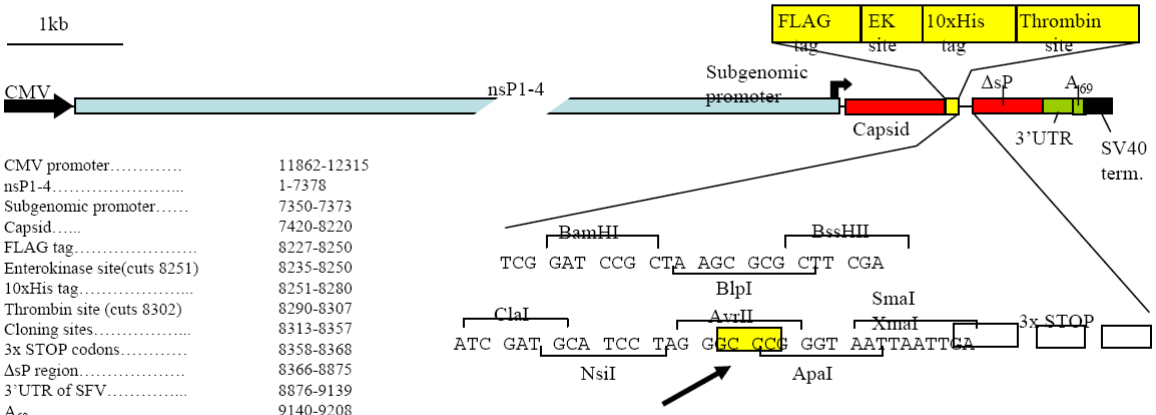
Plasmid map of pSMART Vectors

pSMART2b
(12438 bp)

pSMART2a and 2b are the same, except for the reading frame of the cloning site.

Full sequence of pSMART2b is here (LINK).

When cloning in the pSMART series, YOU MUST INSERT YOUR cDNA SO THAT IT IS IN FRAME WITH THE UPSTREAM CAPSID-FLAG-10xHIS TAG. Ribosomes use the capsid AUG to start translating the subgenomic RNA, so if your cDNA is not placed in frame, your protein will not be made. The Capsid sequence contains a translation enhancer (up to 8-10 fold higher expression, 4-fold in our experience with pSMART-lacZ). Immediately after translation, the Capsid self-cleaves, leaving behind your tagged protein.



CMV promoter……	11862-12315
nsP1-4……..	1-7378
Subgenomic promoter……	7350-7373
Capsid…...	7420-8220
FLAG tag……	8227-8250
Enterokinase site(cuts 8251)	8235-8250
10xHis tag……..	8251-8280
Thrombin site (cuts 8302)	8290-8307
Cloning sites……..	8313-8357
3x STOP codons……	8358-8368
ΔsP region……..	8366-8875
3`UTR of SFV……..	8876-9139
A_{69}……	9140-9208
SV40 termination signal…	9214-9455

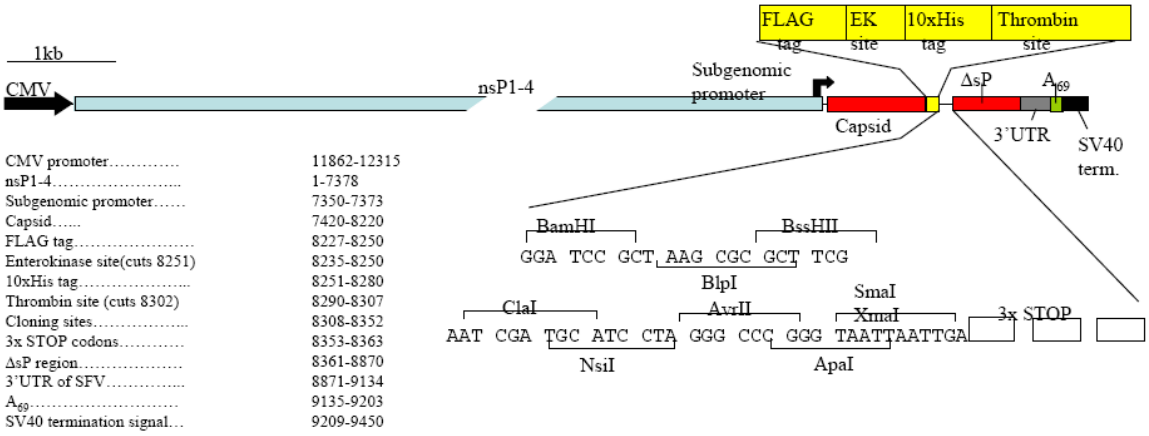
- Drawing is to scale, although full length nsP1-4 region is not shown. Unique cloning sites are indicated.
- The FLAG tag can be cleaved with EK, and both FLAG &10xHis tags can be cleaved off using thrombin.
- The pSMART cloning sequence is shown in triplets, corresponding to the reading frame
- ΔsP is the non-functional structural protein ORF containing a large deletion. Nucleotides 7421-7783 of the SFV genome are repeated twice in pSMART, once in the Capsid segment (pMART 7421-7783) and again at the start of the ΔsP segment (pSMART 8361-8723).

pSMART2a
(12433 bp)

pSMART1 is identical, but lacks cloning sites between Bam and Cla (BlpI and BssHII)

Full sequence of pSMART2a is here (LINK).

When cloning in the pSMART series, YOU MUST INSERT YOUR cDNA SO THAT IT IS IN FRAME WITH THE UPSTREAM CAPSID-FLAG-10xHIS TAG. Ribosomes use the capsid AUG to start translating the subgenomic RNA, so if your cDNA is not placed in frame, your protein will not be made. The Capsid sequence contains a translation enhancer (up to 8-10 fold higher expression, 4-fold in our experience with pSMART-lacZ). Immediately after translation, the Capsid self-cleaves, leaving behind your tagged protein.



CMV promoter……	11862-12315
nsP1-4……..	1-7378
Subgenomic promoter……	7350-7373
Capsid…...	7420-8220
FLAG tag……	8227-8250
Enterokinase site(cuts 8251)	8235-8250
10xHis tag……..	8251-8280
Thrombin site (cuts 8302)	8290-8307
Cloning sites……..	8308-8352
3x STOP codons……	8353-8363
ΔsP region……	8361-8870
3'UTR of SFV……..	8871-9134
A_{69}……	9135-9203
SV40 termination signal…	9209-9450

- Drawing is to scale, although full length nsP1-4 region is not shown. Unique cloning sites are indicated.
- The FLAG tag can be cleaved with EK, and both FLAG &10xHis tags can be cleaved off using thrombin.
- The pSMART cloning sequence is shown in triplets, corresponding to the reading frame
- ΔsP is the non-functional structural protein ORF containing a large deletion. Nucleotides 7421-7783 of the SFV genome are repeated twice in pSMART, once in the Capsid segment (pSMART 7421-7783) and again at the start of the ΔsP segment (pSMART 8361-8723).

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Communications during the work

Research Article

- Geng Li, **Deepak P Assudani**, Aija Line, Fuming Cao, Amanda Miles, Stephanie E B McArdle and Robert C Rees. SEREX analysis of Prostate Cancer cDNA libraries: the identification of MTA1 as a wide-spread tumour associated protein. Submitted to Cancer Immunity

Reviews and Book Chapter

- **Deepak P. Assudani**, Roger B.V. Horton, Morgan G. Mathieu[†], Stephanie E.B. McArdle, Robert C. Rees The Role of CD4+ T Cell help in Cancer Immunity and the Formulation of Novel Cancer Vaccines. Cancer Immunology Immunotherapy, March 2006.
- **Deepak P. Assudani**, Murrium Ahmad, Geng Li, Robert C Rees and Selman A. Ali. Immunotherapeutic potential of DISC-HSV and OX40L in cancer. Cancer Immunology Immunotherapy, *Cancer Immunol Immunother*. 2005, 55, 104-11.
- **Deepak Assudani**, Murrium Ahmad, Selman Ali, Stephanie McArdle, Geng Li and Robert Rees. Cancer Vaccine and Immunotherapy. Book chapter for 'Treatment of Cancer'. In Preparation

Abstracts

- **Deepak Assudani**, Walton T, Li G, Ali S, McArdle S, Ahmad M, Miles A and Rees R. Is MTA1 a good target for immunotherapy? Poster Presentation at the AACR conference, Washington DC, April 2006.
- **Deepak Assudani**, Walton T, Li G, Ali S, McArdle S, Ahmad M, Miles A and Rees R. Validation and Investigation of MTA1 as a potential target for immunotherapy of cancer. Poster Presentation at the CRI Symposium, CANCER VACCINES 2005: Barriers, Endpoints, and Opportunities.