NOTTINGHAM TRENT UNIVERSITY

HAGE, a novel cancer/testis antigen with strong potential as a target for immunotherapy against cancers

Thesis Submitted by

Morgan G. Mathieu

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Copyright statement

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Table of contents

List of Figures

List of Tables

Abbreviations

AAV	Adeno-associated virus
ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukaemia
APC	Antigen-presenting cells
AZAC	5'-aza-2'-deoxycytidine
BM-DC	Bone marrow-derived dendritic cells
CEA	Carcinoembryonic antigen
cDNA	Complementary deoxyribonucleic acid
CLIP	Class II-associated invariant peptide
CML	Chronic myeloid leukaemia
CT	Cancer/testis antigens
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DISC-HSV	Disabled infectious single cycle-Herpes simplex virus
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
EBV	Epstein-Barr virus
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescent assay cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor
HAGE	Helicase antigen
HBV	Hepatitis B virus
HDAC	Histone deacetylase inhibitors
HPV	Human papillomavirus
HSC	Haematopoietic stem cells
HSP	Heat shock protein
HSV	Herpes simplex virus
HTLV	Human T cell leukaemia virus
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activations motif
LCM	Laser capture microdissection
LOH	Loss of heterozygosity
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MICA/B	MHC class I related protein A/B
mRNA	Messenger ribonucleic acid
NK	Natural killer cells
NKG2D	NK cell activating receptor
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor

PI	Propidium iodide
Poly.I.C	Polyinosinic polycytidylic acid
PSA	Prostate-specific antigen
PSMA	Prostate-specific membrane antigen
Rb	Retinoblastoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
RT-Q-PCR	Real time quantitative polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEREX	Serological analysis of recombinant cDNA expression libraries
SiRNA	Small interfering RNA
SFV	Semliki Forest Virus
TAA	Tumour-associated antigens
TAM	Tumour-associated macrophages
TAP	Transporter-associated protein
TBC	Total blood cells
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th	T helper lymphocytes
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cells
Trp-1	Tyrosinase-related protein-1
TSA	Trichostatin A
TSG	Tumour-suppressor genes
VEGF	Vascular endothelial growth factor
VitE	Vitamin E

Abstract

Since van der Bruggen *et al.* (1991) first identified specific human tumour antigens of the MAGE family, numerous potential immunotherapeutic targets have been discovered, often belonging to the so-called cancer/testis (CT) gene family. In a search for novel epitopes from potential tumour target antigens, HAGE, a CT antigen, has been studied. It was first identified in a sarcoma and has since been reported in several carcinomas and leukaemias at the mRNA level only. This study proposed to investigate HAGE as a potential target for immunotherapy in a murine tumour model. HAGE mRNA was found to be expressed in a small proportion of carcinomas, some melanomas and in a strong proportion of chronic myeloid leukaemias as compared to normal tissues, which do not express HAGE with the exception of testis. HAGE protein levels were also confirmed on tissue sections and in cell lines in order to rule out any post-transcriptional modifications. Furthermore, HAGE has been previously described as member of the DEAD-box family of ATP-dependent RNA helicases but very little is known about its actual function. RNA helicases are involved in various steps of RNA metabolism and their over-expression has often been linked with tumorogenesis. Using a combination of silencing and transfection experiments, HAGE was proven to be critical for tumour cell proliferation. Next, the identification of candidate MHC class I and class II immunogenic peptides derived from the HAGE protein was undertaken by combining reverse immunology with the use of HLA transgenic mice. Four HLA-A2 peptides were found to be immunogenic in C57BL/6-HHDII mice with one of them being also naturally processed. Four HLA-DR1/-DR4 peptides were defined as immunogenic in FVB/N-DR1 and C57BL/6-DR4 mice with two of them being also endogenously processed. The discovery of three novel HAGE-derived epitopes may then contribute to the range of immunotherapeutic targets for use in cancer vaccination programs. Finally, potent DNA-based vaccination strategies targeting HAGE were evaluated in an *in vivo* tumour model developed in HHDII-DR1, double transgenic mice. HAGE DNA vaccination by either gene gun or intra-muscular injection led to tumour protection and/or clearance in immunised animals. Also, the use of co-stimulatory molecules to boost the immune response induced by HAGE DNA vaccination was studied in a therapeutic setup and B7.2 appeared to be the most promising one. However, further work is needed to improve this tumour model and assess other methods of vaccination such as syngeneic dendritic cell-based vaccination or viral vaccination and the use of Semliki Forest virus. Collectively, these data demonstrate that HAGE represents a valid candidate target for several cancers and should maybe be included in future immunotherapeutic design.

Chapter 1: Introduction

1.1 Cancer

A tumour or a neoplasm is the result of an excessive and uncontrolled proliferation of a single normal cell, which has been transformed into a cancerous state following multiple cellular alterations and rendered resistant to apoptosis, cell-to-cell contact inhibition, growth factor removal or immune cells (Bertram, 2000). According to the latest figures obtained by the World Health Organisation (on line at http://www.who.int/cancer/en/), cancer is responsible for 13% of deaths worldwide with approximately 8 million deaths a year and at least 11 million new cases diagnosed every year. The appearance of a malignant tumour is associated with multiple interrelated factors relevant to the subject itself (heredity, hormonal state, efficacy of immune defences), but also to its environment (life conditions, alimentary habits, exposition to diverse toxic or infectious agents). Smoking is for example a well known risk factor for lung cancer causation while similarly; ultra violet radiation from sunlight is strongly linked with melanoma, a common form of skin cancer (Ivry *et al.*, 2006). Obesity was shown, by numerous epidemiological studies, to be associated with the development of cancers in a variety of tissues such as the oesophagus, colon, kidney, breast, pancreas, liver or gall bladder (Calle and Thun, 2004). It was recently demonstrated that almost 5% of cancer was directly and indirectly caused by a chronic infection with the likes of human papillomavirus in 98% of cases of cervical cancers, hepatitis B virus in some hepatocarcinoma, as well as Epstein Barr virus having a possible involvement in at least four different types of cancer including Burkitt's lymphoma and nasopharyngeal carcinoma. Finally, spontaneous DNA damage during normal cell division can also lead to cancer.

1.1.1 Cancer and tumoral transformation

Each cell of the organism undergoes the duplication of its genomic content during the cell cycle before continuing its division process (Fig. 1.1). Each of the four phases (G1, S, G2 and M) of the cell cycle contains checkpoints required to ensure the integrity of the genetic material and which allows the cell to either go along with the division process following the repair of any DNA damage or start the complex pathway of programmed cell death (apoptosis) if the damage is simply too important. The conservation of the tumoral phenotype by cellular division is compatible with three mechanisms:

- Alteration of the coding region or the regulation sequence by chemical or physical environmental influences.

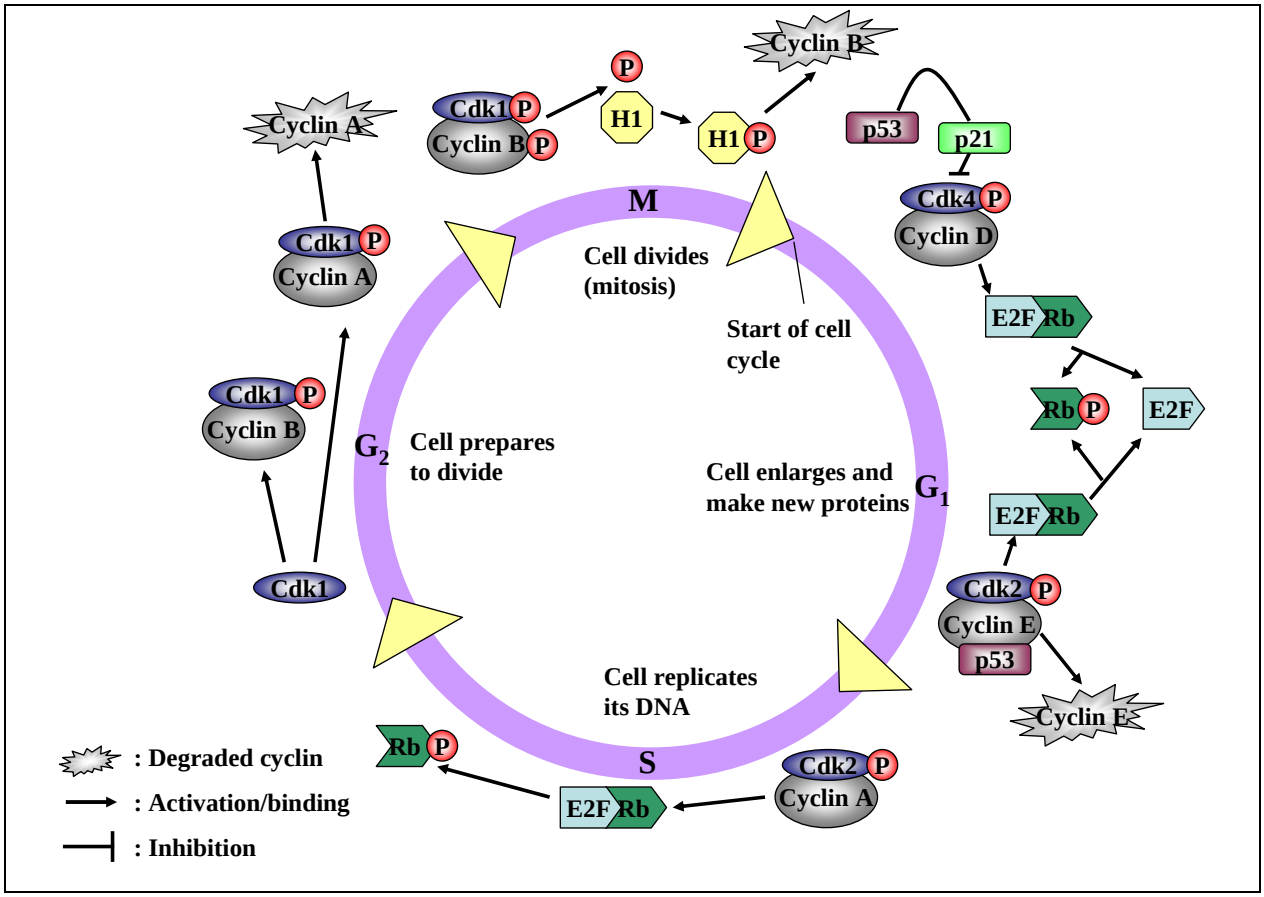

Figure 1.1: Mammalian cell cycle (Adapted from www.cancerline.com). *Cell cycle mechanisms control normal cell growth and division. Progression into each phase is tightly controlled by complexes formed of cyclins and cyclin-dependent kinases (Cdk) as well as tumour-suppressor genes such as p53 and retinoblastoma (Rb). Disruption of these checkpoints leads to uncontrolled cellular growth and development of tumoral phenotype.*

- Alteration of the genome following the addition of foreign genetic materials such as viruses or other infectious agents.
- Alteration of the structure of the chromatin due to epigenetic factors such as ageing mainly through DNA methylation.

In spite of several cell divisions occurring throughout life, the development of a cancer cell remains a relatively rare event, mainly because of the need for multiple alterations to acquire the malignant phenotype. The number of alterations for a cell to become neoplastic is thought to be strictly tumour-dependent. However, this level can be correlated with increasing age and it is generally believed that at least five to six genetic hits are a pre-requisite for the formation of solid tumours in humans and that genetic alterations other than mutations can also transform cells such as translocation, amplification or loss of heterozygosity (LOH)(Cahill *et al.*, 1999).

Depending on the cancer types, genetic alterations affect a wide diversity of genes and it is generally acknowledged that only a small number of them are constantly the target. Akagi (2004) proposed that four phenomena must exist for the tumoral transformation to occur and these include the inactivation of the tumour-suppressor genes Rb and p53, the shortening of telomeres, the constant expression of activated Ras and the activation of protein phosphatase 2A. Any DNA damage occurring on these pathways, which goes undetected by the checkpoints become fixed and is inherited by daughter cells, which can then acquire further mutations. The multistep hypothesis has therefore been advanced (Baak *et al.*, 2003). Sequential mutations of genes in cases of head and neck, prostate or colorectal cancers have been detailed in the literature. The latter has been one of the first described sequences of events leading to the transformation of a normal colon cell into a metastatic carcinoma. The model, proposed by Fearon and Vogelstein (1990), described a mutation on the Ras gene as well as the LOH of chromosome 5q at an early stage of tumour development, followed by two mutations on the p53 gene and LOH of chromosome 18q at a later stage (Fig. 1.2).

More recently, Forastiere *et al.* (2001) described an even more complicated sequence of events occurring in head and neck carcinomas with a succession of losses of heterozygosity on chromosome 9p (locus of cell cycle inhibitor p16) at an early stage, on chromosomes 17p (locus of p53) and 3p at an intermediary stage, and finally on chromosomes 11q, 13q and 14q at a later stage. Although these models were detailed in depth, they are a rather simplified version of what the reality is. Further studies are required for a better understanding of the tumour biological processes such as proliferation, differentiation, apoptosis or invasion, as well as tumour stages in relation with molecular events occurring inside the cells. Thus, by allowing

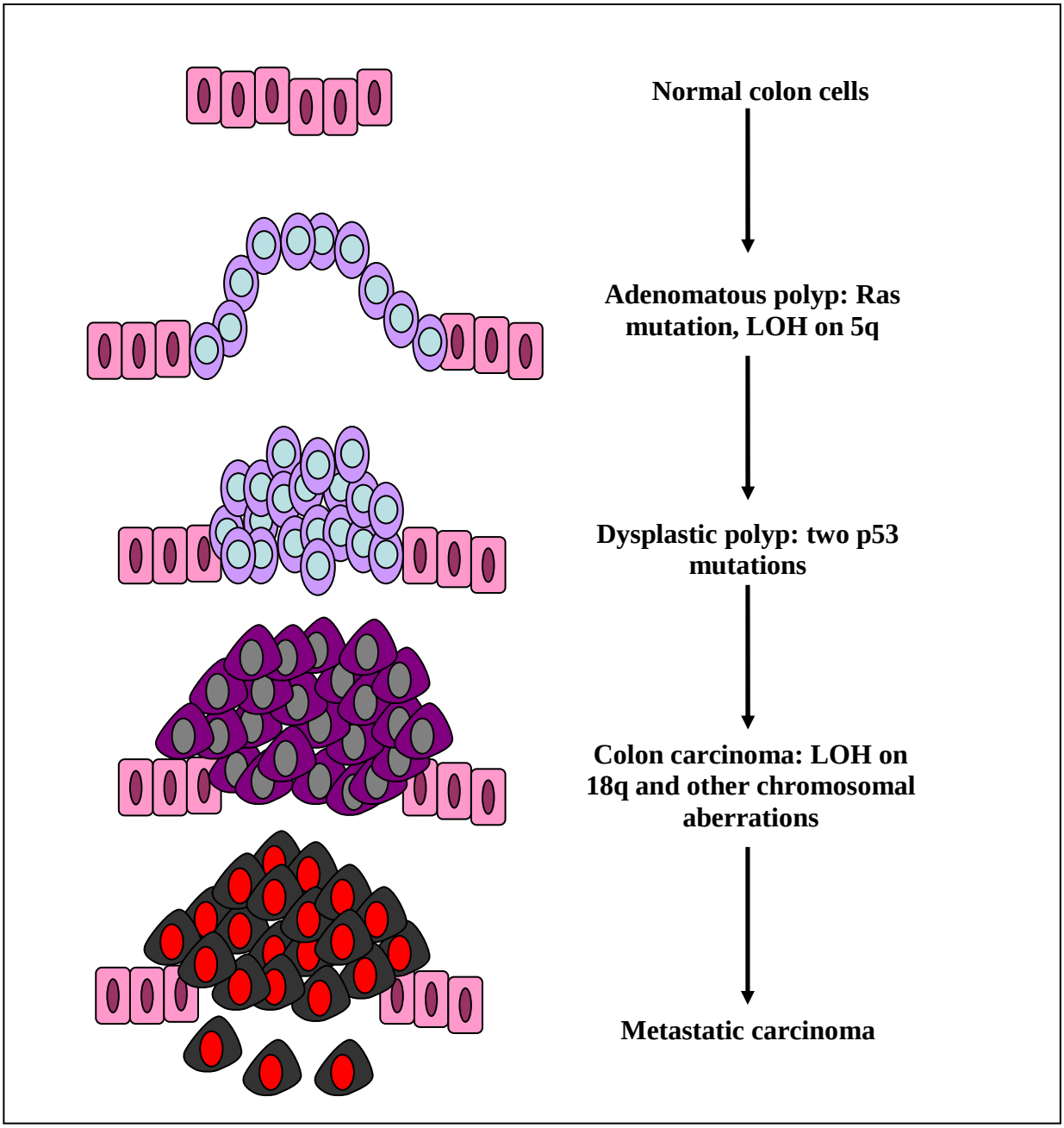

Figure 1.2: Multistep progression hypothesis of tumours in colon carcinoma (Adapted from Knudson., 2001).

a correlation between tumour-specific antigens and aggressiveness of the tumour, direct specific therapies targeting tumour-specific antigens, expressed at certain stages of the tumour-development, could be developed and used accordingly by clinicians (Bertram, 2000).

1.1.2 Tumorogenesis and cellular specialisation

DNA damage, initiated by mutagenic agents and responsible for the disruption of the normal functioning of the cell cycle, affects principally two groups of genes involved in the control of the cellular proliferation and differentiation, and called proto-oncogenes and tumour-suppressor genes (Karpinets and Foy, 2005).

1.1.2.1 Oncogenes

Oncogenes are a class of genes that stimulate cell growth. There are two types of oncogenes: viral oncogenes and cellular oncogenes or proto-oncogenes. Viral oncogenes can initiate and maintain cancers; one of the most studied being the Sarcoma or Src oncogene induced by Rous retrovirus (Martin, 1970; Martin, 2004). Although a rare event, these viral oncogenes derive from actual coding regions of cellular oncogenes, which have been taken up by these viruses and incorporated into their own DNA next to the coding regions that are essential for their survival. The host gene becomes part of the viral genome and is from then transcribed in infected cells under the viral promoter rather than the normal host, which would be highly regulated by a network of transcription factors. Over-expression of the gene causes the infected cells to escape growth regulation.

Proto-oncogenes require to be “switched on” to take part in the induction of cancer. Although these are necessary, they are not sufficient on their own to cause cancer. Each cellular oncogene is involved either in the initiation or the maintenance of tumour development but needs another gene (viral or cellular) in a multistep process to achieve malignant tumour. Activation of proto-oncogenes into oncogenes can occur through various genetic alterations, among which mutations, translocation and amplification can be included. Some viruses can also insert their promoter in front of a proto-oncogene thereby causing the over-expression of the “particular” proto-oncogene and ultimately leading to excessive cell proliferation. Proto-oncogenes encode for proteins, which are involved at different levels of cellular proliferation going from the transmission of extra-cellular signals *via* surface receptors to the intra-nuclear command of replication including growth factors, growth factor receptors, protein kinases, signal pathway transduction mediators and transcription factors (Peters, 1997). At the same time, proto-oncogenes also lead to the uncoupling of tumour cells from external regulatory

signals, meaning that factors secreted by normal surrounding cells cannot control the proliferation of cells homing these oncogenes, hence the neoplastic phenotype (Bertram, 2000; Lucas *et al.*, 2002). The selection of cells having these activities, favoured by the acquired genetic instability, constitutes a pivotal point in tumour development. Molecules involved in this process are varied and include abnormal growth factors (FGF, EGF, VEGF, PDGF, and TGF-α) as well as abnormal growth factor receptors leading to their constitutive activation and/or their deregulation (EGFR)(Cross and Dexter, 1991). It is generally believed that genetic alterations required for invasion, metastasis or angiogenesis can be absent in the first steps of tumoral transformation but they are often acquired at the early stages of tumour formation. Vessels, characterising tumoral angiogenesis, develop from non-tumoral cells but distinguish themselves from normal vessels by their specific expression pattern of growth factors and growth factor receptors. The creation of these new vessels is consecutive to a modification of the balance between inhibitors and activators of angiogenesis secreted by tumour cells such as thrombospondin 1 and VEGF or FGF, respectively, with constitutive activation of Ras (Hanahan and Folkman, 1996). The capacity of tumour cells to invade tissues situated in the vicinity of the tumour constitutes a prime characteristic of aggressive tumours. Clear correlations have also been observed between the pattern of expression of molecules involved in inter-cellular adhesion and metastatic potential. For example, the variation of pattern of integrins can trigger the adaptation of tumour cells to different types of extra-cellular matrices during the metastatic process (Goepel *et al.*, 1991). The second class of proteins involved in the metastatic process, as well as the angiogenic phenomenon, can be described as the family of proteases secreted by tumour or stroma cells and capable of degrading the extra-cellular matrix (Coussens and Werb, 1996). These proteases can be over-expressed and reciprocally, the expression of their respective inhibitors is often diminished.

Cellular proliferation is also due to alterations in the signalling pathway with molecules such as phosphatases involved in the activation or the inhibition of protein kinases triggering constitutive activation of signalling pathways. Among all these pathways, the SOS-Ras-Raf-MAPK cascade has a “privileged seat” as being the first identified (Parada *et al.*, 1982). Indeed, Ras is activated in more than 25% of tumours and is capable, with the co-operation of other genes, to induce transformation by deregulating the Jnk and/or Erk pathways and therefore modifying biochemical and cytoskeletal properties of the transformed cells (Peters, 1997). On the other hand, experiments carried out *in vitro* in mouse cell lines showed that transfection of the Ras gene does not provide a sufficient signal to provoke tumoral transformation on its own

and that immortalisation of the cells is required prior to transfection with the Ras gene to induce the tumoral phenotype (Scholl *et al.*, 2005). That is why mutations affecting the signalling pathway are often accompanied with mutations occurring further downstream of the signalling, mainly transcription factors. Proteins such as Fos, Jun or Myc are often affected by the process of mutagenesis and are normally responsible for the activation or the inhibition of genes involved in the cell cycle. Fos expression is directly modulated by phosphorylation by MAPK following activation of the Ras pathway while its stability and its DNA-binding capacity is affected by other protein kinases such as PKA or PKC (Greenberg *et al.*, 1984; Milde-Langosch., 2005). Moreover, the frequency of its heterodimerisation with the other transcription factor Jun required to provide transcription signals often correlates with the severity of the cancer (Schutte *et al.*, 1989). Myc, whose expression is also due to the activation of PKC (Greenberg *et al.*, 1984), down-regulates the expression of the tumour-suppressor p53, and promotes the expression of cyclin A and cyclin E among others, therefore forcing the cells into cell cycle and cell division (Peters, 1997; Mackay and Williams, 2003; Ponzielli *et al.*, 2005).

1.1.2.2 Tumour-suppressor genes

Mutations also occur on anti-oncogene genes, which limit the cellular proliferation such as the much studied tumour-suppressor p53 and Rb genes (Harris and Levine, 2005). Functions of tumour-suppressor genes are abolished or reduced by mechanisms such as loss of heterozygosity, methylation, cytogenetic aberrations, genetic mutations, and gain of auto-inhibitory function or polymorphisms (Zingde., 2001). In most cases, the normal suppressor allele can function alongside the mutated allele, reinforcing the need for both genes to be inactivated before abolishment or reduction of function is seen. Moreover and contrary to oncogenes which are dominant and normally prevent development of viable embryo, defects in tumour-suppressor genes can be inherited (Bertram., 2000), and loss of heterozygosity, that is to say the loss of function of the second normal allele, will be detrimental as early onset tumours will be able to rise (Bamne *et al.*, 2005; Fearnhead *et al.*, 2004).

The Rb pathway is the second metabolic pathway to be targeted in several cancers. Indeed, retinoblastoma, the most common ocular malignancy occurring early in child development, is due to mutations taking place in the Rb gene. The Rb protein functions as a checkpoint for cells to enter into S-phase. The molecule can be directly altered by mutations affecting the pockets of interactions with the E2F/DP transcription complex, genetic deletion or simple inhibition of its expression. Rb expression can also be modified following mutations on molecules

controlling its phosphorylation leaving the E2F/DP complex to act freely on the cell cycle. Indeed, overexpression of cyclins D, amplification of Cdk4 or inactivation of the ink4 inhibitor family have been linked with reduced or suppressed expression of Rb (Peters, 1997).

The p53 tumour-suppressor gene is an important regulator of genomic stability, replicative senescence and premature senescence *via* the control of telomere length (Bertram., 2000). It controls, at least partially and in close collaboration with the Rb gene, mechanisms of DNA repair and cellular apoptosis and it also controls the blockade of the cell cycle *via* the p21 molecule (Fig. 1.3)(Graeber *et al.*, 1996; Linke *et al.*, 1996)). This pathway, often considered "the guardian of the genome", is inactivated in over 70% of all tumours while most of the genes present upstream or downstream of this pathway are also mutated (Levine., 1997). p53 normally blocks the cell cycle progression by increasing the level of p21, which in return allows the dephosphorylation of the cyclin D/Cdk4 complexes (el-Deiry *et al.*, 1993). Moreover, p53 can be down-regulated by the Mdm2 protein to allow progression into the cell cycle. For these two reasons, p53 represents a critical target in the formation of neoplasms. The inactivation of p53 is, in 90% of cases, due to punctual mutations mostly occurring in the DNA/protein interaction sites. It prevents the formation of hydrogen bonds between the p53 protein and the DNA, and triggers a loss of function or a dominant effect associated with the stabilisation of the protein (Cho *et al.*, 1994). This increase of protein half-life and its potential dominant effect over the wild type p53 protein play an important role in the possible appearance of an immune response against p53, strongly helped by the fact that the occurrence frequency of certain mutations is quite high providing a way of targeting these mutated p53-expressing tumour cells. In summary, key regulatory proteins involved in cell growth and survival are affected by mutations and lead to the uncontrolled cell proliferation.

1.1.3 Tumour-associated antigens and their classification

Mutations occurring in proto-oncogenes and tumour-suppressor genes and the eventual incorporation of viral DNA into the human genome trigger a cascade of reactions affecting the expression or the structure of other genes. Proteins derived from these genes are called tumour-associated antigens (TAA). Most investigations have focussed their attention in identifying them and more specifically the tumour rejection antigens, which are peptides derived from the tumour antigens, presented by major histocompatibility complex (MHC) molecules to T cells and capable of eliciting a tumour-specific T cell response. Bruggen *et al* (1991) identified the first tumour antigen from a melanoma patient tissue (MAGE-1) using gene cloning and demonstrated that it was capable of inducing a cytolytic response.

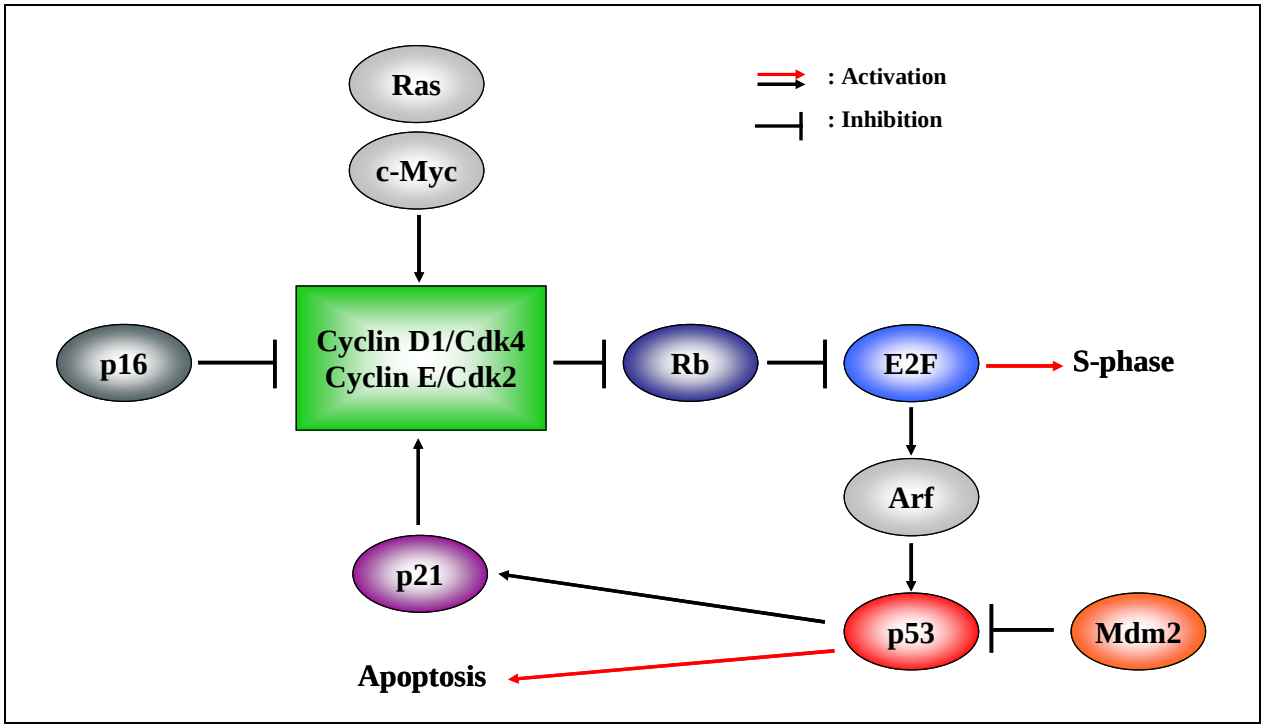

Figure 1.3: Cooperation between the two tumour-suppressor genes p53 and Rb for the regulation of the cell cycle.

Since then, many tumour antigens have been identified using a sequencing (HPLC-mass spectrometry) or a serological approach (SEREX) described in more details in chapter 5. Five major categories were established to classify tumour antigens (Table 1.1):

Table 1.1: Examples of tumour antigens grouped into five categories

Tumour antigens	Tumour expression	Healthy tissue expression
Viral antigens		
EBV	Burkitt's and Hodgkin's lymphoma	None
HBV	Hepatocellular carcinoma	None
HPV	Cervical cancer	None
HTLV	T cell leukaemia	None
Tumour-specific unique antigens		
Bcr/Abl	CML	None
Ig idiotype	B cell non Hodgkin's lymphoma	None
Abnormally expressed antigens		
HER-2/neu	Breast, ovary, lung carcinoma	Ubiquitous
MUC-1	Many carcinomas	Breast
PAP, PSA, PSMA	Prostate carcinoma	Prostate
p53 (mutated)	Breast, colon, other cancers	None
p53 (non-mutated)	Pancreatic, colon, lung cancers	Ubiquitous
Ras (mutated)	ALL, AML, CML	None
WT-1, Proteinase-3	CML, other cancers	None
Differentiation antigens		
gp100, Mart-1, tyrosinase	Melanoma	Melanocytes
Cancer/testis antigens		
MAGE-1, -3 and -6	Many carcinomas	Testis
BAGE, GAGE, XAGE	Bladder, gastric, other cancers	Testis
HAGE, NY-ESO-1	Many carcinomas	Testis
RAGE	Many carcinomas	Retina

_ Viral antigens: The identification of target antigens is conceptually simple when it is linked with tumoral transformation induced by viruses, with their molecules being foreign to the organism and the T cell receptors adapted to their recognition having no reasons to have been deleted and being deprived of auto-reactivity. Epstein-Barr virus (EBV), responsible for different types of lymphomas (Burkitt's lymphoma, nasopharyngeal cancer, Hodgkin's

lymphoma), is a classical example in humans (Murray *et al.*, 1992). In the same fashion, human papillomavirus (HPV) is linked with the development of cervical cancers (Feltkamp *et al.*, 1993), hepatitis B virus (HBV) with hepatocellular carcinoma (Koziel *et al.*, 1995), and human T cell leukaemia virus (HTLV) with adult T cell leukaemia (Koenig *et al.*, 1993). Not all of the viral proteins have the same immunotherapeutic interest as some viruses can go in latent phase expressing only a limited number of proteins. Also, tumour cells can contain only truncated viral genomes, limiting themselves to the expression of transforming genes such as E6 and E7 in HPV cases. Several clinical trials targeting viral antigens such as E6 and E7 in patients with cervical cancers resulted in encouraging results (Mahdavi and Monk, 2005).

_ Tumour-specific unique antigens: The structural alteration of proteins involved in a direct or indirect fashion to the cell cycle and its control can lead to the transformation of a cell when its functional capacity is altered. Genes encoding cell cycle-activating proteins, proto-oncogenes or tumour-suppressor genes can potentially express antigenic proteins because of the presence of different amino acid sequences in these natural proteins. These modifications can result from chromosomal translocations (Bcr/Abl), point mutations or the creation of a chimerical protein by gene fusion (β-catenin, Cdk4, Ig idiotype, p53 and k-Ras)(Yang *et al.*, 2006; Ruiz-Godoy *et al.*, 2006). Modified peptides can bind to some HLA molecules and can therefore be stimulator of both cytotoxic and helper responses. These neo-antigens can have a real therapeutic interest if they are found recurrently in a large number of patients as they are often specific of an individual tumour or shared between specific histological tumour types. For example, Bcr/Abl is a well studied antigen in this group specific of chronic myeloid leukaemia and is characterised by an abnormal tyrosine kinase activity. It results from the translocation of genetic material between chromosomes 9 and 22 and causes the malignant transformation of haematopoietic stem cells (Deininger *et al.*, 2000). Another interesting antigen is the immunoglobulin (Ig) idiotype made of unique heavy and light chains and specific of B cell lymphoma. Specific Igs are produced by gene fusion during B cell differentiation and could be used efficiently as targets (Ruffini *et al.*, 2002). This category is often considered by many as being the most interesting because it provides the largest number of immunogenic epitopes.

_ Abnormally-expressed genes: In a frequent fashion, the tumoral transformation is linked or accompanied with the over-expression, without any structural alterations, of proteins involved in the control of the cell cycle or in the natural senescent evolution of cells. For example, the telomerase enzyme protects the ends of chromosomes by forming DNA/protein complexes to prevent the progressive shortening of chromosome extremities during successive cellular

divisions (Murnane, 2006). Cells fall eventually in replicative cell senescence due to lack of telomerase activity (de-Lange., 2005). However, this enzyme is strongly expressed in most tumours. Tumour cells display considerable telomere shortening and other characteristics such as extensive chromosome fusion, which ultimately lead to the inability of tumour cells to senesce (Romanov *et al.*, 2001). The up-regulation of the proto-oncogene HER-2/neu, coding for a membranous molecule involved in the transduction of activation signals, is often associated with the development of some adenocarcinoma (Tsiambas *et al.*, 2006). Most of the tumour antigens identified till date, such as survivin, p53, Ras, hTERT and WT-1 belong to this group, and represent the most challenging aspect. In fact, it is conceivable to use these molecules as immunotherapeutic targets because this up-regulation often correlates with an over-presentation of the corresponding peptides at the surface of tumour cells *via* the HLA complex. However, there remains a need to design and identify proper peptide targets as they can be at the origin of a state of tolerance by clonal deletion, and in the opposite case, to ensure the absence of auto-reactivity against the organism.

_ Differentiation antigens: These normal molecules of the organism are only expressed in specific tissues. Tyrosinase, MelanA/Mart1 molecule, gp100 glycoprotein are for example only expressed by melanocytes (Romero *et al.*, 2004). In the same way, prostaglandin, prostate acid phosphatase (PAP), prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) are specific of the prostate tissue (Fong and Small, 2007). It is relatively easy to induce a T cell-based response against those proteins. In fact, they are not expressed at the thymus level and consequently, tolerance should not be linked to a clonal deletion but to a simple state of anergy, which can often be reversed (Engelhard *et al.*, 2002). Inducing a T cell-based response against these differentiation antigens can eradicate the tumour, but like other over-expressed antigens, can also have side-effects with concomitant destruction of the healthy tissues expressing the same antigens. The best example is melanoma where tumour regression can be accompanied with the destruction of healthy melanocytes and a loss of cutaneous pigmentation called vitiligo as observed in some clinical trials (Schreiber *et al.*, 1999; Phan *et al.*, 2003). The targeting of a differentiation antigen depends on the acceptable criteria for the organism of the destruction of healthy tissues expressing those as well.

_ Cancer/testis (CT) antigens: In a frequent fashion, the tumoral transformation is accompanied with the re-activation of genes normally expressed at the embryonic level but not by healthy adult tissues, except testis. Examples are numerous such as the melanoma antigen MAGE, the bladder antigen BAGE and the α-fetoprotein over-expressed in some forms of hepatocellular

carcinoma. This category is of major interest as the expression of its members is only found in either germ cells from normal testis or placenta, or tumour cells. Germ cells are characterised by the lack of expression of MHC molecules, making them immuno-privileged. Therefore, finding and targeting CT antigens, which are processed and presented at the surface of tumour cells by MHC molecules to T lymphocytes, would allow the immune response to be directed against the tumour only and would eventually trigger tumour rejection. CT antigens are at the moment one of the most studied family in animal models and in clinical trials because of those characteristics. Melanoma antigen (MAGE) was the first CT antigen (van der Bruggen *et al.*, 1991) described of many others later identified such as GAGE, BAGE, LAGE, XAGE, semenogelin and NY-ESO-1. The latter is of major interest as it has been found that a humoral response against NY-ESO-1 CT antigen is elicited in a wide variety of tumours with high frequency, and was described as one of the most immunogenic CT antigen (Scanlan *et al.*, 2004; Valmori *et al.*, 2005; Zhao et al., 2005; Velasquez and Lipkin, 2007). Helicase antigen (HAGE) also belongs to this category of antigens (Martelange *et al.*, 2000).

1.1.4 Cancer stem cells

In haematological malignancies and in some solid tumours such as brain, breast, colon, pancreas and prostate, there are compelling evidences that a small fraction of the tumour cell population is actually composed of cancer stem cells. Contrary to cancer cells, cancer stem cells have self renewal and proliferation properties of normal stem cells and are able to maintain the tumour mass (Costa *et al.*, 2007). This observation led to the elaboration of new theories on how tumour cells survive and expand. In fact, it was thought that the tumour mass was composed of a heterogeneous population of cells all able to differentiate and proliferate. The new theory proposes that the tumour mass is still composed of a heterogeneous population of cells unable to divide and differentiate, and accompanied with a small unit of cancer stem cells with stem cell properties at the origin of the bulk of tumour cells suggesting that the transplantation of only one cancer stem cell is actually sufficient for the tumour formation (Reya *et al.*, 2001).

Moreover, stem cells are the oldest cells of the organism and it is legitimate to think that they are more prone to genetic mutations and epigenetic defects than any other cells. However, it is still not clear whether cancer arises from a mutated stem cell or from a downstream progenitor (Huang *et al.*, 2007). Fusion of stem cells with cancer cells which have accumulated changes has also been suggested to explain the presence of this population of immortalised cells with transforming potential (Bjerkvig *et al.*, 2005). Finally, genetic defects in tumour cells might

have the differentiation process of the cells rendering the behaviour of these cells very similar to the one of stem cell (Costa *et al.*, 2007).

This model of cancer stem cells would also explain issues of tumour recurrence and even metastases due to eventual chemo-resistant residual cancer stem cells in patients (Brabletz *et al.*, 2005). Chemotherapy drugs challenge rapidly dividing cells and molecular targets that represent the bulk of the tumour but may not affect slowly growing stem cells, which in future can repopulate tumours. In theory, the identification of these cancer stem cells and a better understanding on how these might develop into cancer cells could provide new therapeutic targets to prevent tumour relapses. Interestingly, it was reported that some CT antigens are also expressed in human mesenchymal stem cells of the bone marrow. This indicates that CT antigens are not only a hallmark in gametogenesis but also in stem cells (Cronwright *et al.*, 2005). Because CT antigens are not expressed in normal non-stem cells, it is conceivable to think CT antigen expression found in tumours is actually coming from cancer cells with stem cell properties. Therefore, targeting these antigens by chemotherapy and/or immunotherapy could eventually improve considerably the treatment of cancer but further research will be needed to confirm the validity of the cancer stem cell model and provide efficient therapies.

1.1.5 Current treatments and their limitations

Cancer can be treated by four approaches: surgery, radiotherapy, chemotherapy and hormonal therapy. Surgery, although radical, is by far the most effective treatment for single tumours but could prove useless or barely palliative if the cancer has metastasised to distant sites. Surgery can therefore be used in combination with chemotherapy and/or radiotherapy. Chemotherapy and radiotherapy both target rapidly dividing tumour cells. Chemotherapy relies on the administration of cytotoxic drugs that inhibit cell proliferation and ultimately lead to cell death while radiotherapy is based on the fragility of cancer cells and their inability to repair DNA damages brought on by the radiations. Unfortunately, other cells are also able to proliferate intensely and are affected by these two treatments. This leads to the appearance of severe side-effects often overcoming the benefits of these treatments on tumour cells. Moreover, chemotherapy and radiotherapy are only effective when tumour cells are largely oxygenated and thus making badly perfused or hypoxic tumour cells able to proliferate. It can also be mentioned that, under certain circumstances, chemotherapy and radiotherapy can trigger adverse effects and promote tumour progression rather tumour regression (Kim and Tannock, 2005). Finally, hormonal deprivation can be considered as tumours often rely on the supply of hormones, such as testosterone in prostate cancers, to grow. However, tumour cells often

develop mechanisms of resistance rendering the treatment unsuitable. In summary, cancer therapy requires the development of new treatment modalities allowing prolonged survival of the patients and improvements of life conditions by reducing cytotoxicity and increasing specificity. Immunotherapy can therefore be envisaged as a new pathway as the activation of immune cells to fight cancer cells can be directed and controlled to avoid side-effects.

1.2 Anti-cancer immunity

The concept of immune surveillance was introduced in the seventies by Macfarlane Burnet and Lewis Thomas (Burnet *et al.,* 1970), but was suggested much earlier by Erhlich in 1909. This is based on the hypothesis that the immune system, because of its capacity to recognise tumour cells, could interact to prevent the apparition of tumours or at least limit their growth. Since then, several proofs have been accumulated to prove this concept. In humans, both CD4+ and CD8+ tumour-antigen-specific lymphocytes have been found in the bloodstream, in lymph nodes or infiltrating tumours of diverse origins (Jager *et al.,* 2000)(Zeng *et al.,* 2001). In the same way, natural killer (NK, NKT) cells are attracted by stress molecules secreted by tumour cells and recruited to destroy the latter (Bauer *et al.,* 1999). In the last twenty years, tumour immunology has known considerable advances in the field of cytokines, receptors and their respective ligands directing lymphocyte activation, antigen processing and presentation, lymphocyte migration and molecular bases of tumour recognition by the immune system. Moreover, a large number of tumour antigens as well as some epitopes derived from these tumour antigens have been identified and constitute as many potential targets for cancer immunotherapy. However, despite these discoveries, cancer vaccination did not always lead to success as the tumour system can display means of counteracting the action of the host immune system. Nowadays and because of the increase in knowledge of tumours and tumour immunotherapy, researchers are moving towards a more specific and more adapted anti-tumour immunity.

1.2.1 Innate immunity and cancer

Innate immunity is often referred to as the “non-specific” arm of the immune system. It is constitutively present and does not require immunological memory. Its primary role is to protect the organism by rapidly destroying any foreign pathogens that the body encounters on a day-to-day basis. This response is ensured by several effectors among which the most important are NK and NKT cells, both originating from lymphoid progenitor cells, and phagocytic cells such as macrophages and dendritic cells (DC) originating from myeloid

progenitor cells. These mediators of the innate immune system recognise pathogens through surface receptors, which are able to bind non-specifically a wide variety of ligands. This system was then described as the "pattern-recognition specific" system with receptors able to recognise conserved surface molecules specific for certain bacteria, viruses and other micro-organisms (Janeway *et al.*, 1991). Once recognition has taken place, cells of the innate immune system can engulf the pathogens by the process of phagocytosis, lyse the foreign bodies following the release of complement molecules, or finally activate the adaptive immune system *via* the use of antigen-presenting cells (APC). The response to external threat is rapid without the delays occasioned by clonal expansion (Adam *et al.*, 2003). The differentiation between pathogen and host is mediated by, among others, members of the Toll-like receptor (TLR) family. So far, ten members of this family have been identified in mouse and humans and were found to be expressed by phagocytic cells, mostly macrophages and DC. They are able to recognise a plethora of ligands belonging to most pathogens such as lipoproteins and peptidoglycans (TLR2), double stranded RNA (TLR3), lipopolysaccharide (TLR4), flagellin (TLR5), and unmethylated CpG DNA motifs (TLR9)(Beutler *et al.*, 2003). Immune cells of the innate system are stimulated by the activation of TLR and are responsible for the release of cytokines and chemokines, but also for the induction of the expression of co-stimulatory molecules by APC, critical for engaging the adaptive immune response. Antigen-presenting cells are indeed very important as they act as a link between the innate and the adaptive immune system and allow the development of a long-lasting and potent protection mediated by lymphocytes.

Innate immune system is known to provide the first defence against infectious agents and is capable of initiating the adaptive immune system. It is now believed that it also plays the same role of immunoediting when cancer cells are involved. Effector cells of the innate system, present on the tumour site, are capable of sensing the tumour development and alert the immune system to its presence. It is not entirely clear how these effector cells realise the existence of a tumour cell but it is thought that uric acid released by damaged cells (Shi *et al.*, 2003) or simply the intrinsic biology of the tumour (Seong and Matzinger, 2004) are sufficient to provide the "danger signal" required by the innate system. A pro-inflammatory response can then follow the reception of this danger signal and the presence of TLR ligands such as heat shock proteins (HSP) on the tumour site allows the activation of DC and macrophages expressing TLR, consequently making the link with the adaptive immune system (Dunn *et al.*, 2004; Napolitani *et al.*, 2005). Tumour cells are also able to produce a different type of danger signals, which allows the recruitment of other cells of the innate immune system. As mentioned

earlier, some tumours can develop in an oxygen-free environment leading to the expression of specific molecules, such as MHC class I chain-related proteins A and B (MICA/B) and UL16-binding proteins, normally absent from tumours developing in non-hypoxic conditions (Papamichail *et al.*, 2004). These ligands, present at the surface of tumour cells, can bind receptors found at the surface of NK and NKT cells called NK cell activating receptor or NKG2D, and activate the latter. In return, NK cells secrete interferon-γ (IFNγ), which is an important cytokine not only capable of directly killing tumour cells by blocking angiogenesis and reducing the metastatic potential of the tumour, but also by inducing the secretion of chemokines by tumour cells and surrounding healthy cells to attract lymphocytes, and acting on DC and macrophages to induce maturation and secretion of Interleukin (IL)-12 by these two cells for the activation of the adaptive immune response and more specifically cytotoxic T lymphocytes (CTL)(Degli-Esposti and Smyth, 2005)(Fig1.4). Moreover, molecules present at the surface of NK cells called killer inhibitory receptors (KIR) and C-type lectin-like receptors can specifically recognise and bind MHC class I molecules, hence inhibiting their tumoricidal action (Moretta *et al.*, 1996). This system allows the maintenance of self-tolerance towards the normal cells of the organism, with most of them expressing MHC class I molecules. In an attempt to avoid recognition by cytotoxic T cells, tumour cells can down-regulate the expression of MHC class I molecules (Restiflo *et al.*, 1993). This leads at the same time to the suppression of NK cell inhibition by KIR, causing these cells to be more sensitive to NK cell-mediated lysis operating through the secretion of perforins and other reactive oxygen and nitrogen species. On the other hand, the role of NKT cells against tumour development is still unclear and controversial. This population of cells has characteristics of both NK and T cells and is secreting large quantities of cytokines, mainly IL-13, but research has shown that they can have both negative and positive effects on the immune system and the actual tumour, respectively (Terabe *et al.*, 2000; Godfrey *et al.*, 2000).

The depletion of NK and NKT cells in mice indicated a higher susceptibility to chemically-induced tumours (Dunn *et al.*, 2004). More and more evidences are accumulated showing that these two populations of cells and especially the crosstalk between them and DC are crucial for an immune response, both innate and adaptive, against cancer to take place (Smyth *et al.*, 2005). B lymphocytes, macrophages and DC are the three most interesting subsets of cells involved in antigen capture, processing and presentation *via* MHC molecules to cells of the adaptive immune system in order to initiate a specific immune response against this antigen.

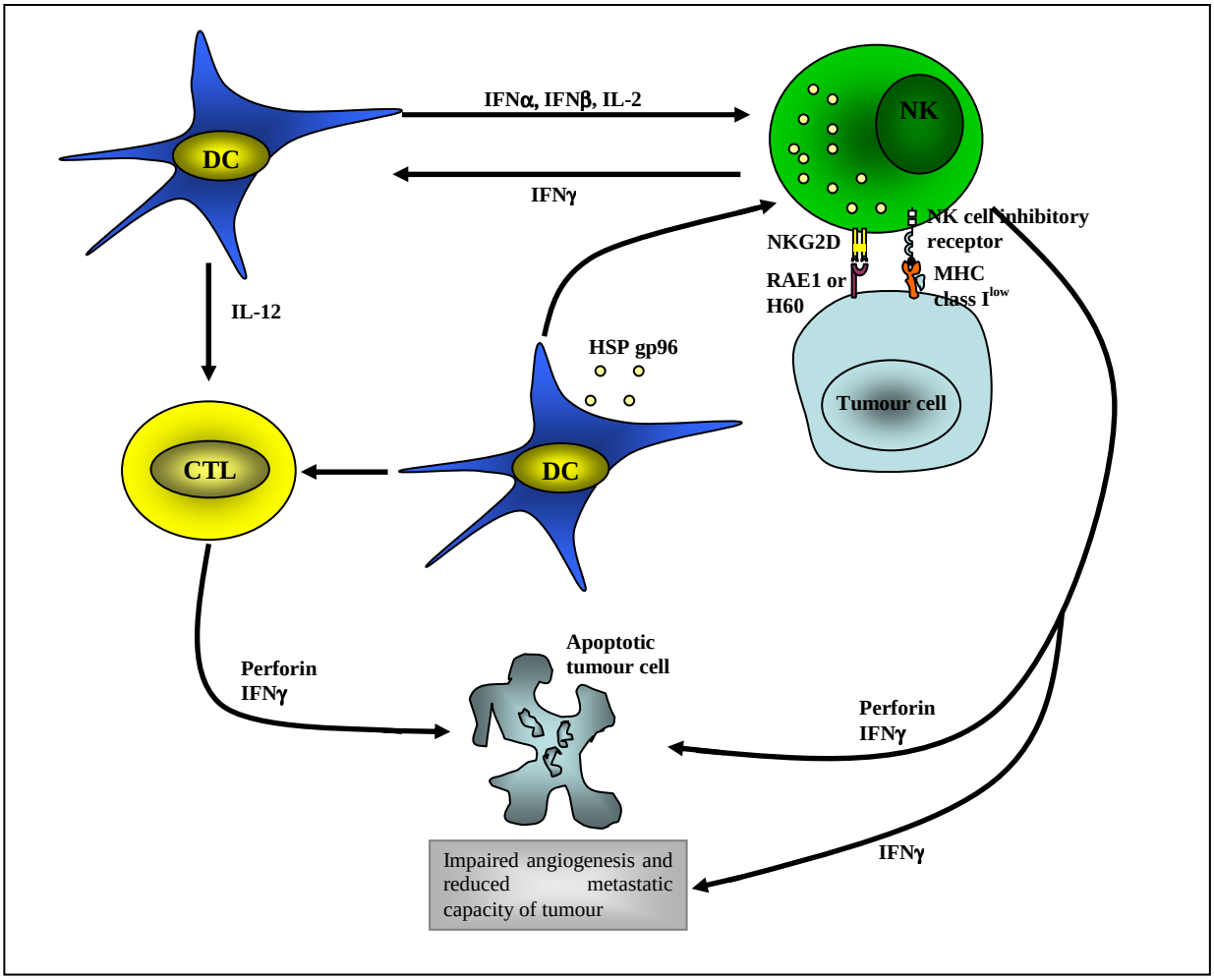

Figure 1.4: Impact of crosstalk between dendritic cells and natural killer cells on immunity to tumours. *Crosstalk between DC and NK cells operates via the secretion of cytokines such as IFNα, IFNβ, IFNγ and IL-2. Secretion of IFNγ by NK cells acts on DC and macrophages to induce maturation and the secretion of IL-12 by these two APC. In turn IL-12 acts as an important mediator of the adaptive immune response. Moreover, NK cells once activated are capable of directly killing tumour cells that do not express inhibitory MHC class I through the secretion of perforins and reactive oxygen and nitrogen species (Taken from Degli-Esposti and Smyth, 2005).*

However, it is generally admitted that although B cells and macrophages are efficient APC, DC are the most potent and important APC in the body, situated at the crossroad between innate and adaptive immune system. DC are capable of rapidly engulfing the antigen at its entry in the system, processing and presenting it to T cells together with the help of co-stimulatory molecules (Adam *et al.*, 2005). It is for this reason that DC are nowadays one of the most talk-about subjects in the research evolving around the study of the immune response and on how they could be used efficiently to treat infections but most of all cancer.

As described earlier, the innate immune system initiates the anti-tumour response, resulting in the attraction of DC which phagocytose the tumour cell debris and apoptotic cell fragments ensuing from the destruction started by the innate mediators. Because of this ability and their natural morphology, DC are described as "the sentinels" of the immune system (Rafiq *et al.*, 2002). They originate from the bone marrow, travel through the blood and are seeded into non-lymphoid tissues. These "immature" or "pre-conditioned" DC can engulf and process large quantities of antigens from cancer cells but have strong difficulties in activating T cells because of low expression levels of major histocompatibility complex (MHC) antigens and co-stimulatory molecules (Gallucci *et al.*, 1999). Following internalisation and processing, DC migrate *via* the bloodstream and afferent lymph to secondary lymph nodes and go through different stages of maturation by up-regulating MHC class I and class II molecules and co-stimulatory molecules such as CD40 and B7. In the lymph nodes, they interact with T helper cells and CTL in the presence of cytokines such as IFNα in a mechanism called cross-presentation. Co-stimulatory molecules bind to their ligands present on the surface of T cells (e.g.: CD40L and CD28, respectively) allowing co-conditioning of both DC and T cells (Ridge *et al.*, 1998). Dendritic cells release several other cytokines such as TNFα, IL-1β and IL-12 and become potent activators of T cell-based responses *via* antigen processing and antigen presentation on the surface in the context of MHC molecules (Girolomoni and Ricciardi-Castagnoli, 1997). This area will be discussed in further details later on in Section 1.2.2.2.

1.2.2 Adaptive immunity

The immune system protects the organism from non-self agents using proteins and cells that circulate the body. As described earlier, this mechanism starts with the innate response and ends with the adaptive system. The latter is mediated by the humoral response involving B cells and antibodies, and/or the cellular response relying mainly on the action of CD4+ T helper and CD8+ cytotoxic T cells. Because of the abnormal pattern of expression of

oncogenes, tumour-suppressor genes and TAA, a tumour cell can be used as a target for the adaptive immune system and although both B and T cell responses can have a critical role for the elimination of cancer cells, it is generally thought that the action of CD4+ and CD8+ T cells is the key factor for an efficient immune response against tumours bearing these antigens.

1.2.2.1 Humoral response and cancer

B lymphocytes are responsible for the humoral response. Upon DC activation, a Th2 response can be elicited allowing the mounting of a B cell-based response, ultimately leading to B cell proliferation and differentiation into memory B cells and antibody-secreting plasma cells. However, in spite of this specific function, they can also internalise, process and present antigens to T lymphocytes. The latter become active and start secreting cytokines ensuring the development and the regulation of the humoral response (differentiation into plasma cells and isotypic commutation of antibodies). Several TAA have been identified through serological analysis of recombinant cDNA expression (SEREX) indicating that the humoral immune system is fully capable of eliciting an antibody response against tumours. The role of B lymphocytes in the anti-tumour response is still controversial. Indeed, it has been demonstrated that the adoptive transfer of B cells in T and B cell-depleted HPV16 mice was leading to the reinstatement of all the parameters required for full malignancy, that is to say, chronic inflammation, angiogenesis and tumour cell proliferation (de Visser *et al.*, 2005). This result is in agreement with the observation that a strict Th2 response is detrimental to the anti-tumour response. Tumours might escape the action of CTL by down-regulating the expression of MHC class I molecules and orientate the adaptive immune system towards a humoral response more beneficial to their development. On the other hand, several monoclonal antibodies are nowadays available to treat certain forms of cancer with success indicating that antibodies are efficient anti-tumour agents, on their own or together with a Th1 response (Pegram *et al.*, 1998; Plunkett and Miles, 2002). In conclusion, it seems that the balance between Th1 and Th2 responses, as well as the presence of all the actors of the adaptive immune system (CD4+, CD8+ and B cells) are actually crucial to promote tumour rejection (Kao *et al.*, 2006).

1.2.2.2 Cellular response and cancer

The cellular response, with the help of APC from the innate system, relies on three subsets of T lymphocytes:

_Cytotoxic T lymphocytes or CD8+ T cells recognise infected or transformed cells such as tumour cells and eliminate them.

_T helper 1 lymphocytes or CD4+ Th1 cells exchange information with antigen-presenting cells and play a crucial role in the activation of CTL.

_T helper 2 cells or CD4+ Th2 cells, as mentioned above, mediate the development of B cells.

Briefly, DC are attracted to the tumour site where they phagocyte tumour cell debris and apoptotic cell fragments ensured by the mediators of the innate response. Upon endocytosis of tumour material, DC migrate to the lymph nodes where they will meet and interact with CD4+ Th1 cells and CD8+ T cells. These three subsets of cells form a trivalent complex that results in the maturation of DC through cytokine and cell-surface receptor interactions between CD4+ T cells and DC, which in turn allows communications between CD8+ T cells and DC leading to the differentiation of CD8+ T cells into CTL. After activation, CTL and T helper cells can migrate back to the tumour site where they exert their antigen-specific killing of any tumour cells presenting the antigen against which they were pre-conditioned through the secretion of cytotoxic granules or by stimulating "death-receptors" expressed by tumour cells. This model of adaptive immune response is a simplified version of the reality. Many cytokines, such as IL-2, IFNγ, IL-12 and IL-18 are also involved in this process but this does not stop the tumour to grow indicating that the system is not infallible, probably because of the tumour's ability to escape recognition by avoiding the induction of "danger signals" whilst the tumour is relatively small and developing, or inducing a state of immune tolerance towards itself and an immunoselection of the tumour cells that can escape due to their genetic instability and are free to expand in an uncontrolled state. This system, although not perfect, underlines the crucial role of the TCR genes rearrangement during T cell differentiation and clonal expansion, the processing and the presentation of tumour-derived peptides by MHC molecules, and finally the interaction between the MHC molecules presenting antigenic tumour-derived peptides to the TCR of CD4+ and CD8+ T cells.

(a) Major histocompatibility complex and its polymorphisms

Specific membranous molecules called major histocompatibility complex (MHC) antigens allow the immune system to do the discrimination between self, modified self and non-self antigens. Firstly discovered on human leukocytes as a result of their effects on transplantations, major histocompatibility complex (MHC) antigens are also termed HLA for human leukocyte antigens (McDevitt., 2000; Shiina *et al.*, 2004). MHC molecules are proteins encoded by a group of genes situated in a specific region of chromosome 6 in humans and the equivalent H2 genes on chromosome 17 in mice. In these regions, a certain number of loci encode proteins

exerting immunological functions very tightly linked to histocompatibility molecules and involved in the formation of MHC: peptide complexes. Indeed, these genes favour the processing (LMP2, LMP7), the transporting (TAP1, TAP2) to the endoplasmic reticulum (ER), or the loading (tapasin) of the antigenic peptides onto MHC class I and class II molecules. Genes coding for MHC molecules are grouped in 6 principal loci (A, B, C, DP, DQ and DR) including for each one a high number of alleles (Fig. 1.5). For each of these 6 genes, each person inherits 2 alleles, one from each parent. Because millions of combinations are possible, it is unlikely for two persons to have identical MHC molecules, except homozygous twins. Furthermore, this diversity provides the organism and the T cells with a plethora of antigenic peptides (Reche and Reinherz, 2003).

These epitopes are presented on MHC molecules to the TCR of T lymphocytes (Neefjes and Momburg, 1993). T cell receptors are composed of two functional subunit clusters, α/β and γ, δ, ε, ζ, which shares the roles of antigen recognition and signal transduction, respectively. The α and β subunits have the property to interact with antigenic peptides loaded onto MHC class I and class II molecules. α and β proteins are each formed of a variable region (Vα and Vβ, V domain) connected by a short segment to a constant region (Cα and Cβ, C domain) having a refolding very similar to immunoglobulins (Ig). Vα and Vβ regions are encoded by a high number of genetic segments (V, D and J), which are juxtaposed through a series of site-specific DNA recombinations during the development of T cells to create a large diversity of binding sites. TCR binding to the appropriate MHC: peptide complex in conjunction with either CD4 or CD8 binding (MHC class II and class I binding, respectively) allows the activation of the TCR intra-cellular domain.

The γ, δ, ε and ζ subunits, like α/β, are transmembranous proteins but with very distinct structures from α/β. γ, δ and ε subunits form the multimeric CD3 complex. They have an extra-cellular domain similar to Ig and an intra-cellular domain each carrying conserved repeated sequences called immunoreceptor tyrosine-based activations motifs (ITAM). These ITAM are essential to the signal transduction and the high number of motifs is an indication of the capacity to quantitatively and qualitatively regulate the signalling (Fig. 1.6).

This signalling, in cooperation with co-stimulatory molecules such as CD28 and CD40, triggers a cascade of reactions allowing full T cell activation. In summary, the quality of the MHC-peptide-TCR interaction is crucial to ensure a proper antigen presentation and a strong T cell activation (Saito and Yamasaki, 2003).

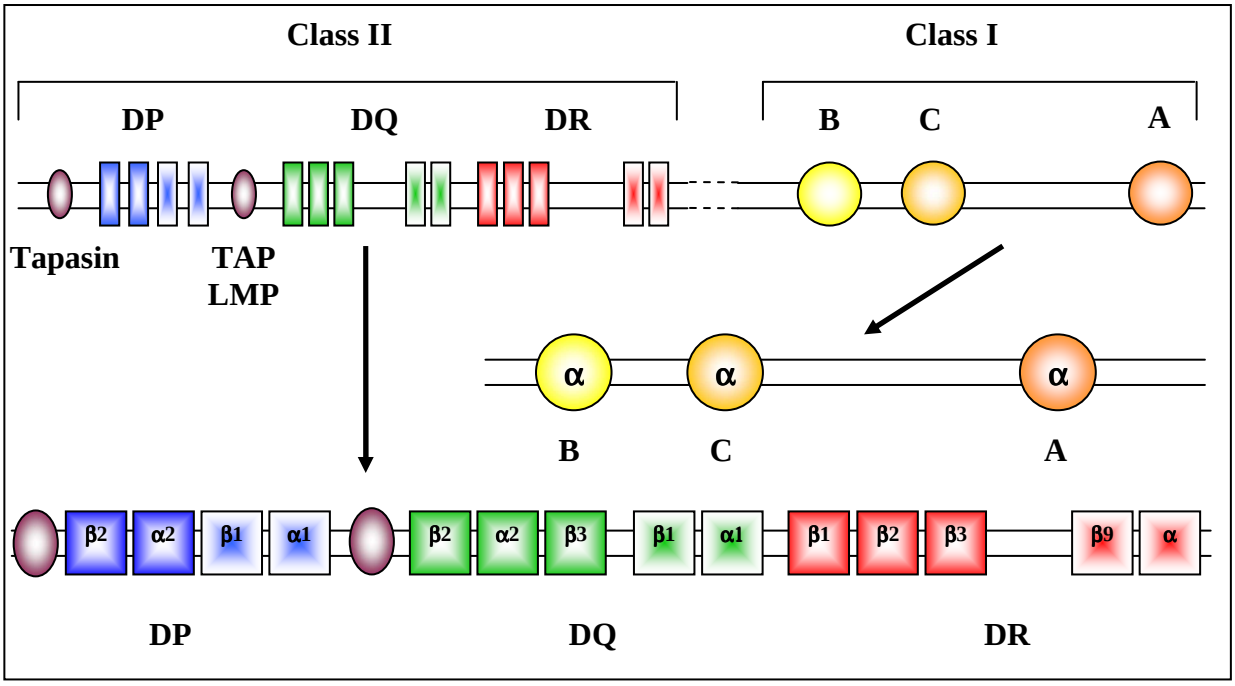

Figure 1.5: Distribution of MHC genes in humans (Adapted from pathmicro.med.sc.edu/ghaffar/mhc2000.htm).

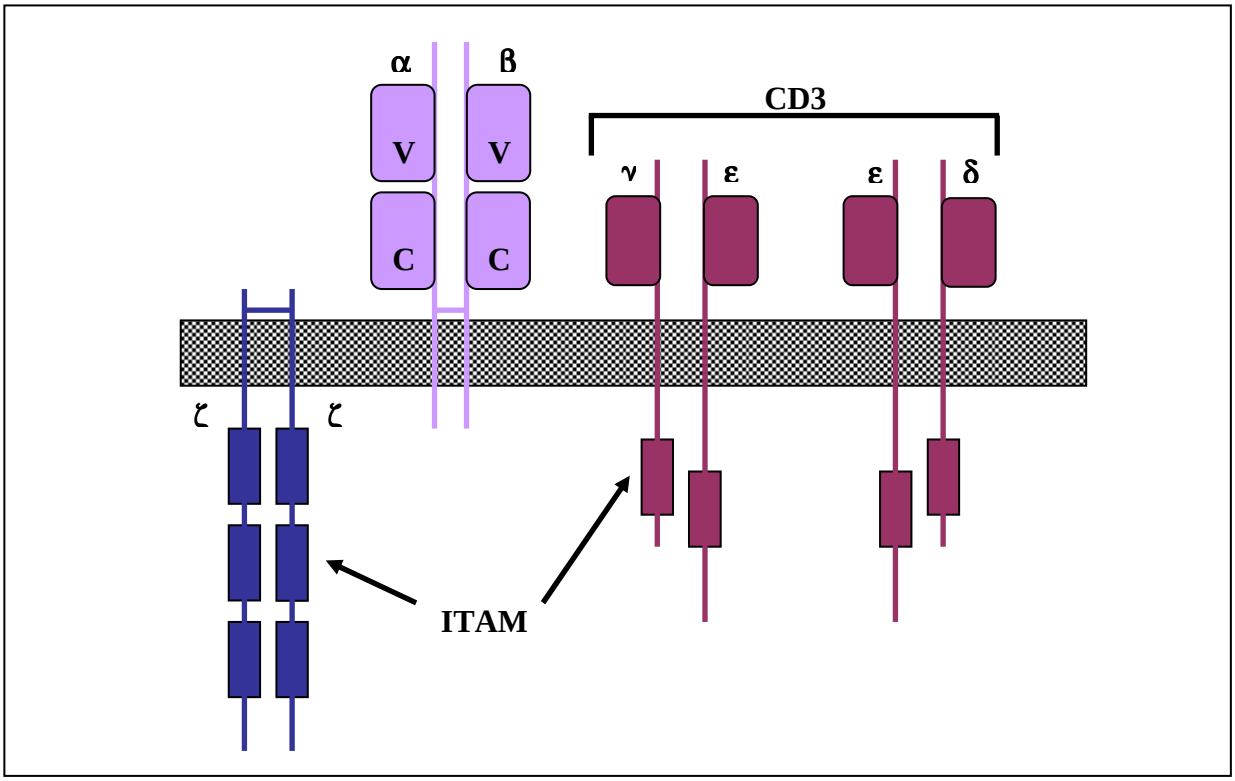

***Figure 1.6: Schematic representation of TCR structure**. The TCR is composed of two chains (α and β), themselves constituted of one variable (V) and one constant (C) region each. The multimeric CD3 complex is formed by the γ, δ, ε and ξ subunits. ITAM, responsible for the signal transduction, are represented by rectangles in the intra-cellular domain.*

MHC glycoproteins are grouped in two classes of MHC class I and MHC class II molecules:

_Genes coding for MHC class I molecules are divided into three loci: HLA-A, -B and –C. Each of these loci has a high number of alleles; 649 alleles for the A locus, 1029 for B and 350 for C have been identified (www.anthonynolan.com/HIG). It is also worth mentioning that, depending on the population of origin, each of these alleles has a different frequency (*e.g.*: 19% and 30% of the african and caucasian populations are HLA-A2-positive, respectively)(www.ashi-hla.org). Encoded molecules are membrane-spanning glycoproteins formed by an integral protein of 45kDa and presenting three external domains, α1, α2 and α3. The latter is sustained non-covalently by a globular protein of 12kDa encoded by a gene on chromosome 15 and termed β2-microglobulin, which is essential to the structure, the activity and the expression of class I antigens (Fig. 1.7a). The specific way that the α1 and α2 domain conform generates the peptide-binding cleft. It is generally admitted that peptides binding to this groove are of 8-11 amino acids in length (Bjorkman *et al.*, 1987; McDevitt., 2000). MHC class I molecules are found in lymphoid tissues and on nucleated cells, even though some tissues such as thymic epithelia, hepatocytes, kidney or brain can show relatively low levels. Their function is to present peptides of predominantly intra-cellular origin to CD8+ T cells. Peptides recognised as a threat by CD8+ T cells, such as bacteria- or virus-derived, will lead to the elimination of the infected cells (Adam *et al.*, 2003).

_Genes coding for MHC class II molecules are divided into three loci: HLA-DP, -DQ and –DR, each of them having several alleles. Like MHC class I alleles, the frequency of each of these MHC class II alleles in a determined population is variable (e.g.: 10% and 17% of the caucasian population are HLA-DR1- and HLA-DR4-positive, respectively)(www.ashi-hla.org). Encoded membrane-spanning glycoproteins are heterodimers formed by two non-covalently linked chains (α and β) of 33kDa and 28kDa, respectively, each of them containing two external domains (α1/α2 and β1/β2)(Fig. 1.7b). Furthermore, there are variations in the MHC repertoire with the production of multiple types of α and β chains for each type of MHC class II molecules. There are five α chains (1 DRα, 2 DPα and 2 DQα) and eight β chains (3 to 4 DRβ, 2 DPβ and 2 DQβ) (Campbell and Trowsdale, 1993).

A high number of combinations of α and β chains and consequently a huge number of binding peptides are therefore available to propose a strong immune protection, providing that the individual is heterozygous (Lipsitch *et al.*, 2003). Unlike the binding groove of MHC class I molecules, α1 and β1 form a peptide-binding cleft with an opened structure allowing the binding of longer peptides (13-18 amino acids in length)(Rudensky *et al.*, 1991).

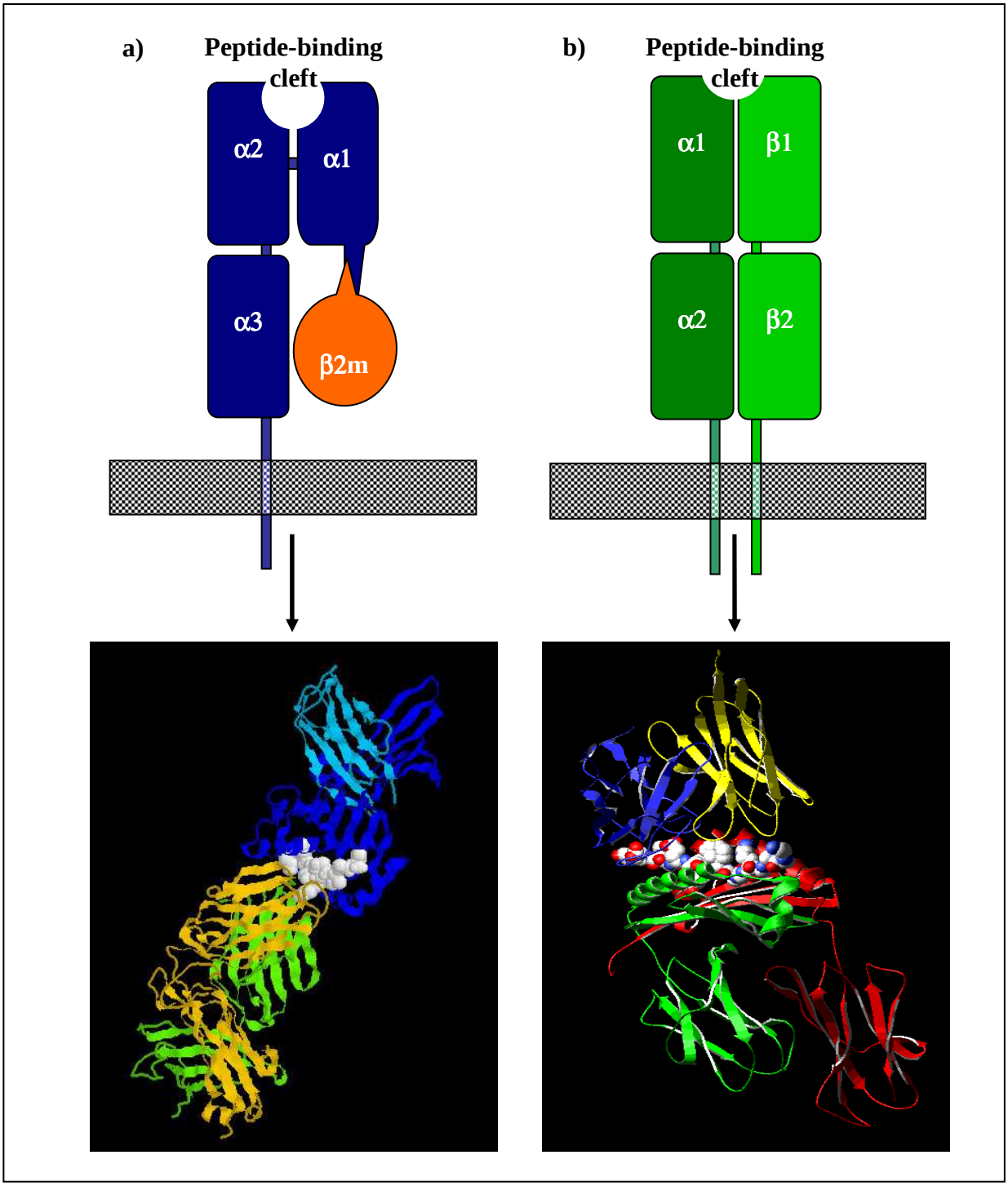

Figure 1.7: Structure of MHC class I and class II molecules. *a) Top: Class I MHC with a membrane-spanning integral protein with three external domains (α1, α2 and α3) associated with β2-microglobulin. Bottom: crystallography representing a MHC class I molecule (α chain: dark blue, β2-microglobulin: light blue) presenting an antigenic peptide (white) to a TCR (α and β chains: yellow and green, respectively)(Taken from pathmicro.med.sc.edu); b) Top: Class II MHC made of two transmembranous chains (α and β) with two external domains for each of them (respectively α1, α2 and β1, β2). Bottom: crystallography representing a MHC class II molecule (α chain: green, β chain: red) presenting an antigenic peptide (white) to a TCR (α and β chains: yellow and blue, respectively)(Taken from www.usm.maine.edu).*

MHC class II expression is found on the surface of professional APC (DC, B cells and macrophages), in lymphoid tissues and can be induced with TNFα or interferons in other nucleated cells (Schartner *et al.*, 2005). Their function is to present peptides of pre-dominantly extra-cellular origin to CD4+ T cells, which orchestrate the adaptive immune response as CD4+ T cells are critical for the activation of other cells and the generation of a long-lasting memory CTL response (reviewed by Assudani *et al.*, 2007).

Disparities in MHC molecules between patients and donors are the main reasons for graft failures and graft-versus-host diseases. The discovery of minor histocompatibility antigens complicated this understanding as they were reported to account for similar consequences in HLA-matched transplantation procedures such as allogeneic bone marrow (Goulmy, 1996) or stem cell (Falkenburg *et al.*, 2003) transplantations between MHC-identical individuals. However, minor antigens, such as HA-1 and HA-2, could be interesting targets for cancer immunotherapy as they were shown to be responsible for a graft-versus-leukaemia effect in leukaemia patients (Hambach and Goulmy, 2005) providing that the right signals are emitted with adapted CD4+ T cell help and lack of inhibition from NKG2/CD94 engagement (Robertson *et al.*, 2007).

(b) Antigen processing and presentation

As described previously, the adaptive immune response relies on the activation of CD4+ and CD8+ T cells following the binding of their TCR to antigenic peptides presented by MHC class II and class I molecules, respectively. These T cell epitopes derived from the target protein are usually between 8 and 18 amino acids in length depending on whether they are restricted to MHC class I or class II molecules. For a peptide to be presented in this context, the protein must undergo a cascade of reactions that will ultimately lead to their transport and cleavage of this peptide. These reactions differ according to the origin of the proteins. Indeed, endogenous proteins follow pre-dominantly the MHC class I pathway, while exogenous proteins undergo pre-dominantly the MHC class II pathway.

(i) MHC class I processing: The proteolysis of endogenous proteins into antigenic determinants takes place within the cytoplasm and is mediated mainly by a multi-catalytic complex called the proteasome and other peptidases (Kloetzel and Ossendorp, 2003). Most of the proteins synthesised by the cell that are no longer required, damaged or “foreign” are eliminated by the proteasome following poly-ubiquitinylation, which allows the unfolding of

the protein and acts as a signal for their degradation (Jariel-Encontre *et al.*, 1995). These peptides are then propelled in an ATP-dependent manner into the ER lumen using the heterodimeric transporter-associated with antigen processing (TAP), consisting of two subunits TAP1 and TAP2 and encoded by genes within the MHC (Ortmann *et al.*, 1997). The α chain and β2-microglobulin of MHC class I molecules are assembled within the ER lumen with the help of three chaperones called calreticulin, calnexin and ERp57 (Neefjes and Momburg, 1993; Williams and Watts, 1995). A fourth chaperone, tapasin, allows the translocation of peptides to the MHC class I binding groove and prevents the degradation of class I - β2-microglobulin dimers (Reits *et al.*, 2000). MHC class I molecules are stabilised through the process of peptide binding and the complex is finally transported from the ER lumen to the Golgi apparatus before finally reaching the cytoplasmic membrane using a Golgi vesicle to present the peptides to the TCR of CD8+ T cells (Fig. 1.8). The cell provides a continuous exposition of a sample of its peptidic content. A fragment, from an abnormal or intruder protein presented by a MHC class I molecule, induces an immune response by reacting with cytotoxic CD8+ lymphocytes and ensuring a true immune surveillance that eliminate cells presenting foreign antigenic determinants.

(ii) MHC class II processing: Extra-cellular antigens that are endocytosed by APC *via* phagocytosis result in antigens being presented in the context of MHC class II for CD4 recognition. Engulfed proteins enter the endocytic pathway, where they will be degraded. Endosomes become more acidic as they mature and finally fuse with lysozymes allowing the degradation of the antigens by acid proteases, reductases and unfoldases activated in the low pH environment. Members of the acid proteases include the cathepsins B, D, S and L (Janeway., 2001). Like MHC class I, the α and β chain are assembled in the ER lumen to form the αβ heterodimer. At this stage, a third chain associates to the heterodimer to form a heterotrimer. This chain is known as the invariant chain (Ii), prevents the binding of any polypeptides present in the ER lumen and is thought to be important for the trafficking of the class II molecules to the endosomal compartments (Bikoff *et al.*, 1993). The endosomal vesicles containing the MHC class II molecules fuse with the ones containing the antigenic peptides. In this new compartment, Ii is cleaved by cathepsin L or S and the class II-associated invariant peptide (CLIP) is left inside the MHC class II groove (Bennet *et al.*, 1992). CLIP is finally exchanged for an antigenic peptide with the help of MHC-like molecules known as HLA-DM and HLA-DO. These molecules are involved in the process called peptide editing,

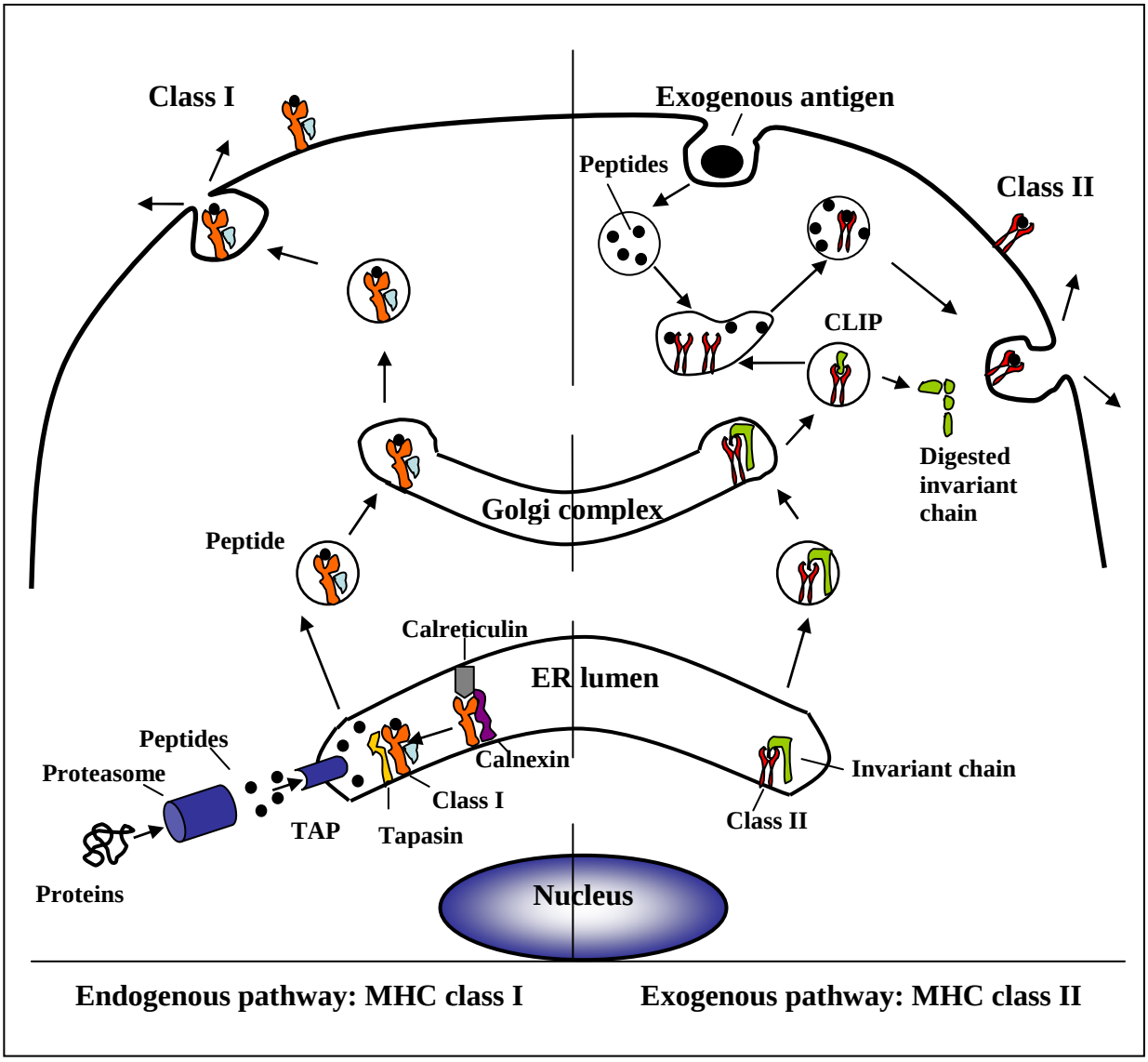

Figure 1.8: MHC class I and class II processing. *On the left hand side: endogenous pathway showing digestion of the cytosolic antigenic protein by the proteasome, integration in the endoplasmic reticulum of antigenic peptides by TAP, binding of these peptides to MHC class I molecules and presentation at the cell surface to CD8+ T-lymphocytes. On the right hand side: exogenous pathway detailing the proteolysis of an exogenous antigen in endocytic compartments, the incorporation of the resulting antigenic peptides in the MHC class II groove instead of CLIP and the presentation at the cell surface to B- and CD4+ T cells (Taken from Neefjes and Momburg, 1993).*

which is based on the stabilisation of the MHC class II: peptide complex and the continuous exchange of antigenic peptides until the higher affinity between the two molecules is found. Upon stabilisation, the complex is finally transported to the plasma membrane to present the peptide to the TCR of CD4+ T cells (Fig. 1.8). Interestingly and although the endocytic pathway and exogenous proteins remain the major providers of peptides to class II molecules, endogenous antigens can also enter the MHC class II pathway and elicit a T helper response. Indeed, it was shown that the generation of MHC class II-restricted endogenously synthesised epitopes is independent of the proteasome and the TAP complexes, and might overlap with the classical endosomal pathway for presentation of exogenously synthesised peptides (Dissanayake *et al.*, 2005). This is an important factor when considering the antigen-specific recognition of class II positive tumour cells by CD4+ T cells.

(c) The pivotal role of CD4+ T cells in anti-tumour immunity

Over the past two decades, most of the tumour immunotherapy research has focused on developing a way to stimulate CD8+ T cells as they can exert specific cytotoxicity against tumour cells. However, more and more evidence has accumulated showing the importance of a CD4+ T cell help in anti-tumour immunity. CD4+ T lymphocytes act upon recognition by their TCR of antigenic peptides presented in the context of MHC class II. These peptides come from tumour-derived proteins that have been either endogenously processed by the tumour itself or by mature APC that have ingested and digested exogenous tumour-derived proteins. The recognition of antigenic peptides occurs through the TCR and is assisted by a new accessory protein, the CD4 molecule, which binds to the proximal domains of the α and β chains of the MHC class II molecules. This cell population is specialised in the production of cytokines (e.g.: IFNγ, IL-2, -4, -5, -6, -10, -12 or -13), which stimulate or regulate the activation of CD8+ cytotoxic T cells or B lymphocytes. Therefore, CD4+ T cells are essential for the development of strong effector responses, orientating the immune system according to the produced cytokine profile towards a cytolytic response (cellular differentiation of Th1 CD4+ T cells, IFNγ, IL-2 and -12 production), or a production of antibodies by differentiated B lymphocytes (cellular differentiation of Th2 CD4+ T cells, IL-4, -5, -6, -10 and -13 production).

CD4+ T lymphocytes can therefore regulate virtually all antigen-specific immune responses. Indeed, the Th1 subset can be characterised by the secretion of IL-2 required for the proliferation of cytotoxic T cells, and IFNγ, which can have many effects such as the up-regulation of the immunoproteasome and MHC class I and class II molecules in both APC and

tumour cells resulting in increase presentation for the CTL. On the other hand, the Th2 subset of CD4+ T cell population can facilitate the uptake of antigens by promoting the generation of antibodies. Antibodies may bind to tumour antigens and other apoptotic bodies and assist to their opsonisation *via* the binding of the Fc fragment of antibodies to the Fc receptors present on the surface of APC, which ultimately leads to cross-presentation. However, the involvement of antibodies in the anti-tumour response remains unclear. Moreover, Th2 cells are often associated with tumour growth by both facilitating angiogenesis and inhibiting cellular responses and although Th2 cells have also been linked with tumour-infiltrating granulocytes such as eosinophils, which exert an anti-tumour cytotoxicity (Ellyard *et al.*, 2007), it is generally believed that the modulation of the balance between the two subsets is more important for the elimination of cancer cells than one subset or the other on its own, and is required to generate the appropriate response against an altered self threat (Tanaka *et al.*, 2000). Also, a sub-population of CD4+CD25+ T cells, also called T regulatory cells (Treg), with an immunosuppressive activity and an involvement in peripheral tolerance mechanisms was identified (Thornton and Shevach, 1998). T regulatory cells can occur either naturally following selection in the thymus as an anti-self repertoire or induced in the periphery with the expression of the CD25 marker upon the action of immunosuppressive cytokines such as IL-10, TGFβ (Von Boehmer H, 2005) or more controversially IL-2 secreted by Th1 cells in the tumour environment (Antony *et al.*, 2006). These antigen-specific cells can decrease the anti-tumoral response by altering the ability of APC to activate CD4+ and CD8+ T cells. Their mechanism of suppression is not yet fully understood but evidence of their involvement in the anti-tumour response accumulate and imply a careful study to find a balance between maintenance of tissue integrity by avoiding autoimmune response to take place and induction of a potent tumour-specific immune response allowing successful tumour regression (Assudani *et al.*, 2006).

The majority of the help provided by CD4+ T cells comes from the Th1 subset of CD4+ T lymphocytes, which interacts with professional APC to induce a strong CTL response (Assudani *et al.*, 2007). This CTL "licensing" by Th1 cells can be summed up in three consecutive steps. First, CD4+ T cells interact with pre-conditioned DC through the CD40 pathway and take part in the maturation of both cell types (O'Sullivan and Thomas, 2003). This pathway and more specifically the levels of CD40 were shown to correlate with the efficacy of the anti-tumour response. Indeed, the higher level, the higher chances are for the tumour to regress underlining the critical role of Th1 cells (Murugaiyan *et al.*, 2007). At this

stage, mature and conditioned DC can activate naive CD8+ T cells on their own (Ridge *et al.*, 1998). Secondly, mature DC process and present high quantities of peptides in the context of MHC class I and class II molecules to both CD8+ and CD4+ T cells, respectively. Following formation of a trivalent complex between DC, CD4+ and CD8+ T cells and upon antigenic recognition by the TCR, Th1 cells allows the long-lasting persistence of CTL and the generation of memory CD8+ T cells (Smith *et al.*, 2004; van Mierlo *et al.*, 2004). Indeed, it seems that cognate recognition of the same antigen on the same DC by CD4+ and CD8+ T cells is the best way to generate an efficient CTL response composed of long-lasting effector CTL and CD8+ T cells with a memory phenotype that can be readily re-activated upon re-exposure to the antigen. More recently, it was demonstrated that CD4+ Th1 cells can acquire MHC class I: peptide complexes from DC and directly present them to CD8+ T cells for their activation (Xiang *et al.*, 2005). Finally, Th1 have been shown to be crucial for the re-activation of memory CD8+ T cells (Gao *et al.*, 2002). This model of CD4+ T cell help varies from one study to another depending on the model and the nature of the antigen used. Indeed, a pathogen may lead to the immediate activation of CD8+ T cells without extemporaneous conditioning of DC because of the danger signals it brings, while the chronic state of cancer may require a primary signal through CD40-CD40L ligation to induce an anti-tumour immune response. That is why, CD4+ T cell help remains a subject of research and a source of controversy.

Apart from providing a strong long-lasting CTL response, CD4+ Th1 cells can also mediate tumour regression on their own (Daniel *et al.*, 2005). Indeed, some CD4+ T cells are capable of directly recognising antigenic peptides presented by MHC class II molecules on the surface of tumour cells and exerting cytotoxic activity. A number of mechanisms have been proposed to explain this phenomenon. Upon antigen recognition, Th1 cells can secrete TNFα (Tite *et al.*, 1990). They can mediate tumour cell apoptosis by engaging Fas ligand (FasL) (Schattner et al., 1996; Bagot *et al.*, 1998; Echchakir *et al.*, 2000; Dicker *et al.*, 2005) or TNF-related apoptosis-inducing ligands (TRAIL)(Thomas and Hersey, 1998; Dicker *et al.*, 2005) with their respective receptors present on the surface of target cells. Like CD8+ T cells, some Th1 cells were shown to be capable of excreting granzymes and perforins to destroy tumour cells (Echchakir *et al.*, 2000; Dorothee *et al.*, 2002). Finally, secretion of IFNγ by CD4+ T cells was also proven to have pro-apoptotic and anti-proliferative effects on tumour cells through the activation of macrophages, the release of reactive oxygen and nitrogen species by the latter, and the secretion of IL-12 by the macrophages, which appears to induce apoptosis when they are directly in contact with the tumour cells (Tsung *et al.*, 1997; Tsung *et al.*, 2002; Ikeda *et al.*,

2002). However, these CD4+-specific cytotoxicity only represents a minor mechanism of the anti-tumour immunity as it relies on the expression of MHC class II molecules, which, depending on the tumour type, are often absent from the surface of tumour cells.

Models of adoptive transfer have largely undermined the role of CD4+ T cells as when these therapies were transferred to clinic, a clinical response was rarely obtained despite the generation of tumour-specific CTL (Cormier *et al.*, 1997; Wang *et al.*, 1999). On the other hand, co-transfer of CD4+ T cells with the CD8+ T cells has been shown to prolong the survival of adoptively transferred TIL (Blattman *et al.*, 2003) and improve the direct clinical outcome (Rosenberg and Dudley, 2004). Altogether, these data suggest that CD4+ T cell help is required to generate a correct anti-tumour response by allowing the DC to license CTL, efficiently activating CTL, recruiting other immune cells and mediating direct or indirect tumour cell killing (Fig. 1.9).

(d) The effector role of CD8+ T cells in anti-tumour immunity

Research has focused over the years on priming CTL to specifically lyse and eradicate tumour cells after recognition of the MHC: peptide complex (Pardoll *et al.*, 1998). Using adoptive transfer of T cells in tumour-bearing hosts, CTL can invade at the initial tumour site as well as at distal locations and trigger potent anti-tumour immunity as shown in mice (Ryan *et al.*, 2001) and in humans (Dudley *et al.*, 2001). It has been proved that there is an inverse correlation between the amount of CD8+ tumour-infiltrating lymphocytes (TIL) and the tumour outcome, and this was observed in a variety of cancer such as melanoma, breast, prostate or colon carcinoma (Dunn *et al.*, 2004). Cytotoxic T cells exert their function using two main lysis pathways: one based on the release of granzymes and perforins and the other one based on the triggering of apoptosis *via* Fas or TRAIL signalling (Andersen *et al.*, 2006). A third pathway can also be considered with the recruitment of macrophages at the tumour site after the release of IFNγ by activated CTL. Release of complement proteins at the tumour site will then help in the phagocytosis of cancer cells by these macrophages (Dredge *et al.*, 2002)(Fig. 1.10).

Naive CD8+ T lymphocytes differentiate in cytotoxic effectors following engagement of their TCR and recognition of peptides associated with class I antigen on APC (de Haan *et al.*, 2000). The activation of their effector function depends strongly on the quality of the TCR/peptide interaction and on the number of TCR engaged in a sequential fashion. These lymphocytes generally end their functional maturation with both co-stimulatory and cytokine signals.

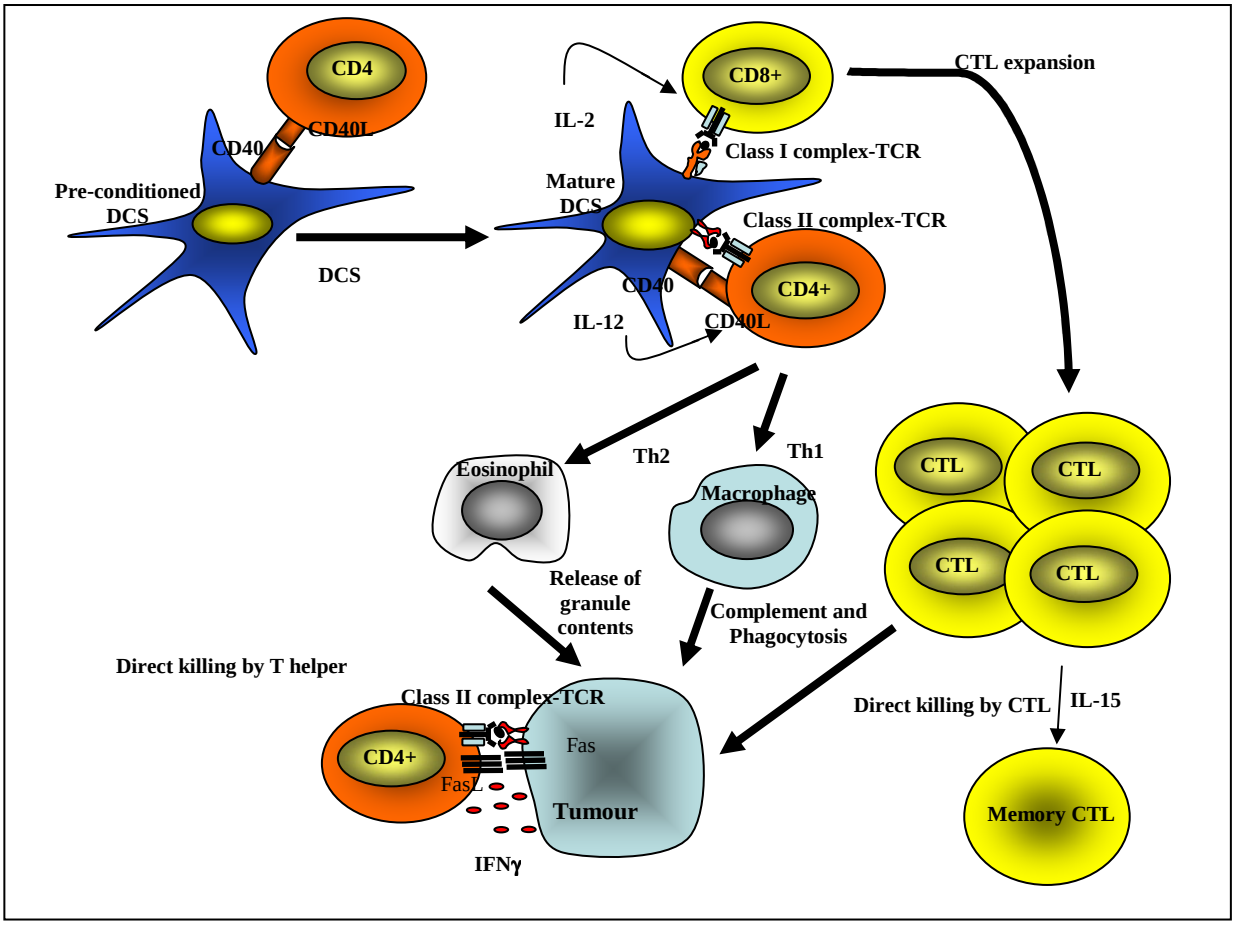

Figure 1.9: Model of the functions of CD4+ T cells in anti-tumour immunity. *The priming phase of CD4+ T cells involves antigen uptake and processing by BM-DC. The latter presents class I and class II peptides to CD8+ and CD4+ T cells, respectively. This triggers a chain of reactions whereby Th1 and Th2 cytokines are secreted to recruit eosinophils and macrophages, respectively. In parallel, this complex of CD4+-CD8+-DC will also activate both CD4+ and CD8+ T cells, which will directly kill tumour cells.*

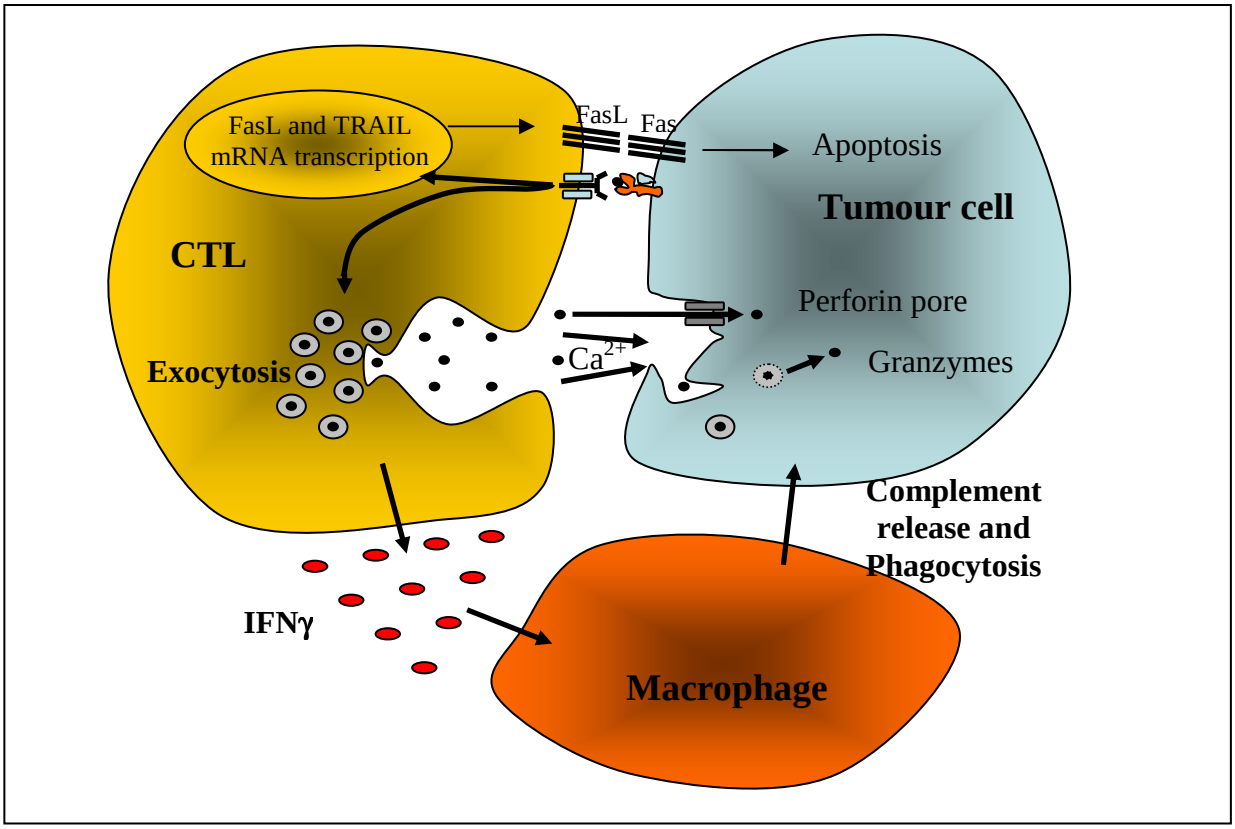

Figure 1.10: Mechanisms of action of cytotoxic T cells against tumour cells. *The three pathways of cytolysis are initiated by the presentation of a tumour-derived class I peptide to the TCR. Transcription of genes encoding for FasL and TRAIL takes place. FasL-Fas connection is made with the tumour cell to trigger an apoptotic cascade. IFNγ is released to attract macrophages, which, in return will attack the tumour cell. Finally, the MHC class I-TCR interaction stimulates the release of perforins and granzymes by exocytosis allowing the tumour cell to lyse.*

Indeed, the activation of CD8+ T cells is strongly dependent on the help brought by T helper cells recognising an antigen on the same APC (Ridge *et al.*, 1998). Activated T helper cells release co-stimulatory molecules such as IL-2, which then contributes to the activation of CD8+ T cells. CD8+ T cell activation is still largely studied and remains controversial as other pathways of activation have been found. In fact, depending on the affinity of the antigenic peptide for class I molecules and its avidity for TCR, a CD8+ T cell-based response can occur in the absence of CD4+ T cells (Schoenberger *et al.*, 1998). Activation of CD8+ T cells can also occur sequentially with first, the recognition of an antigen by a T helper cell delivering a signal to the APC and secondly, the stimulation of CD8+ T cells following the reception of that signal (Ridge *et al.*, 1998). Finally, priming of CD8+ T cells can take place using the important CD40-CD40L co-stimulatory pathway with an APC without the help of CD4+ T cells (van Mierlo *et al.*, 2004)(Fig. 1.11). Altogether, these data suggest that activation of CD8+ T cells is critical for successful immunotherapy against certain types of malignancies.

1.3 Tolerance, tumour evasion and their relevance to cancer

1.3.1 T-cell selection and tumour immunology

The immune system is composed of a society of cells mostly originating from the bone marrow. The inventory of T lymphocytes is built in two steps. In a first step, lymphoid progenitors reach the thymus where they enter different stages of development. T cells, originally negative for CD4 and CD8 molecules become positive for either CD4 or CD8. Following this evolution, T cells undergo central tolerance mechanisms. A diversified inventory is constituted in which TCR genes are rearranged by a unique combination mechanism along the cellular differentiation process (See Section 1.2.2.2). This new repertoire of TCR allows the organism to face any possible antigen. Secondly, T cells undergo negative and positive selection processes. T cells, having no or too high affinity for self-antigens, are negatively selected and deleted by apoptosis. However, potentially auto-reactive T cells can escape this surveillance mechanism and attack cells presenting self-antigens in the periphery (Anderton *et al.*, 2002). On the other hand, T cells with medium affinity for MHC: peptide complexes are positively selected upon reception of survival signals. T cell development and primary T cell selection processes occur mainly in the cortical region of the thymus. T cells then migrate to the medulla where they undergo further negative selection. Indeed, T cells meet thymic epithelial cells, which have been shown to present self antigens expressed in all cells as well as tissue-specific antigens, and upon self-antigen recognition, auto-reacting T cells are deleted.

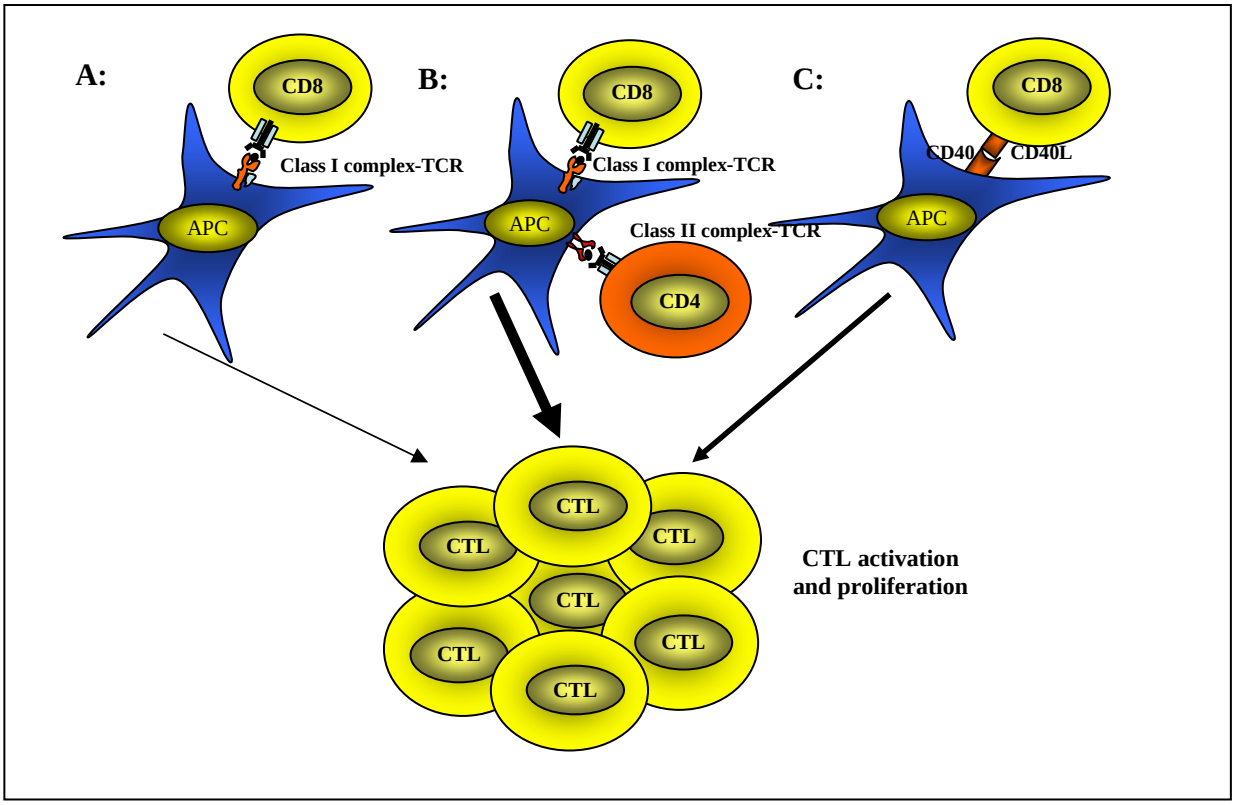

Figure 1.11: Activation of CD8+ T lymphocytes. *The priming phase of CD8+ T cells, strongly relying on the presence of bone marrow-derived dendritic cells to allow their activation and proliferation, can be summarised by three major mechanisms. A: a DC presents an antigenic epitope with strong binding affinity and strong avidity to a CD8+ T cell. B: a DC presents at the same time or sequentially a class II and a class I peptide to a CD4+ and a CD8+ T cell, respectively. C: A CD40-CD40L connection is made between a DC and a CD8+ T cell.*

Finally, it can also be mentioned that some of the cells that never meet any of the antigens they are specific for, die by neglect due to the lack of survival signals. Positively-selected CD4+ and CD8+ T cells migrate to peripheric lymph nodes where they browse the antigen pool presented by APC and other cells to identify anything abnormal. In summary, these stringent central tolerance mechanisms allow the emergence of an immune system capable of answering efficiently and appropriately to the large diversity of antigens, while limiting the danger to develop auto-immune diseases. Once the maturation of T lymphocytes has taken place, apoptosis continues to have a major role in the homeostatic control of the immune system. Indeed, following antigen presentation and recognition, T lymphocytes are activated, triggering a phase of clonal expansion of reactive cells. Once the antigen is no more present, the activated clones start a phase of decline in order to come back to a state of equilibrium. This come-back is ensured by the apoptotic elimination of the T cell excess, leaving the remaining cells to constitute a pool of memory T cells (Krammer, 2000). The immune system is therefore equipped with a large plasticity over the T cell development and activation because of a tight control of apoptosis.

The deregulation of this process can have harmful consequences. Indeed, the defects of certain pro-apoptotic genes such as CD95, CD95L or Bim can provoke the development of auto-immune diseases while a massive apoptosis can lead to the disappearance of T lymphocytes (Zhang and Insel, 2004). Altogether, these facts indicate that tolerance is a well-controlled mechanism preventing developed T cells to react against self antigens. That is why overcoming tolerance to generate an immune response against over-expressed or differentiation antigens, represents a difficult task.

1.3.2 Mechanisms of tumour evasion

Over the last two decades, tumour immunology has undergone real changes with the discovery of cytokines and more recently the identification of tumour-associated antigens making immunotherapy a fourth modality of cancer treatment (surgery, radio- and chemotherapy being the first three). It has now become clear that the activation of natural and/or specific effector cells is an essential step to the anti-tumour response. However, the latter does not seem sufficient to allow tumour eradication. In fact, these effectors undergo alterations and modifications compelled by the tumour. In a more general manner, the tumour is capable of fighting, almost paralysing the host immune system. Besides classical mechanisms of tumour escape such as the loss of MHC expression and the alteration of the antigen presentation, the tumour develop strategies allowing it to acquire resistance to anti-tumour lysis factors by

creating an environment, which can deviate the local immune reaction and facilitate tumour proliferation. Therefore, tumour escape concerns the tumour target itself as well as the immune system (Müller *et al.*, 2002).

(i) Escape mechanisms associated with the tumour cell: The decrease or the loss of expression of MHC molecules by tumours is quite frequent, particularly in metastases (Garrido *et al.*, 1997; Marincola *et al.*, 2000) with several studies showing a direct correlation between the weak MHC class I expression and the poor prognosis or a high frequency of metastatic lesions (Ferrone and Marincola, 1995; Gudmundsdottir *et al.*, 2000). The alteration of MHC class I expression takes place mostly during their synthesis. Mutations in β2-microglobulin, α chain or transcriptional factors responsible for the transcription of MHC genes have all been described (Paschen *et al.*, 2003; Garcia-Lora *et al.*, 2003). Glycosylation and MHC class I transport inhibitions, as well as decrease of their expression by viruses have also been reported (Benitez *et al.*, 1998). Decreased or absent HLA expression has been observed in about 16 to 50% of human solid tumours including breast carcinoma, colorectal carcinoma, prostate adenocarcinoma and melanoma (Khong and Restifo, 2002). In the well-studied DISC/GM-CSF/CT26 tumour model, down-regulation of MHC class I molecules was found in 60% of mice with progressive tumour growth but absent in mice with regressive tumours (Ali *et al.*, 2002; Ahmad *et al.*, 2004). In another murine model, it has been proved that the transfection of a MHC class I molecule in tumour cells is capable of inducing tumour eradication and loss of invasive capacities (Tanaka *et al.*, 1985). It is also worth mentioning that in certain cases, the loss of MHC class I expression can make the tumours more susceptible to the lysis by NK cells because of the suppression of the inhibition exerted by KIR and NKG2A molecules.

The loss of expression of tumour antigens also corresponds to a mechanism of tumour escape, often resulting from genetic modifications in one or several tumour cells. This loss of antigen recognised by specific CTL has been observed in many murine and human tumours (de Plaen *et al.*, 1988; Lehmann *et al.*, 2000). The loss or the decrease of MHC class I molecules is often associated with the actual processing of the tumour antigen. Therefore, antigen processing can be affected by mutations occurring on LMP2 and LMP7 genes, which are important subunits in the immunoproteasome, or TAP genes, which are involved in the transport of antigenic peptides from the cytosol to the ER lumen. This leads to a flawed antigen presentation. That is why the loss of antigen expression and the selection of tumour variants, which are escaping the immunosurveillance, constitute a real issue at which anti-tumour vaccination is confronted. It is

easy to think that the simultaneous vaccination against several different tumour antigens could reduce the likelihood of selecting tumour variants having lost the antigen.

Several studies also underline the importance of the down-regulation of co-stimulatory molecules such as B7.1 or B7.2 by tumour cells rendering the latter less immunogenic by preventing a more efficient tumour-specific CTL generation. Transfection of B7 molecules in tumour cells can lead to tumour regression in animal models (Bueler and Mulligan, 1996) and there are also evidences that the transfection of tumour cell lines with B7 molecules allows the generation of a stronger CTL response, *in vitro* (Heuer *et al.*, 1996). These results strongly prove that TCR engagement without the appropriate co-stimulatory signal provided by their respective ligands (in case of B7 molecules, CD28) can lead to hyporeactivity or even lymphocyte anergy. Another more controversial way for the tumour to escape is to express FasL at their surface. This molecule binds its receptor, Fas, on the surface of T cells and triggers their apoptosis. This phenomenon has been observed in different types of cancers such as leukaemia (Tanaka *et al.*, 1996), colon carcinoma (O'Connell *et al.*, 1999) and melanoma (Hahne *et al.*, 1996). However, it has also been observed that transfection of tumour cells with FasL makes the anti-tumour response more efficient with an improved tumour reject (Arai *et al.*, 1997). Finally, tumour cells can secrete several immunosuppressive factors such as the well described IL-10, TGFβ and indoleamine 2,3-dioxygenase (IDO) contributing to tumour tolerance by suppressing T cells and activating Treg (Uyttenhove *et al.*, 2003; Munn and Mellor, 2007).

Nitrogen monoxide (Bogdan *et al.*, 2000), prostaglandins E (Roper and Phipps, 1994) and gangliosides (McKallip *et al.*, 1999) all participate with different mechanisms, such as effector cells, apoptosis or antigen processing and presentation inhibitions, to the tumour escape phenomenon. Tumour cells can acquire resistance to lysis mediated by CTL or NK cells. In fact, these anti-tumour effectors use two major pathways to exert their cytotoxic activity against target cells: the release of cytotoxic granules or the triggering of apoptosis involving death receptors (section 1.2.2.2). Certain tumour cells lose their capacity to bind perforins, preventing the piercing of the tumour cell membranes by the latter (Lehmann *et al.*, 2000). Tumour cells can also start producing a serine protease inhibitor called PI9, which specifically inhibit the action of granzyme B stopping the induction of apoptosis by the latter (Medema *et al.*, 2001). Tumour cells can become resistant to apoptotic signals triggered by the caspase or the mitochondrial pathways. This resistant mechanism is quite complex. It involves membranous, cytoplasmic and nuclear determinants, and affects Fas (Medema *et al.*, 1999),

TRAIL (Ashkenazi *et al.*, 1999) and TNFα signalling (Lejeune *et al.*, 1998). Finally, tumour cells can be rendered resistant to cellular cytotoxicity following chemotherapy treatments. Certain drugs lead to the production of anti-apoptotic transcription such as NF-κB, consequently preventing the transmission of apoptotic signals initiated by death receptors in contact of effector cells (Baldwin, 2001). This kind of crossed resistance represents a major handicap for the development of cancer vaccination as immunotherapy is often used following failure or together with chemotherapy.

(ii) Escape mechanisms associated with the immune system: Several studies showed that TIL can present functional alterations that are translated by a weak proliferation under the action of cytokines (e.g.: IL-2) and a decreased cytotoxic activity (Maccalli *et al.*, 1999). This can be explained by anomalies in the ζ chain of the CD3 complex and in certain protein kinases involved in the primary steps of TCR signalling (Lai *et al.*, 1996). Cytotoxic T lymphocyte associated molecule 4 (CTLA-4) is another molecule linked with the actual down-regulation of the immune response. This molecule is structurally very similar to CD28 and binds also CD28 ligands (i.e.: B7 molecules) but with higher affinity. Its expression is limited to T and B lymphocytes but by binding B7 molecules, the CTLA-4 molecule brings a negative control on activated T cell proliferation by transmitting an inhibitory signal (Carreno *et al.*, 2000).
It is generally admitted that a specific anti-tumour response results from equilibrium between immunoregulatory and immunosuppressive cytokines present in the tumour environment. It was described earlier that tumour cells can secrete immunosuppressive cytokines to defend themselves against the immune response but paradoxically, certain T lymphocytes are capable of secreting cytokines with identical functions and auto-inhibit themselves. Tumour-infiltrating lymphocytes and more specifically Th2 cells have been shown to produce IL-10 in non-small cell lung carcinoma (Asselin-Paturel *et al.*, 1998). Regulatory T cells have been also described at the tumour site inhibiting tumour-specific CTL *via* the secretion of immunosuppressive cytokines (IL-10 and/or TGFβ) or the expression at their surface of inhibitory molecules such as CTLA-4 (Von Boehmer., 2005). Treg cells account for 2 to 5% of the total population of CD4+ T cells in humans and are generally characterised by a CD4+CD25+Foxp3+ phenotype although other variants have also been identified. Naturally occurring Treg are likely to be selected in the thymus and capable of maintaining peripheral tolerance to self antigens. Generation of these suppressor cells depends on the ectopical expression of organ-specific antigens in the thymus and in fact, leads to the protection of tumours expressing self antigens.

Moreover, Treg can also be induced by a whole new range of peptide ligands specifically presented by tumour cells (Wang *et al.*, 2004; Wang *et al.*, 2005) suggesting the idea that these Treg may come from CD4+CD25- T cells which have been converted to CD4+CD25+ Treg because of the surrounding suppressive cytokines present at the tumour site or after direct interaction with naturally occurring Treg according to the "infectious" tolerance theory (Dieckmann *et al.*, 2002). They have been extensively studied in cancer patients and there are increasing evidence of their presence within lymphoid tissues, peripheral blood and tumour-infiltrated lymphocytes (Ichihara *et al.*, 2003). Several studies demonstrated the link between presence of Treg in lymph nodes surrounding the tumour and impaired cell-mediated immunity as well as their contribution to tumour growth (Curiel *et al.*, 2004; Kawaida *et al.*, 2005). Treg have also been pointed out as the major reason for failed immunotherapies. Recently, a study showed that a CTL response was obtained after immunisation of melanoma patients with peptide- or tumour lysate-pulsed APC which peaked at seven days post-immunisation. The CTL response was then in decline reaching the pre-vaccine level 28 days after administration of the vaccine. This decrease correlated with the expansion of IL-10-secreting Treg in post-vaccine peripheral blood lymphocytes (Chakraborty *et al.*, 2004), emphasising the involvement of Treg not only in the maintenance of tolerance but also in the down-regulation of the anti-tumour response.

Finally, another study has described the involvement of activation induced cell death (AICD) in tumour escape (Malmberg *et al.*, 2001). This mechanism normally occurs at the end of the T cell response. However, in some cases, AICD could be deregulated and leads to the elimination of the T cell compartment. Although several strategies of tumour escape have been described, our knowledge of the conflict between the tumour and the immune system remains hypothetical and fragmentary. A better understanding of these two systems should allow the development of more adapted and more efficient cancer immunotherapy.

1.4 Cancer vaccines

The immune system, because of its capability to actively destroy emerging tumour cells, represents a formidable tool to use in order to eliminate tumour cells that have escaped immunosurveillance. Until now, a large variety of mechanisms have been developed to achieve this goal and thwart tumour invasion. These can be summed up in three main headings: active immunotherapy which activates the anti-tumour response directly within the patient; adoptive immunotherapy which relies on *ex vivo* T cell stimulation before transfer back to the patient;

and finally passive immunotherapy mostly based on the administration of immune-effectors molecules such as Ig.

1.4.1 Active immunotherapy

(i) Whole cell vaccines: Immunotherapy against cancer started with the development of whole cell vaccines. Irradiated autologous or allogeneic tumour cells were administered to patients together with an adjuvant (e.g.: BCG), modified with a hapten to make the cells more immunogenic (Berd., 2001), or cultured in a cocktail of cytokines such as IL-2, GM-CSF and IL-6 to promote MHC class I and class II presentation prior to re-injection (Zhang *et al.*, 2005). The frequency of positive responses remains however low with tumour regression observed in only 10 to 30% of patients (Weber., 2000; Zhang *et al.*, 2005). Subsequently, cells were engineered to express cytokines or co-stimulatory molecules to improve their immunogenicity (Ali *et al.*, 2000). Several cytokine genes have been introduced in melanoma cell lines and other human tumours such as prostate or kidney in order to immunise patients with metastatic tumours. Clinical trials in melanoma or kidney cancer were carried out using autologous tumour cells transfected to express cytokines such as IL-2, -7, IFNγ or GM-CSF. Only a small proportion of patients responded partially or completely to these treatments. Indeed, only 20 to 30% of the patients showed delayed hypersensitivity (DTH) and this result varies little when each cytokine were compared (Parmiani *et al.*, 2000). In another phase I study, autologous melanoma tumour cells have been transfected with IL-12 gene in order to generate primarily a Th1-orientated response. A DTH reaction has been obtained in 2 out of 7 patients only, while only one of them showed tumour-specific CD4+ and CD8+ TIL (Sun *et al.*, 1998). It is worth mentioning that only a small number of autologous tumour cells can be generated and that the standardisation of allogeneic tumour cell lines, easier to prepare and this in large quantity, should be undertaken. They also present the advantages of being administrable to all patients and that the cross-presentation by MHC molecules of the tumour cell lines to T cells does not need to match the MHC of the recipient to induce an immune reaction. Nevertheless, the use of cell lines for immunotherapy should be strictly controlled to ensure consistency of the vaccine as the genetic material, hence the expression of tumour-associated antigens and the immunogenicity, can evolve considerably over multiple passages.

(ii) Heat shock protein vaccines: Heat shock proteins (HSP) are considered natural adjuvants appearing to be promoters of the anti-tumour vaccination because of their ability to bind

antigenic peptides and chaperone these peptides to APC in order to stimulate naive T cells in lymph nodes. This system has been proved to generate potent immune response in mice (Srivastava *et al.*, 1998), and HSP96, HSP70, HSP90, HSP110, grp170 and calreticulin have all been found able to generate an anti-tumour response (Binder *et al.*, 2004). They generate considerable interests as they are the first natural mammalian adjuvants to have been identified and are able to activate all the arms of the immune response going from CTL, B cells, NK cells to DC (Schild *et al.*, 2000). Moreover, the ability of HSP to overcome tolerance by stimulating APC and generating co-stimulatory signals have been suggested (Li *et al.*, 2002). Consequently, clinical trials have been initiated in patients carrying histologically-varied tumours with autologous HSP96 vaccines. No toxicity was observed and 50% of the patients tested developed a tumour-specific CTL response. One of them even showed marked reduction of the lesion size by more than 50% after the third immunisation and four other patients demonstrated stabilisation of the disease from 3 to 7 months (Janetzki *et al.*, 2000). However and despite their immunologic interest, this approach needs to check whether autologous HSP do present epitopes recognised by the patient CTL.

(iii) Peptide vaccines: The arrival of new technologies allowed the construction of new types of vaccination founded on the identification of genes encoding TAA and peptide epitopes derived from these TAA and recognised by CTL in the context of MHC class I. Advantages of using peptides are numerous as patients can be screened for the expression of TAA by PCR or immunohistochemistry; it is relatively easy to synthesise and cost-effective; it is easy to administrate in clinic and monitor *in vitro* the presence of an epitope-specific immune response. Peptides derived from melanoma antigens such as the differentiation antigens (e.g.: MelanA/MART-1, gp100 and tyrosinase) and CT antigens (e.g.: MAGE-1 and -3) were the first used in phase I/II clinical trials. Marchand *et al.* (1999) showed significantly different results: 28% of HLA-A1 patients treated with a HLA-A1 MAGE-3 peptide responded clinically with complete remission while a different epitope from the same protein presented in the context of HLA-A2 or a HLA-A1 peptide from MAGE-1 induced a clinical response in only 11% of tested patients. In another study, MART-1-derived peptides injected together with an adjuvant, in that case incomplete Freund's adjuvant (IFA), allowed the production of peptide-specific CTL but no clinical response (Rosenberg *et al.*, 1998). Clinical response was then achieved in 42% of patients when MART-1 vaccine was associated with strong systemic administration of IL-2 (Rosenberg *et al.*, 1998). In order to increase the efficiency of peptide presentation, melanoma antigens have been co-administrated with GM-CSF. Although this

approach appeared successful at short term, most of the tumours re-appeared with only 11% of the patients showing beneficial responses and increased peptide-specific CTL (Jager *et al.*, 1996). Another recently identified antigen belonging to the CT antigen family, NY-ESO-1 has proved to be strongly immunogenic with at least 50% of patients with melanoma mounting B and T cell responses. When administrated with GM-CSF, a specific CTL response was accompanied with a stabilisation of the disease and objective regression of a few metastases in 60% of patients (Jager *et al.*, 2000). Moreover, peptides have also been used to immunise patients with cancers from different histological origin such as colon adenocarcinoma with Ras-derived peptides (Gjertsen *et al.*, 2001), breast carcinoma with HER2/Neu-derived peptides (Knutson *et al.*, 2001), or chronic myeloid leukaemia (CML) with Bcr/Abl-derived peptides (Pinilla-Ibartz *et al.*, 2000). Like previously described, peptide vaccination generated specific CTL responses but only a very small proportion of them led to noticeable tumour regression.

Tolerance, immune escape, lack of MHC class II peptides, the importance of the adjuvant and the dose/schedule used for immunisation can all explain the very low clinical efficacy of peptide vaccination. Fortunately, a lot of efforts are put into improving these results by combining CTL epitopes with T helper epitopes to induce and sustain a more potent CTL response. Results achieved in animal models have been encouraging (Rojas *et al.*, 2005) and clinical trials using combination of peptides or single peptides with dual class I and class II binding abilities are currently ongoing (Zeng *et al.*, 2002). Others have tried to modify the anchor residues of CTL epitopes to improve their affinity to their respective MHC class I molecules and TCR and induce a stronger immune response. Rosenberg *et al.* (1998) proved that this hypothesis could have beneficial applications. Several studies are also looking at delivery systems such as liposomes (Ludewig *et al.*, 2001; Malik *et al.*, 2007), nanobeads (Kalkanidis *et al.*, 2006) or in combination with stronger adjuvants such as CpG oligonucleotides (Davila *et al.*, 2003; Peng *et al.*, 2005) to increase T cell avidity upon better presentation to the APC without severe and toxic side-effects (Fifis *et al.*, 2004). Finally, there is a common understanding that peptides are more efficient in eliciting a specific T cell response than whole proteins (Disis *et al.*, 1996). However, anti-idiotype antibody-based vaccines raised a lot of interests. Indeed, by displaying partial amino acid sequence homology, anti-idiotype antibodies are proteins capable of mimicking the activity of tumour antigens such as HER2/neu and CEA, and effectively overcome unresponsiveness of the adaptive immune system to tumour cells over-expressing the nominal antigen. Promising results were obtained in

mice (Mohanty *et al.*, 2007; Pignatari *et al.*, 2007) and clinical trials in humans are currently ongoing to assess this revolutionnary protein-based vaccine modality.

(iv) Dendritic cell vaccines: The knowledge of DC physiology and their potent antigen presenting capacities allowed their use for the vaccination of mice with tumours. Dendritic cells bring numerous advantages as they can act as nature's own adjuvants. Their utilisation became also possible in humans because of new technologies allowing the generation of high quantities of autologous DC from monocyte origin or deriving from CD34+ cells of patients by culturing them *in vitro* in the presence of GM-CSF and IL-4 (Siena *et al.*, 1995). It has then been demonstrated that autologous DC loaded *ex vivo* with peptides or tumour cell lysates were capable of efficiently presenting TAA to T cells (Nieda *et al.*, 1998; Moyer *et al.*, 2006). Several clinical studies have been initiated to analyse the therapeutic value of this approach in patients with cancer. Patients with melanoma were vaccinated with autologous DC loaded with MHC class I MART-1, tyrosinase or gp100 peptides, or with tumour cell lysates. A clinical response was obtained in 25% of patients treated out of 32 patients, with approximately 50% of them developing specific CTL responses (Schadendorf *et al.*, 2000). Another study using a MAGE-3 peptide loaded to autologous DC without any adjuvants showed a high frequency of CTL responses (Marchand *et al.*, 1999). Nevertheless, no significant clinical regression of metastatic lesions has been obtained even though few cutaneous metastases disappeared (Thurner *et al.*, 1999). This approach of vaccination did not prove successful when autologous DC were loaded with prostate antigens such as PSA, PSMA or PAP with or without GM-CSF administration for the treatment of prostate cancer with less than 2% of clinical responses, even though PSA levels decreased by up to 50% in at least 30% of the 107 patients tested (Lodge *et al.*, 2000). It is interesting to underline the use of tumour cell lysates to provide DC with TAA. Despite containing also self antigens and hence increasing the risk of auto-immunity, it corresponds to a practical source of antigens for vaccinations and this approach has been used in melanoma (Schadendorf *et al.*, 2000). However, complementary information on the tumour itself are required to define the TAA present in these lysates in order to be able to monitor and evaluate their immunogenicity. Another interesting approach is the fusion between DC and tumour cells. The end product is an APC capable of presenting all the repertory of TAA present in the tumour cells (Rosenblatt *et al.*, 2005). After studies in animals (Weigel *et al.*, 2006), this technique has been utilised in patients with melanoma or kidney cancer. Unfortunately, vaccination with these hybrids led to contradictory results with 13% of clinical responses in melanoma while this same approach allowed tumour regression in 40% of patients

with renal cell carcinoma (Kugler *et al.*, 2000). Dendritic cells play a critical role in vaccination strategies as it was shown in whole cell tumour vaccine engineered to express GM-CSF, which is a potent mitogenic factor required by DC for the induction of an intermediate stage of differentiation whereby antigen uptake and presentation is improved (Caux *et al.*, 1992). The genetically-modified tumour cells, in comparison with wild-type tumour cells, were shown to trigger a potent systemic anti-tumour immunity as a result of DC infiltration, lymph node migration and activation of CTL (Dranoff *et al.*, 1993). However, no significant clinical results have really been obtained in a number of phase III clinical trials. In order to overcome the issues associated with traditional DC vaccines, different strategies were adopted. Dendritic cells can indeed be transfected with mRNA derived from the tumour cells (Heiser *et al.*, 2000; Peng *et al.*, 2006), or with an expression vector encoding the full cDNA sequences of TAA (Yang *et al.*, 2000) together or not with a stimulating cytokine such as IL-18 to increase CTL activity (Cao *et al.*, 2007), enabling targeted loading of TAA-derived peptides in the antigen processing pathways. Another way of circumventing these difficulties is to improve the maturation status of the prepared DC by transfecting them with co-stimulatory molecules or maturation factors, such as CD40L or GM-CSF, so that they can be co-administrated to patients with antigen-loaded DC and strong maturation factors such as anti-CD40 monoclonal antibodies or CpG oligonucleotides (Hanks *et al.*, 2005). This system should allow the DC to provide sufficient levels of T cell activation signals in order to overcome tolerance and hopefully lead to better clinical responses. Finally, DC can be modified to secrete immunostimulatory molecules such as IL-2, -12 or IFNγ to improve CTL response levels. Murine models and clinical trials have shown promising results using this methodology (Murphy *et al.*, 1996; Huttner *et al.*, 2005). Recently, different subsets of DC have been identified capable of inducing different levels of CTL activation depending on the cocktails of cytokines used for their maturation and activation. For example, it seems that IL-15 can have a more potent effect on the final CTL stimulation than IL-4 (Mohamadzadeh *et al.*, 2001; Feili-Hariri *et al.*, 2005). Interestingly, another study reported that depending on the activating factors used, interferon-secreting killer DC with tumour killing capabilities could be generated in mice (Chan *et al.*, 2006). In summary, DC potentially represents a formidable tool for cancer immunotherapy but so far, clinical trials have proven to be inconclusive. Therefore, it seems that a better understanding of the maturation and activation processes of DC are required to enhance potential immune responses and standardise the immunisation procedures such as site of vaccination, dose, schedule and injection.

(v) DNA vaccines: Another mean allowing the elaboration of vaccines against defined antigens is based on the utilisation of genes coding for TAA. The advantage of this method is the possibility to induce the expression of an entire antigenic protein including both MHC class I and class II epitopes that are not restricted to the specific MHC haplotypes, and activate both arms of the adaptive immune system (i.e.: B and T cells, respectively)(Ulmer *et al.*, 1993). DNA vaccines were first used in animal models before being applied to humans. They can be administrated by different routes such as intramuscular, intramucosal, intranasal or into the dermis using gene gun technology after coating of the DNA with gold. DNA doses have been shown to be independent of the species with fairly similar amounts used to raise responses. However, the route of administration seems to be critical as injection of DNA extra-cellularly requires 100 to 1,000 times more DNA to induce immune responses than gene gun targeting introducing DNA directly inside cells (Fynan *et al.*, 1993). This is quite relevant as it allows easy standardisation of the technique and an easy transfer of this mode of immunisation from animal models to humans. Moreover, the immunogenicity of DNA vaccines is increased because of the presence of bacterial CpG oligonucleotide sequences, which are known for their capacity to promote cytokine secretion and danger signalisation leading to optimum activation of the APC (Pisetsky, 1996). Finally, DNA vaccines are unlikely to integrate the host's genomic DNA or be neutralised by anti-DNA antibodies (Nichols *et al.*, 1995; Gilkeson *et al.*, 1996). Depending on the route of immunisation, the DNA can either be picked up by epidermal Langherans cells following gene gun bombardments or be moved by blood flow from the muscles to the spleen or other lymph nodes (Winegar *et al.*, 1996). More recent studies have shown that gene gun bombardment with DNA-gold particles predominantly generates Th2 responses while intramuscular immunisation preferentially leads to Th1 responses emphasising the importance of the route of immunisation for DNA vaccines (Weiss *et al.*, 2002). Furthermore, single or multi-insert plasmids can be used for DNA vaccinations meaning that a TAA gene could eventually be combined with modulators of the immune response such as co-stimulatory molecules (e.g.: B7-1, -2) or cytokines (e.g.: GM-CSF, IL-12) in order to increase the immunogenicity of the receiving cells, which process and present the encoded TAA (Iwasaki *et al.*, 1997). However, DNA vaccines have been proved to be so far weakly immunogenic but they represent a real interest when combined in prime-boost vaccinations protocols. Indeed, prime-boosting strategies involving DNA followed by virus vaccinations showed promising results by allowing expansion of TAA-specific T cells and selection of T cells with the highest avidity against the respective TAA (Palmowski *et al.*, 2002; Woodland, 2004).

(vi) Viral vector vaccines: This approach was first carried out in animals with a variety of DNA vectors able to bring DNA to APC. They represent a very interesting and promising possibility as they can initiate a strong immune response and break tolerance, which is of a major interest regarding immunisation against self or differentiation antigens. Retroviruses, lentiviruses, adenoviruses, alphaviruses, poxviruses and Herpes simplex viruses have all been tested in animal models with promising results and are in clinical trials with tumour antigens such as PSA or CEA or with immunoregulatory molecules such as GM-CSF (Aarts *et al.*, 2002). Many of them can be disabled, have limited toxicity upon systemic or intratumour injection, infect dividing and non-dividing cells and integrate large inserts of genetic information (Ali *et al.*, 2002; Stripecke *et al.*, 2003). However, they can have limiting factors as hosts can already have produced blocking antibodies against some of these viruses. Immunodominance can also take place as viral antigens are often more immunogenic than tumour antigens. In order to circumvent these difficulties, immunomodulatory molecules can be combined with TAA to enhance the potency of such vectors. Among all these families, alphaviruses and herpes simplex viruses are of particular interest. Indeed, recombinant alphaviruses such as Semliki Forest Virus require the co-transfection of a helper plasmid for the making of infective viral particles (DiCiommo and Bremner, 1998; Atkins *et al.*, 1999). Following viral infection, the cells die by apoptosis facilitating cross-presentation by DC. Disabled infectious single cycle-Herpes simplex virus (DISC-HSV) and its immunotherapeutic have been largely studied in murine tumour model (Boursnell *et al.*, 1997; Rees *et al.*, 2002; Ahmad *et al.*, 2005). Intratumour injection of DISC/GM-CSF has been shown to lead up to 70% of the mice rejecting the tumour and once combined with other modalities, further therapeutic advantages could be achieved (Ali *et al.*, 2002; Ali *et al.*, 2004).

Each of these families of viruses mentioned earlier have relevant properties for gene therapy and more and more efforts are put into trying to blend these properties into a single viral vector in order to develop the “perfect” viral vector that could be used on its own as a potent tool for cancer immunotherapy or in prime-boost strategies (Kay *et al.*, 2002; Woodland., 2004).

1.4.2 Adoptive immunotherapy

Adoptive immunotherapy is based on the observation that specific T lymphocytes with a therapeutic activity are absent or in insufficient numbers in patients. Tumour infiltrating cells isolated from autologous tumours or allogeneic donor cells can be amplified and activated *in vitro*, and injected to the deficient host in large quantities to obtain the desired therapeutic effect. Allogeneic transplantation has shown to be very successful in haematological

malignancies. Indeed, T cells seem to mediate a graft-versus-leukaemia reaction following ablation of tumour cells, high doses of chemo- or radiotherapy and allogeneic bone marrow or haematopoietic stem cell transfer (June, 2007). Autologous adoptive immunotherapy has more applications when solid tumours are involved. The first application of adoptive cell transfer in immunotherapy was against melanoma in combination with high doses of IL-2. Objective tumour responses were successfully obtained in 35% of the vaccinated patients (Rosenberg *et al.*, 1994). This frequency was at this moment higher than any other tumour vaccinations undertaken so far. The same group later improved this result to 50% by performing lymphoablative chemotherapy followed by adoptive transfer of polyclonal T cell lines containing CD4+ and CD8+ T cells specifically recognising the tumour (Dudley *et al.*, 2002). Subsequent trials using the same immunisation protocol and larger cohorts of melanoma patients with metastases in distant sites such as lung, brain, liver or lymph nodes, showed similar figures (Rosenberg and Dudley, 2004, Dudley *et al.*, 2005). By removing homeostatic cytokines such as IL-7 or IL-15 and by depleting Treg, lymphoablation probably encourages the expansion of tumour-reactive T cells. These results were confirmed when tumour-bearing mice were depleted from CD4+ T cells, re-infused with tumour-specific CD8+ T cells and CD4+CD25- T helper cells only, and showed noticeable tumour regression. Regression was however abrogated when CD4+CD25+ Treg were re-infused (Antony *et al.*, 2005). Rosenberg and colleagues also developed a novel technology involving the transfer of tumour antigen-specific TCR gene into autologous T cells prior to T cell re-infusion. This technique was applied to different tumour antigens such as gp100 (Morgan *et al.*, 2003), MART-1 (Rosenberg and Dudley, 2004) or NY-ESO-1 (Zhao *et al.*, 2005), and led not only to the expansion of tumour antigen-specific T cells with high avidity for their respective antigen, but also to significant clinical regressions in some patients. Altogether, these results are very promising as they show that high avidity tumour-specific T cells can induce tumour regression when used in the right context and that the development of genetically engineered T cells for adoptive immunotherapy can only improve the outcome.

1.4.3 Passive immunotherapy

Tumour antigen-specific monoclonal antibodies (mAb) are the main actors of passive immunotherapy and have demonstrated a high degree of success in some forms of cancer. The identification of several surface expression markers on tumour cells allowed their development even though their mechanisms of action remain hypothetic. The first theory behind the effects of mAb is the triggering of the antibody-dependent cellular cytotoxicity (ADCC). Monoclonal

antibodies bind to the antigens they are specific for and lead to the mechanism of opsonisation by phagocytes expressing the complement receptors, the immediate killing of tumour cells and an inflammatory reaction. One of the most widely used mAb is an anti-CD20 mAb (Rituximab) for the treatment of B cell lymphoma and appears to function through ADCC. The other hypothesis suggests that the effects of mAb are due to the variable regions, which bind to the tumour cell surface and might block the access to growth factors required for tumorogenesis or inhibit a signalling cascade. Therefore, mAb might stop tumour growth and induce apoptosis of target cells. Trastuzumab, commercially known as Herceptin, specifically targets the membrane-bound HER2 protein, a tyrosinase kinase receptor. Upon binding, the mAb leads to a cessation of the signalling cascade that normally follows HER2 activation and tumour cell death by apoptosis (Viani *et al.*, 2007). Other activity of mAb that have been suggested is an anti-angiogenic activity. Finally, mAb can be conjugated with a cytotoxic agent such as a radioactive compound or a cytotoxic drug. The clinical trials with an anti-CD20 mAb coupled with a radioisotope demonstrated that an antibody can efficiently bring the radioisotope in contact or at proximity of tumour cells (Witzig *et al.*, 2007). Further work is being carried out to develop new mAb conjugated to all sorts of toxins, radioisotopes or pro-drugs that can be activated by systemic administration of activator compounds, or able to cross-link effector cells of the immune system so that the latter can exert their action directly at the tumour site.

1.5 HAGE as a target for immunotherapy

There is an ever increasing need for new targets to treat cancer considering the limitations of some of the current therapies (section 1.1.5). Therefore, it is valuable to identify new immunogenic TAA that could be potentially targeted by immunotherapy for late stage diseases or in a combination therapy with more traditional treatments at early stages to prevent tumour cell proliferation and invasion. HAGE might be such antigen. It was first identified with sarcoma antigen (SAGE) using representational difference analysis, a technique that basically relies on the subtraction of the total RNA expression from normal tissues such as uterus, breast, colon and heart with the total RNA expression from a sarcoma cell line. The two cDNA clones were identified as tumour-specific with pattern of expression very similar to the genes from the MAGE family (Martelange *et al.*, 2000). HAGE gene was mapped on chromosome 6 (6q12-q13) by radiation hybrid analysis and encodes a putative 73kDa protein. Analysis of the protein sequence of HAGE revealed that it has a DEAD box characteristic of the family of ATP-dependent RNA helicases. The sequence also displayed 55% homology with DDX5 (p68), another member of this family. RNA metabolism, control of cell cycle, spermatogenesis and

embryogenesis are among the possible processes that HAGE might be involved in. However, little is known about its localisation or its function (Martelange *et al.*, 2000; Rocak and Linder, 2004). Northern blot analysis showed that HAGE transcripts were present at a level that was 100-fold higher in many tumours of various histological types than in normal tissues except testis (Martelange *et al.*, 2000). HAGE was later found to be over-expressed in a small number of normal salivary glands but in a higher proportion in benign and malignant salivary gland neoplasms (Nagel *et al.*, 2003). HAGE is also over-expressed in more than 50% of CML, in 20% of acute myeloid leukaemia (AML)(Adams *et al.*, 2002) and more than 40% of multiple myeloma (Condomines *et al.*, 2007), and although HAGE is present in a small numbers of lung cancers (Martelange *et al.*, 2000), there is no correlation between HAGE gene expression and clinicopathological factors indicating that the detection of HAGE in this type of cancer has limited usefulness (Sasaki *et al.*, 2003). A recent study described the methylation status of the HAGE gene in CML patients and cell lines, and showed that like most CT antigens, hypomethylation of the HAGE gene promoter correlated with increase of HAGE expression, and that its expression was strongly associated with advanced disease and poor prognosis suggesting a potential role of HAGE in cellular proliferation and as a marker of disease progression (Roman-Gomez *et al.*, 2007).

Considering the expression pattern and the diversity of HAGE expression in different tumours, HAGE might be potentially used as a target for immunotherapy. This study proposes to investigate the immunotherapeutic potential of HAGE in a murine model as a pre-requisite for using HAGE as a potential cancer vaccine in patients. Firstly, HAGE will be validated as a target for immunotherapy by confirming its tumour-specific expression at the mRNA and protein level in various cell lines, as well as normal and abnormal human tissues. Following its validation, this study aims at the identification of immunogenic class I and II peptides derived from HAGE for immunotherapy and immunomonitoring. Ultimately, potent vaccination strategies such as DNA or viral vaccines will be developed and evaluated in prophylactic and therapeutic experiments.

Chapter 2: Materials and Methods

2.1 General laboratory consumables and equipments

2.1.1 Reagents and list of producers

Reagents were stored as per manufacturer instructions and used before the expiry date.

Culture media	**Supplier**
DMEM, IMDM, RPMI	Bio Whittaker Europe
Opti-MEM	Gibco Life Technologies

Supplements to culture media	**Supplier**
Fungizone	Promega
Geneticin (G418)	Promega
HEPES	Bio Whittaker Europe
Foetal calf serum	Bio Whittaker Europe
Glutamine	Bio Whittaker Europe
Penicillin/Streptomycin	Bio Whittaker Europe
2-mercaptoethanol	Sigma

Other cell culture reagents	**Supplier**
Dimethyl sulfoxide (DMSO)	Sigma
Incomplete Freund's adjuvant (IFA)	Sigma
Lipopolysaccharide (LPS)	Sigma
Phosphate buffer saline (PBS)	Bio Whittaker Europe
Polyinosinic polycytidylic acid (Poly I.C)	Sigma
Trypan blue	Sigma
Trypsin/Versene	Bio Whittaker Europe

Chemical reagents	**Supplier**
Acetic acid	Fischer Scientific
Acetone	Acros Organics
Acrylamide-bis	Geneflow
Agar	Oxoid Ltd
Agarose	Bioline
α-chymotrypsin	Sigma
Ampicillin	Sigma
Anhydrous ethanol	Sigma
Aprotinin	Sigma
Aza-deoxycytidine (AZAC)	Sigma
Bovine serum albumin (BSA)	ICN Biomedicals
Calcium chloride ($CaCl_2$)	Sigma
Chromium-51	Amersham Biosciences
Dextran sulphate	Sigma
Dithiothreitol (DTT)	Sigma
DNA ladder (1kp Plus)	Gibco Life Technologies
dNTP	Bioline
ECL Western Blotting reagents	Amersham Biosciences
Ethanol	BDH
Ethidium bromide	Sigma
Ethyldiamine tetraacetic acid (EDTA)	Sigma

Fluorescent mounting media	Dako
Gill haematoxylin solution	Sigma
Glucose	Sigma
Glutaraldehyde	Sigma
Glycerol	Sigma
Goat serum	Sigma
Gold microcarriers (1.0μm)	BioRad
Harris haematoxylin solution	Sigma
Hydrochloric acid (HCl)	Fischer Scientific
Hydrogen peroxide (H_2O_2)	Sigma
Interferin	Polyplus Transfection
Isopropanol	Sigma
Isoton	Beckman-Coulter
Lipofectamine 2000	Invitrogen
Marvel	Premier Brands
Magnesium chloride ($MgCl_2$)	Fischer Scientific or Promega
Methanol	Acros Organics
Murine GM-CSF	Biosource
Murine IL-2	Biosource
1.5M Tris-HCl, pH 6.8 or pH 8.8	Geneflow
Orange G	Sigma
Paraformaldehyde	Sigma
Phenol/Chloroform/Isoamyl alcohol	Sigma
Phosphate buffer saline (PBS)(1X)	Bio Whittaker Europe
Phosphate buffer saline (PBS)(Tablets)	Oxoid
Phusion HF buffer	Finnzymes
Phusion DNA polymerase	Finnzymes
Poly vinyl pyrrolidone (PVP)	Sigma
Ponceau S solution	Sigma
Potassium acetate (KOAc)	Sigma
Potassium ferricyanide ($K_3Fe(CN)_6$)	Sigma
Potassium ferrocyanide ($K_4Fe(CN)_6$)	Sigma
Propidium iodide	Sigma
Rabbit liver powder	Sigma
Restriction enzymes	Promega or New England Biolabs
RNA-STAT 60	Biogenesis
Sodium azide (NaN_3)	Sigma
Sodium chloride (NaCl)	Fischer Scientific
Sodium dodecyl sulphate (SDS)	Sigma
Sodium hydroxide (NaOH)	Fischer Scientific
Sodium phosphate dibasic (Na_2HPO_4)	Sigma
Sodium phosphate monobasic (NaH_2PO_4)	Sigma
Spermidine	Sigma
SYBR green supermix	BioRad
Trichostatin A (TSA)	Sigma
Tris	Fischer Scientific
Trizma base	Sigma
Tryptone	Oxoid Ltd
Tween 20	Sigma
2-methylbutane (Isopentane)	Acros Organics

Vitamin E	Sigma
Xylene	Acros Organics
Yeast extracts	Oxoid Ltd
Zeocin	Invitrogen

Immunochemical reagents	**Supplier**
Goat anti-hamster-FITC	Serotec
Goat anti-mouse-FITC	Sigma
Goat anti-rabbit-biotin	DAKO
Goat anti-rabbit-HRP	DAKO
Goat anti-rat-FITC	Sigma
Hamster anti-mouse CD11c	BD Biosciences
HB54 (mouse anti-HLA-A2.1)	Hybridoma
L243 (mouse anti-HLA-DR)	Hybridoma
Mouse anti-FLAG-M2-FITC	Sigma
Rabbit anti-HAGE polyclonal serum	Dr. Ashley J. Knights (Tuebingen University)
Rat anti-mouse CD45R	Serotec
Rat anti-mouse CD80(B7.1)-PE	BD Biosciences
Rat anti-mouse CD86(B7.2)-PE	BD Biosciences
Rat anti-mouse DEC205	Serotec
Rat anti-mouse F4/80	Serotec
Rat anti-mouse 4.1BBL-PE	BD Biosciences
Streptavidin-HRP	Zymed

Plasmids	
pcDNA3.1, pBudCE4.1	Invitrogen
pSCAb, pSCAhelper, pSHAME2a	Dr. Rod Bremner (University of Toronto)

Kits	**Supplier**
Reverse transcription:	
M-MLV Reverse Trancriptase kit	Promega
Immunohistochemistry:	
ABC and DAB kits	Vector Laboratories
Cloning:	
T4 ligase kit	Promega
DNA extraction from agarose gel:	
Geneflow DNA isolation kit	Geneflow
Generation of SFV viruses:	
ProFection Mammalian transfection system	Promega
Lymphocyte depletion:	
Mouse CD4: Dynabeads mouse CD4	Dynal
Mouse CD8: Dynabeads mouse CD8	Dynal
ELISA:	
Mouse IFNγ or IL-5	R&D Systems

2.1.2 Equipment

Glassware
Pyrex glassware was washed in teepol, rinsed twice in distilled water and autoclaved

Disposable equipment and plastic-ware	**Supplier**
BD microlance 3 needles (0.5ml, 1ml)	Becton Dickenson
Bijou tubes (5ml)	Sterilin
Cryovials (1.2ml)	TPP
8-chamber slides	
ELISA plates (96-well)	Costar
Eppendorf tubes (0.5ml, 1.5ml)	Sarstedt
FACS tubes	Elkay
Filter tips (10µl, 200µl, 1ml)	Sarstedt
Flat bottom culture dishes (6-, 24-, 96-well)	Sarstedt
Hyperfilm ECL films	Amersham
96-well plate harvester filters	Perkin Elmer
Pasteur pipettes	Sarstedt
PCR tubes	Micronic Systems
Petri dishes	Sarstedt
Round bottom culture dishes (96-well)	Sarstedt
Scalpels	Swann Morton
Screw top tubes (15ml, 50ml)	Sarstedt
Serological pipettes (5ml, 10ml, 25ml)	Sarstedt
Syringes (10ml)	Becton Dickenson
Tefzel tubing	BioRad
Tips (20µl, 200µl, 1ml)	Sarstedt
T25, T75, T175 tissue culture flasks	Sarstedt
Universal tubes (20ml)	Sterilin
0.2µm filters	Sartorius

Equipment	**Supplier**
Centrifuge, microcentrifuge	MSE
Class II safety cabinets	Walker
Confocal microscope	Leica
Cryostore	Forma Scientific
Drying cabinet	Scientific Laboratory Supplies
Dynabeads separation unit	Dynal
Electrophoresis gel tanks	BioRad
Flow cytometer	Beckman-Coulter
Helios gene gun	BioRad
Microscope	Nikon
Microwave	Matsui
-80°C freezer	Revco
96-well plate reader	Tecan
96-well plate harvester	Packard
PCR thermal cycler	Thermo Hybaid
Power packs	BioRad
Real time PCR thermal cycler	BioRad
Refrigerated microcentrifuge	Hettich Zentrifugen

Semi-dry transfer apparatus	BioRad
37°C, 5% CO_2 incubator	Forma Scientific
Top count scintillation counter	Packard
Tubing prep station	BioRad
UV spectrophotometer	Sanyo
UV transilluminator	Ultra Violet Products
Water baths	Grant instruments

2.1.3 Buffers

(i) Buffers for tissue cultures

Trypan Blue:
0.1% (v/v) solution of Trypan blue in PBS

White cell counting solution:
0.6% (v/v) acetic acid in PBS

(ii) Buffers used for DNA or protein analysis and polymerase chain reaction (PCR)

50X TAE:
242g tris
57.1ml glacial acetic acid
100ml 0.5M EDTA (pH 8.0)
Completed to 1000ml with ddH_2O

1.4% agarose gel electrophoresis:
1.4g agarose
100ml 1X TAE
10µl Ethidium bromide

Orange G DNA loading buffer:
0.25% (w/v) orange G
30% (v/v) glycerol
Completed to final volume with ddH_2O

Wash buffer:
25ml 10X Wash solution
100ml Ethanol
Completed to 250ml with ddH_2O

Tris buffer saline (TBS):
1.21g Tris
22.33g NaCl
pH adjusted to 7.5 with HCl
Completed to 1000ml with ddH_2O

TBS-Tween 20-(Marvel):
0.05% Tween 20
(5% (w/v) Marvel)
Completed to final volume with TBS

Resolving gel buffer:
1165µl 30% (w/v) Acrylamide-bis
875µl 1.5M Tris-HCl, pH 8.8
1460µl ddH_2O
35µl 10% (w/v) APS
3.5µl TEMED

Stacking buffer:
466.5µl 30% (w/v) Acrylamide-bis
875µl 1.5M Tris-HCl, pH 6.8
2158.5µl ddH_2O
35µl 10% (w/v) APS
3.5µl TEMED

Running buffer:
0.25M Trizma base
2M Glycine
1% (w/v) SDS
Completed to 1000ml with ddH_2O

Transfer buffer:
48M Tris
39mM Glycine
20% (v/v) methanol
Completed to 1000ml with ddH_2O

Reducing sample buffer:
0.5M Tris-HCl, pH 6.8
2% (w/v) SDS
10% (v/v) glycerol
1% DTT

ECL chemioluminescence reagent:
1 volume solution A
1 volume solution B

Completed to final volume with ddH_2O

(iii) Buffers for flow cytometry

Permeabilisation solution:
1% (v/v) paraformaldehyde in PBS

Fixation solution:
70% (v/v) ethanol in PBS

FACS buffer:
0.1% (w/v) BSA
0.02% (w/v) NaN_3
1X PBS

(iv) Buffers for immunohistochemistry

Primary antibody
100µg/ml rabbit anti-HAGE antibody
5% (v/v) goat serum
Completed to final volume with PBS

Secondary antibody:
10µg/ml goat anti-rabbit IgG-biotin
1.5% (v/v) goat serum
Completed to final volume with PBS

ABC reagent:
2.5ml PBS
1 drop reagent A
1 drop reagent B

DAB reagent:
2.5ml ddH_2O
1 drop buffer
2 drops DAB
1 drop H_2O_2

(v) Buffers for bacteria culture

LB broth:
5g NaCl
10g tryptone
5g yeast extracts
pH adjusted to 7.0 with NaOH
Completed to 1000ml with ddH_2O

LB Agar:
5g NaCl
10g tryptone
5g yeast extracts
15g agar
pH adjusted to 7.0 with NaOH
Completed to 1000ml with ddH_2O

(vi) Buffers for alkaline lysis

0.2M Tris-HCl buffer:
12.1g Tris
pH adjusted to 8.0 with HCl
Completed to 1l with ddH_2O

GTE solution:
50mM glucose
25mM Tris-HCl (pH 8.0)
10mM EDTA

KOAc solution:
60ml 5M potassium acetate
11.5ml glacial acetic acid
28.5ml ddH_2O

NaOH/SDS solution:
0.2M NaOH
1% (w/v) SDS
ddH_2O

0.2M Tris-HCl buffer:
12.1g Tris
pH adjusted to 8.0 with HCl
Completed to 1000ml with ddH_2O

(vii) Buffers for ELISA

Wash buffer:
0.05% (v/v) Tween 20 in PBS

Stop solution:
2N H_2SO_4

Block buffer:
1% (w/v) BSA
5% (w/v) sucrose
0.05% (w/v) NaN_3
Completed to final volume with PBS

Substrate solution:
1 volume colour reagent A (R&D Systems)
1 volume colour reagent B (R&D Systems)

Reagent diluent for IL-5:
1% (w/v) BSA
Completed to final volume with PBS

Reagent diluent for IFNγ:
0.1% (w/v) BSA
0.05% (v/v) Tween 20
Completed to final volume with PBS

(viii) Buffers for X-Gal assay

X-Gal solution :
0.2% (v/v) X-Gal
1mM $MgCl_2$
150mM NaCl
3.3mM $K_3Fe(CN)_6$
3.3mM $K_4Fe(CN)_6$
40mM NaH_2PO_4
60mM Na_2HPO_4

Glutaraldehyde solution:
0.1M sodium phosphate pH 7.0
1mM $MgCl_2$
0.25% (v/v) Glutaraldehyde

2.1.4 Primary culture media

Culture media were prepared and used within a month. Complete BM-DC medium was prepared fresh just before use.

T cell media:
RPMI
10% (v/v) FCS
2mM L-glutamine
20mM HEPES
50μM 2-mercaptoethanol
50U/ml Penicillin/Streptomycin
0.25μg/ml Fungizone

BM-DC media:
RPMI
5% (v/v) FCS
2mM L-glutamine
10mM HEPES
50μM 2-mercaptoethanol
25U/ml Penicillin/Streptomycin
0.25μg/ml Fungizone

Complete BM-DC media:

BM-DC media + 1ng/ml mGM-CSF

2.1.5 Cell lines and media

Various cell lines in this study are described below in table 2.1.

Table 2.1: Cell lines and their descriptions

Name	Description	Media	Source
ALC	Murine lymphoma	RPMI+10% (v/v) FCS+2mM L-glutamine 500µg/ml G418	Dr. C. Baxevanis (Saint Savas Cancer hospital, Athens)
ALC/HAGE	Murine lymphoma, HAGE-positive	RPMI+10% (v/v) FCS+500µg/ml G418+50µg/ml Zeocin	Nottingham Trent University
BHK-21	Syrian hamster kidney	DMEM+10% (v/v) FCS	ATCC
CMLT-1	Chronic myeloid leukaemia	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. I. Dodi (Anthony Nolan Institute, London)
ESTDAB-07	Human melanoma	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. D. Schadendorff (Tuebingen University)
ESTDAB-17	Human melanoma	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. D. Schadendorff (Tuebingen University)
ESTDAB-27	Human melanoma	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. D. Schadendorff (Tuebingen University)
ESTDAB-34	Human melanoma	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. D. Schadendorff (Tuebingen University)
ESTDAB-95	Human melanoma	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. D. Schadendorff (Tuebingen University)
KCL-22	Chronic myeloid leukaemia	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. I. Dodi (Anthony Nolan Institute, London)
K812	Chronic myeloid leukaemia	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. I. Dodi (Anthony Nolan Institute, London)
K562	Chronic myeloid leukaemia	RPMI+10% (v/v) FCS+2mM L-glutamine	ATCC
KYO-1	Chronic myeloid leukaemia	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. I. Dodi (Anthony Nolan Institute, London)
NALM-1	Chronic myeloid leukaemia	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. I. Dodi (Anthony Nolan Institute, London)
RMA/S-A2	Transgenic lymphoblastoid	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. F. Lemonnier (Institut Pasteur, Paris)
293	Human embryo kidney	DMEM+10% (v/v) FCS	ICRF
UCHM-1	Chronic myeloid leukaemia	RPMI+10% (v/v) FCS+2mM L-glutamine	Prof. I. Dodi (Anthony Nolan Institute, London)
U937	Human histiocytic lymphoma	RPMI+10% (v/v) FCS+2mM L-glutamine	University of Sheffield

2.2 Methods

2.2.1 Expression analysis

2.2.1.1 RNA extraction and cDNA synthesis

Total RNA was isolated from cell lines and tissues using RNA STAT-60 following manufacturer's instructions. Briefly, tissues were grounded to a powder in liquid nitrogen and 1ml of RNA STAT-60 added to them and stored at room temperature (RT) for 5 minutes. 0.2ml of chloroform was added and the homogenate shaken vigorously for 60 seconds and left at RT for 3 minutes. Samples were then centrifuged at 14,000rpm for 10 minutes. The aqueous phase was transferred to a fresh tube and 0.5ml of isopropanol was added to them. Samples were incubated at RT for 10 minutes followed by centrifugation at 12,000rpm for 15 minutes. Supernatant was discarded and the pellet washed with 70% (v/v) ethanol. RNA pellet was then dried and re-suspended in molecular grade water and the concentration and purity of the RNA was measured on a UV spectrophotometer.

RNA was then reverse transcribed into cDNA as follows. 2µg of RNA was taken in a tube along with 0.5µg of Oligo-d(t_{15}) primers. Tube was heated at 70°C for 5 minutes and then placed on ice. The following mix was then added to the tube: 5µl of 5X Reaction buffer, 1µl of dNTP (12.5mM), 25 units of RNasin ribonuclease inhibitor, 200 units of M-MLV reverse transcriptase. Nuclease free water (ddH_2O) was then added to make the final volume to 25µl. Content of the tube was gently mixed and heated at 39.2°C for 80 minutes followed by cooling on ice and heating at 95°C for 5 minutes before storing at -20°C.

2.2.1.2 PCR amplification

PCR was performed on a DNA thermal cycler. Primers were supplied by MWG Biotech. Cell lines and tissues were all pre-screened for housekeeping gene 18S ribosome, which yielded a 110bp product. For HAGE screening by conventional RT-PCR, primers used were 5'-CCTTTCAATGTTATCCTGAG-3' and 5'-TATTCTTCAGATTGACGAAG-3', which yielded a 432bp product. For amplification by PCR, 2µl of cDNA was supplemented with 4µl of 5X Phusion HF buffer, 0.4µl of 10mM dNTP, 20pmol of each of primer solutions, 1 unit of Phusion DNA polymerase, and ddH_2O to a final volume of 20µl.

PCR for HAGE was initiated by a melting step at 98°C for 30 seconds, followed by 32 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 20 seconds. It was followed by a final extension step at 72°C for 10 minutes. All the primers used for the study are listed below in table 2.2.

Table 2.2: Primers used for PCR of HAGE

Conventional PCR primers of GAPDH and HAGE	
GAPDH Forward	5'-ACCACCAACTGCTTAGCACC-3'
GAPDH Reverse	5'-CCATCCACAGTCTTCTGGGT-3'
HAGE Forward	5'-CCTTTCAATGTTATCCTGAG-3'
HAGE Reverse	5'-TATTCTTCAGATTGACGAAG-3'

PCR products were visualised using 1.4% (w/v) agarose gel containing 1µg/ml of ethidium bromide.

2.2.1.3 Real time quantitative PCR

Samples of breast, gastric and colon carcinoma mRNA, along with patient-matched normal tissue mRNA, were kindly provided by Dr. Aija Line (Latvia). Samples of melanoma and head & neck carcinoma were kindly provided by Prof. D. Schadendorff (Germany) and Dr. R. Ferris (USA), respectively. Samples of CML were kindly provided by Dr. R. Clark. Samples of mRNA from normal tissues were purchased from Clontech. Human cell lines are described above. Primers were supplied by MWG Biotech. Reverse transcription of the mRNA was performed as described earlier with exception of using Random primers instead of Oligo-d(t_{15}) primers. For preparing the standard curve, cDNA from a HAGE-positive cell line (ESTDAB-27) was serially diluted. Primers for various genes (GAPDH, h18S, HAGE, p53, Bcr/Abl, OAS1 and STAT1) were designed with the assistance of the primer Vs program at the following website: (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All the primers, described in table 2.3, were designed to generate PCR products of under 250bp in size to optimise the RT-Q-PCR.

RT-Q-PCR was performed using a BioRad real time Thermocycler using SYBR green fluorescent dye. Thermocycling for each reaction was done in a final volume of 12.5µl containing 0.5µl of cDNA template, 6.5µl of SYBR green supermix containing high fidelity DNA polymerase, and 20pmol of each of the gene-specific primer solutions. This was then made up to 12.5µl with ddH_2O. RT-Q-PCR was initiated by a melting step at 98°C for 30 seconds, followed by 45 cycles of denaturation at 98°C for 10 seconds, annealing at the corresponding annealing temperatures indicated in table 2.3 for 30 seconds and extension at 72°C for 20 seconds. It was followed by a final extension step at 72°C for 10 minutes and a stop step at 98°C for 1 minute. Finally, a dissociation curve was created by doing 0.5°C increment going from the annealing temperature to the denaturation temperature.

Table 2.3: Sequences of primers used for RT-Q-PCR and respective annealing temperatures

Real time PCR primers and respective annealing temperature		
Bcr/Abl Forward	5'-TCCACTCAGCCACTGGATTTAA-3'	62°C
Bcr/Abl Reverse	5'-TGAGGCTCAAAGTCAGATGCTACT-3'	
h18S Forward	5'-CAACTTTCGATGGTAGTCG-3'	54°C
H18S Reverse	5'-CCTTCCTTGGATGTGGTA-3'	
GAPDH Forward	5'-ACCACCAACTGCTTAGCACC-3'	54°C
GAPDH Reverse	5'-CCATCCACAGTCTTCTGGGT-3'	
HAGE Forward	5'-GGAGATCGGCCATTGATAGA-3'	66°C
HAGE Reverse	5'-GGATTGGGGATAGGTCGTTT-3'	
OAS1 Forward	5'-CAAGCTCAAGAGCCTCATCC-3'	59°C
OAS1 Reverse	5'-TGGGCTGTGTTGAAATGTGT-3'	
p53 Forward	5'-GTGGAAGGAAATTTGCGTGT-3'	66°C
p53 Reverse	5'-CCAGTGTGATGATGGTGAGG-3'	
STAT1 Forward	5'-AAATTCCTGGAGCAGGTTCA-3'	59°C
STAT1 Reverse	5'-TGGCCCCAGTCACTTAATC-3'	

2.2.1.4 Cell lysate preparation

ESTDAB-07 and -27 melanoma cell lines have been described in section 2.1.4. Cells were harvested and washed twice in ice cold PBS at 1,500rpm for 3 minutes at 4°C. Cell pellets were re-suspended in 100µl of ddH_2O for lysis. Tubes were sonicated for 5 minutes before being agitated for 30 minutes at 4°C. Tubes were than centrifugated at 14,000rpm for 30 minutes at 4°C and supernatants transferred to fresh tubes. Samples were stored at -80°C until analysis by protein assay and SDS-PAGE.

2.2.1.5 Protein assay for SDS-PAGE samples

Protein concentration was determined in the lysates preparation by performing a BioRad Dc protein assay as described by the manufacturer's protocol. The standard was made of BSA diluted in water in serial dilution. Briefly, 25µl of Reagent A was added to 5µl of samples. To each well, 200µl of Reagent B was then added. Each sample was run in duplicate. The reaction was left to develop for 15 minutes at RT and the plate was read at 750nm on a Tecan 96-well plate reader.

2.2.1.6 SDS-PAGE and transfer

Reducing sample buffer was added to all samples and boiled for 5 minutes at 95°C to denature proteins, before being loaded (30µg) on the polyacrylamide gel. As a standard, 30µg of BSA was run with the samples. The gel ($100cm^2$) was run at 90V through the 4% stacking gel and

120V through the 10% resolving gel using electrophoresis. Proteins were then transferred at 13V onto nitrocellulose membrane for 40 minutes using a semi-dry transfer unit according to manufacturer's instructions.

2.2.1.7 Immunoprobing

Membranes were stained with Ponceau S, and the standard lane was cut from the rest of the membrane. The membrane blocked overnight in TBS-Tween 20-Marvel under constant agitation. At the same time, the primary rabbit anti-HAGE antibody (a kind gift from Dr. A. Knights, Tuebingen University), was first adsorbed with rabbit liver powder overnight at 4°C. After centrifugation, the supernatant was then added at 1:1000 dilution in TBS-Tween 20-Marvel and incubated for 2 hours at RT. After washing the membrane four times for 15 minutes in TBS-Tween 20 at RT, the secondary HRP-conjugated antibody was added to the membrane at a 1:1000 dilution in TBS-Tween 20-Marvel and incubated for 2 hours at RT. The membrane was washed four times for 15 minutes in TBS-Tween 20 at RT, and revealed using ECL chemioluminescence kit. Hyperfilm ECL films were used to detect the luminescence.

2.2.1.8 Fluorescent activated cell sorting (FACS)

2-5*10^5 cells were used per tube. Cells were washed twice in FACS buffer and incubated on ice for 30 minutes with primary antibody. Rat anti-mouse CD80, CD86, 4.1BBL, Macrophage/Monocyte marker (F4/80), DEC205, Ia/Ie (murine class II), CD45R, mouse anti-HLA-A2, HLA-DR, FLAG, rabbit anti-HAGE and hamster anti-mouse CD11c antibodies were used in these experiments. Appropriate isotype controls were used in each experiment. Following incubation with the primary antibody, cells were washed twice in FACS buffer and incubated for 30 minutes on ice with FITC- or PE-conjugated goat anti-rat IgG, goat anti-mouse IgG, goat anti-rabbit IgG or goat anti-hamster IgG as secondary antibody accordingly. Finally, cells were washed twice in FACS buffer and re-suspended in 400µl of Isoton and FACS analysis was performed on the flow cytometer. In cell cycle analysis, 2-5*10^5 cells were used per tube. 100µl of 10µg/ml of propidium iodide (PI) was added to the cells. After 5 minutes at 37°C, FACS analysis was performed on the flow cytometer.

2.2.1.9 Immunofluorescence

A day prior to staining, 2*10^4 adherent cells or 1*10^5 non-adherent cells were plated in 8-chamber slides. Media were removed and cells were consecutively permeabilised and fixed

with 1% (w/v) paraformaldehyde and 70% ethanol, respectively, each for 10 minutes at RT. Rat anti-mouse CD80, CD86, 4.1BBL, mouse anti-HLA-DR, FLAG and rabbit anti-HAGE were used in these experiments. Appropriate isotype controls were used in each experiment. Following incubation with the primary antibody, cells were washed twice in FACS buffer and incubated for 30 minutes on ice with FITC- or PE-conjugated goat anti-rat IgG, goat anti-mouse IgG or goat anti-rabbit IgG as secondary antibody accordingly. Finally, cells were washed twice in FACS buffer. Slides were dried at 37°C, mounted with fluorescent mounting media and left overnight at 4°C. Slides were finally studied under confocal microscope the following day.

2.2.1.10 Immunohistochemistry

Paraffin-embedded multiple normal and tumour tissue microarrays were purchased from US Biomax. Frozen melanoma and head & neck carcinoma tissue sections were kindly provided by Prof. D. Schadendorff (Germany) and Dr. R. Ferris (USA), respectively. Prior to immunohistochemistry, antigen retrieval was carried out on paraffin-embedded tissues. Briefly, sections were de-waxed in xylene and re-hydrated in graded ethanol (100%, 100% and 70% (v/v)). Slides were consecutively rinse in tap water and in ddH_2O before being heated up in 0.01M citrate buffer pH 6.0 for 10 minutes in the microwave in order to perform antigen retrieval. Once again, slides were consecutively rinsed in tap water and in ddH_2O. At this stage, both frozen and paraffin-embedded tissues can undergo immunohistochemistry. 0.03% (v/v) H_20_2 diluted in 1X PBS was added to the tissue sections for 5 minutes before being washed with PBS. Sections were then blocked for 10 minutes with 10% (v/v) serum made from the species of the secondary antibody. In the case of HAGE, goat serum was used. Following incubation, the serum was removed and 100µg/ml of the primary antibody (adsorbed rabbit anti-HAGE) was added for overnight incubation at 4°C. Slides were then washed in 1X PBS and 10µg/ml of the secondary antibody (biotin-conjugated goat anti-rabbit IgG) was added. Slides were incubated for 30 minutes at RT and washed thoroughly with 1X PBS. The ABC reagent was laid onto the slides, left to react with secondary antibodies for 30 minutes at RT and washed off with PBS. The DAB reagent was added to the sections in order to react with the ABC reagent for 10 minutes at RT and washed off with ddH_2O. Finally, frozen and paraffin-embedded sections were counterstained in Gill's and Harris' staining, respectively, and fixed consecutively in graded ethanol (70%, 100%, 100% (v/v)) and in xylene. Slides were mounted, air-dried and observed under the microscope. Staining intensities were semi-

quantitatively evaluated as positively stained according to the following categories: -, no staining; 1+, weak but detectable; 2+: moderate, 3+, intense. For each tissue, a histologic score (H-score) value was derived by summing the percentage of cells that stained at each intensity category and multiplying that by the weighted intensity of the staining. In each slide, five different areas were assessed under the microscope (x40 magnification) and the average score was used.

2.2.2 Gene induction or silencing

2.2.2.1 DNA transfection

Lipofectamine 2000 was used to transfect cells as electroporation was found to result in very high cell mortality. Transfection was done as per manufacturer's instructions. Briefly, $6*10^5$ cells in suspension or $5*10^4$ adherent cells in 500µl of culture media were plated per well in a 24-well plate. 24 hours later, 1.2µg of expression plasmid DNA was diluted in 50ml of Opti-MEM medium for each well. At the same time, 2ml of lipofectamine was diluted in 50ml of Opti-MEM medium for each well. After 5 minutes at RT, DNA and lipofectamine were combined, gently mixed and incubated for 20 minutes at RT in order to allow the formation of DNA-lipofectamine complexes. 100µl of the required mixture was then added to each of the wells containing cells and medium. Wells were mixed by gently rocking the plate back and forth. 24 hours later, medium in the wells was removed and 1ml of fresh culture medium containing 500µg/ml G418 or 50µg/ml zeocin was added in its place. The selective antibiotic was chosen in agreement with the mammalian resistance gene expressed by the plasmid used for transfection. Transiently-transfected cells were then left to grow to confluency for 48 hours for expression analysis by RT-PCR, FACS or immunofluorescence. 48 hours after transfection, stably-transfected cells were made by plating 100 cells per well in 200µl of selective medium in a 96-well plate. Upon repeated splitting in 96-well plates, cells growing in the selective media were harvested and put back into culture in a 24-well plate to bulk-up. Cells were then left to grow to confluency after which time; the cells were pooled and put into culture in T25 flasks for expression analysis by RT-PCR and storage in liquid nitrogen.

2.2.2.2 Small interfering RNA (siRNA) transfection

SiRNA transfection was carried out using Interferin following manufacturer's protocol. Briefly, $2*10^4$ adherent cells in 500µl of culture media were plated in a 24-well plate. On the day of transfection, 1µl of 40µM HAGE siRNA (sense: 5'-AUUAGAGAGGAAGGUUUGA-3'; anti-

sense: 5'-UCAAACCUUCCUCUCUAAU-3') from Eurogentec was diluted in 100µl of Opti-MEM media and vortexed. 5µl of Interferin was then added and the mixture was thoroughly vortexed for 10 seconds before being incubated for 10 minutes at RT. While incubating, the medium was removed and replaced with 100µl of fresh media. 100µl of siRNA-Interferin mixture was given to cells while rocking the plate back and forth. After 6-hour incubation at 37°C, 5% CO_2, 500µl of fresh medium was added. 24 hours later, media in the wells was removed and 1ml of fresh culture media was added in its place. Expression analysis was then carried out by real time PCR and immunofluorescence to observe HAGE silencing and its effects were evaluated by performing proliferation assays using Trypan blue exclusion and thymidine incorporation, and cell cycle analysis using propidium iodide DNA staining.

2.2.2 Cloning of human HAGE and murine co-stimulatory molecules

2.2.3.1 Enzyme digestion

Plasmids or PCR amplicons were digested with two restriction enzymes, in order to generate the correct overhangs for ligation, depending on the sequence of the cDNA cloned and the restriction enzymes available in the multiple cloning sites of the original and the receiving expression vectors. Briefly, 6µl of DNA was mixed with 1µl of restriction enzyme-specific buffer, 1µl of 1X BSA and 1µl of each restriction enzyme for a total volume of 10µl. Tubes were incubated for 1-2 hours at the restriction enzyme's optimal temperature. A negative control was included whereby the restriction enzymes had been omitted from the reaction mix and replaced with ddH_2O. The restriction enzyme digestion products were then run on a 1.4% (w/v) agarose gel and visualised under UV light.

2.2.3.2 Band extraction of DNA

As described above, DNA was run on a 1.4% (w/v) agarose gel. Bands were visualised by placing the gel on a UV transilluminator. The DNA of interest was then excised from the gel and extracted using Geneflow DNA isolation kit following the manufacturer's protocol. Briefly, the gel was dissolved in a solution of NaI at 55°C for 5 minutes. 30µl of glass powder was then added to the re-suspended DNA and incubated for 5 minutes at RT with intermittent mixing. Following incubation, the DNA, now bound to the glass powder, was washed three times in wash buffer. After the last wash, the DNA was centrifuged at 14,000rpm and the supernatant was discarded. The pellet was then re-suspended in 30µl of ddH_2O. DNA was eluted from the

glass powder by incubation at 55°C for 5 minutes. The DNA present in the supernatant was finally harvested following centrifugation at 14,000rpm for 45 seconds.

2.2.3.3 DNA ligation

Ligation of HAGE, mB7.1, mB7.2 and m4.1BBL cDNA into their new expression vectors was done using T4 ligase as per the manufacturer's instructions. Briefly, 7µl of the required digested insert was added to 1µl of the double digested plasmid with 1µl of T4 buffer and 1µl of T4 ligase for a total volume of 10µl. This mixture was then left overnight at 4°C in order to complete the ligation process.

2.2.3.4 Transformation into XL1-Blue E.coli and bulking up

XL1-Blue is a competent strain of *Escherischia coli* used for routine nucleotide transformation and cloning procedures. For transformation, a previously prepared aliquot of XL1-B was taken and defrosted on ice. 10µl of the ligation mix was added to the bacteria and incubated on ice for 30 minutes. Following incubation, cells were heat-shocked at 42°C for 3 minutes and cooled on ice. 250µl of LB media was then added and cells were incubated at 37°C in a shaker for 1 hour. 150µl of transformed cells were then plated onto LB agar plates containing either 50µg/ml ampicillin or 30µg/ml zeocin depending on the resistance gene expressed by the plasmid used for transformation. Plates were left on the bench for 5 minutes to allow the absorption of media before being inverted and put into a 37°C incubator overnight. The following morning 10 universals containing 3ml of LB broth plus 50µg/ml ampicillin or 30µg/ml zeocin were set up. Isolated colonies from the agar plates were then picked using a pipette tip and cells were placed in a 37°C shaker to grow overnight.

2.2.3.5 DNA isolation and sequencing

1.5 ml of each of the overnight cultures, prepared as above, was harvested and the bacteria pelleted by centrifuging at 14,000rpm for 5 minutes. Pellets were re-suspended in 100µl of cold GTE and incubated for 5 minutes. Following this incubation, 200µl of 1% (w/v) SDS/0.2M NaOH was added and the pellets incubated on ice for 5 minutes. 150µl of KOAc was added, the samples vortexed and incubated on ice for a further 5 minutes. Samples were centrifuged as before and supernatants collected. 800µl of chloroform-isoamyl alcohol was then added and the samples were vortexed then centrifuged as before for 5 minutes. Upper layers were collected and 1ml of absolute ethanol added to each sample. These were then

incubated at room temperature for 15 minutes. Samples were centrifuged at 14,000 rpm for 15 minutes in order to pellet DNA. Supernatants were discarded and the DNA pellets washed with 500µl of 70% ethanol. Samples were centrifuged at 14,000rpm for 5 minutes and the tubes inverted onto tissue paper to dry out the DNA. Samples were then air-dried for 30 minutes. Finally the DNA was re-suspended in 50µl of ddH_2O with 1µl RNase (final concentration of 20µg/ml). Sequencing by MWG Biotech was used to confirm sequences of the cloned cDNA.

2.2.4 Animals and immunisation

2.2.4.1 Animals

Mouse class I (H-2) knockout C57BL/6 HLA-A2.1 mice (HHDII) and C57BL/6 HLA-A2.1/-DR1 (HHDII-DR1) animals were received as a generous gift from Dr. F. Lemonnier (Institut Pasteur, Paris). FVB/N-DR1 animals were received as a generous gift from Dr. M. Altmann (MRC Clinical Sciences Center, London). Mouse class II ($I\text{-}A^b$) knockout C57BL/6-DR4 mice were purchased from Taconic, USA. HHDII, HHDII-DR1, FVB/N-DR1 or C57BL/6-DR4 F_2 mating positive animals were maintained inbred by ensuring they have a common F_0 ancestor. Colonies were bred at Nottingham Trent University animal house in accordance with the Home Office Codes of Practice for the housing and care of animals.

2.2.4.2 Peptides and peptide immunisation

HAGE protein sequence was screened for peptides binding to HLA-A2.1 using a web-based algorithm (www.syfpeithi.de). Peptides (Table 2.4) were chosen based on their binding score, synthesised (Alta Biosciences) and dissolved in 100% DMSO to a working concentration of 10mg/ml and then stored at -80°C. Each HHDII mouse was immunised at the base of the tail with 100µl of a peptide/IFA emulsion containing 100µg of peptide and 140µg of helper peptide (HepB) in 50% IFA. One round of immunisation was undertaken.

Table 2.4: HAGE-derived HLA-A2 peptides to test for cytotoxicity

Peptide name	Sequence	HLA-A2 binding score
HAGE 103	IIQEQPESL	24
HAGE 126	AVIDNFVKKL	24
HAGE 296	YLMPGFIHLV	30
HAGE 506	DLILGNISV	25
HAGE 507	LILGNISVE	17
HAGE 508	ILGNISVESL	28
HAGE 509	LGNISVESL	19

HAGE 551	LDVHDVTHV	16

HAGE protein sequence was also screened for peptides binding to both HLA-DR1 and -DR4 using the same algorithm as before. Peptides (Table 2.5) were chosen based on their binding score, synthesised and re-suspended as described above. Each FVB/N-DR1 or C57BL/6-DR4 mouse was immunised at the base of the tail with 100µl of a peptide/IFA emulsion containing 100µg of peptide in 50% IFA. Two rounds of immunisation with the same peptide were undertaken at 7-day intervals.

Table 2.5: HAGE-derived HLA-DR1 and –DR4 peptides used in proliferation assays

Peptide name	Sequence	HLA-DR1 binding score	HLA-DR4 binding score
HAGE 109	ESLVKIFGSKAMQTK	28	26
HAGE 195	KKNFYKESTATSAMS	26	28
HAGE 338	KYSYKGLRSVCVYGG	33	28
HAGE 505	SDLILGNISVESLHGD	22	20
HAGE 506-II	DLILGNISVESLHGD	22	20
HAGE 545	DLASRGLDVHDVTHV	18	16

2.2.4.3 Plasmid, DNA bullets and DNA immunisation

Expression vectors encoding HAGE and/or murine co-stimulatory molecules were coated onto 1.0µm gold microcarriers using manufacturer's instructions. Briefly, 36µg of DNA was mixed with 200µl of 0.05M spermidine containing 16.6mg of gold. After sonication, 200µl of 1M $CaCl_2$ were added dropwise to the mix whilst sonicating and the mixture was incubated for 10 minutes at RT. The DNA-gold mixture was then washed three times in anhydrous ethanol and re-suspended in 2ml of 0.025mg/ml PVP. Whilst the tube was sonicating, the sample was loaded into a dried Tefzel tubing and left to stand for 30 minutes in a Tubing Prep Station. The dry ethanol was gently removed using a syringe leaving the gold undisturbed. Nitrogen was turned on and the tubing was left spinning for five minutes. Once totally dried, the tubing was removed from the station and cut using a guillotine. DNA bullets were stored at 4°C until used for immunisation. Each HHDII, HHDII-DR1, FVB/N-DR1 or C57BL/6-DR4 mouse was immunised with one bullet containing human and/or mouse DNA using a Helios gene gun. Three rounds of immunisation were undertaken at 7-day intervals. Naked DNA immunisation was also used in *in vivo* challenge experiments. 100µg of DNA in serum free media per HHDII/DR1 mice was administrated twice intra-muscularly at 7-day interval.

2.2.4.4 Dendritic cells, transfection and DC immunisation

The preparation of dendritic cells used was adapted from Inaba *et al*. (1992). Briefly, mouse hind limbs were harvested and bones were flushed with BM-DC media after removing muscles and knuckles. Cells were washed, re-suspended in 2ml of BM-DC media and counted in 0.6% (v/v) acetic acid and 0.1% (v/v) Trypan blue. Cells were plated out in 24-well plates at $1*10^6$ cells per ml per well of BM-DC media containing 1ng/ml of murine GM-CSF. After two days, cells were harvested and re-plated in a 24-well plate at $1.2*10^6$ cells per ml of BM-DC media containing 1ng/ml of mGM-CSF, and transfected with the pSHAME2a/HAGE plasmid using lipofectamine as described in section 2.1.1.1.

On the fourth day, 1µg/ml of LPS and 500µg/ml of G418 were added to induce complete maturation and selection of positively-transfected BM-DC. On the fifth day, cells were harvested, washed, re-suspended in serum free media and counted. Dendritic cell immunisation was then induced by intra-dermal injection of $2*10^6$ cells in the right flank of HHDII and HLA-DR4 transgenic mice.

2.2.4.5 Recombinant Semliki Forest Virus and SFV immunisation

(i) Generation of SFV virus

pSCAβ, pSCAhelper and pSHAME2a plasmids were kindly provided by Dr. Rod Bremner from the University of Toronto. For production of infective viral particles, pSCAβ or pSHAME2a and pSCAhelper plasmids were co-transfected in 293 cells at different molar ratios. Calcium phosphate transfection combines with 1:3 molar ratio of expression plasmid: helper plasmid was used in all experiments. Calcium phosphate transfection was performed using the ProFection mammalian transfection system following manufacturer's instructions. Briefly, 293 cells were plated at 5*105 cells per 3ml per well in a 6-well plate a day before transfection. On the day of transfection, cells were washed once and media was replaced 3 hours before transfection. DNA and HBS solutions were prepared in two separate 1.5ml tubes. 12µg of DNA was diluted in ddH_2O and 37µl of 2M $CaCl_2$ for a final volume of 300µl. This DNA mixture was added to 300µl of 2X HBS solution in another tube with constant vortexing. The DNA-HBS-$CaCl_2$ mix was incubated for 30 minutes and added to the cells dropwise. After incubating the plate for 16-18 hours at 37°C, 5% CO_2, medium was removed from transfected cells. Cells were then washed once with PBS and fresh medium was added to the cells. After a further 48-hour incubation, medium containing the lysed cells and virus was harvested following a freeze-thaw cycle and spun at 2,000rpm for 10 minutes at 4°C. The supernatant

containing the virus was transferred into a fresh tube and stored at -20°C until use. Generated viruses were inactive and prior to use, they were activated by adding 1-20th of total volume of 10mg/ml of α-chymotrypsin to cleave the p62 glycoprotein into E2 and E3 proteins. After 1-hour incubation at RT, 10mg/ml of aprotinin was added to 1/15th of total volume to stop the protease activity.

(ii) Viral titration

In order to calculate the virus titre, BHK-21 cells were infected with different volumes of activated virus for 60 minutes at 37°C. After incubation, cells were washed once with PBS and incubated for 24 hours after addition of fresh media. To visualise cells infected by SFV-βgal, X-gal assay was performed. Briefly, cells were washed twice with PBS and fixed with glutaraldehyde for 15 minutes at 37°C. Cells were washed twice with PBS followed by the addition of 1ml per well of X-gal solution. Colour was allowed to develop overnight at RT and blue cells were counted. Titre 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.

For titration of SFV/HAGE virus, after infection of BHK-21 cells as above, infected cells were visualised by immunohistochemistry. 24 hours after infection, cells were fixed with -20°C methanol for 1 minute followed by the addition of mouse anti-FLAG primary antibody. Following 1-hour incubation at RT, cells were washed twice and secondary HRP-conjugated goat anti-mouse antibody was added. After further 30-minute incubation at RT, cells were washed and stained with DAB solution. Colour was allowed to develop and dark brown infected cells were counted to determine the titre.

2.2.4.6 In vivo challenge experiments

In prophylactic experiments, HHDII/DR1 mice were challenge with $6*10^5$ ALC/HAGE cells one week after the last DNA immunisation. In therapeutic experiments, HHDII/DR1 were challenged first with $6*10^5$ ALC/HAGE cells and then immunised three to seven days later with DNA vaccines. Animals were monitored three times a week for tumour development and size, and were sacrificed when the tumour reached a size of 1cm^2 according to the Home Office guidelines.

2.2.5 T cell preparation and cytotoxicity in mice

2.2.5.1 Preparation of LPS blasts

Spleens were harvested from naïve mice and cells were flushed out with T cell medium. LPS blasts were set up in a T75 flask by culturing $60*10^6$ spleen cells in T cell medium supplemented with 1mg of LPS, 7μg/ml of dextran sulphate and 40μg/ml of Vitamin E. After 48 hours, cells from LPS blasts were harvested, washed, re-suspended in 5ml of T cell medium and irradiated with caesium for 8 minutes at the University of Nottingham. These LPS blasts were washed again and pulsed with 100μg/ml of the relevant or irrelevant peptide for 1 hour at 37°C. After washing, these cells were used for *in vitro* re-stimulation of the splenocytes harvested from immunised mice.

2.2.5.2 In vitro re-stimulation of murine splenocytes with LPS blasts

One week after the last immunisation, spleens were harvested from the immunised mice and single cell suspensions were prepared in sterile conditions. Cells were counted, re-suspended and set up in a T25 flask at $25*10^6$ cells/5ml. Finally, $5*10^6$ irradiated and peptide-pulsed LPS blasts per 5ml were added to the splenocytes to make a final volume of 10ml in each T25 flask. Supernatants were usually collected on day 3 and 5 of the re-stimulation for cytokine testing.

2.2.5.3 Chromium release cytotoxicity assay

On day 5 of the *in vitro* re-stimulation, splenocytes were harvested, washed twice in serum free media, re-suspended in T cell media, counted and used as effector cells. RMA/S-A2 target cells were pulsed with 10μg/ml of the relevant or irrelevant peptide a day prior to the cytotoxicity assay. On the day of the assay, RMA/S-A2 target cells were harvested, washed and labelled with 1.85MBq of chromium-51. A standard 4-hour chromium-release assay was performed and the percentage specific cytotoxicity was determined using the following equation:

$$\text{Percentage of cytotoxicity} = \frac{(\text{Experimental release} - \text{Spontaneous release})}{(\text{Maximum release} - \text{Spontaneous release})}$$

2.2.6 BM-DC, T cell generation and proliferation assay in mice

2.2.6.1 Murine BM-DC generation for proliferation

Bone marrow-derived dendritic cells were generated as described in section 2.2.4.4. Cells were washed with fresh media every two days and on day 7, BM-DC were re-plated at $5*10^5$ cells

per ml per well and pulsed with 10µg/ml of the peptide of interest for 4 hours. LPS was added at 1µg/ml to induce complete maturation. The following day, BM-DC were washed twice in T cell media, re-suspended in 2ml and then pulsed with 10µg/ml of relevant or irrelevant peptide for 4 hours at 37°C, 5% CO_2. These cells were plated at 5 x 10^3 per well together with the responder cells in a round bottom 96-well plate.

2.2.6.2 In vitro re-stimulation of murine splenocytes with peptides

Spleens of immunised animals were harvested seven days after the last immunisation and cells were flushed out with T cell media. The cell suspension was then washed, counted and plated in 24-well plates at 2.5x10^6 cells per well in 1ml of T cell media containing 10µg/ml of the relevant peptide. Splenocytes were cultured for 6 days at 37°C, 5% CO_2 prior to CD8+ T cell depletion. On day 13, cells were used at 5x10^4 cells per well as responders for the proliferation assay. In parallel, splenocytes were also cultured with irrelevant peptide as a control for specific cytokine release.

2.2.6.3 Murine CD8+ T cell depletion

On day 7, depletions were done using CD8-specific dynabeads by following the manufacturer's instructions. Briefly, CD8+ T cells attached to the beads were depleted using a magnet. The remaining cells were collected, counted, re-plated at 2.5*10^6 per ml in T cell media containing 20U/ml of murine IL-2 and incubated for another week at 37°C, 5% CO_2. Purity was assessed by FACS analysis and the preparations were about 98% free of the depleted T cell population (data not shown).

2.2.6.4 Proliferation assay for murine T cells

Responder T-cells were counted and plated at 5x10^4 cells per well in 96-well plates. Peptide-pulsed syngeneic BM-DC were used as APC 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*10^3 cells per well. To ascertain the MHC restriction of the response, a MHC blocking antibody (2mg/ml L243 anti HLA-DR) was added to the culture when possible. A matched isotype control antibody was also used in these experiments. Each culture was performed in quadruplicate for approximately 60 hours. Tritiated thymidine was added 18 hours prior to harvesting at a final concentration of 0.037MBq/ml. Cells were harvested using a 96-well

harvester onto a 96-well filter plate and 40µl of scintillation fluid was added to each of the filter wells. Filters were counted for 1 minute per well with a Top-Count scintillation counter.

2.2.6.5 Enzyme-linked immunosorbent assay (ELISA)

Splenocytes from the animals were prepared as outlined in Sections 2.2.5.1 and 2.2.6.2. Supernatants were collected into a 1.5ml tube and stored at 4°C until required. IFNγ and IL-5 ELISA were respectively carried out using mouse IFNγ and IL-5 R&D ELISA kits by following the manufacturer's instructions. Briefly, a 96-well ELISA plate was coated with 100µl of the diluted capture antibody, sealed and incubated overnight at RT. The plate was washed with wash buffer three times and blotted against tissue paper. The plate was then blocked by adding 300µl of block buffer to each well and left for incubation for 1 hour at RT before being washed as before. 100µl of samples or standards in reagent diluent were added to each well and the plate was incubated for 2 hours at RT. The washing step was repeated as before. 100µl of the detection antibody, diluted in reagent diluent, was added to each well and the plate was incubated for 2 hours at RT. The washing step was repeated as described previously. 100µl of the working dilution of Streptavidin-HRP was added to each well and the plate was covered and incubated for 20 minutes at RT. The washing step was repeated as before. 100µl of the substrate solution was added to each well and the plate was incubated for 20 minutes at RT. 50µl of stop solution was added to each well and the plate was gently tapped to ensure thorough mixing. Optical density of each well was determined at 450nm on a Tecan 96-well plate reader.

Chapter 3: Validation of HAGE as a potential antigen for immunotherapy

3.1 Introduction

Since van der Bruggen *et al.* (1991) first discovered specific human tumour antigens of the MAGE family, numerous other TAA have been identified forming a large pool of potential immunotherapeutic targets against cancer. Tumour antigen-based vaccination had different degrees of success whether newly designed vaccines were administrated to mice or humans. Indeed, antigen-specific vaccinations targeting MAGE, MelanA/MART-1, tyrosinase, gp100, and HER2/neu were shown to be very successful in both prophylactic and therapeutic experiments in murine models (Goldberg *et al.*, 2005; Prins *et al.*, 2003; Lachman *et al.*, 2001) but very elusive in human clinical trials (Rosenberg *et al.*, 1998; Anichini *et al.*, 1999; Knutson *et al.*, 2001). One of the reasons for their failure may partly be due to the choice of antigens targeted. Indeed, these antigens might simply be weakly immunogenic and the discovery of strongly immunogenic tumour-related proteins such as the cancer/testis antigen NY-ESO-1, capable of inducing an immune response in more than 50% of patients, is essential for the future development of immunotherapy strategies (Jäger *et al.*, 2000).

Virally-induced cancers have been targeted with known viral antigens, which are proving to be successful in clinical trials, such is the case of E6/E7 cancer vaccines against HPV in cervical cancers (Adams *et al.*, 2001). Viral antigens are highly immunogenic and have not been previously encountered by the immune response, therefore thwarting the problem of tolerance. Furthermore, only infected normal and cancer cells express these antigens, thereby preventing the generation of an auto-immune response. Unfortunately, most human cancers are caused by environmental and genetic factors. Identification of tumour-specific unique non-viral antigens would provide good targets for immunotherapy. However, this approach cannot be applied to patients with cancer as it is time-consuming and expensive. Therefore, shared tumour antigens, which are not expressed in normal cells but expressed in a variety of tumours, represent ideal targets. That is why a major focus has been the study of the so-called CT antigen family. Cancer/testis genes are usually normally expressed in some immunoprivileged normal tissues including testis and placenta, exhibit highly tumour tissue-restricted expression and were shown to be immunogenic in cancer patients. In a search for new potential tumour target antigens, a CT antigen, first identified in a human sarcoma and called HAGE, has been studied. HAGE over-expression was reported in different types of cancer (*e.g.* breast, lung, prostate, colon or skin) and at various frequencies (5%, 32%, 22%, 31% or 17% respectively) (Scanlan *et al.*, 2004). It was also found in 50% of CML (Adams *et al.*, 2002).

HAGE, also named DDX43, is a member of the DEAD-box family of ATP-dependent RNA helicases and despite the description of more than 500 members across species (Boeckmann *et al.*, 2003), consensus sequences characterising this family remain unchanged. Indeed, members of this family are characterised by the presence of nine conserved motifs among which the Q-motif, motifs I, II (also called D-E-A-D box as a single letter code of Asp-Glu-Ala-Asp) and IV bind and hydrolyse ATP molecules, while motifs Ia, Ib, IV and V are thought to interact with the RNA substrate. These sequences form two distinct domains connected by a linker region called SAT, which is thought to allow conformational changes of the helicase core to adapt to its substrate (Caruthers and McKay, 2002)(Fig. 3.1). RNA helicases are often described as the driving forces behind RNA metabolism. They have various functions but because of their large similarity, one can assume that the enzymatic activity will also be quite similar. Functions of RNA helicases in RNA metabolism are still not clear but they seem to be involved in processes such as transcription, pre-mRNA splicing, ribosome biogenesis, cytoplasmic transport, translation initiation/elongation, organelle gene expression and RNA decay (Rocak and Linder, 2004). Because of the importance the interactions between RNA and helicases seem to fulfill, it is critical that the assembly occurs in an energy-dependent manner to ensure appropriate complexation and correct triggering of forthcoming events. Furthermore, RNA helicases have very low affinity for RNA *in vitro* suggesting that they probably need the help of other ribonucleoprotein complexes in order to have optimal enzymatic activity and substrate specificity (Silverman *et al.*, 2003). This low specificity towards RNA *in vitro* does not easily render the identification of a specific role or a functioning mechanism for each of these molecules *in vivo*. It is now globally hypothesised that RNA helicases browse RNA in bidirectional fashion (*i.e.*: either 5' to 3', or 3' to 5') using the energy gained from the ATP hydrolysis until they encounter ribonucleoprotein-RNA complexes. The helicase activity then allows the dissociation of RNA from the ribonucleoproteins to which they have high affinity (Cordin *et al.*, 2006). Consequently, it becomes legitimate to think that splicing, RNA export, ribosome biogenesis and translation constitute an ideal "playground" for these enzymes. These RNA helicases have already been suggested to play a major role in cancer development based upon their deregulation of expression and/or their involvement in the regulation of molecules associated with cancer. Indeed, DDX1 is an example of RNA helicases reported in different tumours. DDX1 was shown to be regularly co-amplified with the transcription factor MycN in neuroblastoma and retinoblastoma (Amler *et al.*, 1996; Godbout et *al.*, 1998) leading the authors to imply that transcription of some DEAD-box genes could be regulated through interaction with members of the proto-oncogenic Myc family.

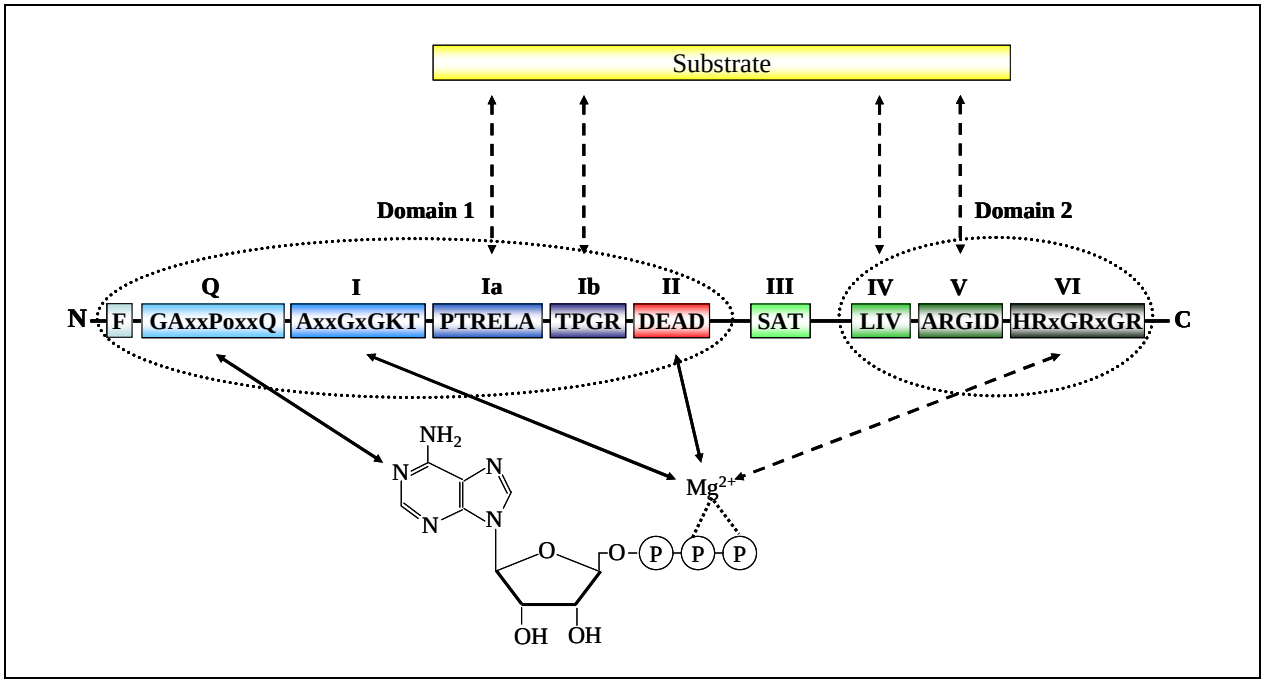

Figure 3.1: Conserved motifs of DEAD proteins and their interactions with ATP. *Nine conserved motifs have been identified. The Q-motif and motifs I and II (also called DEAD motif) are required for the binding of ATP and its hydrolysis. Motif III seems to be involved in the conformational changes required for helicase activity. Finally, the remaining motifs (Ia, Ib, IV and V) might be involved in RNA binding, however further evidence are needed to confirm this hypothesis (Taken from Rocak and Linder, 2004).*

DDX2 is over-expressed in melanoma and the induced down-regulation of this gene resulted in the inhibition of the proliferation (Eberle *et al.*, 2002). DDX5 (p68), which showed 55% homology with HAGE, is normally implicated in growth regulation by acting as a transcriptional co-activator of estrogen-receptor α(Wilson *et al.*, 2004). It was also demonstrated to be associated with the histone deacetylase 1 molecule, a well-known transcriptional repression protein (Wilson *et al.*, 2004). However, once phosphorylated at its tyrosine residues, DDX5 stops exerting its normal functions and was associated with abnormal cell proliferation in colorectal carcinoma (Yang *et al.*, 2005). Like HAGE, RCK gene encodes a protein, named DDX6 or rck/p54 protein, belonging to this family of RNA helicases. Despite DDX6 mRNA being ubiquitously found in all normal tissues, the protein is not expressed in normal lumbar and lung tissues. However, DDX6 was constantly present at moderate levels in neuroblastoma, glioblastoma, rhabdomyosarcoma and lung cancer cell lines suggesting an eventual role of DDX6 in the process of tumorogenesis (Akao *et al.*, 1995).

One can be critical of some of these studies as the actual translation of the mRNA into a protein in tumour tissues has not always been demonstrated. Indeed, mRNA expression does not always correlate with protein expression in normal (Rogel *et al.*, 1985) and abnormal tissues (Chen *et al.*, 2002) emphasising the need to determine whether TAA such as HAGE or DDX1 are not only expressed at the message level but also at the protein level in those tissues. In order to validate HAGE as a potential target for immunotherapy, the first step was to confirm that the HAGE gene was silent in normal tissues, except testis. Expression at the mRNA level was quantified using real time PCR and protein levels were compared with mRNA levels using immunohistochemistry. The second step was to confirm previous publications describing the actual over-expression of HAGE in some cancer tissues. Cancer samples, cancer tissues with patient-matched normal tissues and cancer cell lines were used in real time PCR, immunohistochemistry and immunofluorescence experiments. Furthermore, RNA helicases seem to play a critical role in RNA metabolism but also in cancer cell transformation and proliferation. The eventual implication of HAGE in tumorogenesis offers a major advantage in targeting this product for immunotherapy, namely that tumours may be unable to escape an adoptive immune response by downregulating the target molecule. Hence, a series of experiments relying mainly on immunofluorescence, gene silencing and gene induction were designed to determine the localisation of HAGE inside the cell and give an insight on HAGE function and its relationship with tumorogenesis.

3.2 Results

3.2.1 Expression of HAGE in human normal tissues

Although few studies have reported the pattern of HAGE expression in a wide array of tumour types, a more complete study is required before HAGE can be considered a potential candidate for immunotherapy. Therefore, the analysis of HAGE expression included a large number of normal tissues as well as a larger cohort of tumours of different tissue origins. Moreover, unlike previous studies describing HAGE expression at the mRNA level, a more thorough analysis was undertaken combining the determination of the expression of HAGE at both mRNA and protein levels. Normal tissue mRNA were purchased and conventional RT-PCR analysis was carried out using primers originally described by Martelange *et al.* (2000) with the RT-PCR conditions detailed in the Materials and Methods section. Briefly, 2μg of RNA was used to reverse transcribe and generate cDNA using random primers from the samples and semi-quantitative RT-PCR was performed for 32 cycles. Normalisation of the experiment was carried out using 18S ribosome RNA. In contrast to previous reports (Martelange *et al.*, 2000; Adams *et al.*, 2002), normal tissues such as brain, heart, kidney, liver, prostate, and peripheral blood mononuclear cells (PBMC) all tested positive for the expression of HAGE, although to a much weaker extent than seen in testis (Fig. 3.2). In addition, expression of the tumour-suppressor gene p53 was found at equal levels in all normal tissues.

To enable the validation of this analysis, the more sensitive technique of real time quantitative RT-PCR (RT-Q-PCR) was performed. Primers for HAGE and p53 were designed to distinguish between genomic DNA and the cDNA templates so that no genomic DNA amplification could occur and that the PCR amplicons would be under the 250bp limit as suggested by Bustin (2000). Primers for HAGE, p53 and Bcr/Abl give a product size of 220bp, 184bp and 90bp, respectively. Similarly, 2μg of RNA was used to reverse transcribe and generate cDNA using random primers. Maximum volume was used for samples with very low concentration of RNA. Real time PCR was performed for 45 cycles and relative gene expression was estimated by dividing the starting quantity of the studied gene by that of housekeeping genes. It is noteworthy that two different housekeeping genes were used, GAPDH and ribosomal 18S RNA as suggested by several published studies (Bustin., 2000). High values of 18S RNA were observed leading to low relative gene expression, whereas GAPDH values were the most consistent and were therefore used for the determination of relative gene expressions in all future analyses.

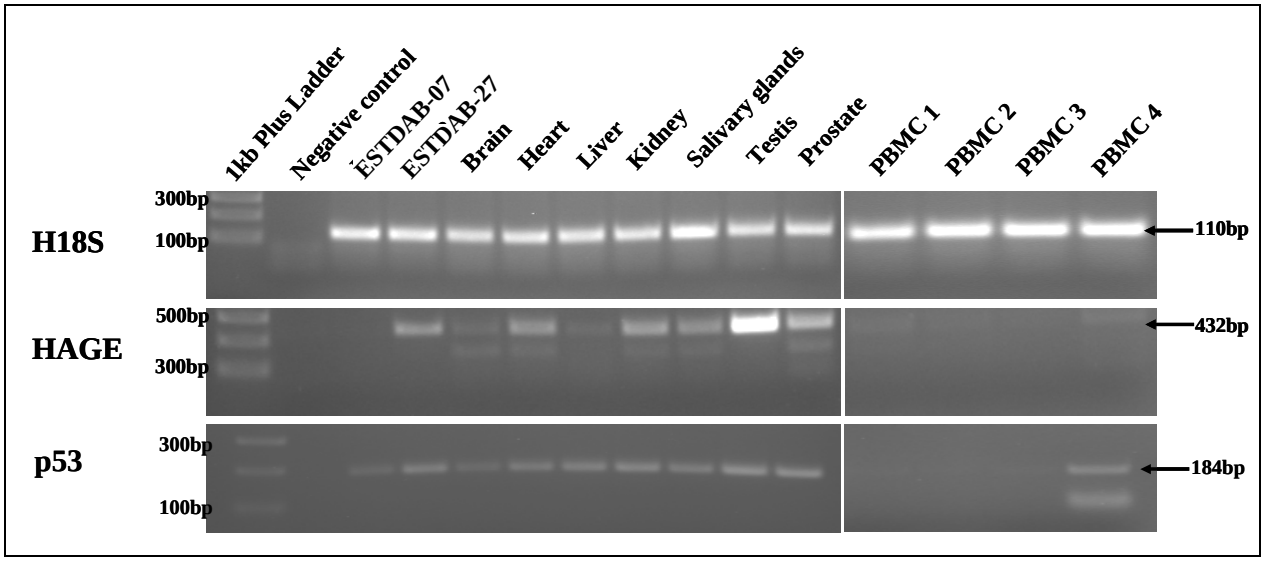

Figure 3.2: Expression of HAGE and p53 in human normal tissues by RT-PCR. *This figure represents the semi-quantitative analysis of expression of HAGE and p53 in normal tissues and in PBMC, using water and a HAGE-negative cell line (ESTDAB-07) as negative controls, as well as a HAGE-positive cell line (ESTDAB-27) and testis as positive controls. PCR products for 18S ribosome, HAGE and p53 should be of 110, 432 and 184bp, respectively.*

As seen in Fig. 3.3, relative HAGE expression was proved to be undetectable or extremely low in all the normal tissues tested when compared to testis, which value was arbitrarily set as one. Importantly, relative HAGE expression was also found to be undetectable in normal PBMC from four healthy donors. In this experiment, p53 mRNA was present in all the normal tissues tested but at various levels. These RT-Q-PCR results are in contradiction with the results achieved by semi-quantitative RT-PCR and will be discussed later. However, and because of its sensitivity, its specificity and the low levels of mRNA from tumour tissues obtained through different collaborations, RT-Q-PCR was chosen as the method of choice for all subsequent analyses of HAGE expression in tumour tissues and for the validation of this study.

To date, HAGE has only been described as a putative protein due to the fact that this CT antigen has only been detected at the message level. As no commercial antibodies were available at the time of the study, antisera were produced in rabbits against HAGE peptides predicted to be antigenic *in silico* (Dr. A. Knights, Tuebingen University). These sera were controlled by Western-blotting on cell lysates generated from a previously described HAGE positive melanoma cell line and resulted in a band on the developed membrane with a mass in the region of 73kDa, the expected mass for the HAGE gene product. Bands of different sizes also showed up and this unspecificity was largely resolved by incubating the antisera overnight with rabbit liver powder at 4°C while shaking. Upon centrifugation, supernatants were harvested, tested and showed much improved specificity although further work is needed to investigate as to the nature of the contamination by 2D gel analysis, tryptic digestion and tandem mass spectrometry (Fig. 3.4). Once the quality and the specificity were confirmed, antisera were applied to normal tissue microarrays, multiple tumour tissue microarrays and tumour sections in immunohistochemistry staining procedures, and to melanoma cell lines in immunofluorescence protocols.

Multiple normal tissue microarrays were stained for HAGE expression following antigen retrieval, antiserum incubation and DAB staining. Fig. 3.5 demonstrates that whilst no significant staining of normal tissues was observed (Fig. 3.5A-K); normal testis tissues showed positive staining with the antiserum (Fig. 3.5L). Therefore, protein expression was shown to correlate with mRNA levels observed earlier by RT-Q-PCR. Importantly, HAGE could not be detected in the thymus by immunohistochemistry suggesting that HAGE-specific T cells would not be deleted by central tolerance mechanisms (Fig. 3.5J).

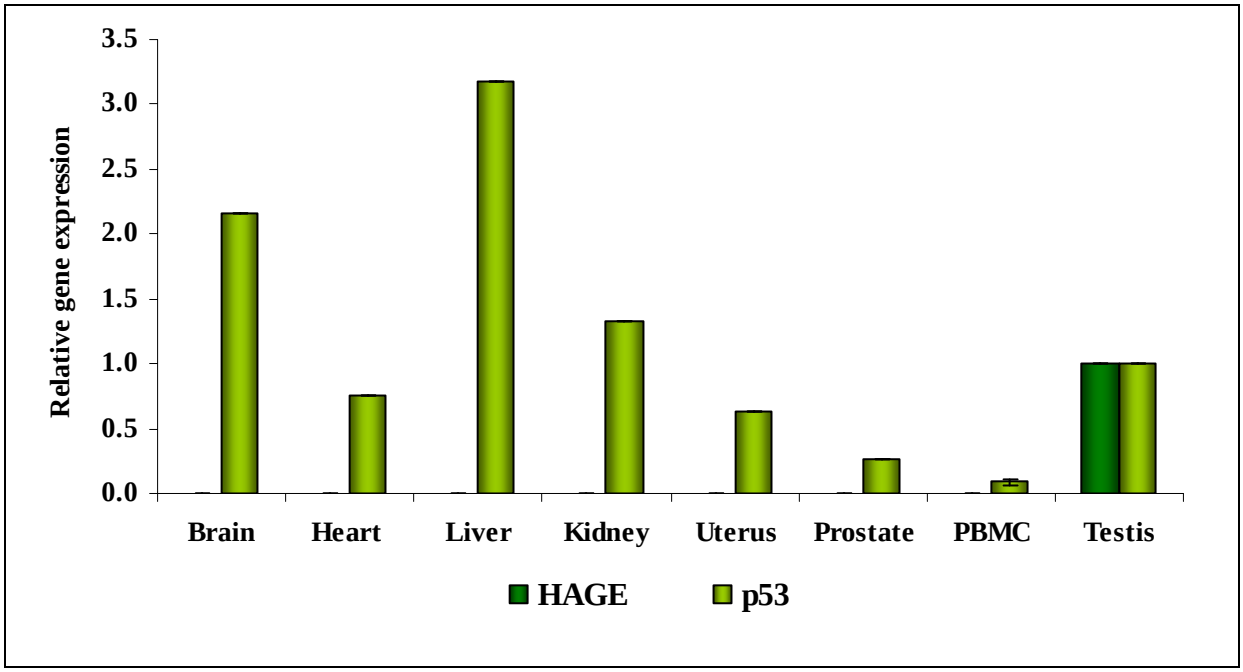

Figure 3.3: Expression of HAGE and p53 in human normal tissues by RT-Q-PCR. *Real time PCR analysis was carried out on 7 human normal tissues and 4 PBMC samples from healthy donors. The experiment was carried out once and data are expressed relative to the mRNA level of normal testis, arbitrarily set as 1.*

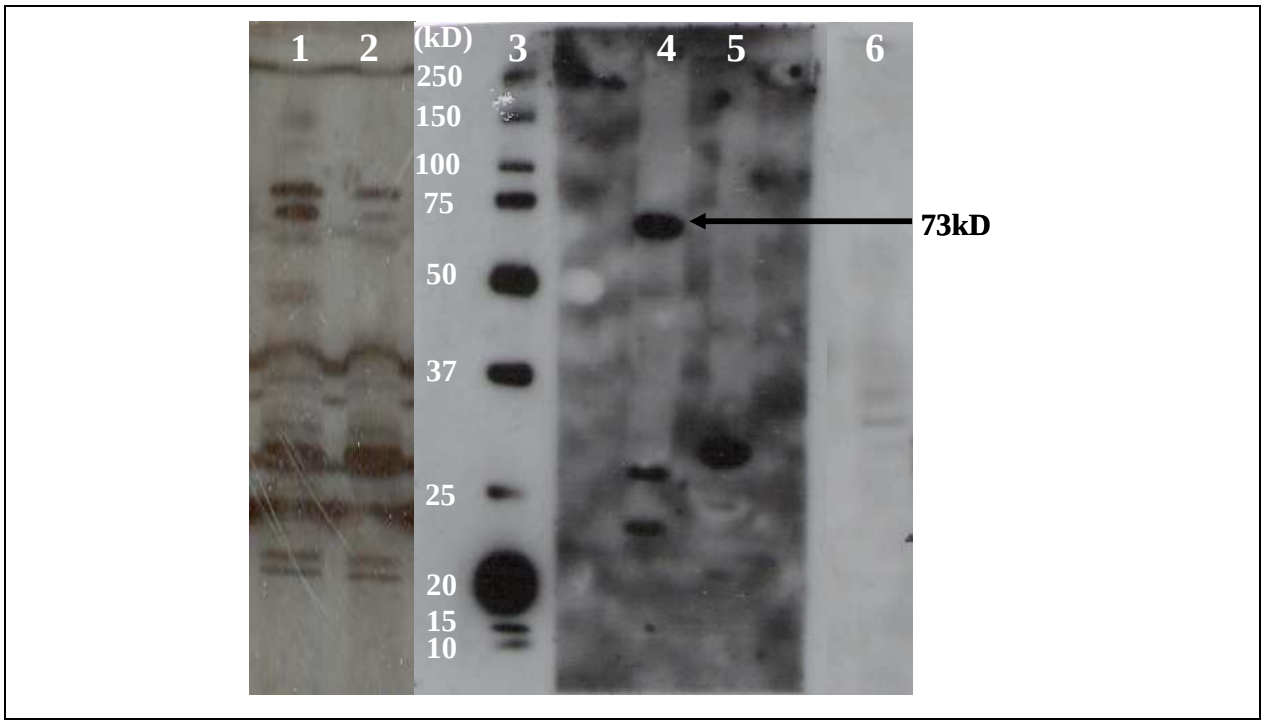

Figure 3.4: Western-blot of human melanoma cell lines. *30μg of protein was loaded in each lane and Western-blot performed by probing with anti-HAGE antibody, either neat (1 and 2) or after adsorption with rabbit liver powder (4, 5, and 6). (1) ESTDAB-27, (2) ESTDAB-07, (3) Protein marker, (4) ESTDAB-27, (5) ESTDAB-07 and (6) secondary antibody only.*

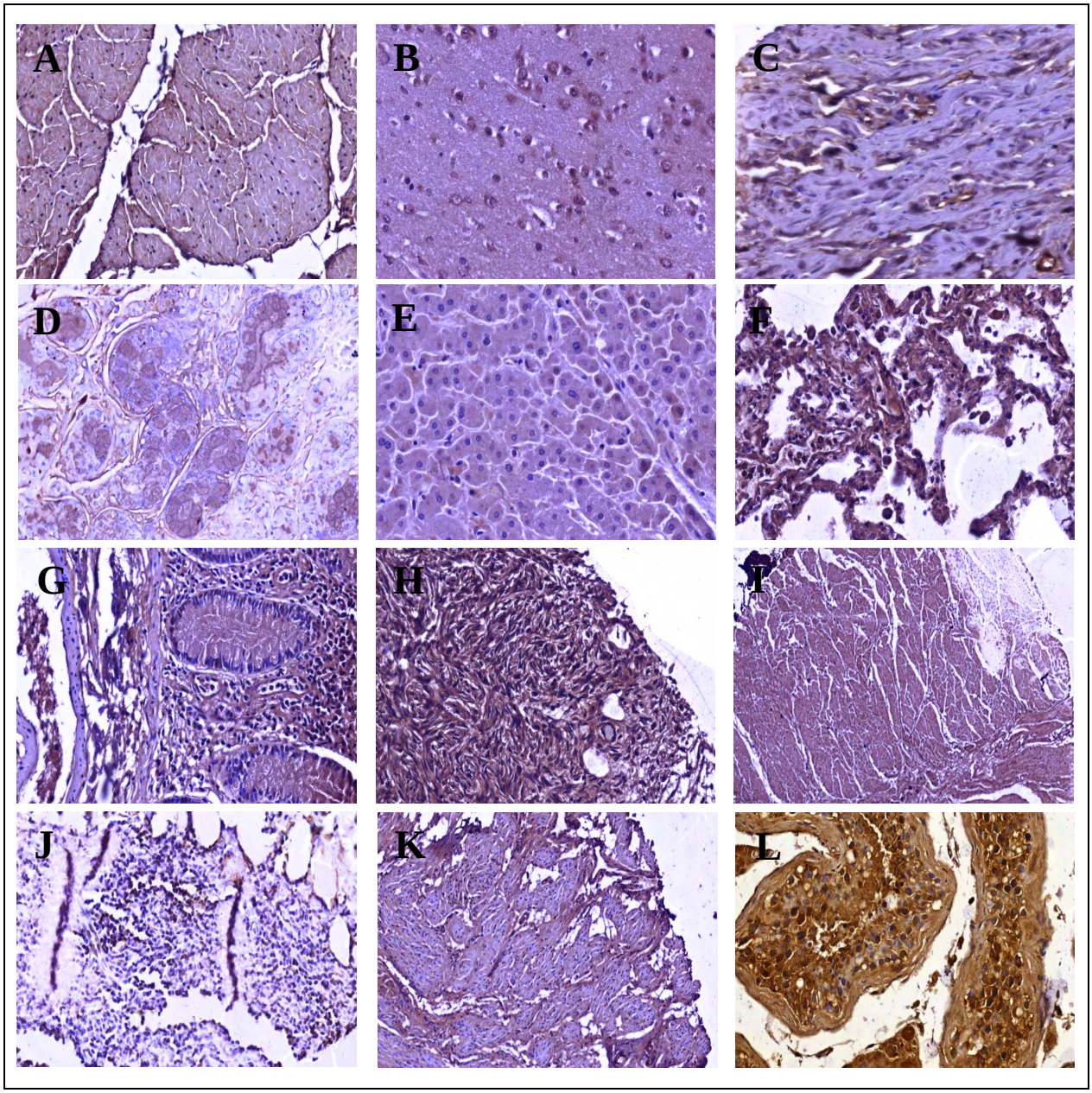

Figure 3.5: Immunohistochemistry staining for HAGE protein expression in multiple normal tissue microarrays. *Immunohistochemistry staining demonstrates the in vivo expression of HAGE at the protein level in testis (L), but not in bladder (A), brain (B), larynx (D), liver (E), lung (F), kidney (G), skin (I), thymus (J) or uterus (K). Some staining was obtained in oesophagus (C) and in ovaries (H). Objective magnification: x20.*

3.2.2 Expression of HAGE in human CML samples

Previously published studies have shown HAGE over-expression in human CML samples using conventional RT-PCR. In an attempt to confirm these results HAGE as a target for immunotherapy, HAGE expression was also investigated in CML samples using RT-Q-PCR. Two groups of ten patients with high and low Bcr/Abl:Abl ratio were assessed (Fig 3.6A and B, respectively). As seen in Fig. 3.6A, the relative expression of HAGE is 10 to 180 times the one observed in normal total blood cells (TBC) in 90% of CML samples with high Bcr/Abl:Abl ratio. On the other hand, the relative expression of HAGE was only 5 to 20 times that observed in TBC in 50% of CML samples with low Bcr/Abl:Abl ratio (Fig. 3.6B). It is also worth noting that the relative expression of p53 was quite low and constant in all these samples while the relative expressions of HAGE and Bcr/Abl were generally very similar. However, HAGE expression at the protein level remains to be confirmed in TBC of healthy and CML donors, as mRNA and protein expression was shown to be discordant in some cases (Rogel *et al.*, 1985; Chen *et al.*, 2002). The eventual correlation between HAGE expression and Bcr/Abl levels in CML patients will be discussed further in the discussion.

3.2.3 Expression of HAGE in solid tumour tissues

Other studies have not only focused on haematological malignancies but included solid tumours. Indeed, HAGE expression has been demonstrated to a lower proportion in brain, colon, lung and prostate cancers, among others (Martelange *et al.*, 2000; Nagel *et al.*, 2003). In this study, mRNA from breast, colon, gastric, and head & neck carcinoma were available with mRNA from patient-matched normal tissues allowing direct comparison of the levels of expression in both tissue types. Real time PCR was carried out and results were summarised in Fig. 3.7. The overall relative expression of HAGE is higher in normal breast tissues than in breast carcinoma. However, this might be largely influenced by the low number of samples provided (n=10) as well as the very low and/or poor quality RNA in six of these samples in which either the housekeeping gene and/or HAGE could not be detected. Individual patient variation could only be compared in four samples with two of them (Br6 and Br9) having a dramatic increase in HAGE expression of more than 10 times the expression in normal breast tissues (Fig. 3.7A). Increased number of breast samples might be required to conclusively suggest HAGE over-expression in breast carcinoma (See Appendix 2).

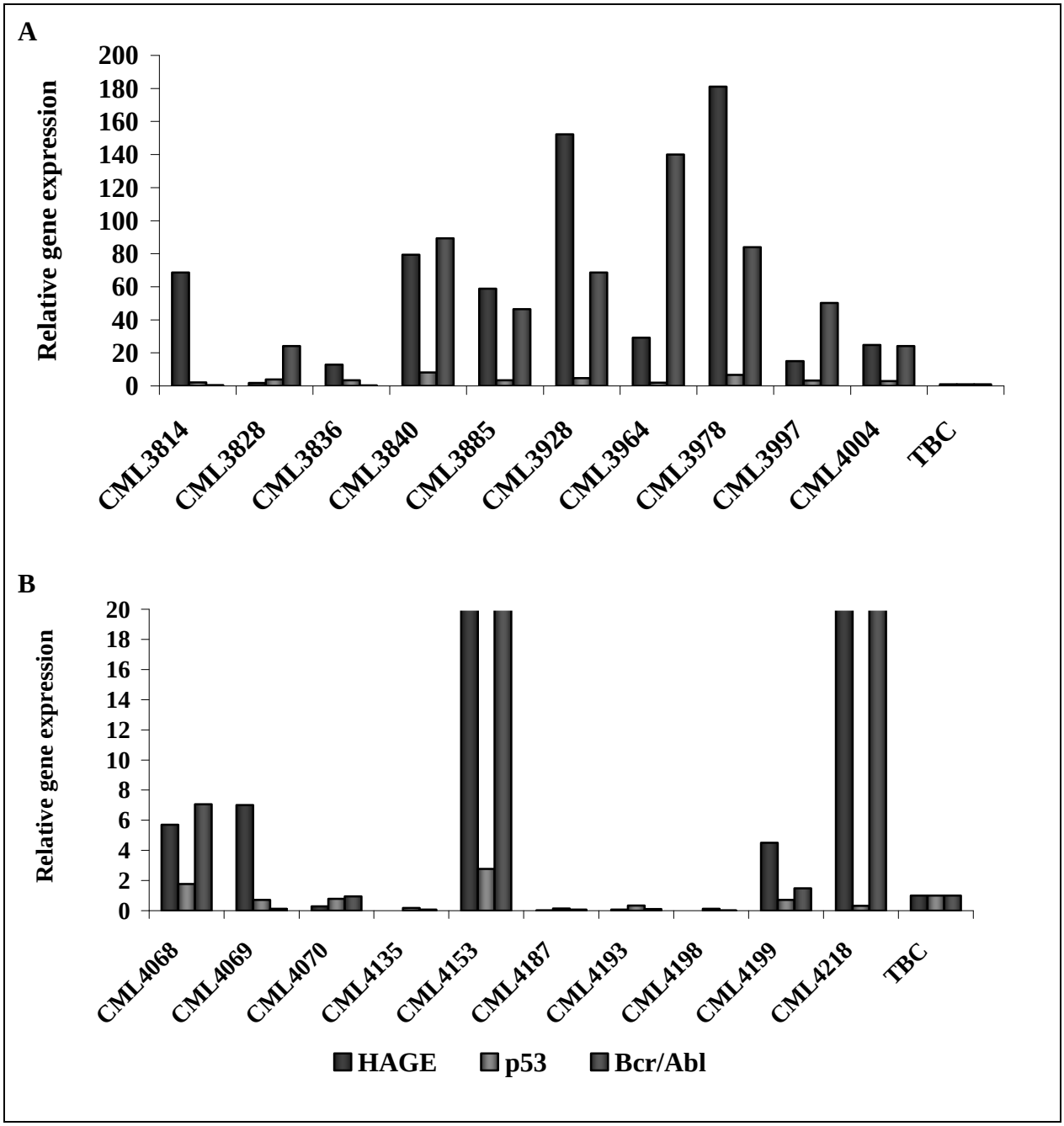

Figure 3.6: Expression of HAGE, p53 and Bcr/Abl in chronic myeloid leukaemia. *Real time PCR analysis was carried out on 20 CML samples and normal total blood cells (TBC). In A: 10 CML samples from patients with high Bcr/Abl:Abl ratio and in B: 10 CML samples with low Bcr/Abl:Abl ratio. The experiment was carried out once and data are expressed relative to the mRNA level of TBC, arbitrarily set as 1.*

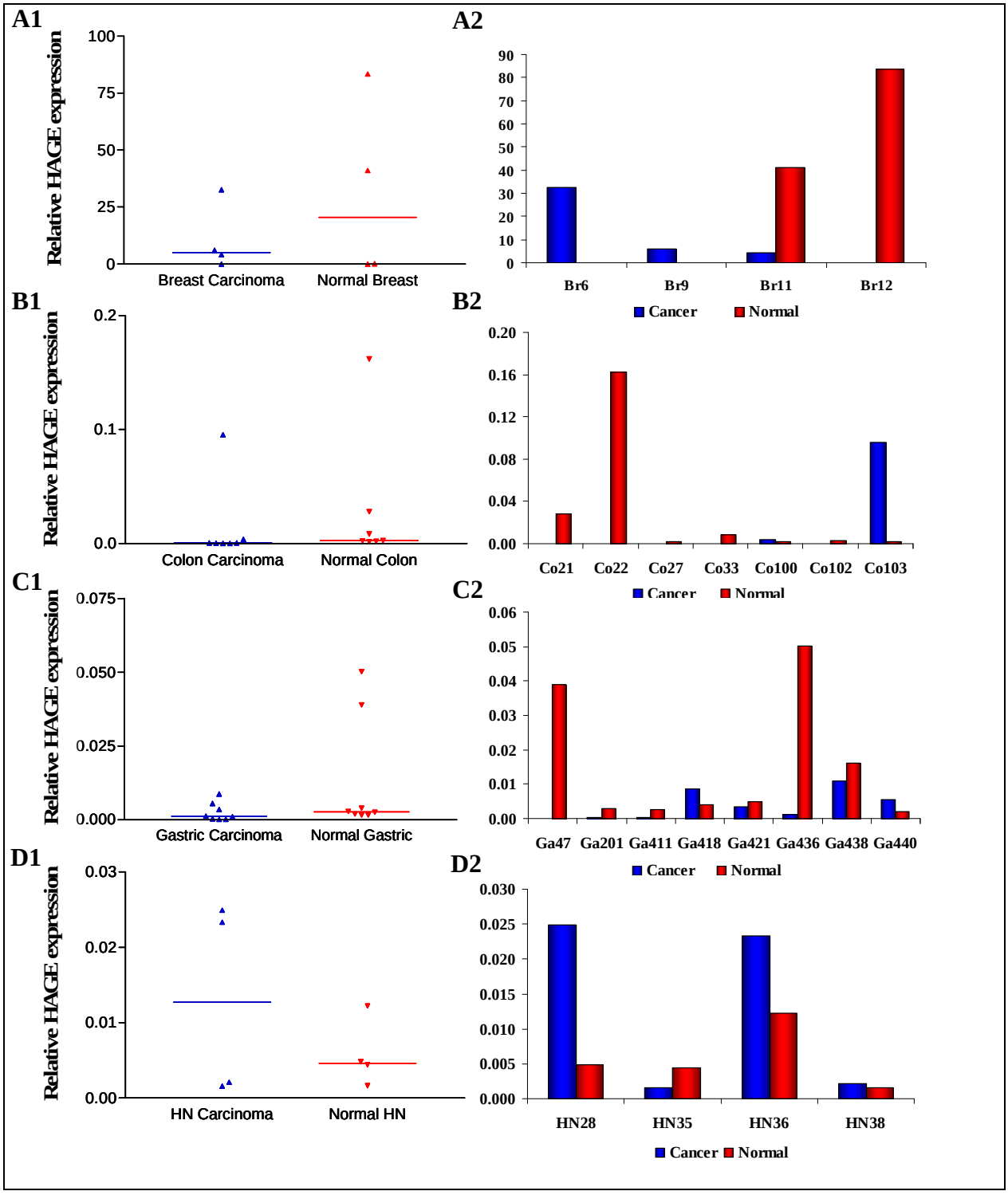

Figure 3.7: Expression of HAGE in different carcinoma and patient-matched normal tissues. *(1) Graph analysing tumour and normal tissues from patients. (2) Individual patient variation of HAGE expression. Analyses were carried out using breast (A), colorectal (B), gastric (C) and head & neck (HN) (D) carcinoma samples. Paired Student T tests were performed to compare statistical differences between carcinoma and patient-matched normal tissues. This experiment was carried out once.*

The relative expression of HAGE was not found to be statistically different between colorectal carcinoma and patient-matched normal colon tissues. Similarly to breast cancer tissues, this might be due to the low number of samples available (n=10) and the fact that HAGE expression could not be detected in some cancers. Indeed, individual HAGE expression levels showed an increase in two out of seven samples (Co100 and Co103), out of which patient Co103 had a clear increase (>10 times) (Fig. 3.7B). Interestingly, these two patients' colorectal carcinoma did not show any differences with the other patients in term of cancer stage or differentiation but in term of localisation as they are mainly situated in both the rectum and the sigmoid (See Appendix 2). However, more samples would be required to link HAGE expression with cancer localisation in patients with colorectal carcinoma. Similarly, results in gastric cancers and normal tissues did not show significant differences. However, a clear increase of HAGE expression can was observed in two out of nine samples. Indeed, only Ga418 and Ga440 respectively presented a doubling and a trebling of HAGE expression in gastric cancer tissues (Fig. 3.7C). Unfortunately, HAGE expression level could not be correlated with cancer grade, differentiation status or localisation (See Appendix 2).

Finally, four head & neck carcinoma with patient-matched normal tissues were also available and results showed that average HAGE expression was increased in head & neck cancer samples as compared to normal head & neck tissues but did not reach the level of statistical significance. Moreover, HAGE expression in the patient-matched normal tissues was multiplied by two to five folds in the cancer tissues of patients HN28 and HN36 (Fig. 3.7D). These results led us to further investigate the expression of HAGE at the mRNA level in head & neck carcinoma. Through collaboration with Dr. Robert Ferris (Pittsburgh University), we were able to obtain a further ten patient cDNA samples in order to confirm and extend these results. Interestingly, 40% of these samples showed a dramatic increase of HAGE expression in HN03-1036, HN04-1649, HN05-1833 and HN05-1842 samples being 1.5, 14, 6.5 and 2 times the HAGE expression in testis, respectively (Fig. 3.8). It is noteworthy to say that p53 was also over-expressed but to a lesser extent than HAGE in 40% of the samples tested. Following these results, paraffin-embedded tissue sections from these four patients plus one negative control (HN05-1903) were obtained and immunohistochemistry was carried out in order to confirm not only the expression of HAGE at the message level but also at the protein level. Positive staining was obtained in HN04-1649, HN05-1833 and HN05-1842 with the staining intensity correlating with the message intensity observed earlier. On the other hand, both HN03-1036 and the negative control HN05-1903 did not demonstrate any staining at all (Fig. 3.9).

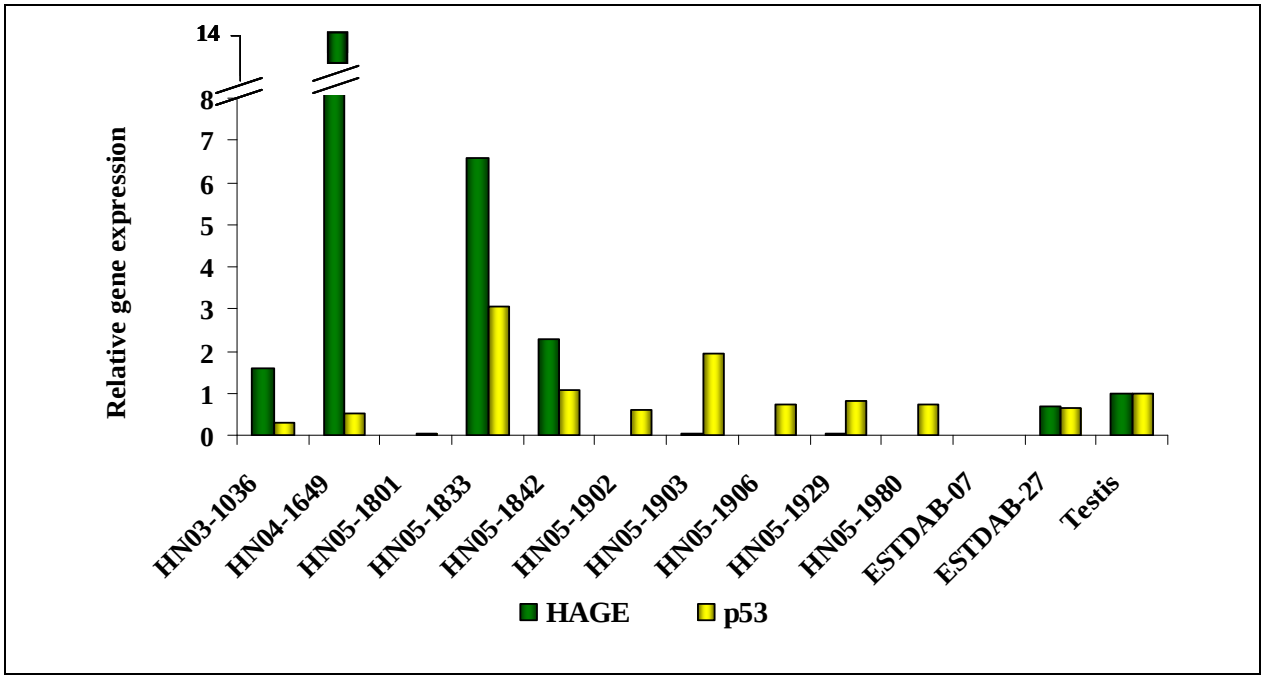

Figure 3.8: Expression of HAGE and p53 in head & neck carcinoma. *Real time PCR analysis of 10 primary tumours from patients with head & neck carcinoma, ESTDAB-07 (negative control), ESTDAB-27 and testis (positive controls). The experiment was carried out once and data are expressed relative to the mRNA level of normal testis, arbitrarily set as 1.*

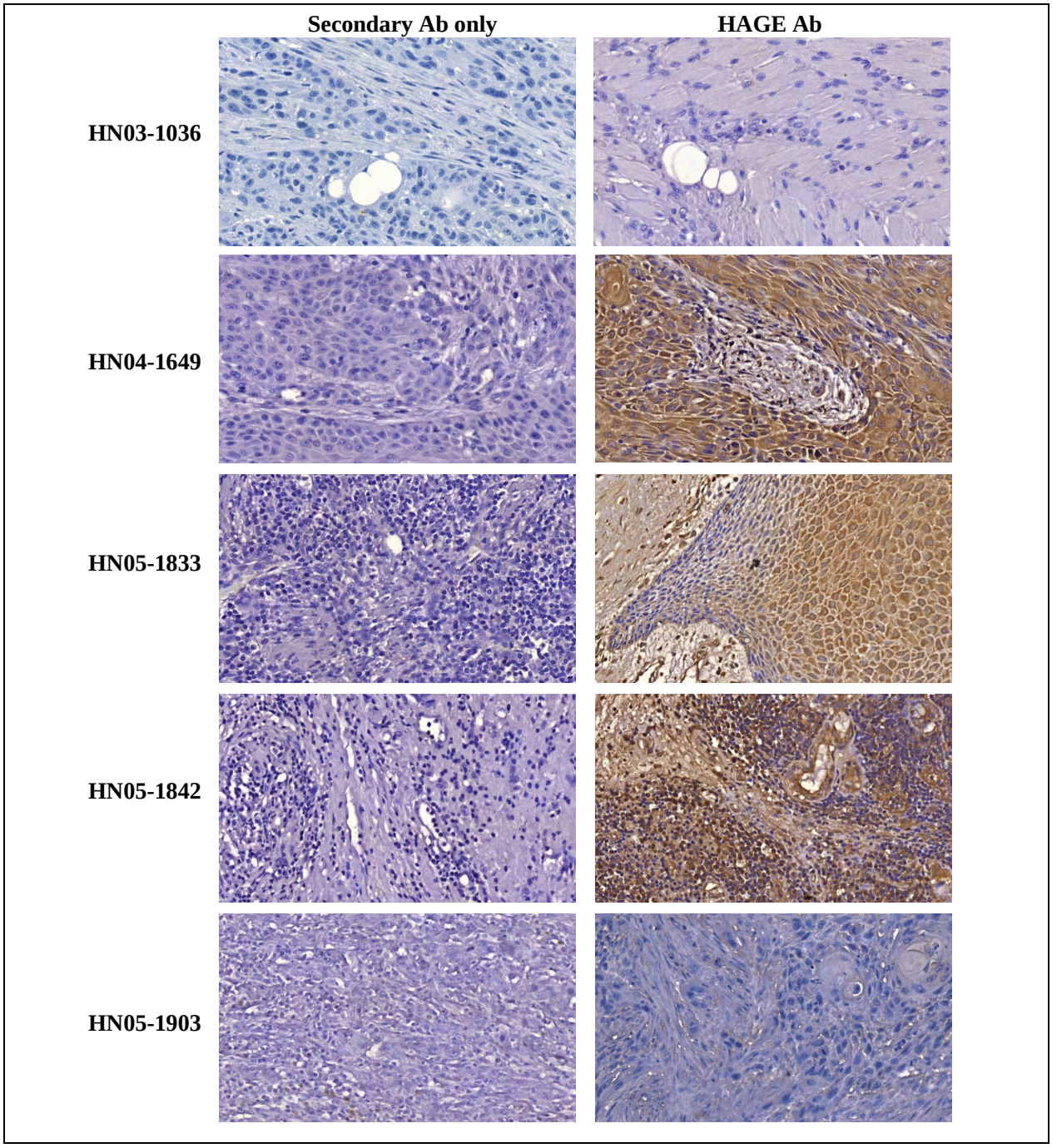

Figure 3.9: Immunohistochemistry staining for HAGE protein expression in head & neck carcinoma sections. *Immunohistochemistry staining demonstrates the in vivo expression of HAGE at the protein level in HN04-1649, HN05-1833 and HN05-1842, but not in HN03-1036 and HN05-1903 section. No non-specific secondary staining was obtained in all samples. Objective magnification: x20.*

The lack of HAGE expression at the protein level in HN03-1036 could be explained by the fact that this sample showed the lowest HAGE expression at the message level or that not all the mRNA was translated into protein. Further samples and clinical data will therefore be needed to confirm this hypothesis and investigate the link if any between cancer stage, differentiation or localisation and HAGE expression.

Using an identical study process, ten tissues from melanoma patients were analysed. Total RNA was extracted for subsequent RT-Q-PCR analysis and frozen tissue sections were prepared using a cryostat when possible for immunohistochemistry staining. Relative HAGE expression in these samples was compared with the relative HAGE expression in a HAGE-negative melanoma cell line (ESTDAB-07), HAGE-positive melanoma cell line (ESTDAB-27) and testis, which value was arbitrarily set as one. In two of these samples (Mel781 and Mel793), relative HAGE expression almost reached two times the one in testis and three times the one in ESTDAB-27. Moreover, relative HAGE expression was also detected in three other samples but to a lesser extent (Mel429, Mel488 and Mel512)(Fig. 3.10). HAGE staining by immunohistochemistry was performed on six of these tissue samples and demonstrated positive coloration in Mel429, Mel488 and Mel793. Samples that were negative for HAGE expression at the message level remained negative at the protein level (Mel789, Mel791 and Mel816)(Fig. 3.11).

Finally, immunohistochemistry was performed on multiple cancer microarrays in order to find out whether HAGE is also expressed at the protein level in other forms of cancers. Cancers that have not been described earlier in the litterature or that are difficult to obtain were selected and studied. Bladder, liver, lung, thymus, uterus and testis cancers all showed positive staining. Interestingly and in correlation with earlier experiments, upper jaw cancer, which is a form of head & neck carcinoma, colon and skin cancers were also positively stained. In contrast, kidney and two other forms of head & neck carcinoma (cheek and larynx) did not indicate any staining (Fig. 3.12). Expression differences were evaluated and scored on an inverted microscope using the H-score system representing levels of staining intensity (See Section 2.2.1.10). H-scores of normal and abnormal tissues as well as head & neck carcinoma and melanoma samples were summarised in table 3.1.

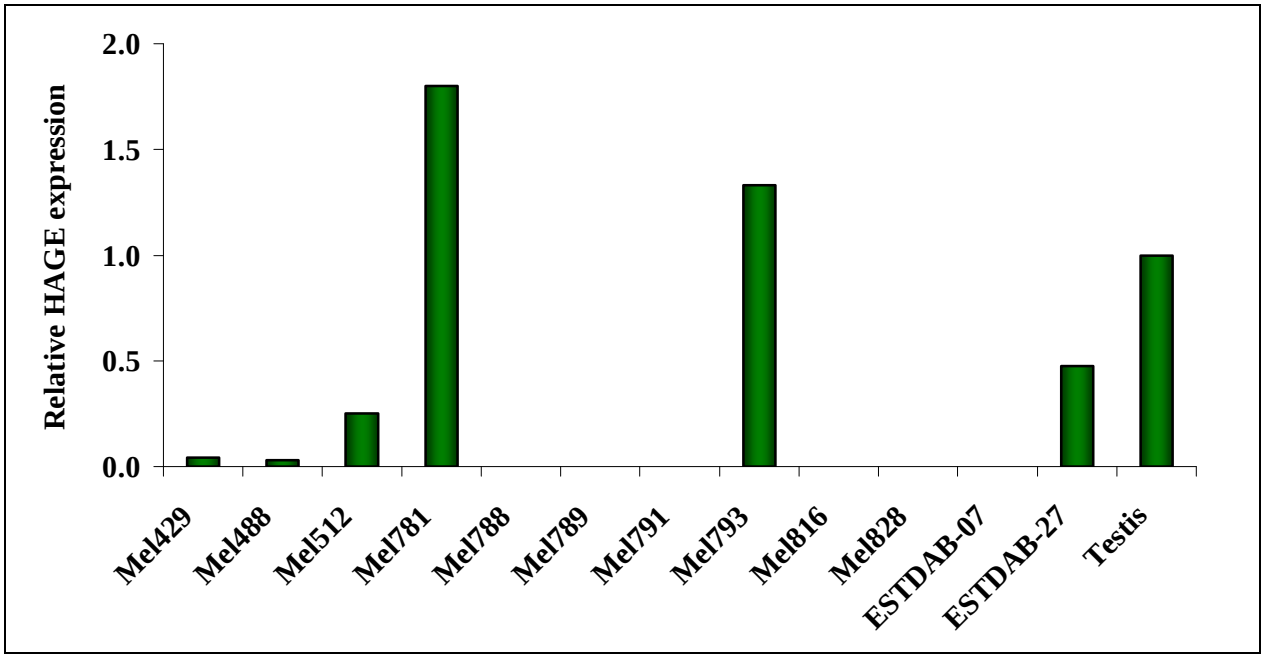

Figure 3.10: Expression of HAGE in melanoma. *Real time PCR analysis of 10 primary tumours from patients with melanoma, ESTDAB-07 (negative control), ESTDAB-27 and testis (positive controls). The experiment was carried out once and data are expressed relative to the mRNA level of normal testis, arbitrarily set as 1.*

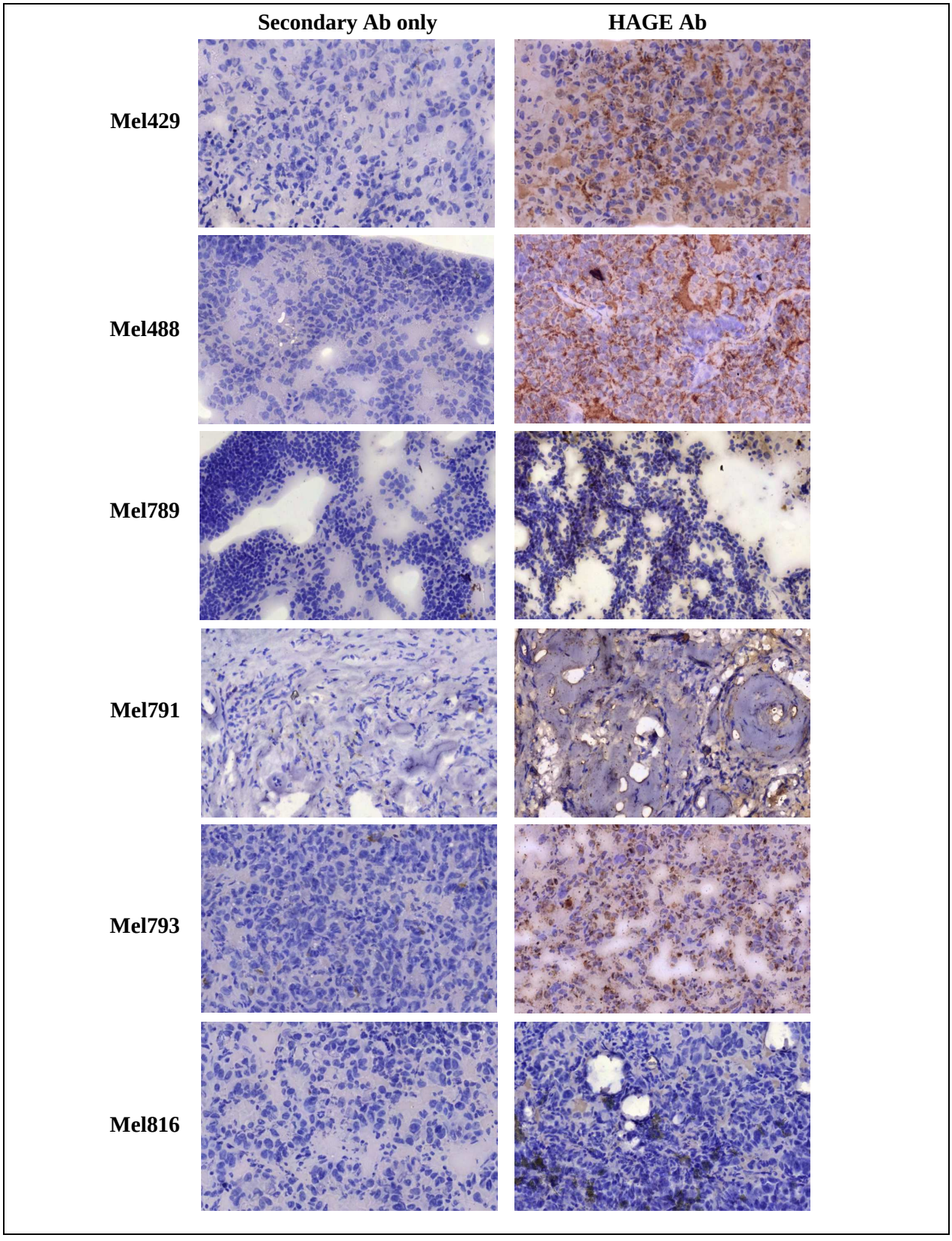

Figure 3.11: Immunohistochemistry staining for HAGE protein expression in melanoma sections. *Immunohistochemistry staining demonstrates the in vivo expression of HAGE at the protein level in Mel429, Mel488 and Mel793, but not in Mel789, Mel791 and Mel816 samples. No non-specific secondary staining was obtained in all samples. Objective magnification: x20.*

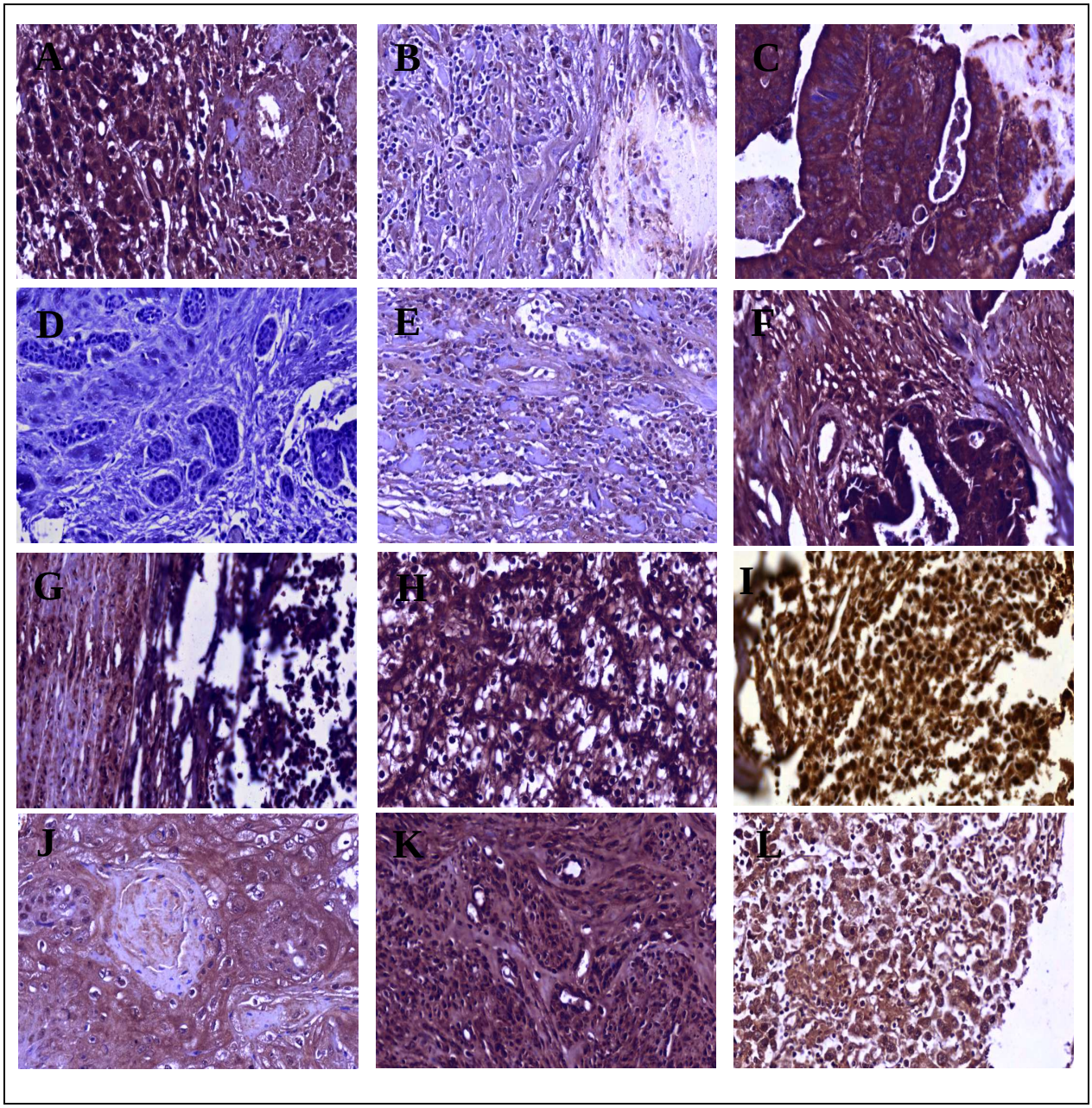

Figure 3.12: Immunohistochemistry staining for HAGE protein expression in multiple cancer tissue microarrays. *Immunohistochemistry staining demonstrates the in vivo expression of HAGE at the protein level in bladder tumour (A), colon tumour (C), liver tumour (F), lung tumour (G), melanoma (H), thymus tumour (I), upper jaw tumour (J), uterus tumour (K) and seminoma (L), but not in cheek tumour (B), kidney tumour (D) or larynx tumour (E). Objective magnification: x20.*

Table 3.1: Scoring of immunohistochemistry sections

Normal tissues (Tissue/H-score)	Malignant tissues (Tissue/H-score)	Head&neck carcinoma (Samples/H-score)	Melanoma (Samples/H-score)
Bladder / 0	Bladder / 150	HN03-1036 / 0	Mel429 / 10
Brain / 0	Cheek / 0	HN04-1649 / 210	Mel488 / 5
Larynx / 0	Colon / 240	HN05-1833 / 100	Mel789 / 0
Liver / 0	Kidney / 0	HN05-1842 / 160	Mel791 / 0
Lung / 0	Larynx / 0	HN05-1903 / 0	Mel793 / 20
Kidney / 0	Liver / 150		Mel816 /0
Oesophagus / 0	Lung / 150		
Ovaries / 0	Skin / 300		
Skin / 0	Thymus / 300		
Thymus / 0	Upper jaw / 50		
Uterus / 0	Uterus / 300		
Testis / 300	Testis / 300		

Thus, HAGE seems to be expressed at higher levels in tumour cells than in normal cells and it is also noteworthy to mention that HAGE seems to be for the majority located in the nucleus according to the immunohistochemistry study carried out here, although granular staining inside the cytoplasm could also be observed when sections were looked at higher magnification. This suggests that HAGE could be exploited as a target for immunotherapy where the immune response would be predominantly directed against tumour cells with no serious risk of autoimmune reactions.

3.2.4 Expression of HAGE in human cancer cell lines

Taking advantage of the availability of a large number of CML, melanoma and head & neck carcinoma cell lines, analysis of HAGE expression was carried out using RT-Q-PCR. Seven CML, five melanoma and four head & neck carcinoma cell lines were grown under similar culture conditions and total RNA was extracted. Upon reverse transcription, real time PCR was performed and showed interesting results. Indeed, all CML cell lines expressed HAGE at the same level as normal total blood cells (Fig. 3.13). However, expression of HAGE in these cell lines proved to be extremely low when compared to testis (Data not shown).

This result is in correlation with a recent study by Roman-Gomez *et al.*, (2007), which investigated the methylation status of the HAGE promoter in CML cell lines and CML patients in chronic or blast crisis. HAGE promoter appeared to be strongly hypermethylated in CML cell lines in contrast with CML cells from patients, which would influence its mRNA expression.

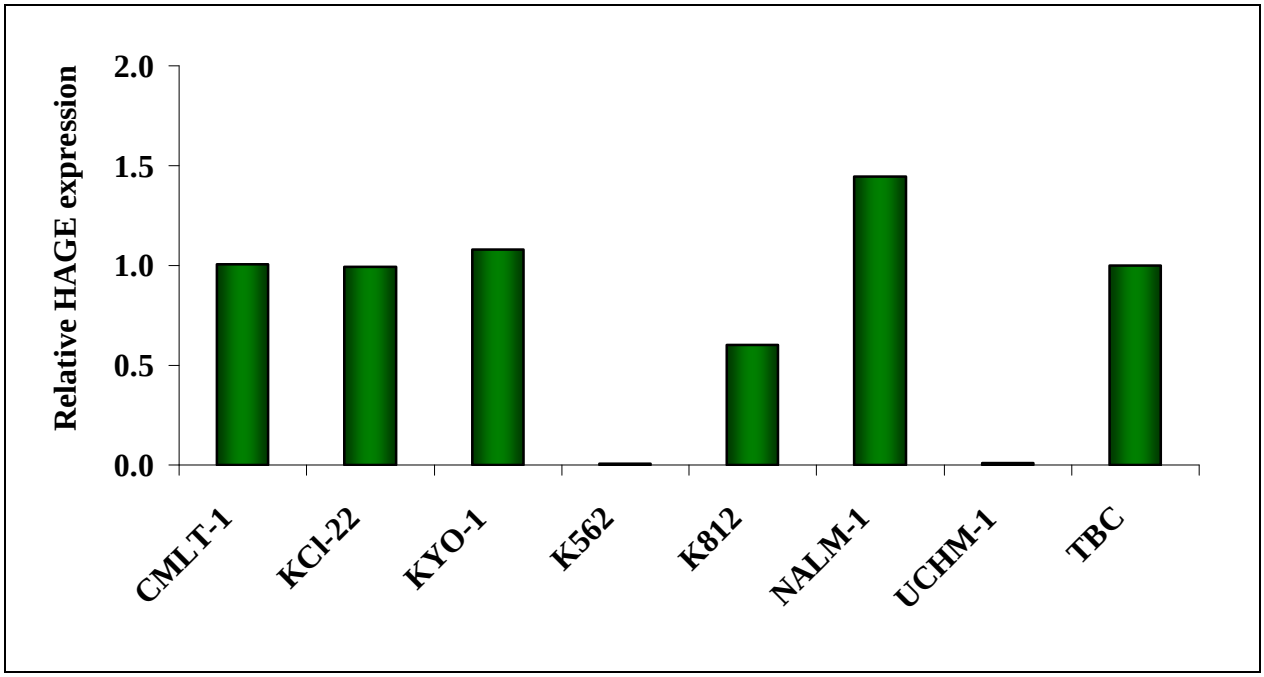

Figure 3.13: Relative expression of HAGE in CML cell lines. *Real time PCR analysis of 7 CML cell lines. The experiment was carried out once and data are expressed relative to the mRNA level of TBC, arbitrarily set as 1.*

Following this observation, K562 and Kyo-1 cells were treated with a demethylating agent 5'-aza-2'-deoxycytidine (AZAC), together or not with a deacetylase inhibitor called trichostatin A (TSA) in order to determine whether HAGE repression could be reversed, at least at the mRNA level. The demethylating agent increased considerably the expression of HAGE in the CML cell lines tested. Trichostatin A did not have any effects on its own but seemed to act synergistically when co-incubated with AZAC leading to an even more impressive increase of HAGE expression (Fig. 3.14). Moreover, differences between cells treated with AZAC and TSA and cells treated with diluent only were statistically significant. It is also worth mentionning that because of the nature of these treatments, cells stopped proliferating as demonstrated by tritiated thymidine incorporation (Data not shown).

High levels of HAGE mRNA expression was observed in ESTDAB-17, -27, -34 and -95 although to a lower extent than in testis, whereas ESTDAB-07 did not express HAGE at all. Interestingly, HAGE mRNA could not be detected in most of the head & neck cell lines tested (BB30, BB49 and ILUS) or only at a very low level (SENY) when compared with testis (Fig. 3.15). Again, the specific promoter methylation status of cancer/testis antigens such as HAGE in solid tumour cell lines might be involved in its expression or rather its lack of expression and explain these results. These results might lead us to be careful when working with cell lines as opposed to tumour tissues. This finding will be addressed further in the discussion.

As mentionned previously, the cancer/testis antigen HAGE had been described as a putative protein and no studies have actually demonstrated the translation of HAGE mRNA into protein. In order to determine the localisation pattern of HAGE inside the cells, HAGE-negative and HAGE-positive melanoma cell lines were grown in a multi-chamber slide, fixed, permeabilised and observed by immunofluorescence after staining for HAGE. Secondary antibody tagged with fluorescein highlights the expression and localisation of HAGE within the cells. High levels of protein could be detected in ESTDAB-17, -27, -34 and -95 (Fig. 3.16C, 3.16D, 3.16E and 3.16F, respectively) but none in ESTDAB-07 (Fig. 3.16B) and none in the secondary alone (Fig. 3.16A). HAGE was observed to be localised mainly in the cytoplasm, at least in this melanoma cell line, which again seems to be in opposite to the results obtained with fresh tissues.

3.2.5 Role of HAGE

Little is known about HAGE function in the cell. Roles in the control of the cell cycle or in the RNA metabolism have been so far suggested and hypotheses have mainly relied on the study of its protein structure and its conserved domains.

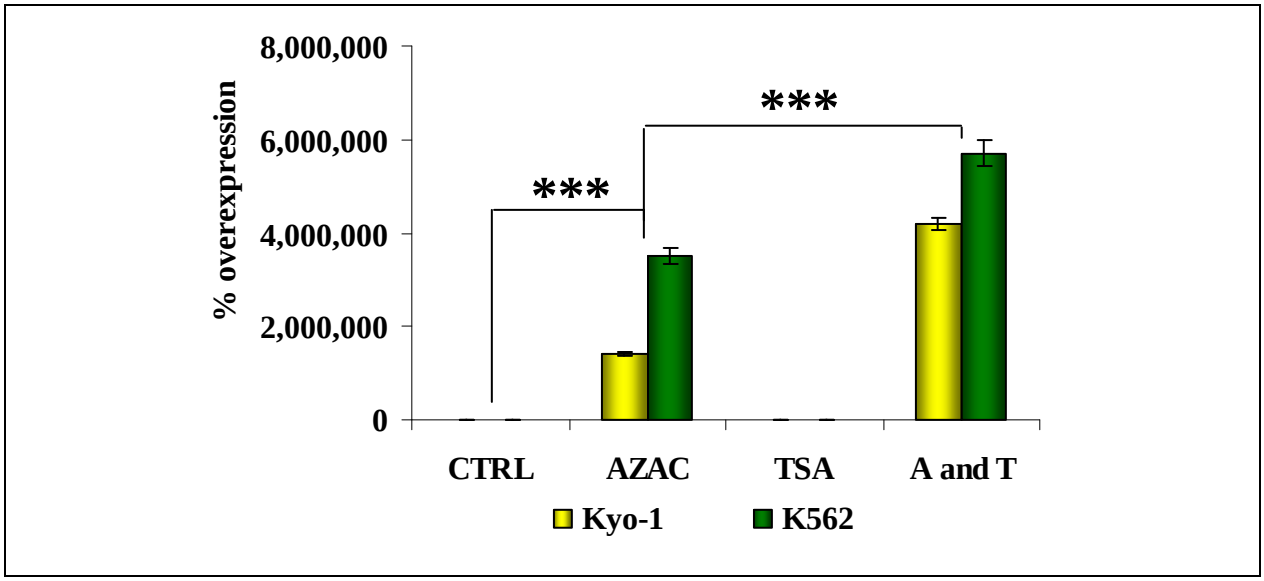

Figure 3.14: Relative HAGE expression in CML cell lines following demethylation and/or inhibition of deacetylase. *Real time PCR analysis showed that treatment with the demethylating agent AZAC (A) of CML cell lines increased dramatically the relative expression of HAGE, and that this effect was synergistically enhanced when cells were also treated with the deacetylase inhibitor TSA (T). This experiment was carried out once in triplicates (n=1). *** $p<0.001$ is the statistical difference between control cells and cells treated with AZAC, and between cells treated with AZAC and cells treated with both AZAC and TSA determined by paired Student T test.*

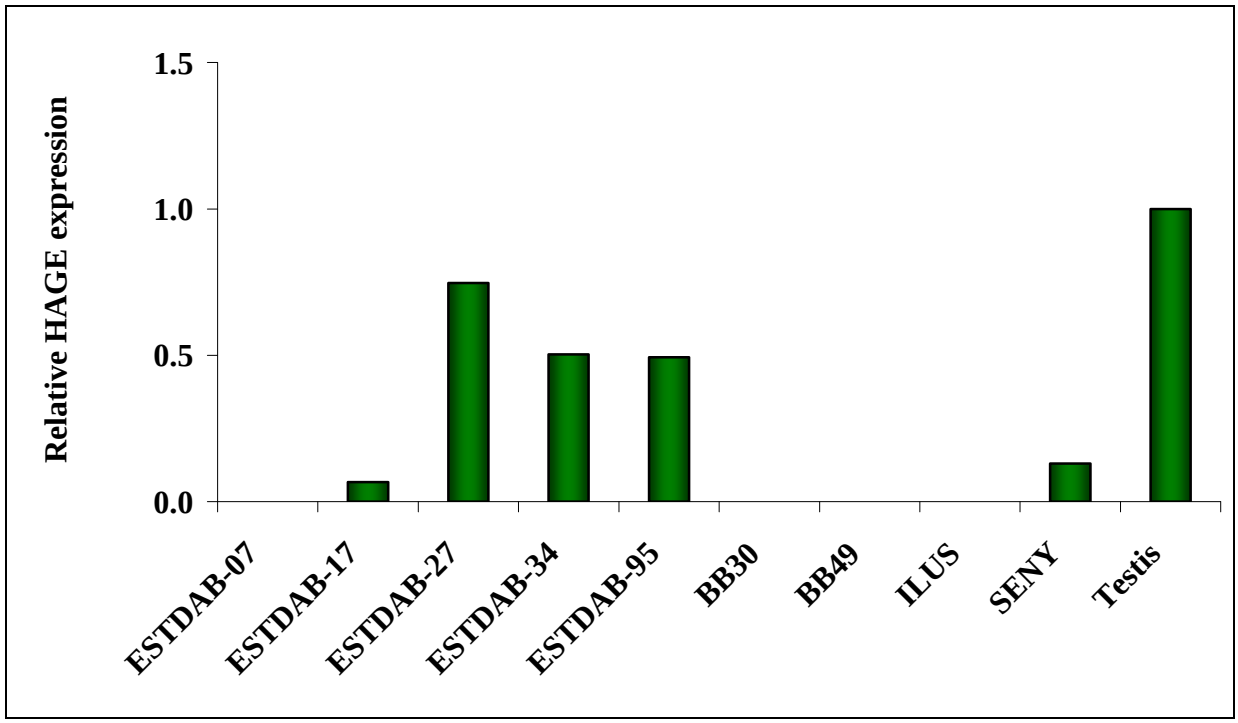

Figure 3.15: Expression of HAGE in melanoma and head & neck carcinoma cell lines. *Real time PCR analysis of 5 melanoma cell lines (ESTDAB-07, -17, -27, -34 and -95) and 4 head & neck carcinoma cell lines (BB30, BB49, ILUS and SENY). The experiment was carried out once and data are expressed relative to the mRNA level of normal testis, arbitrarily set as 1.*

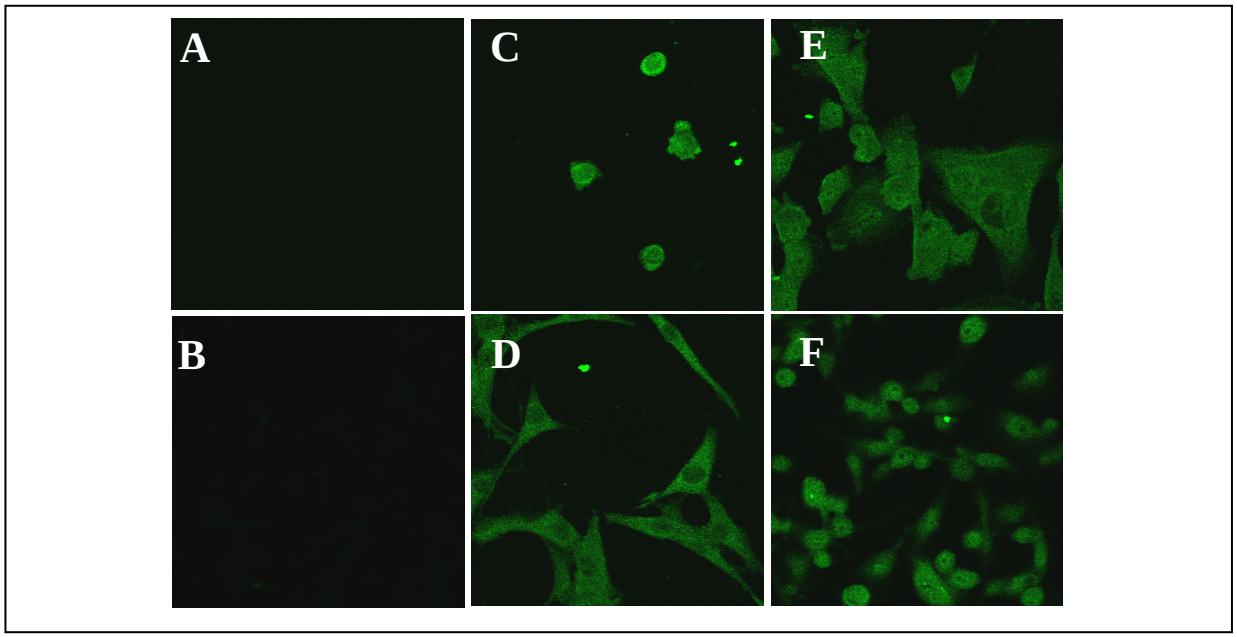

Figure 3.16: Immunofluorescence assay for HAGE protein expression analysis and localisation in melanoma cell lines. *Immunofluorescence was observed under confocal microscope in HAGE-positive ESTDAB-17 (C), -27 (D), -34 (E) and -95 (F) but not in HAGE-negative ESTDAB-07 (B). No non-specific secondary antibody staining was obtained (A). Cytoplasmic HAGE protein localisation can be observed. Objective magnification: x40.*

In order to further investigate the eventual function of HAGE inside the cells, a series of experiments based on the use of siRNA to silence HAGE expression or the use of plasmid encoding HAGE to express HAGE have been designed. Briefly, HAGE-positive ESTDAB-27 melanoma cells were grown in a 24-well plate. Once 60% confluence was reached, cells were transfected with either control siRNA or HAGE siRNA mixed with the transfection reagent Interferin. Total RNA was extracted after one, three and seven days in order to check HAGE silencing at the message level by RT-Q-PCR. As seen in Fig. 3.17A, two HAGE siRNA were tested and both appeared equally efficient in silencing HAGE as about 90% knockdown was achieved after 24 hours. Moreover, HAGE expression was not affected by the control siRNA or the treatment of the cells with the transfection reagent only. A time course was then carried out in order to see the duration of the silencing and a decrease to 60% and 50% knockdown was observed after three and seven days, respectively (Fig. 3.17B). In the same period, cells in culture were fixed, permeabilised, stained for the expression of HAGE and observed under confocal microscope as described in section 3.2.2 in order to confirm HAGE silencing at the protein level.

Twenty-four hours after HAGE siRNA transfection, cells still showed fluorescent staining under confocal microscope but this signal dramatically decreased to almost total disappearance three days after HAGE siRNA transfection (Fig. 3.17C). This indicates that not only HAGE mRNA knockdown after 24 hours was successfully obtained but also total HAGE protein silencing after three days, offering a window of opportunity to observe the effects of HAGE silencing on the cells.

HAGE silencing was therefore repeated and cell morphology was recorded under microscope. The cells did not show any structural alterations and did not seem to go under either apoptosis or necrosis. They remained adherent and only their proliferation seemed to be changed (Data not shown). Following these observations, a proliferation assay relying on the incorporation of tritiated thymidine was performed three and seven days following HAGE siRNA transfection. Proliferations were then compared with cells treated with the transfection reagent only and with cells transfected with a control siRNA. Fig. 3.18 shows a statistically significant decrease of the proliferation of cells transfected with HAGE siRNA when compared with cells transfected with the control siRNA. This decrease occurred three days after transfection but not after seven days. This is probably due to the reduced effect of HAGE siRNA on HAGE mRNA expression and the reinstallement of the normal production of HAGE proteins.

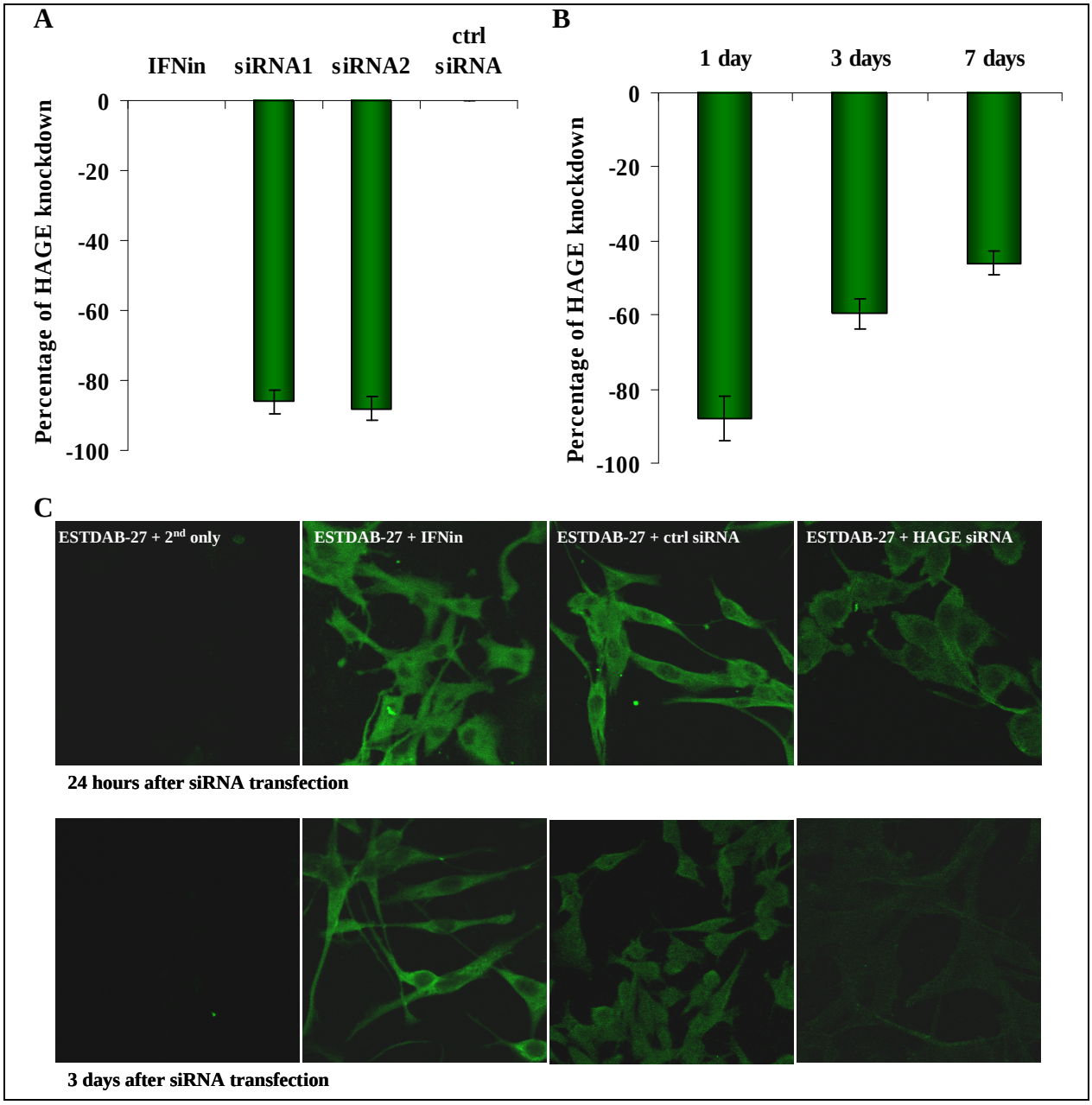

Figure 3.17: HAGE silencing of a HAGE-positive melanoma cell line. *(A) Real time PCR analysis showing the percentage of HAGE knockdown following transfection of a HAGE-positive melanoma cell line with 2 different HAGE siRNA. (B) Time course carried out by real time PCR indicating HAGE silencing evolution against time. RT-Q-PCR results shown are representative of two independent experiments carried out in triplicates (n=2). (C) Immunofluorescence assay demonstrating reduced HAGE protein expression three days after transfection, but not after 24 hours. Objective magnification: x40.*

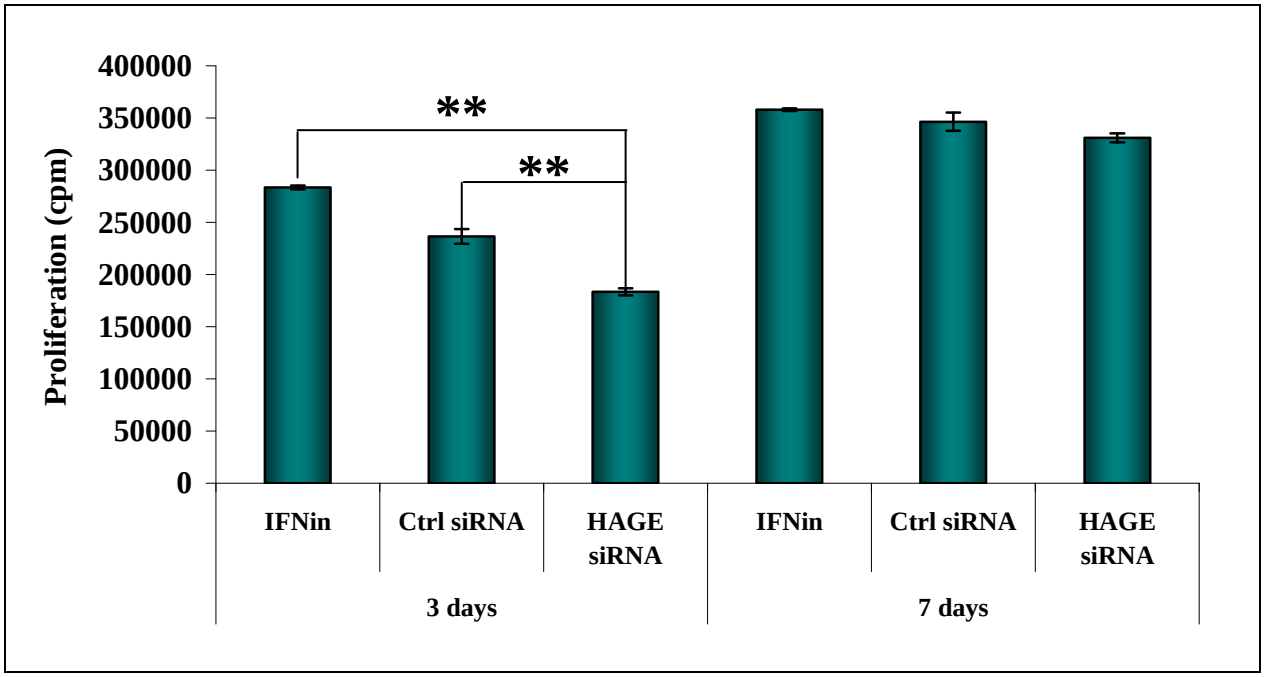

Figure 3.18: Proliferation assay following HAGE silencing. *Reduced proliferation of HAGE-positive melanoma cell line was observed upon HAGE silencing. This experiment was carried out twice in triplicates (n=2). ** $p<0.01$ is the statistical difference between cells transfected with a control siRNA and HAGE siRNA determined by paired Student T test.*

Moreover, siRNA transfection with or without the help of cationic lipids such as the transfection reagent Interferin used in this experiment can potentially induce an interferon-based response affecting the expression of both target gene and interferon-responsive genes, and the interpretation of the results. Oligoadenylate synthase 1 (OAS1) and signal transduced and activator of transcription 1 (STAT1) are two interferon-responsive genes, the expression of which were shown to be altered upon siRNA transfection (Fish *et al.*, 2004; Ma *et al.*, 2005). In this experiment, both OAS1 and STAT1 showed no or very little changes in expression by real time PCR (Data not shown). These results indicate that the effects observed earlier with RNA interference on HAGE-positive cells are not interferon-related or due to the transfectant but are indeed caused by HAGE silencing.

Following on from these results, a reverse experiment was carried out whereby HAGE-negative ESTDAB-07 melanoma cells were grown in a 24-well plate and transiently transfected with the pBudCE4.1 plasmid encoding HAGE cDNA mixed with the transfection reagent Lipofectamine 2000. Upon transfection, ESTDAB-07 cells expressed HAGE at a much higher level than HAGE-positive ESTDAB-27 cells (Fig. 3.19A). Moreover, the lack of HAGE expression was not affected by the treatment of the cells with Lipofectamine 2000. In the same way as described earlier, a time course was carried out, the results of which indicated that HAGE mRNA was strongly present one and three days following transfection. On the other hand, HAGE could barely be detected seven days following transfection (Fig. 3.19B). Immunofluorescence then confirmed the translation of HAGE mRNA into a protein two days after transfecting ESTDAB-07 cells with no real difference in term of fluorescence intensity when compared to control ESTDAB-27 cells (Fig. 3.19C); the efficiency of HAGE cDNA expression was therefore confirmed. The experiment was repeated and, non-transfected and transfected ESTDAB-07 cells were this time labelled with tritiated thymidine to measure their proliferation. Fig. 3.20 shows a statistically significant increase of the proliferation of ESTDAB-07 cells transfected with HAGE cDNA after seven days. This result correlates with the results obtained when HAGE was silenced suggesting that HAGE is not only expressed in some tumour cells but also involved in the proliferation of the latter. Martelange *et al.* (2000) suggested an eventual function of HAGE in the control of the cell cycle and this would correlate with the results described above. Propidium iodide was therefore used to stain cells transfected with either control or HAGE siRNA in order to establish the consequences of HAGE silencing on the cell cycle. Following staining, FACS was used for the analysis of the cells. Interestingly, HAGE silencing did not affect any of the phases composing the cell cycle (Data not shown).

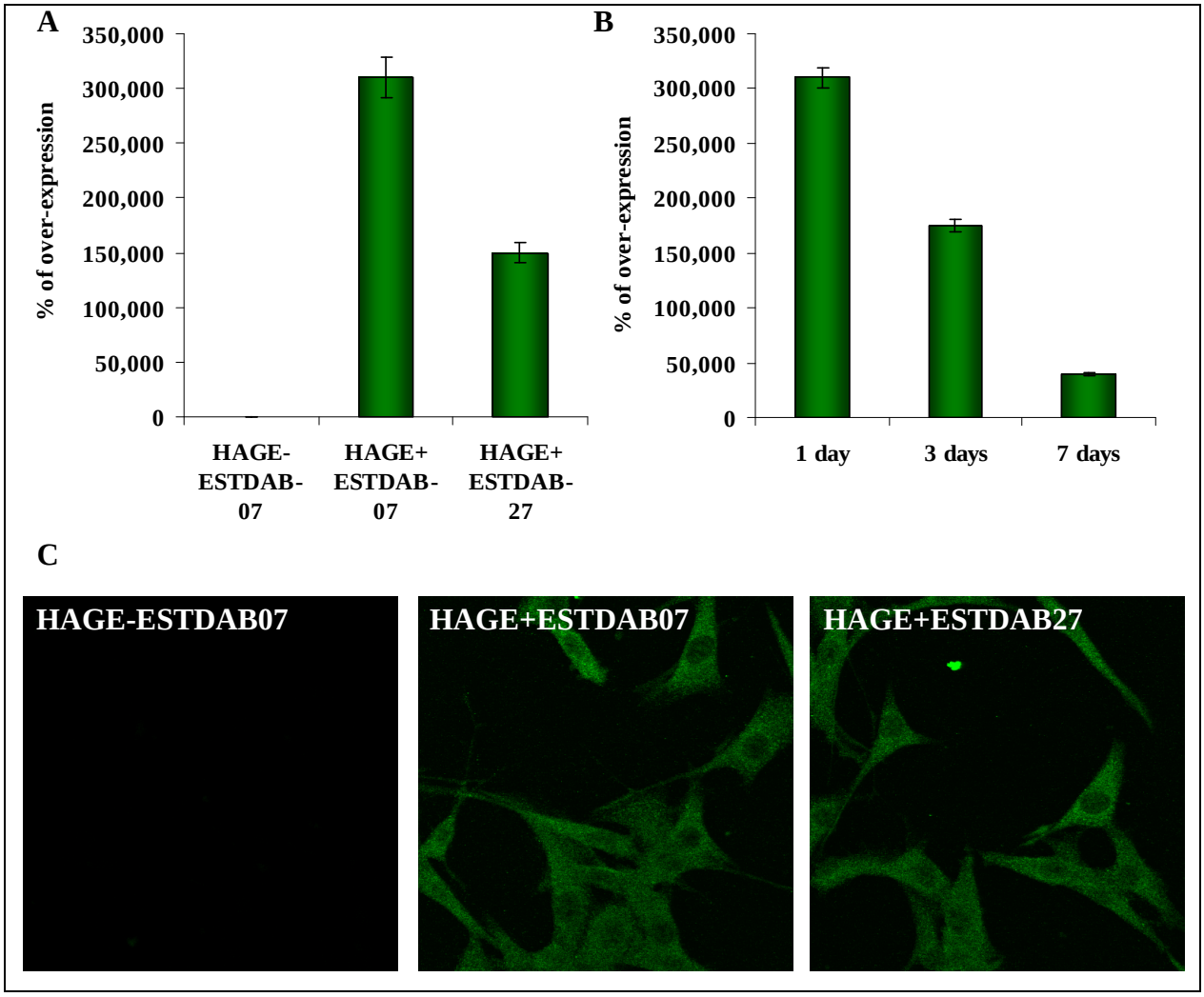

Figure 3.19: HAGE transfection of a HAGE-negative melanoma cell line. *(A) Real time PCR analysis showing the percentage of HAGE over-expression following transfection of the HAGE-negative ESTDAB-07 melanoma cell line. (B) Time course carried out by real time PCR indicating HAGE over-expression evolution against time. RT-Q-PCR results shown are representative of two independent experiments carried out in triplicates (n=2). (C) Immunofluorescence assay demonstrating increased HAGE protein expression two days after transfection of ESTDAB-07 cells with HAGE cDNA. Objective magnification: x40.*

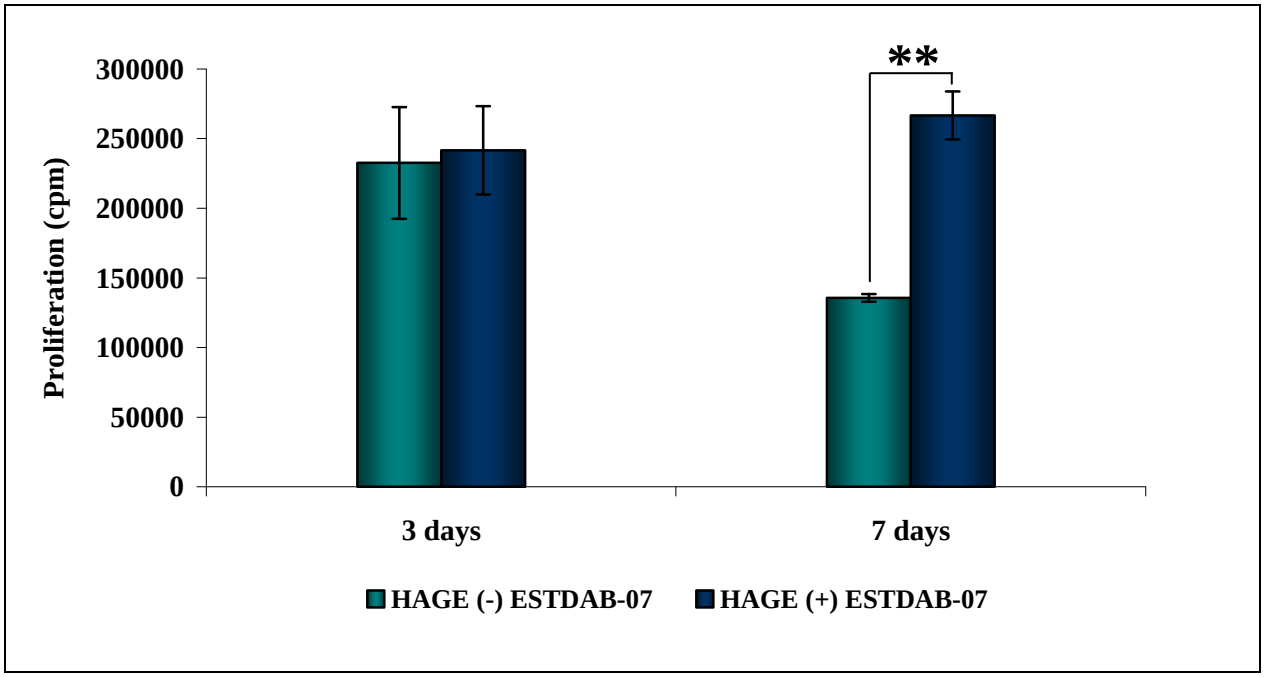

Figure 3.20: Proliferation assay following HAGE transfection. *Increased proliferation of the HAGE-negative ESTDAB-07 melanoma cell line was observed upon HAGE cDNA transfection. This experiment was carried out twice in triplicates (n=2). ** $p<0.01$ is the statistical difference between non-transfected ESTDAB-07 and HAGE-transfected ESTDAB-07 determined by paired Student T test.*

3.3 Discussion

In this chapter, an attempt to validate the tumour-associated gene, HAGE, as a potential antigen for cancer immunotherapy was described. HAGE was initially identified by Martelange *et al.*, (2000) who applied the technique of cDNA substraction analysis to identify novel genes with tumour-specific expression. HAGE was discovered on a wide range of tumour tissues at levels at least 100-fold that of normal tissues, with the exception of testis where this gene was shown to be highly expressed. Furthermore, the expression of this gene could be induced with the demethylating agent AZAC, a classical feature of cancer/testis genes (Scanlan *et al.*, 2002). Therefore, the authors defined this gene as being a member of the CT family and concluded that based on their tumour-specific expression; they may represent potential targets for immunotherapeutic vaccination of cancer. However, in previous reports, the expression of HAGE was not studied in normal tissues. This information is critical if one wants to use HAGE as a target for immunotherapy. mRNA HAGE expression was therefore first determined using conventional semi-quantitative RT-PCR. Results showed that HAGE was expressed in normal tissues such as brain, heart, liver or even PBMC, although to a much lesser extent when compared with testis. HAGE expression was also detected at low levels in 66% and 80% of AML and CML PBMC, respectively, suggesting an over-expression of HAGE in cancer tissues (See Appendix 1).

Conventional PCR may be misleading because the signal intensity is not directly proportional to the amount of amplified DNA and optimised PCR conditions may result in increased sensitivity. Indeed, a study by Nagel *et al.* (2003) into CT gene expression in benign and malignant neoplasms of the salivary glands, detected HAGE expression in 2/5 healthy salivary gland tissues; a discrepancy from previous findings that they also attributed to the use of a more sensitive RT-PCR method. Thus, the question of the level of HAGE expression in healthy versus malignant tissues still remained and therefore was addressed through the use of real time quantitative PCR. This technique did not detect HAGE expression in a wide array of normal tissues or normal PBMC with the exception of testis where HAGE expression was found to be at least a 1,000-fold higher than in any normal tissue tested.

Subsequent to studies by Martelange *et al.* (2000), Adams *et al.* (2002) analysed a large cohort of myelogenous leukaemia by conventional RT-PCR to investigate the question as to whether haematological malignancies express CT genes. They detected HAGE expression in 23% and 57% of the AML and CML samples tested, respectively. In this study, the expression of HAGE was then assessed by real time PCR on 20 CML samples. Real time PCR detected HAGE levels from five to almost 180-fold higher than in normal total blood cells in 70% of samples

tested, a proportion of HAGE-positive patients largely superior to the one described by Adams *et al.* (2002). Interestingly, these CML samples were composed of two groups of 10 having either low or high Bcr/Abl:Abl ratios respectively, namely that samples with low Bcr/Abl:Abl ratio relates to patients successfully responding to treatment with Imatinib and reciprocally. Real time PCR showed that Bcr/Abl:Abl ratio correlated with HAGE expression with the latter being between five to 20-fold higher than in TBC in 50% of samples with low Bcr/Abl:Abl ratio, and 20 to 180-fold higher than in TBC in 90% of samples with high Bcr/Abl:Abl ratio. These results suggest that HAGE is not only associated with advanced CML disease and poor prognosis as shown in a recent study by Roman-Gomez *et al.* (2007) but is also possibly involved in the pathogenesis of CML.

HAGE was previously shown to be over-expressed in various solid tumours such as bladder, brain, breast, colon, esophagus, liver, lung and skin cancers (Martelange *et al.*, 2000). In order to validate HAGE as a target for therapy, we confirmed the expression of HAGE in various solid tumours obtained from patients and compared it to normal tissues whenever possible. HAGE was found to be over-expressed in breast, colon and gastric carcinoma with a tumour to normal ratio superior to two, in 2 out of 4 breast cancers, in 2 out of 7 colorectal cancers and in 2 out of 9 gastric cancers. However, mean expression level analyses were not statistically different for any of these three types of cancer. In individual breast cancers, this result was much higher than the previously published study by Martelange *et al.* (2000), where approximately only 5% of breast cancers were shown to over-express HAGE. This discrepancy might be due to the low number of samples with good RNA quality but also to the sensitivity of the real time PCR technique. With a larger number of individual colorectal cancers with good quality RNA samples, the HAGE expression frequency obtained (28%) correlated with the previously published study (31%) by Martelange *et al.* (2000). Finally, the largest cohort of individual cancers with patient-matched normal tissues was carried out in gastric tissues and showed increased HAGE expression in only 22%. Unfortunately, there are no previous reports of HAGE in gastric tissues. Further samples would be required to really demonstrate an over-expression of HAGE in gastric carcinoma. Although clinical data were available for all these samples, no correlation between HAGE expression and cancer stages or cancer invasion involving lymph nodes could be drawn from these results. However, more relevant results were obtained with head & neck carcinoma. Indeed, HAGE over-expression was obtained in 75% of individual head & neck cancers. Despite the lack of comparison with previously published studies and the small number of samples tested, results were convincing enough to pursue this analysis on a larger cohort. Ten further samples were obtained and in spite of the lack of

patient-matched normal tissues, the analysis was carried out in comparison with testis. The frequency of HAGE-positive individual head & neck carcinoma was reduced to about 40% with a tumour to testis ratio superior to two. Similarly, 10 melanoma tissues became available for HAGE expression analysis and the frequency of HAGE-positive individual melanoma reached 50%. However, in these HAGE-positive melanoma samples, only two of them demonstrated expression of HAGE of about two-fold the expression in testis and three-fold the expression in a HAGE-positive melanoma cell line. This observation reduced the frequency to 20%, a result in agreement with the previously published study (17%) by Scanlan *et al.* (2004). However, considering that the expression of testis is at least a 1000-fold higher than in any normal tissue, it is legitimate to consider that HAGE over-expression in head & neck carcinoma and melanoma would achieve a larger number when compared to patient-matched normal tissues, hence increasing considerably the frequencies obtained in this study.

The difficulty in the interpretation of the results strongly depends on the referentials used in the analysis. The use of normal testis as a tool of comparison for solid tumours can be criticised as it does not offer a correct representation of the actual expression of a tumour-associated antigen in a given normal tissue. Unfortunately, because of the rarety of cancer samples from patients and the difficult justification for harvesting both malignant and healthy surrounding tissues, it is often impossible to always rely on the provision of patient-matched normal tissues for a comprehensive and true analysis. Moreover, the source of RNA as well as the methodology used for obtaining the tissue is also another essential aspect to consider during analysis of tumour antigens. Radical surgery was used to harvest all the tissues tested in this study. It results in a tissue consisting of different cell types and in a total RNA considerably diluted as a consequence of the presence of heterogenous cell populations. A critical breakthrough came from the development of laser capture microdissection (LCM) allowing for the specific isolation of foci of tumour cells from a heterogenous tumour population. A brief laser pulse is directed towards a specific area to specifically select and isolate individual areas or cells. Recently, two tumour-associated antigens, MTA1 and T21, were investigated in prostate cancer samples resulting from radical prostatectomy and LCM-isolated prostate tissues. Expression analysis of these two antigens gave significantly different results (Walton T., unpublished data; Miles *et al.*, 2007).

Knowing that the HAGE promoter, like any other cancer/testis gene promoter, is strongly susceptible to the methylation status of its CpG islands (Scanlan *et al.*, 2004), it became of interest to determine whether tumour cell lines deriving from either haematological malignancies or solid tumours do express HAGE and at what level. Seven CML, five

melanoma and four head & neck carcinoma cell lines were therefore evaluated. Unfortunately, this experiment failed to demonstrate expression of HAGE in CML and head & neck carcinoma cell lines and only melanoma cell lines were able to demonstrate HAGE expression levels comparable to testis, although to a lower extent. Furthermore, treatment of some of these CML cell lines with the DNA methyltransferase inhibitor AZAC confirmed this hypothesis with a sudden raise of HAGE expression synergistically enhanced when the deacetylase inhibitor TSA was also added to the cells. Certain cancer cell lines have an increased rate of hypermethylation of certain CpG islands and less global genomic hypomethylation, while others are perfectly capable of retaining and presenting the same epigenetic profile of the original tumour cell type (Roman-Gomez *et al.*, 2005), which would explain the discrepancies between the different cell lines used in this experiment. The latters are of interest as they can provide a viable model for *in vitro* experiments but also a good tool of comparison for expression analysis in clinical samples.

However, these data are all restricted to the detection of HAGE on the message level and discrepancies between RNA and protein levels are common finding due to factors such as the varying stability of different mRNA molecules, the regulatory process of translation, post-translational modifications and proteasomal degradation (Rogel *et al.*, 1985; Chen *et al.*, 2002). Therefore, to truly evaluate the expression of HAGE in normal and malignant tissues, analysis on the protein level was required. Using a polyclonal antiserum generated against HAGE peptides predicted *in silico* to be antigenic and exposed on the surface of the protein, immunohistochemistry and immunofluorescence were respectively carried out on tumour tissues and tumour cell lines to underline the putative 73kDa HAGE protein. This should also provide further details regarding the protein's subcellular localisation. The antiserum allowed the confirmation of the non-expression of HAGE at the protein level in all the normal tissues tested, except in testis where the intensity of the staining is quite high. It also allowed checking whether there is a correlation between mRNA expression and protein expression whenever tissues and RNA from the same tissues were both available. Immunohistochemistry staining was carried out on five head & neck carcinoma and six melanoma tissue sections. Only three of the four HAGE-positive head & neck carcinoma sections and all four of the HAGE-positive melanoma sections demonstrated positive staining. Samples negative for HAGE expression by real time PCR remained negative after staining of their respective tumour sections. Moreover, the intensity of HAGE staining was not proportional to the intensity of the message level obtained by real time PCR. Indeed, one head & neck carcinoma sample (HN04-1649) shows a mRNA expression 14-fold higher than in normal testis. However, the intensity of the staining

of this same tissue was much lower to the one of normal testis probably suggesting that HAGE might present a different protein turnover tissue to tissue or that the translation of HAGE mRNA into a protein is somehow strongly down-regulated in certain tumour tissues. Evidence obtained by the staining of a multitumour microarray supported the second hypothesis as the staining intensity in other forms of cancers occurring in bladder, colon, liver, lung, thymus or testis matches the intensity observed in normal testis. Since the ultimate goal of this study was to induce an immune response against the HAGE protein, high protein expression levels are not necessarily a prerequesite for a CTL-mediated response. P53-specific CTL were previously shown to be able to recognise tumour cells expressing moderate or low levels of p53, suggesting that the protein turnover is more important for a CTL-mediated response than the actual steady-state level of expression (Vierboom *et al.*, 2000). Therefore, protein expression study should be followed by a degradation study in order to find out the turnover of the protein. The next major question that comes to mind is: "is HAGE involved in the tumorogenesis process and to what extent?" Immunohistochemistry and immunofluorescence showed that HAGE protein can be found in both nuclear and cytoplasm with a characteristic granular expression. Some RNA helicases are able to play a role in different processes of the RNA metabolism in spite of taking place in very different cellular compartments (Charroux *et al.*, 1999; Charroux *et al.*, 2000). In these studies, the DEAD-box putative RNA helicase Gemin3 was thought to be involved in the formation of the survival of the motor neuron complex, which leads to spinal muscular atrophy if compromised. It was found in both cytoplasm, gems (*i.e.*: nuclear bodies) and nucleoli suggesting roles in the spliceosomal assembly of small ribonucleoproteins with RNA, preribosomal RNA processing and ribosome assembly. This statement would explain the ubiquitous expression of HAGE inside the cells with HAGE being expressed depending on the cellular needs or the cellular state at the moment of the tumour section or tumour cell fixation.

Gene silencing mediated by small interference RNA (siRNA) has been extensively used in the past few years for gene function studies. Two HAGE siRNA were therefore designed and then synthesised, and transfection of a HAGE-positive melanoma cell line with siRNA was optimised. Both siRNA led to a satisfactory knockdown of HAGE expression at the message and at the protein levels one day and three days after transfection, respectively. Cell morphology and cell cycle were not affected but cell proliferation was noticeably reduced. Proliferation assays confirmed that observation. Furthermore, real time PCR of OAS1 and STAT1 confirmed that the reduced proliferation observed following HAGE silencing was not interferon- or transfection reagent-related as both gene expressions remained unaltered upon

siRNA transfection. A HAGE-negative melanoma cell line was then transfected with HAGE cDNA and the reverse reaction happened with increased tritiated thymidine incorporation and therefore increased proliferation. Like DDX1 (Godbout et *al.*, 1998), DDX2 (Eberle *et al.*, 2002) and DDX5 (Yang *et al.*, 2005), evidence suggests that the DEAD-box RNA helicase HAGE is involved in tumour cell proliferation. In support of this hypothesis, Roman-Gomez *et al.* (2007) showed that the hypomethylation status of HAGE promoter directly correlates with HAGE expression and high levels of HAGE mRNA are generally linked with advanced CML disease, *i.e.* CML patients in blast crisis, and poor clinical outcome. Is HAGE at the origin of poorly responding CML or at the receiving end of the genomic instability that reigns in patients with high Bcr/Abl:Abl ratio? The evidence argues against the second alternative as HAGE expression was found in both groups of CML samples tested in this study although at a different degree.

Proteins that are overexpressed in tumours or show tumour-specific expression as well as having roles believed to be associated with tumoral transformation make attractive vaccination targets for tumour immunotherapy, as this may prevent the tumour from down-regulating the gene as a method of tumour escape. Although more refined studies will be required to pinpoint the exact function of HAGE, mRNA and protein studies were sufficient to safely consider HAGE as an ideal antigen in several cancers.

Chapter 4: Cloning of HAGE and murine co-stimulatory molecules

4.1 Introduction

Gene therapy can be defined as the introduction of a gene that has been cloned or manufactured in a cell in order to treat a disease, be it in an infectious disease or in cancer. Gene-based immunisation represents a convenient entity as it is widely applicable to the majority of the population, without the requirement of identifying naturally processed peptides for different MHC haplotypes. Theoretically, the transfer of a gene encoding a tumour antigen should allow both the endogenous process and presentation of this antigen to T lymphocytes, and an adjuvant effect coming from the vector itself through the CpG sequences included in the bacterial DNA. They can be very easily synthesised in clinical grade and at low costs. Moreover, a large panel of routes of administrations can be used allowing the activation of both innate and adaptive arms of the immune system (Pavlenko *et al.*, 2004). Two gene-based therapies can be characterised: naked DNA and viral/bacterial vectors. Furthermore, these vectors can be directly used in immunisation procedures *in vivo* or to augment the immunogenicity or the antigen-presenting ability of tumour cells, APC and immune cells *ex vivo*.

Before considering the use of viral vectors, it is important to mention that naked DNA can have a surprising efficiency when injected *in vivo* in some tissues such as muscles or subcutaneous tissue. Indeed, the efficiency of gene transfer is superior to other non-viral vectors in the tissues quoted earlier with long term protein expression detectable up to two months after immunisation, even though the entry mechanism of naked DNA into the cell remains unknown (Wolff *et al.*, 1990). Plasmid vaccination consists of a bacteria-derived plasmid backbone encoding the gene of interest. Plasmids used for immunisation are usually composed of an expression cassette including a promoter and a polyadenylation signal, an antigen encoding gene sequence, an origin of replication and a selection marker for propagation of plasmids in bacteria. With the help of molecular biology tools and the knowledge of human genome sequences, it is now possible to virtually clone any gene from the human genome. Furthermore, many routes of administrations are available with each having different properties or leading to different immunological outcomes. Naked DNA immunisation can take place intramuscularly, intraperitoneally or intradermally where it is thought to be taken up by resident APC either directly after immunisation or following cross-priming and the release of proteins from transfected muscle cells having undergone apoptosis. Administration can also occur orally through the mucosal route or be coupled with physical methods of transfer such as

electroporation (Rizzuto *et al.*, 1999) or gene gun allowing the subcutaneous injection of gold-coated DNA particles (Yang *et al.*, 1990). These techniques allow the local skin APC (Langerhans cells) to be directly transfected. Moreover, the delivery of DNA doses as low as 1 μg by gene gun were shown to generate potent immune responses, which is 100 to 1,000 less DNA than any other modes of vaccination. Interestingly, it seems that routes of administration influence the polarisation of the immune response (Kwissa *et al.*, 2000). Indeed, intra-muscular or intra-dermal saline-DNA immunisation has been reported to generate a Th1 response and the production of IgG2a antibodies in mice, whereas cutaneous gene gun immunisation tends to lead to a Th2 response and IgG1 antibody production. These results might largely be influenced by the different populations of cells integrating the DNA (Feltquate *et al.*, 1997; Torres *et al.*, 1997) and have since been contradicted by multiple evidences indicating CTL-mediated anti-tumour protection following gene gun-mediated DNA immunisation in mice (Bowne *et al.*, 1999; Gold *et al.*, 2003). Unfortunately, although clinical trials have demonstrated that gene-based immunisation was capable of inducing tumour-specific T lymphocyte responses, these did not lead to an objective positive clinical response (Tsang *et al.*, 1995). One possible explanation is that most tumour antigens used in these trials are self antigens and therefore the level of tolerance exceeds that of stimulation induced by these methods.

By nature, viruses represent an excellent mean of introducing genetic material in a cell mimicking natural viral infection and thereby generating more potent immune responses. However, the possibility of progressive viral infection, especially in immuno-compromised patients, still raises a major concern. Nowadays, for most of viral systems used, the target cell is infected by a virus unable to replicate and therefore non-pathogenic. In practice, the virus is rendered unable to replicate by removing replicating sequences and substituting them with a therapeutic gene cassette. Cell lines, which have been genetically altered or have the ability in their genome to replicate the attenuated virus, are used to produce and expand the number of viral copies *in vitro*. Also, natural host restriction can prevent the multiplication in human cells of some viruses such as avipox virus derived vectors (Kay *et al.*, 2001). Finally, it is worth mentionning that in order to have a prolonged effect on the immune system, viruses must have a very low intrinsic immunogenicity to avoid their neutralisation by circulating antibodies, which would hinder their efficacy as therapeutic vectors. Seven major families have been so far used in cancer immunotherapy: adenovirus (Guse *et al.*, 2007), adeno-associated virus (Sun *et al.*, 2002), alphavirus (DiCiommo and Bremner, 1998), avipox virus (Chakraborty *et al.*, 2007),

herpes simplex virus (Ali *et al.*, 2000; Ali *et al.*, 2002), retrovirus (Heemskerk *et al.*, 1999; Chan *et al.*, 2005) and vaccinia virus (Chakraborty *et al.*, 2007). These families present different modes of functioning, different duration of gene expression or different safety levels. Depending on these characteristics summarised in table 4.1, these viruses offer both advantages and disadvantages.

Table 4.1: Characteristics of different viral vectors (Adapted from Bonnet et al., 2000)

Vector	Biology	Pre-existing immunity in humans	Duration of gene expression	Safety
Adenovirus	Double-stranded DNA	Yes	Transient	Good
Adeno-associated virus	Single-stranded DNA	Yes	Transient	Risk of insertional mutagenesis
Alphavirus	RNA viruses with replicon	No	Good	Safe in animals, not fully characterised in humans
Avipox virus	Double-stranded DNA non –replicative in mammalian cells	Yes	Transient	Very good
Herpes simplex virus	Double-stranded DNA	Yes	Transient	Neurovirulence, insertional mutagenesis
Retrovirus	Diploid RNA strand	No	Good	Risk of insertional mutagenesis
Vaccinia virus	Double-stranded DNA replicative in mammalian cell	Yes	Transient	Well documented safety

According to these characteristics, the alphaviruses represent an interesting option as there are no signs of pre-existing immunity in humans and thus, avoiding an eventual humoral response, which would immediately suppress the beneficial effects of the vaccination. Furthermore, the gene transduced by the virus is readily expressed once inside the cells with so far no documented risks of insertional mutagenesis, at least in animals. Sindbis virus, Venezuelan equine encephalitis virus and Semliki forest virus (SFV) are three commonly used alphaviruses with proven delivery abilities. They are characterised by a single-stranded RNA genome of about 12kb surrounded by an icosahedral capsid protein shell. In this study, SFV was chosen as a vector for the development of new vaccine strategies. Semliki forest virus' single-stranded RNA is enveloped by a nucleocapsid. The latter is composed of 240 copies of the capsid

protein attached together. This shell is surrounded by an extra lipid bilayer from the host cells spiked with glycoproteins encoded by the E1, E2 and E3 nucleotide sequences of the viral genome. The two open reading frames of the SFV RNA encode four non-structural proteins, as well as capsid and envelope proteins, respectively. Semliki forest viruses bind to different cell surface receptors to infect cells and upon transduction, the production of SFV proteins comes in place of the host cell protein synthesis (Lundstrom., 2003).

With the help of genetic manipulation, the infection efficiency, the applicability to a wide range of hosts, the safety and the complexity of the system were improved considerably. Indeed, large amount of work has been carried out on SFV to enhance its original features in order to create a more efficient recombinant SFV vector for immunotherapy. In the original SFV expression vector, structural protein coding regions were substituted with the gene of interest and following co-transfection of the gene coding RNA with a helper RNA encoding the viral structural proteins, recombinant SFV particles were produced (Liljestrom and Garoff, 1991). As a safety mechanism during *in vitro* transfection experiments or *in vivo* therapeutic applications, the p62 structural protein coding region was also mutated rendering the generated viruses inactive if not proteolytically treated with α-chymotrypsin. However, the handling of a RNA-based SFV expression system requires specific conditions, which were overcome by constructing a DNA-based system (DiCiommo and Bremner, 1998). The SP6 promoter was replaced with a RNA polymerase II-dependent cytomegalovirus enhancer/promoter to drive the transcription *in vivo*. Helper and replicon plasmids with the above characteristics were engineered and further improved with the expansion of the multiple cloning site and the addition of FLAG and HIS10 epitope and affinity tags (DiCiommo *et al.*, 2004). Co-transfection with both helper and replicon plasmids allows the generation of a large quantity of recombinant SFV vectors, the transduction of a large variety of mammalian and non-mammalian cells, the rapid production of high levels of proteins, programmed cell death in host cells and protection against tumour challenges. Tumour protection with recombinant SFV vectors was achieved in several animal models (Daemen *et al.*, 2000; Daemen *et al.*, 2003; Huckriede *et al.*, 2003; Ni *et al.*, 2004) and immune responses were also generated in immuno-tolerant mice indicating the ability of SFV to break tolerance (Riezebos-Brilman *et al.*, 2005) This efficiency towards tumours can also be further enhanced following encapsulation of SFV particles into liposomes (Lundstrom and Boulikas, 2003). Altogether, these facts led us to the elaboration of new SFV-based strategies targeting specifically the HAGE antigen.

In order to investigate application of plasmid DNA vaccination for generating immune response to HAGE, this study describes the construction of a plasmid expression vector (pBudCE4.1) encoding HAGE. Also, naive T cells respond poorly to antigen unless secondary signals are provided by co-stimulatory molecules expressed on the surface of APC. Expression of co-stimulatory molecules offers several advantages as it allows the activation of pathogen-specific T cells and minimises the risks of mounting an adaptive immune response against self (Fig. 4.1). In most cases, these signals are cell-to-cell contact-dependent, although some soluble factors have also demonstrated co-stimulatory abilities (Weaver and Unanue., 1990). Over the years, B7.1 and B7.2 ligands and their common receptor CD28 have emerged as crucial molecules for T cell proliferation and IL-2 secretion. Unfortunately, both molecules can lead to opposite effects as they are also capable of binding CTLA-4 with greater affinity, a receptor homologuous to CD28 but with inhibitory properties (Chambers and Allison, 1997). That is why several studies have looked at other co-stimulatory molecules such as 4.1BBL, which binds with high affinity to its receptor 4.1BB. Like B7 molecules, 4.1BBL is inducible and found on APC. Upon binding to its receptor, 4.1BBL allows activation and differentiation of both CD4+ and CD8+ T cells (Vinay and Kwon, 1998). Interestingly, CD28 co-stimulatory signalling can be potentiated by 4.1BB signalling (Hurtado *et al.*, 1995). In this study, murine genes of these three co-stimulatory molecules (mB7.1, mB7.2 and m4.1BBL) were each cloned into pBudCE4.1/HAGE in order to obtain a single vector encoding simultaneously a TAA and a co-stimulatory molecule and investigate the co-stimulation of the immune response. Finally, HAGE was cloned into a SFV vector and viruses were generated. Following each cloning, protein production by the various vectors generated was confirmed.

4.2 Results

4.2.1 Cloning of HAGE into the mammalian expression vector pBudCE4.1

The making of a tumour model to target HAGE in HHDII-DR1 double transgenic mice requires the transfection with HAGE DNA of a murine cancer cell line (ALC) already stably transfected to express a HLA-A2 construct. Unfortunately, the HLA-A2 construct contains a resistance cassette (neomycin), which is identical to the one proposed by the pcDNA3/HAGE expression vector kindly provided by Prof. Thierry Boon (Ludwig Institute, Brussels). Therefore, there is a need to subclone the full open reading sequence of HAGE from pcDNA3 into another expression vector, which has a different resistance mechanism in order to be able to positively select HAGE-expressing ALC transfectants. HAGE cDNA was then cloned into the mammalian expression vector pBudCE4.1 containing a zeocin selection marker.

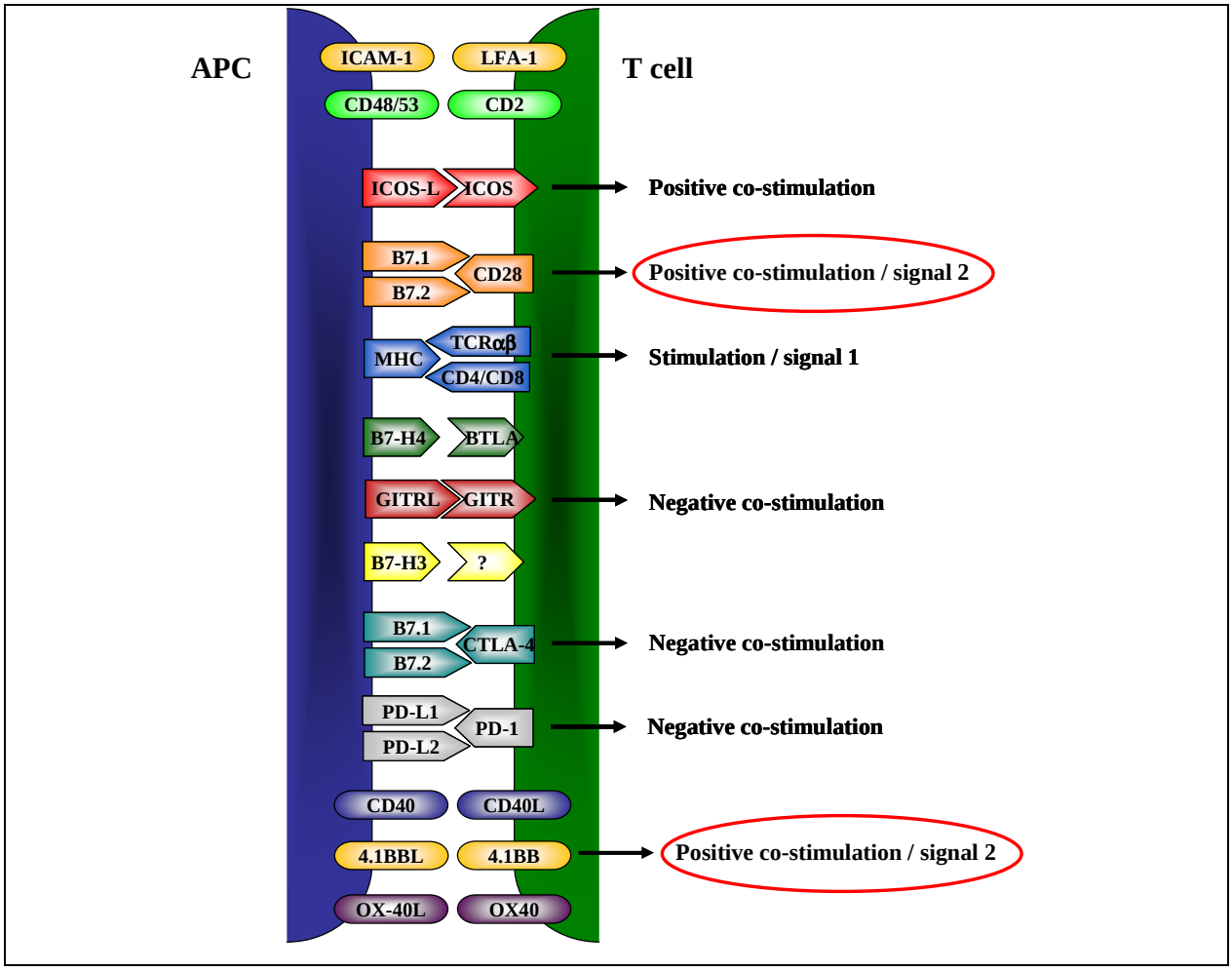

Figure 4.1: Co-stimulation of the T cell response. *At least two signals are required for optimal T cell activation: antigen recognition provided by the formation of MHC-peptide-TCR complexes and co-stimulation provided by the ligation of co-stimulatory molecules expressed on the surface of APC to their receptors found on the surface of T cells. This T cell activation can also be negatively regulated to induce T cell tolerance and limit the immune response (Adapted from www.ebioscience.com/ebioscience/whatsnew/costim.htm). Highlighted in red are the two modes of positive co-stimulation investigated in this project.*

The pcDNA3/HAGE and pBudCE4.1 expression vectors were first double digested with HindIII and XbaI restriction enzymes and run on agarose gel. Digested corresponding bands of HAGE and pBudCE4.1 were gel extracted and ligated overnight at 4°C with T4 DNA ligase enzyme, followed by plating on LB agar with zeocin and selection of clones (Fig. 4.2). After three attempts, clones were confirmed to contain HAGE by restriction digestion. Indeed, a band corresponding to 1,950bp could be detected in 10 out of 10 clones (Fig.4.3). This plasmid was then used for stably transfecting ALC cells as well as immunising transgenic mice with HAGE DNA.

4.2.2 Cloning of murine co-stimulatory molecules into the mammalian expression vector pBudCE4.1/HAGE

The pcDNA3.1/Zeo-B7.1, -B7.2 and -4.1BBL expression vectors were kindly provided by Dr. Barbara Guinn (King's college). The pBudCE4.1 expression vector contains two multiple cloning sites allowing the association of a tumour-associated antigen such as HAGE with a co-stimulatory molecule for different vaccine strategies. Murine B7.1, B7.2 and 4.1BBL were therefore each cloned into pBudCE4.1/HAGE. Primers including restriction sites were specifically designed to allow PCR amplification and cloning of each co-stimulatory molecule into pBudCE4.1/HAGE. Primer sequences and respective annealing temperatures were summarised in table 4.2.

Table 4.2: Sequences of primers used for cloning of murine co-stimulatory molecules into pBudCE4.1/HAGE and their respective annealing temperatures

Primers for cloning and respective annealing temperature		
B7.1F with KpnI site	5'-TCTAGGTACCATGGCTTGCAATTGTCAG-3'	66°C
B7.1R with BstBI site	5'-TCTATTCGAACTAAAGGAAGACGGTCTG-3'	
B7.2F with KpnI site	5'-TCTAGGTACCATGGACCCCAGATGC-3'	67°C
B7.2Rwith BstBI site	5'-TCTATTCGAATCACTCTGCATTTGGTTTTGC-3'	
4.1BBLF with XhoI site	5'-TCTACTCGAGATGGACCAGCACACACTT-3'	67°C
4.1BBLR with BglII site	5'-TCTAAGATCTTCATTCCCATGGGTTGTC-3'	

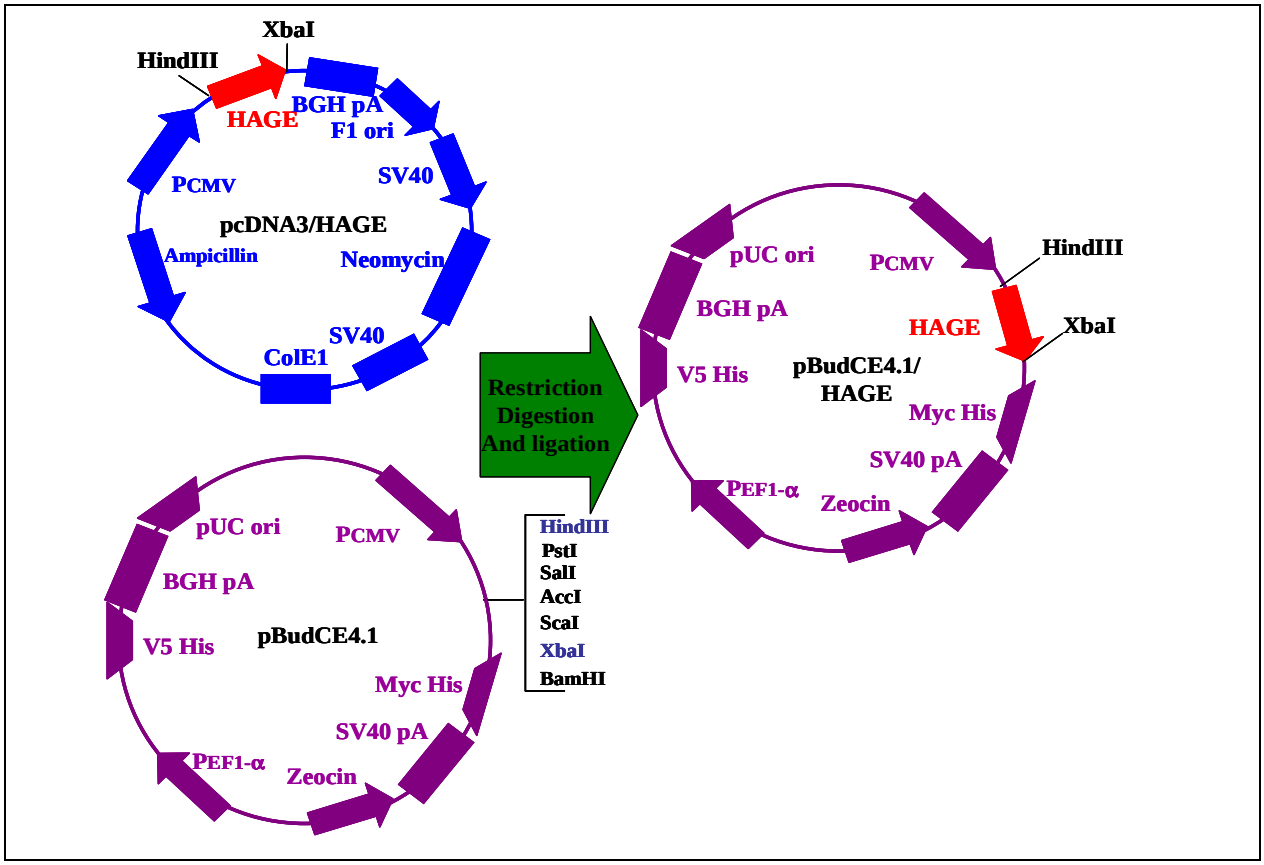

Figure 4.2: Diagrammatic representation of the construction of the plasmid pBudCE4.1/HAGE. *The pcDNA3/HAGE and pBudCE4.1 vectors were first digested with restriction enzymes HindIII and XbaI. The digested products were purified by gel purification and desired fragments were selected for ligation. After ligation, the cDNA for HAGE was inserted into the pBudCE4.1 plasmid.*

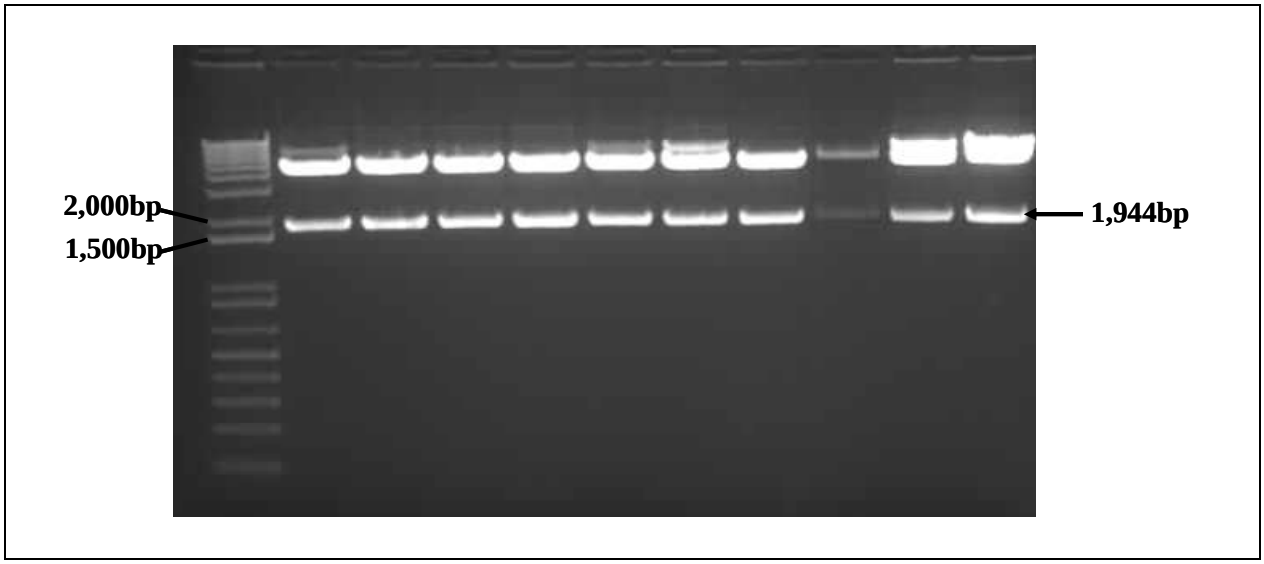

Figure 4.3: Agarose gel electrophoresis after double digestion of pBudCE4.1/HAGE clones. *After double digestion with HindIII and XbaI, a band corresponding to 1.9kb could be detected in 10 out of 10 clones picked, indicating successful cloning of HAGE into the pBudCE4.1 mammalian expression vector.*

4.2.2.1 Cloning of mB7.1 into pBudCE4.1/HAGE

Full length murine B7.1 was first amplified from pcDNA3.1/Zeo-B7.1 using RT-PCR for 32 cycles using a high fidelity Taq polymerase (Phusion, Finnzymes). After amplification, PCR amplicons and pBudCE4.1/HAGE were double digested with BstBI and KpnI restriction enzymes. Restriction digests were run on an agarose gel and a band corresponding to 921bp (mB7.1) was clearly visible and was gel extracted together with the band corresponding to the linearised pBudCE4.1/HAGE plasmid. Upon overnight ligation at 4°C with T4 DNA ligase enzyme, competent XL1-Blue *E.coli* were transformed and plated on LB agar with zeocin (Fig. 4.4). Clones were selected and screened for murine B7.1 by restriction digestion. As seen in Fig. 4.5, band corresponding to 921bp could be detected in 7 out of 10 clones picked and full length mB7.1 was obtained with 100% identical sequence to published mB7.1 sequence in two of them upon sequencing.

4.2.2.2 Cloning of mB7.2 into pBudCE4.1/HAGE

Full length murine B7.2 was first amplified from pcDNA3.1/Zeo-B7.2 using RT-PCR for 32 cycles using a high fidelity Taq polymerase (Phusion, Finnzymes). After amplification, PCR amplicons and pBudCE4.1/HAGE were double digested with BstBI and KpnI restriction enzymes. Restriction digests were run on an agarose gel and a band corresponding to 930bp (mB7.2) was clearly visible and was gel extracted together with the band corresponding to the linearised pBudCE4.1/HAGE plasmid. Upon overnight ligation at 4°C with T4 DNA ligase enzyme, competent XL1-Blue *E.coli* were transformed and plated on LB agar with zeocin (Fig. 4.6). Clones were selected and screened for murine B7.2 by restriction digestion. As seen in Fig. 4.7, band corresponding to 930bp could be detected in 9 out of 10 clones picked and full length mB7.2 was obtained with 100% identical sequence to published mB7.2 sequence in four of them upon sequencing.

4.2.2.3 Cloning of m4.1BBL into pBudCE4.1/HAGE

Finally, full length murine 4.1BBL was first amplified from pcDNA3.1/Zeo-4.1BBL using RT-PCR for 32 cycles using a high fidelity Taq polymerase (Phusion, Finnzymes). After amplification, PCR amplicons and pBudCE4.1/HAGE were double digested with BglII and XhoI restriction enzymes. Restriction digests were run on an agarose gel and a band corresponding to 930bp (m4.1BBL) was clearly visible and was gel extracted together with the band corresponding to the linearised pBudCE4.1/HAGE plasmid.

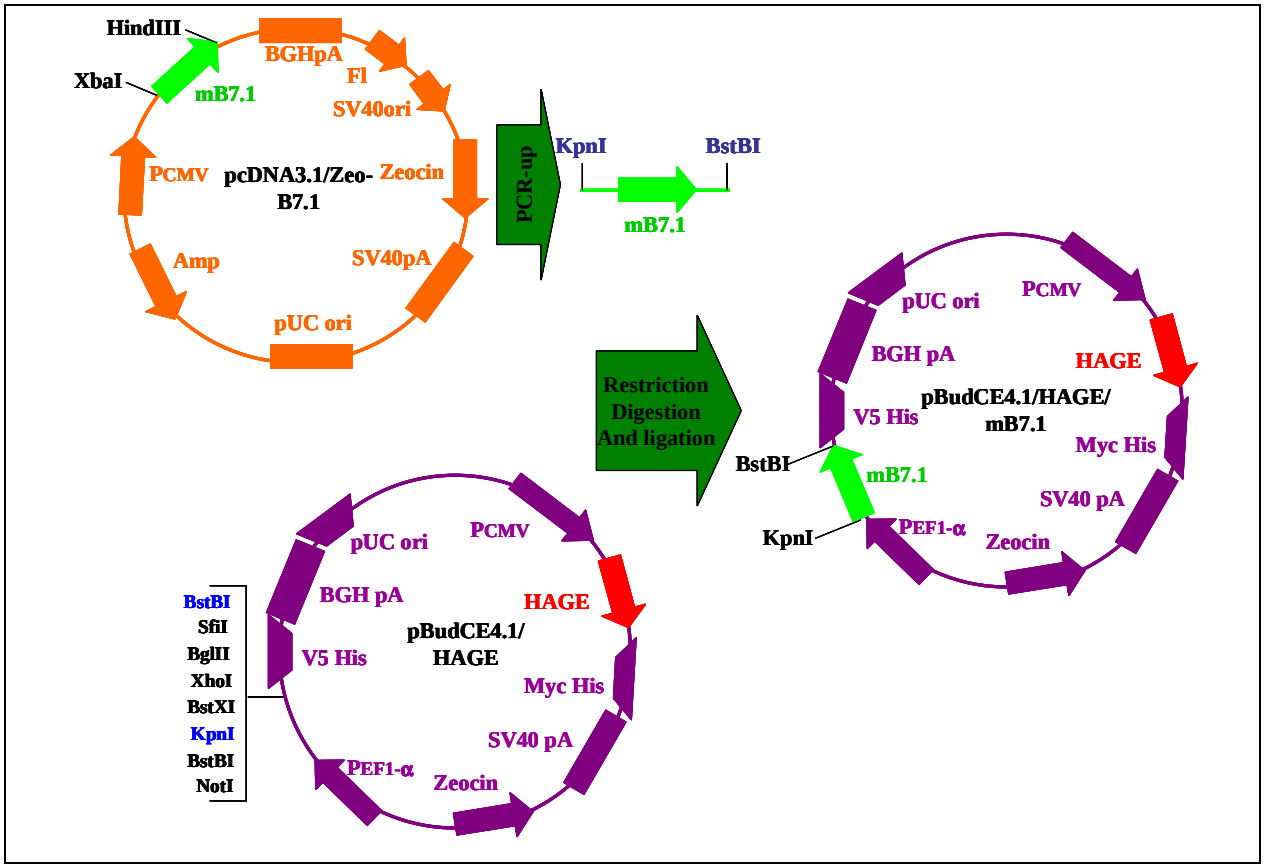

Figure 4.4: Diagrammatic representation of the construction of the plasmid pBudCE4.1/HAGE/mB7.1. *The mB7.1 cDNA was first PCR up. The PCR amplicons and the pBudCE4.1/HAGE vector were digested with restriction enzymes BstBI and KpnI. The digested products were purified by gel purification and desired fragments were selected for ligation. After ligation, the cDNA for mB7.1 was inserted into the pBudCE4.1/HAGE plasmid.*

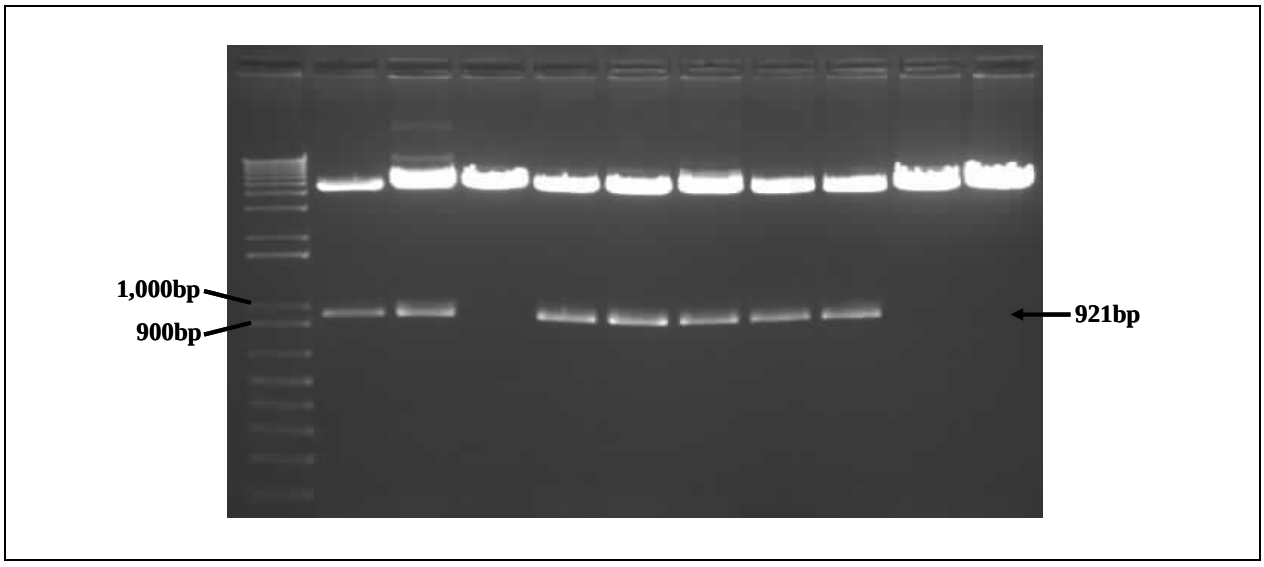

Figure 4.5: Agarose gel electrophoresis after double digestion of pBudCE4.1/HAGE/mB7.1 clones. *After double digestion with BstBI and KpnI, a band corresponding to 921bp could be detected in 7 out of 10 clones picked, indicating successful cloning of mB7.1 into the pBudCE4.1/HAGE mammalian expression vector.*

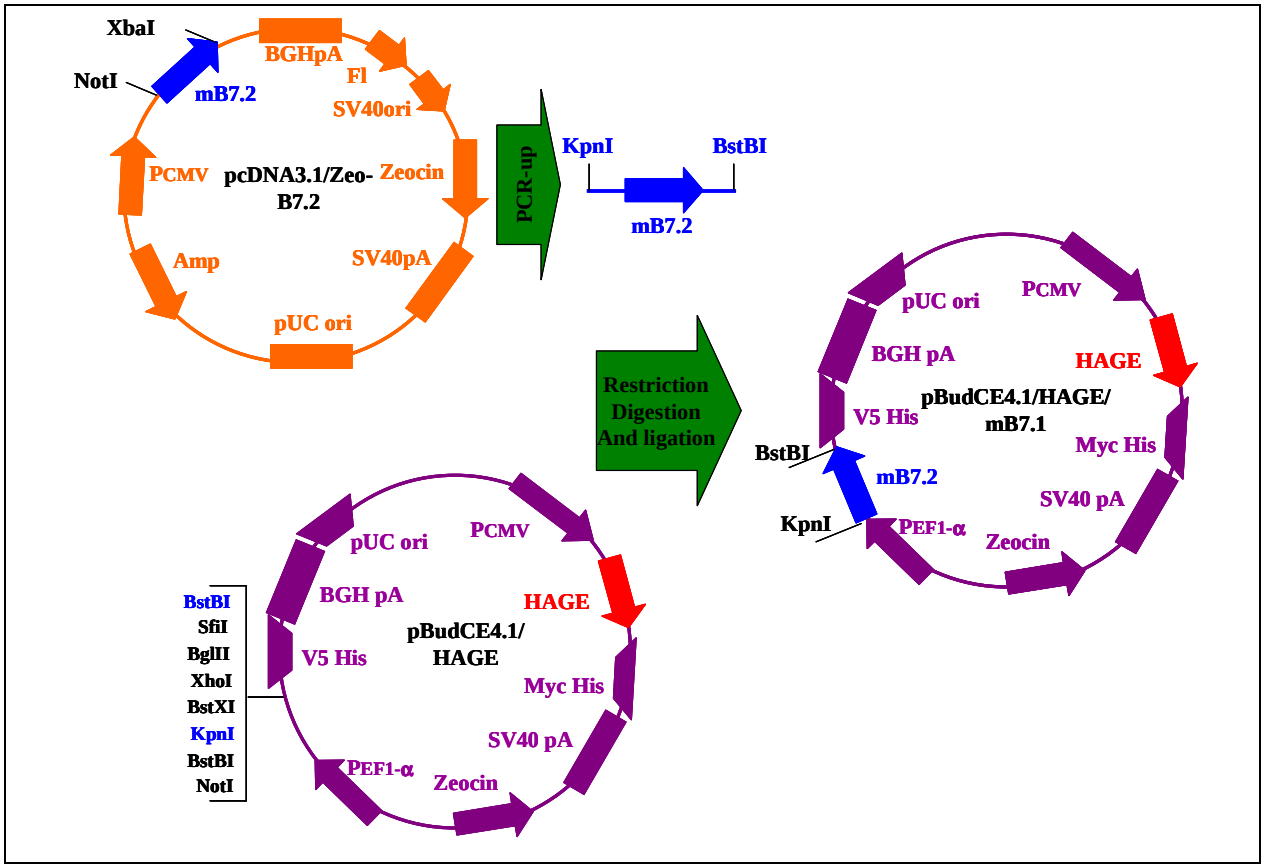

Figure 4.6: Diagrammatic representation of the construction of the plasmid pBudCE4.1/HAGE/mB7.2. *The mB7.2 cDNA was first PCR up. The PCR amplicons and the pBudCE4.1/HAGE vector were digested with restriction enzymes BstBI and KpnI. The digested products were purified by gel purification and desired fragments were selected for ligation. After ligation, the cDNA for mB7.2 was inserted into the pBudCE4.1/HAGE plasmid.*

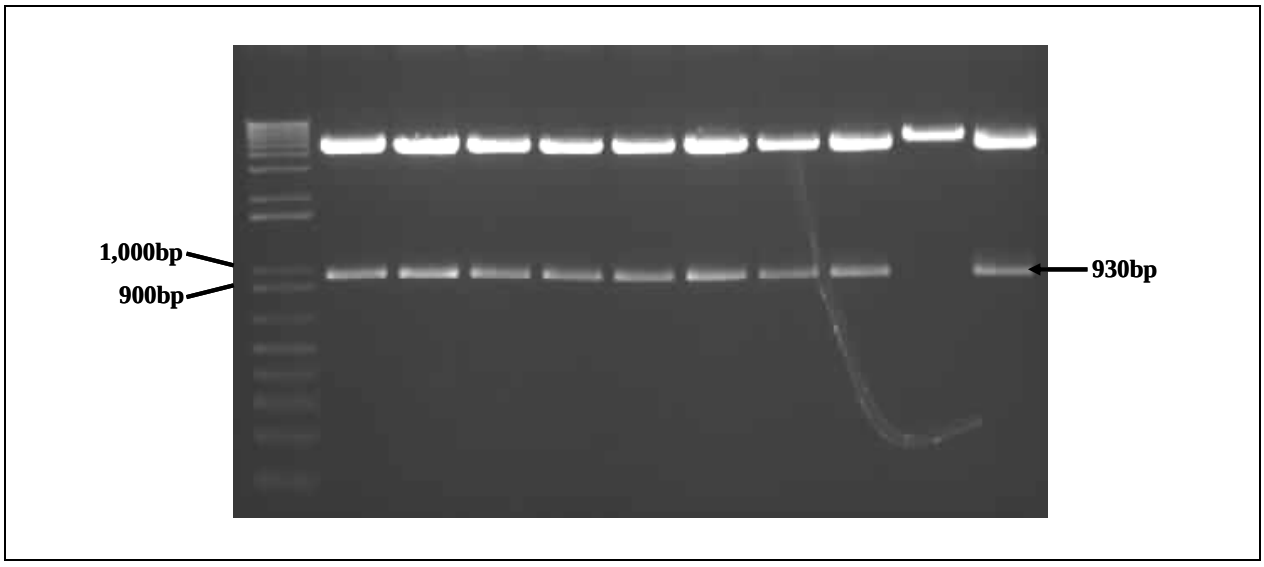

Figure 4.7: Agarose gel electrophoresis after double digestion of pBudCE4.1/HAGE/mB7.2 clones. *After double digestion with BstBI and KpnI, a band corresponding to 930bp could be detected in 9 out of 10 clones picked, indicating successful cloning of mB7.2 into the pBudCE4.1/HAGE mammalian expression vector.*

Upon overnight ligation at 4°C with T4 DNA ligase enzyme, competent XL1-Blue *E.coli* were transformed and plated on LB agar with zeocin (Fig. 4.8). Clones were selected and screened for murine 4.1BBL by restriction digestion. As seen in Fig. 4.9, band corresponding to 930bp could be detected in 2 out of 10 clones picked and full length m4.1BBL was obtained with 100% identical sequence to published m4.1BBL sequence in one of them upon sequencing.

4.2.2.4 Expression of cloned mB7.1, mB7.2 and m4.1BBL at the protein level

To confirm HAGE, mB7.1, mB7.2 and m4.1BBL protein expression by pBudCE4.1 vectors, ESTDAB-07 cells were grown in a multi-chamber slide and transfected with pBudCE4.1 encoding for both HAGE and mB7.1, mB7.2 or m4.1BBL using lipofectamine 2000 reagent and following manufacturer's instructions (See Section 2.2.2.1). Two days after transfection, cells were fixed, permeabilised and stained for HAGE and either mB7.1, mB7.2 or m4.1BBL expression for confocal analysis. Secondary antibody tagged with fluorescein (FITC) highlights the expression and localisation of HAGE within the cells. High level of HAGE protein expression, mainly localised in the cytoplasm, was achieved in about 90% of transfected cells when ESTDAB-07 cells were transfected with any of the plasmids containing HAGE. Primary antibodies conjugated with phycoerythrin (PE) highlight the expression and localisation of the murine co-stimulatory molecules within the cells. Expression of murine co-stimulatory molecules could be detected at different levels of intensity on the cell membrane of about 20% of transfected cells. On the other hand, neither cells transiently transfected with the empty plasmid nor cells incubated with FITC- and PE-conjugated secondary antibodies showed staining (Fig. 4.10).

4.2.3 Cloning of HAGE into pSHAME2a vector for SFV generation

Plasmids for SFV generation were kindly provided by Dr. Rod Bremner (University of Toronto). In order to generate SFV/HAGE viruses, HAGE sequence had to be cloned into pSHAME2a vector. To subclone HAGE sequence from pcDNA3/HAGE expression vector, pcDNA3/HAGE was double digested with BamHI and NsiI restriction enzymes along with pSHAME2a vector. Digested pSHAME2a vector and HAGE band were gel extracted and ligated overnight as described before (Fig. 4.11). Clones of vector from transfected XL1-Blue bacteria were isolated and screened for insertion of HAGE cDNA by repeating the BamHI-NsiI double digestion. As seen in Fig. 4.12, four clones with HAGE insertion were detected and were further used for confirmation of protein production after transfection.

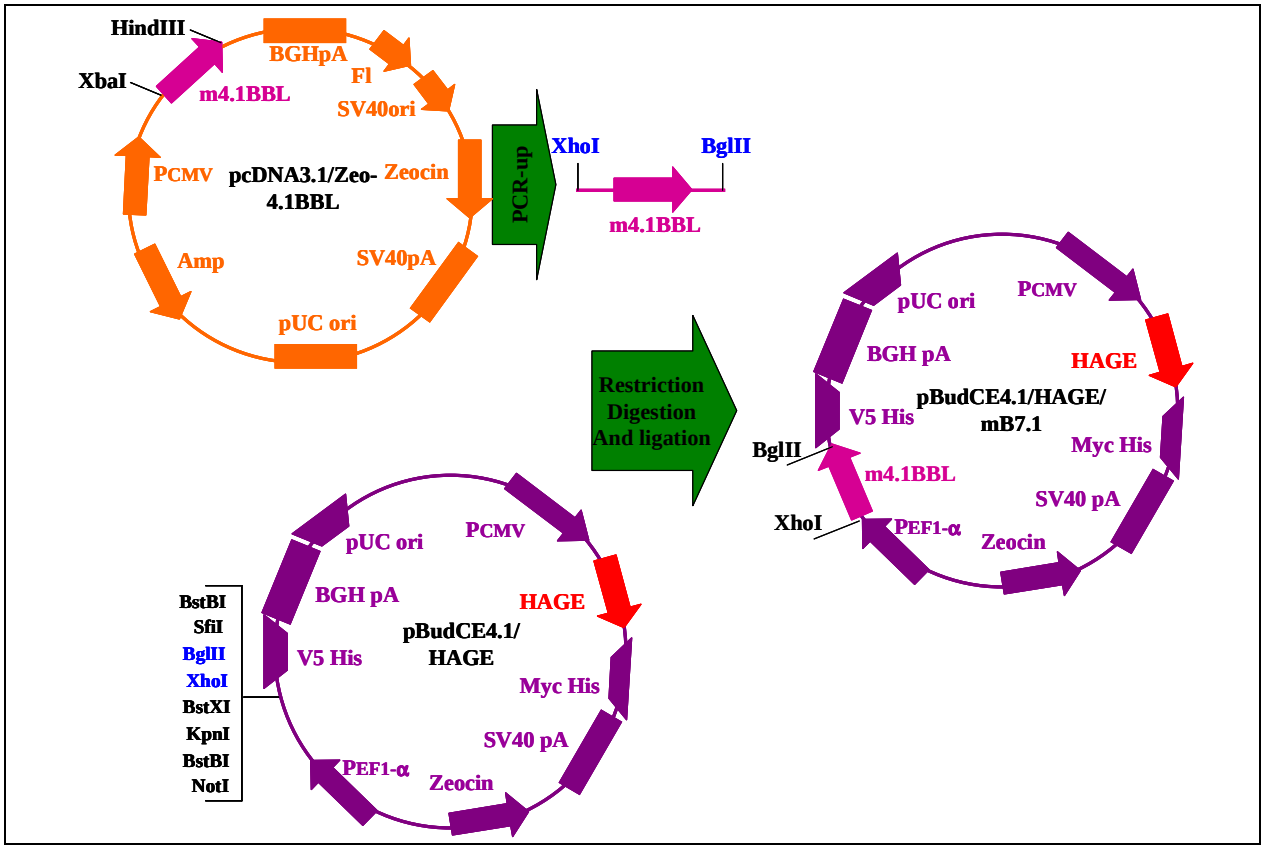

Figure 4.8: Diagrammatic representation of the construction of the plasmid pBudCE4.1/HAGE/m4.1BBL. *The m4.1BBL cDNA was first PCR up. The PCR amplicons and the pBudCE4.1/HAGE vector were digested with restriction enzymes BglII and XhoI. The digested products were purified by gel purification and desired fragments were selected for ligation. After ligation, the cDNA for m4.1BBL was into the pBudCE4.1/HAGE plasmid.*

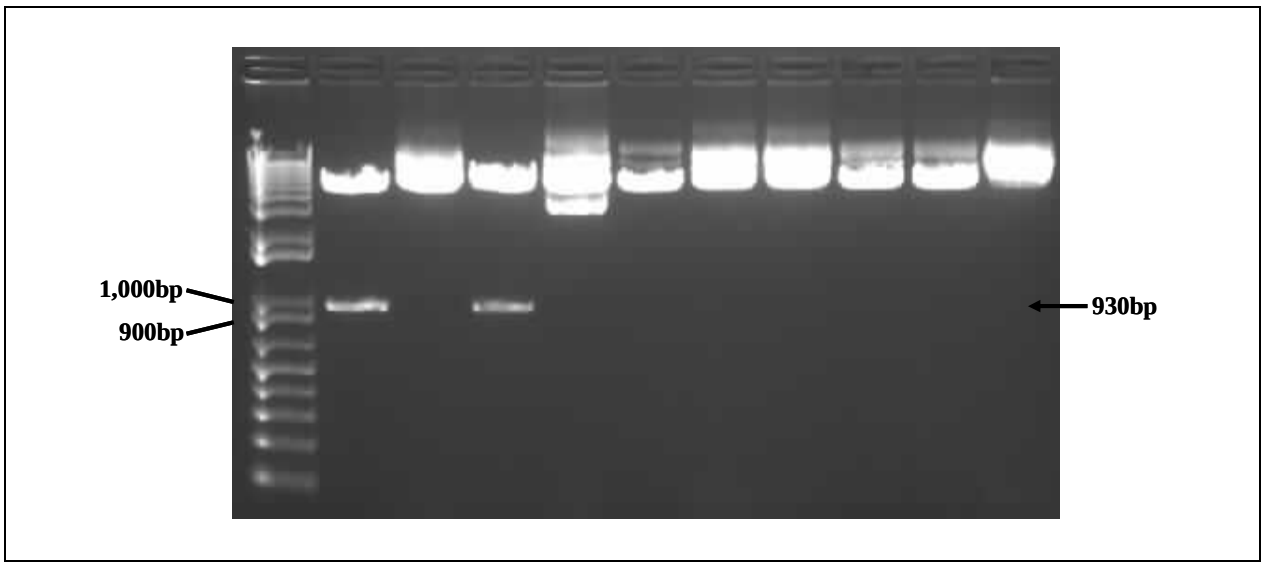

Figure 4.9: Agarose gel electrophoresis after double digestion of pBudCE4.1/HAGE/ m4.1BBL clones. *After double digestion with BglII and XhoI, a band corresponding to 930bp could be detected in 2 out of 10 clones picked, indicating successful cloning of m4.1BBL into the pBudCE4.1/HAGE mammalian expression vector.*

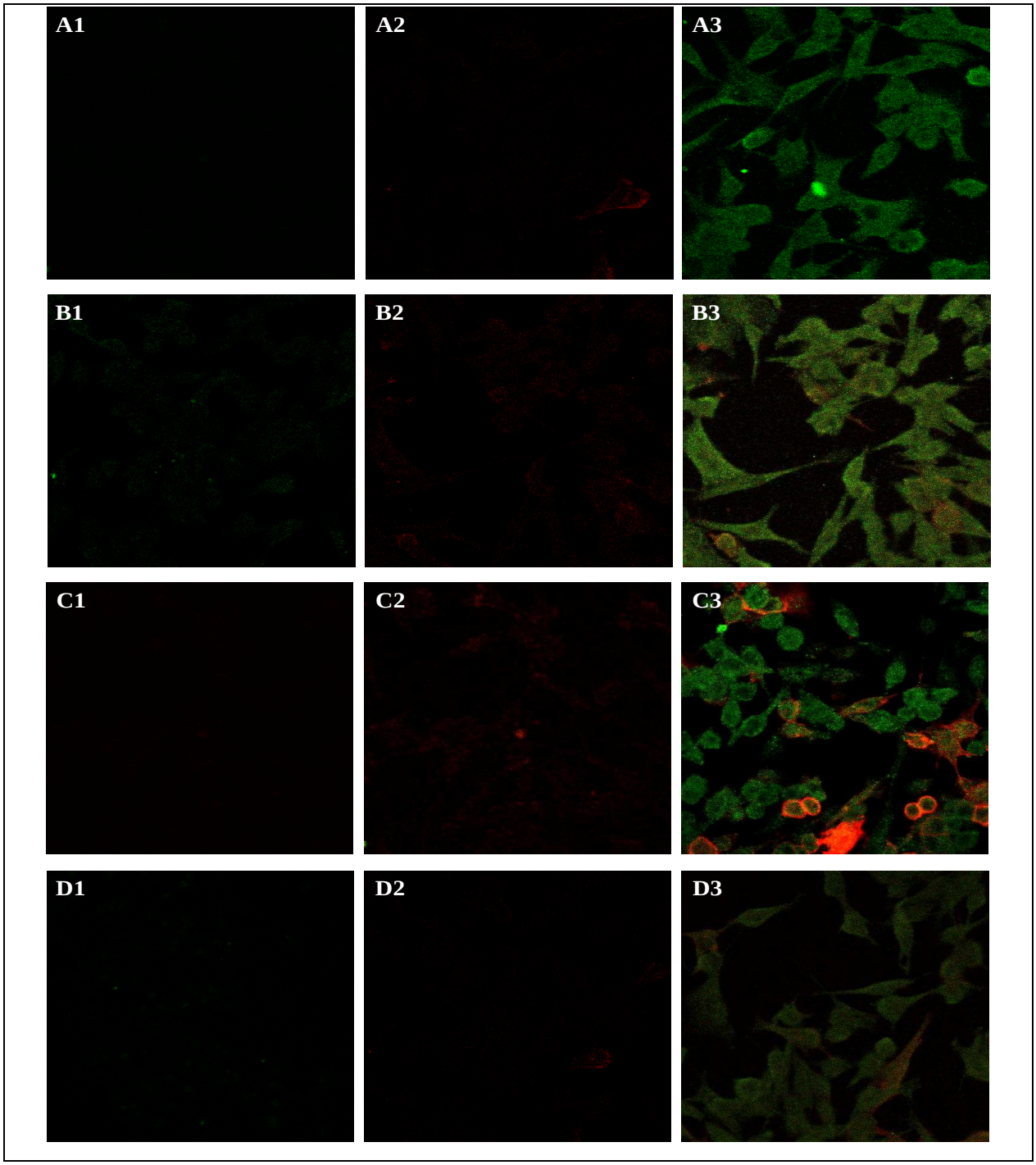

Figure 4.10: Immunofluorescence assay for HAGE and murine co-stimulatory molecules expression analysis and localisation in a melanoma cell line. *Immunofluorescence was observed under a confocal microscope in ESTDAB-07 cells transiently transfected with pBudCE4.1/HAGE (A), pBudCE4.1/HAGE/mB7.1 (B), pBudCE4.1/HAGE/mB7.2 (C) and pBudCE4.1/HAGE/m4.1BBL (D). No non-specific staining was obtained when transfected cells were incubated with both FITC- and PE-conjugated isotype control antibodies only (1) or when cells were transiently transfected with the empty plasmid and stained for protein expression (2). HAGE protein expression is mainly localised in the cytoplasm (green) while murine co-stimulatory molecules are found on the membrane (red)(3). It is worth mentioning that staining of mB7.1 and m4.1BBL molecules will have to be repeated with increased concentrations of primary antibodies and functioning confocal microscope.*

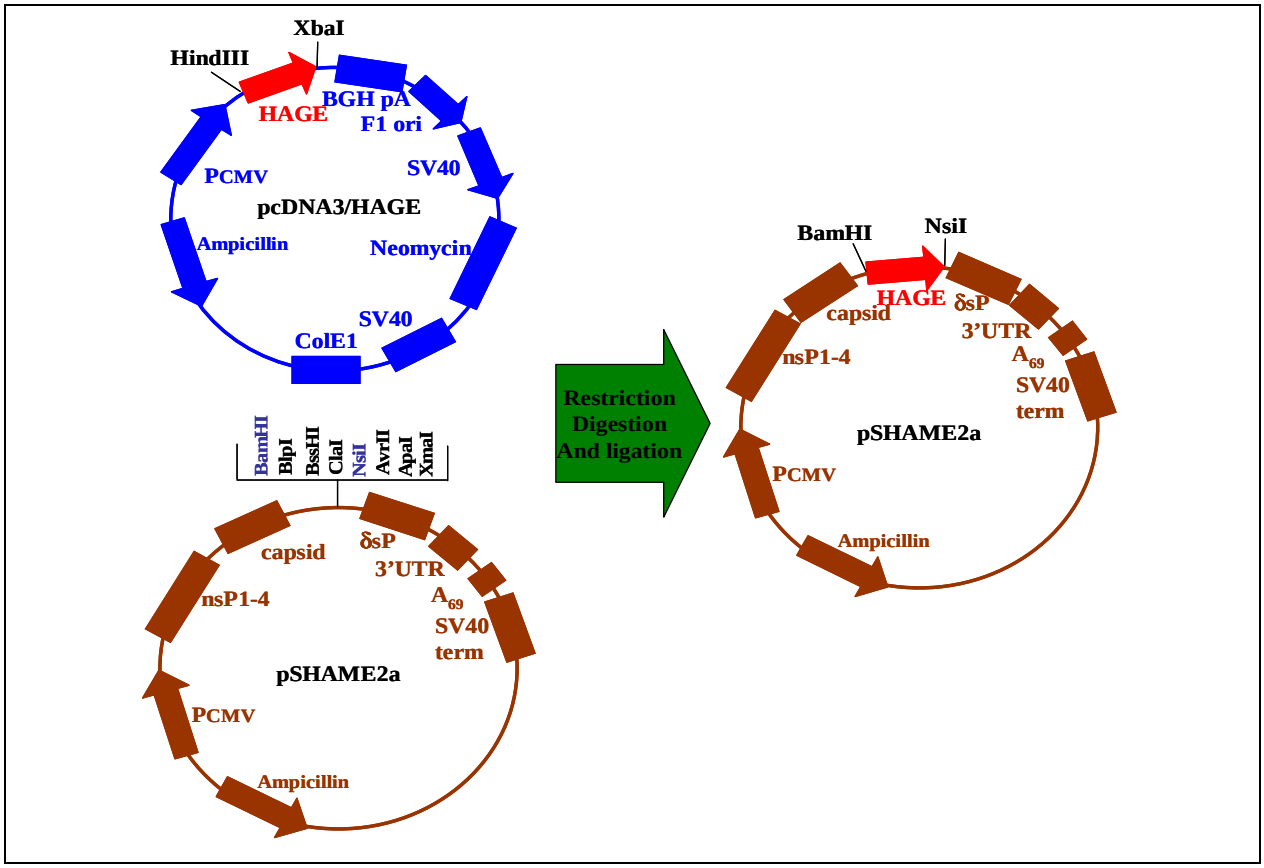

Figure 4.11: Diagrammatic representation of the construction of the plasmid pSHAME2a/HAGE. *The pcDNA3/HAGE and pSHAME2a vectors were first digested with restriction enzymes BamHI and NsiI. The digested products were purified by gel purification and desired fragments were selected for ligation. After ligation, the cDNA for HAGE was inserted into the pSHAME2a plasmid.*

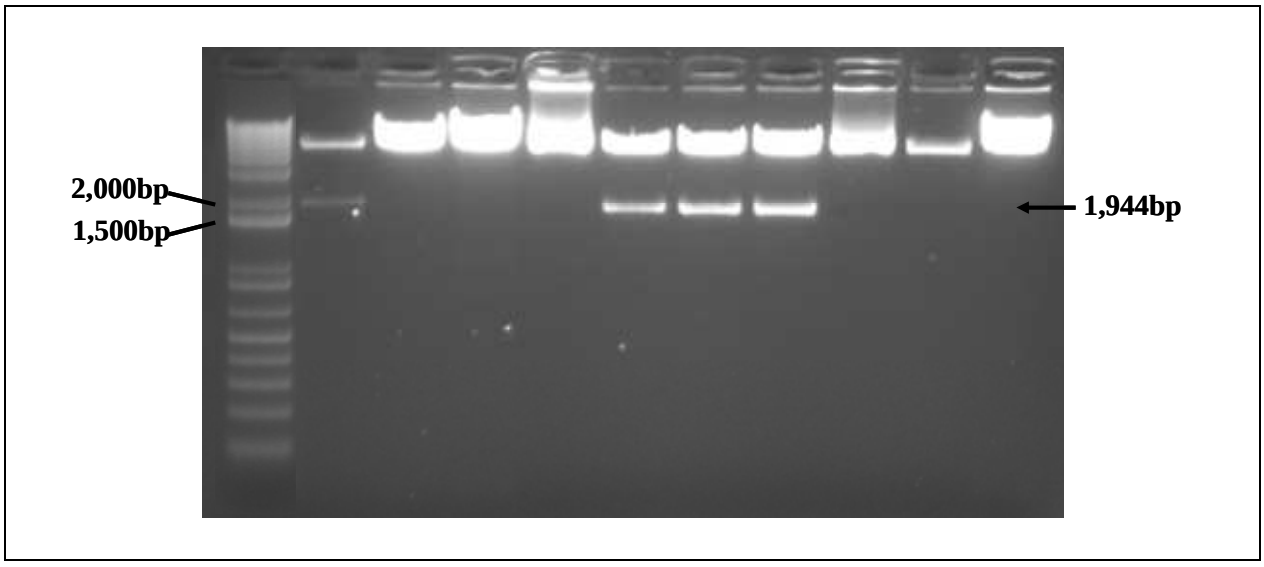

Figure 4.12: Agarose gel electrophoresis after double digestion of pSHAME2a/HAGE clones. *After double digestion with BamHI and NsiI, a band corresponding to 1.9kb could be detected in 4 out of 10 clones picked, indicating successful cloning of HAGE into the pSHAME2a mammalian expression vector.*

To confirm protein production of HAGE protein by pSHAME2a/HAGE vector, murine bone marrow-derived dendritic cells (BM-DC) were transfected with the vector using Lipofectamine 2000 and protein production was tested 48 hours later after staining for the FLAG tag. Secondary antibody tagged with fluorescein highlights the expression and localisation of HAGE within the cells. HAGE was found to be mostly localised in the nucleus as seen in Fig. 4.13 and expressed at the protein level in about 70% of transfected BM-DC. This result confirmed the ability of BM-DC to express HAGE at the protein level upon transfection as well as the validity of the utilisation of BM-DC transiently transfected to express HAGE in immunisation procedures.

4.2.4 Generation of SFV viruses

Semliki Forest Virus generation requires co-transfection of the helper plasmid as well as the replicon plasmid containing the gene of interest (β-gal or HAGE). Factors, such as method of transfection and molar ratios of the two plasmids can affect the titre of virus generated. Calcium phosphate precipitation (Promega) and electroporation were both available as transfection methods. Using 293 cells, it appeared that transfection with calcium phosphate offered the best virus yield (Data not shown). Viral titration appeared to be slightly improved when 1:2 and 1:3 molar ratios were used, although no significant differences were observed. However, a molar ratio of 1:3 generated the highest titrations (up to $1.7*10^6$ IU/ml) and it was decided to use this ratio for all future experiments (Fig. 4.14).

Finally, to confirm HAGE protein production in cells infected with SFV-HAGE, BHK-21 cells were infected with $1*10^6$ IU/ml of the activated virus for one hour and generation of FLAG-tagged protein was visualised using immunohistochemistry. Briefly, anti-FLAG antibody and HRP-conjugated anti-mouse secondary antibody were sequentially added to infected BHK-21 cells. DAB solution was then added to the cells with brown staining indicative of the production of the protein. As seen in Fig. 4.15, dark brown staining was achieved in infected BHK-21 cells suggesting the production of the FLAG-tagged HAGE protein whilst no or only little background staining was observed in control cells. Moreover, immunohistochemistry revealed HAGE to be mostly localised in the nucleus, which agrees with earlier results demonstrating by immunofluorescence the expression of HAGE in pSHAME2a/HAGE-transfected BM-DC.

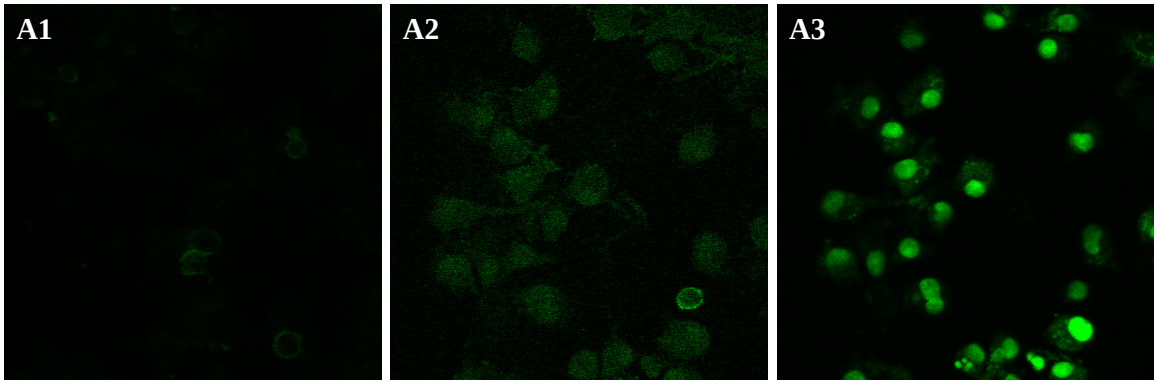

Figure 4.13: Immunofluorescence assay for HAGE protein expression analysis and localisation in bone marrow-derived dendritic cells. *Immunofluorescence was observed under a confocal microscope in BM-DC transiently transfected with pSHAME2a/HAGE (A). pBudCE4. No non-specific or very limited staining was obtained when cells were incubated with no antibody (1) or with FITC-conjugated secondary antibody only (2). HAGE protein expression is mainly localised in the nucleus.*

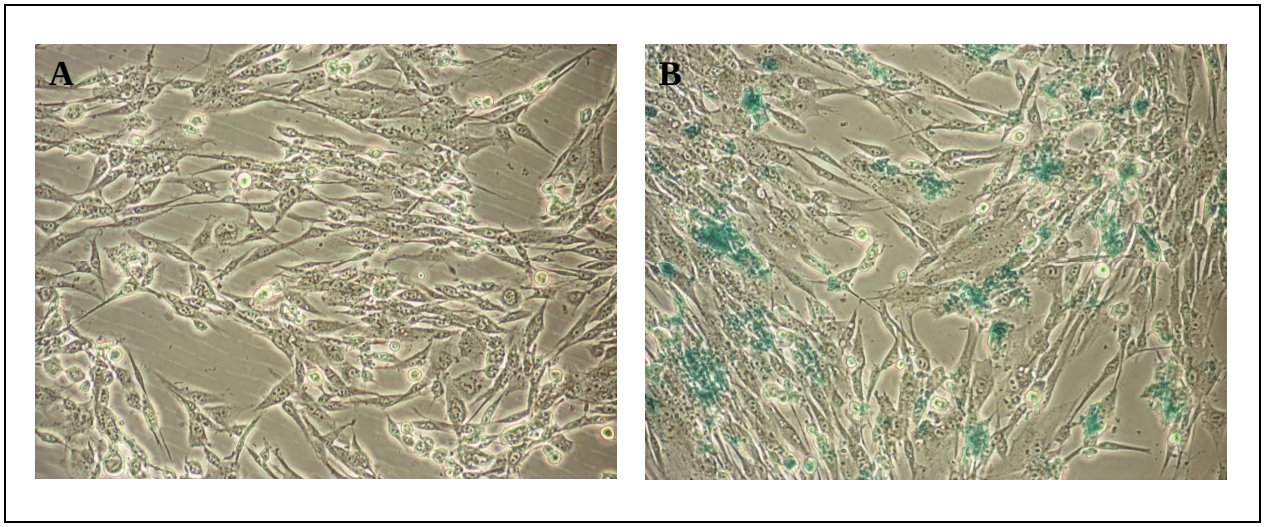

Figure 4.14: X-gal assay on BHK-21 cells 48 hours after infection with SFV/β-gal. *The X-gal assay demonstrates the infection of BHK-21 cells by SFV/β-gal appearing in blue (B). No non-specific staining was obtained in non-infected cells (A).*

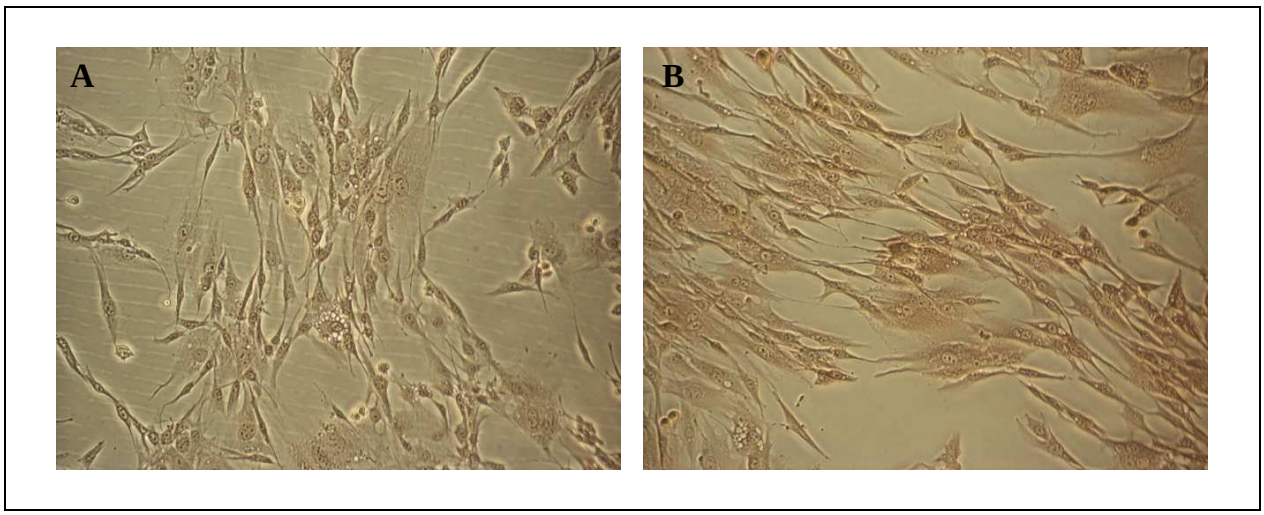

Figure 4.15: Immunohistochemistry staining of BHK-21 cells 48 hours after infection with SFV/HAGE. *The immunohistochemistry demonstrates the infection of BHK-21 cells by SFV/HAGE appearing in brown (B). Little non-specific secondary staining was obtained in non-infected cells (A).*

4.3 Discussion

Nucleic acid vaccination presents numerous advantages, among which the variety of forms such as plasmid or viral DNA depending on the targeted application, the flexibility and the specificity of generating immune responses against multiple antigens and in certain cases polarising that response, and finally the duration of the effects with direct protein expression *in vivo* for several months contrary to whole protein vaccination often considered to be expensive, time-consuming and short-lived as injected proteins are likely to be degraded in a few days.

Immune prevention and rejection responses have been generated with various modes of vaccination such as peptide, DNA or virus. Identification of immunogenic and naturally processed peptides from tumour antigens can be now relatively quick *via* the use of reverse immunology and transgenic mice. However, it still remains difficult in the case of self antigens. Indeed, deletion or tolerisation *via* central and peripheral tolerance mechanisms suppresses most T cells specific for high affinity epitopes from self antigens leaving several medium affinity peptides to be identified, which is expensive and time-consuming. DNA-based vaccination with whole gene or only sequences of it allows the overcoming of this situation. DNA vaccines also present another advantage as they are not restricted to HLA haplotypes of patients meaning that the whole population can be targeted. The efficiency of these vaccines can also be further enhanced by associating antigen genes with various co-stimulatory molecule or cytokine genes (Guinn *et al.*, 1999; Liu *et al.*, 2004), by orientating the antigens to a preferred processing pathway with specific signal sequences (Liu *et al.*, 2004; Stevenson *et al.*, 2004) or by adding a pathogen-derived sequence to provide critical CD4+ T cell help (Buchan *et al.*, 2005).

In this study, HAGE cDNA was cloned in a mammalian expression vector in order to investigate endogenous process of immunogenic HAGE-derived peptides and xenogeneic immunisation in a HAGE-positive tumour model. Upon successful assembly and construction of the expression vector, it was critical to confirm that it performed accordingly in *in* vitro experiments before moving into *in vivo* studies. A HAGE-negative melanoma cell line was therefore transiently transfected with the expression vector using Lipofectamine 2000. HAGE expression at the mRNA level was confirmed by RT-Q-PCR (data not shown). However, mRNA level of a gene does not always correlate with its protein expression. Transfection of the same cell line was then repeated and protein expression of HAGE was determined after two days by immunofluorescence. High level of HAGE expression was observed in transfected cells compared to control cells transfected with the empty plasmid or stained with secondary antibody only. The expression vector used for cloning HAGE is characterised by two multiple

cloning sites with two independent promoters, therefore enabling the cloning of another antigen, a cytokine or a co-stimulatory molecule in order to enhance the efficiency of the elaborated vaccine. In this study, the murine co-stimulatory molecules mB7.1, mB7.2 and m4.1BBL were selected and cloned into the remaining free multiple cloning site. Following sequencing and confirmation that sequences cloned were 100% identical to published sequences, expression of these co-stimulatory molecules at the protein level was studied by immunofluorescence using PE-conjugated primary antibodies 48 hours after cellular transfection. Expression of mB7.1, mB7.2 and m4.1BBL was observed on the surface of transfected cells compared to cells stained with PE-conjugated isotype control antibody only, or cells transfected with the empty plasmid and stained for the expression of the murine co-stimulatory molecules. Altogether, these data confirmed that expression vectors encoding HAGE only or together with a murine co-stimulatory molecule could be used in a xenogeneic tumour model.

Furthermore, tumour vaccines based on naked DNA only do not always lead to beneficial effects depending on the immunogenicity of the antigen used. The immune system often requires a stronger signal and this could be provided by viral or bacterial vectors allowing not only the activation of both arms of the immune system and the infection of different cell types, but also the breakage of tolerance whenever antigens with low immunogenic potential such as self antigens are used in tumour vaccination procedures. Viral and bacterial vectors have been extensively used for *in vitro* modification of cells or *in vivo* immunotherapeutic experiments in animals and humans. Adenovirus and poxvirus have been so far the most widely used viral vectors but like other viral and bacterial vectors, they present a certain number of limitations. Among them, there is always the risk of generating replication-competent vectors *in vivo*, which could be detrimental for cancer patients with immuno-suppressed organisms. Several studies have described the utilisation of alphaviruses such as Semliki Forest virus in immunotherapeutic strategies. Indeed, SFV offers a certain level of safety in vaccination approaches as their production requires the co-transfection of both helper and replicon plasmids, as well as an activation brought by the α-chymotrypsin-dependent cleavage of the p62 structural protein (DiCiommo *et al.*, 2004).

As an alternate to naked DNA immunisation, HAGE cDNA was then cloned in the SFV replicon vector in order to investigate the potential of SFV-based vaccines to target HAGE-expressing tumour cells. Upon successful assembly and construction of the expression vector pSHAME2a/HAGE, transient transfection of murine BM-DC and immunofluorescence

analysis confirmed the expression of HAGE at the protein level, a result critical before performing *in vivo* studies. Also, the generation of recombinant SFV viruses strongly depends on various parameters such as the method of transfection or the molar ratio of the two required plasmids. The co-transfection of β-gal reporter plasmid with the helper plasmid allowed easy optimisation of virus production and titration. Firstly, the efficiency of two transfection techniques (calcium phosphate precipitation and electroporation) was compared. Higher viral titres were reached when calcium phosphate precipitation was used to transfect 293 cells. Indeed, this method was proved to be the cheapest and the most reliable, which is in correlation with previous publications (DiCiommo and Bremner, 1998) and earlier experiments carried out in our laboratory comparing the calcium phosphate method with Lipofectamine 2000 and GeneJuice (Assudani D., unpublished data). Thereafter, different molar ratios of helper to replicon plasmids were trialled in order to determine the optimum mixture capable of generating the highest viral titre. Although differences were not significant, results showed that a molar ratio of 1:3 was slightly better and used for virus generation. Such a ratio ensures that all the cells transfected with the replicon plasmid will also be transfected with the helper plasmid being in higher concentration. All these new parameters were considered and applied to the production of recombinant SFV/HAGE viruses. Upon activation, BHK-21 cells were infected and high levels of HAGE expression were detected by immunohistochemistry staining indicating both viral activity and efficacy.

In conclusion, expression vectors for HAGE alone or combined with a murine co-stimulatory molecule, as well as the SFV vector for HAGE were constructed and their capacity to produce proteins *in vitro* were confirmed using transfection experiments. However, the efficiency of developed DNA and viral vectors to generate an immune response and prevent or reject tumour cells *in vivo* requires further investigation.

Chapter 5: Epitope identification from HAGE

5.1 Introduction

Since the discovery of the first human tumour-associated antigen and CTL epitope derived from this TAA, several differents strategies have been used for the identification of MHC class I and class II epitopes. They can now be grouped in two major categories termed direct immunology and reverse immunology.

(i) Direct immunology: The first category can be sub-grouped into a genetic or a biochemical approach for the discovery of TAA-derived epitopes. Indeed, cell lines expressing the relevant MHC molecules and transfected with DNA or cDNA libraries from tumour cells can be created and then tested by measuring the *in vitro* reactivity of tumour-infiltrating lymphocytes. Using this technique, MAGE-1 was the first TAA to be identified by T. Boon and colleagues (van der Bruggen *et al.*, 1991). The same group applied this technique to identify the BAGE and GAGE antigens. Adapting this method *in vivo*, the group of S. Rosenberg identified several other tumour antigens such as MART-1, gp100 and tyrosinase (Kawakami *et al.*, 1994). Later improvement of this technique based on the utilisation of truncated regions of MAGE-1 DNA allowed the identification of the first CTL peptide target (Traversari *et al.*, 1992). Since then, several CTL and T helper targets contained within these TAA have been identified using this technique (Halder *et al.*, 1997, Smith *et al.*, 2001). However, the improvement with more efficient expression libraries to discover relevant epitopes does not compensate for the difficulties encountered in generating CD4+ and/or CD8+ T cell clones from TIL and the time spent in completing these experiments. That is why researchers have looked into a more direct approach using the biochemical properties of MHC molecules. Three major techniques were developed. The first one extracted total cellular peptides with a very acidic solution to provoke total cell lysis. Unfortunately, only a fraction of the extracted peptides were actually class I-related (Falk *et al.*, 1990). The second technique developed is highly specific and relies on the purification of MHC class I complexes from cell lysates using class I allele-specific monoclonal antibodies. This method has to date been the most commonly applied for the identification of MHC class I epitopes, although not only surface bound MHC class I:peptide complexes were isolated but also those trafficking from the endosomal pathway as well as those still contained within the ER (Nikolic-Zugic and Carbone, 1991). The third and last biochemical approach relies on the brief exposure of viable cells to a low pH buffer (*i.e.*: citrate phosphate buffer at pH 3.3). Acid stripping exploits the property of the non-covalent link

between the α1 domain of the MHC class I heavy chain and β2-microglobulin and that low pH can weaken and destabilise MHC: peptide complexes (Storkus *et al.*, 1991). These MHC peptides, also referred to as "HLA peptidome", can then be separated by means of reverse phase chromatography and sequenced using tandem mass spectrometric techniques. Interestingly, this approach was improved by transfecting tumour cell lines with a truncated version of the MHC genes and resulting in the secretion of soluble MHC:peptide complexes easily purifiable and analysable (Barnea *et al.*, 2002). This technique has been applied to the identification of MHC class I peptides from both cultured tumour cell lines (Ramakrishna *et al.*, 2003) and primary tumour material (Flad *et al.*, 1998) and several tumour-associated peptides have since been discovered (Clark *et al.*, 2001; Bonner *et al.*, 2002). There are several advantages in using a biochemical approach as both high and low-to-moderate affinity peptides can be isolated and sequenced. Also, this is the only current method revealing eventual post-translational modifications carried by MHC epitopes such as phosphorylation (Zarling *et al.*, 2000), deamidation (Skipper *et al.*, 1996) and glycosylation (Haurum *et al.*, 1999). Moreover, a recent study described a new form of post-translational modifications that could also be detected by these approaches, resulting from the generation of peptide-splice variants from short MHC-restricted peptides comprised of two non-contiguous sections of the same protein within the proteasome, although the frequency of this phenomenon remains to be defined (Hanada *et al.*, 2004; Vigneron *et al.*, 2004). With these methods, a large amount of peptides needs to be isolated from tumour cell lines and/or tumour masses for analysis and sequencing, but the constant development of mass spectrometry technology and the improvement in term of sensitivity of the equipments diminishes the importance of this limiting factor and increases the capacity to identify low number of tumour-specific peptides among thousands of irrelevant normal peptides. Mild acid elution has so far largely been used for the identification of MHC class I peptides but proved inefficient for the identification of MHC class II peptides as MHC class II molecules remain stable at this mild pH and can only be eluted at much lower pH, which is generally detrimental to the cells (D. Barry, personal communication). Interestingly, this direct approach can be used in combination with reverse immunology. Indeed, potential epitopes from CEA and p53 have been identified following prediction of an epitope, calibration of the analysis apparatus with the synthetic peptide to assist in its identification and finally, detection of the suspected epitope in the actual tumour samples (Schirle *et al.*, 2000). However, identification of peptides bound to MHC molecules on the surface of tumour cells does not

guarantee their immunogenicity (Rojas *et al.*, 2005) and therefore, requires further *in vivo* and *in vitro* analyses.

(ii) Reverse immunology: Reverse immunology, on the other hand, starts from a known sequence and attempts to predict potential CTL or T helper epitopes using a combination of known MHC binding motifs and the sequence of the antigen of interest. Tumour antigen amino acid sequences can be analysed *in silico* by computer-based algorithms to predict antigenic determinants from tumour antigens that could be useful for immunotherapy. These determinants are predicted by the presence or absence of key anchor residues in positions known to favour interactions with the MHC binding groove of interest. For example, positions 2 and 9 are the key anchor motifs for HLA-A0201 and binding would be enabled by the presence of aliphatic amino acids such as leucine, isoleucine, valine or methionine at these positions. However, these algorithms tend to have low levels of accuracy as other positions are not taken into account and the presence of a large and bulky amino acid in the middle of sequence between positions 2 and 9 might actually interfere with the binding. That is why models considering the whole amino acid sequences have been developed. These algorithms or "motif matrices" attribute negative or positive values to each amino acid depending on the MHC binding cleft it will interact with. BIMAS (www.bimas.cit.nih.gov) and SYFPEITHI (www.syfpeithi.de) are the two most commonly used of these programs and can calculate binding affinity to various MHC haplotypes of peptides derived from a given tumour antigen with greater accuracy. The major drawback of these two algorithms remains the lack of natural processing prediction and later versions are now taking proteasomal cleavage prediction into account enhancing considerably the likelihood of identifying a "true" CTL or T helper epitope. However, proteasomal cleavage prediction and binding affinity determination do not guarantee the immunogenicity of a given peptide (Rojas *et al.*, 2005). So, depending on their scores, peptides are synthesised and screened for their immunogenicity, their natural processing and their presentation in the context of MHC molecules in both *in vivo* and *in vitro* experiments with human PBMC and transgenic mouse models, respectively.

Several MHC class I and class II peptides from various TAA such as gp100, MAGE, MART-1 or p53 have been so far described based on this approach (Touloukian *et al.*, 2000; Zarour *et al.*, 2000; Bar-Haim *et al.*, 2004; Rojas *et al.*, 2005). The combination of transgenic mouse models expressing single human MHC molecules or double class I and class II HLA molecules with or without knockout murine MHC molecules, with reverse immunology brings several advantages as it shortens considerably time and resources, allows pre-clinical investigations to the

applicability of predicted peptides for vaccination programs, in addition to peptide immunogenicity and natural processing that are determined *in vitro* in highly controlled and artificial settings after *in vivo* challenge with peptides and genes, respectively. It is also worth mentionning that several class I and class II peptides have been shown to be processed identically in transgenic mouse models and in humans (Theobald *et al.*, 1995; Theobald *et al.*, 1997; Rojas *et al.*, 2005), although not all peptides are necessarily processed in a same manner (Street *et al.*, 2002). The only major drawback of this technique is the availability of mice transgenic for the HLA molecules with the highest frequencies such as HLA-A0201, -DR0101 and –DR0401. Interestingly, one further method relies on the power of artificial neural networks, a self-trained system capable of determining peptides with immunological interest with very high accuracy. Indeed, peptides predicted to be immunogenic were detected in melanoma patients and found to be able to potentiate a peptide-specific CTL response (Bredenbeck *et al.*, 2005), the only disadvantage being the large amount of data required to train the system. Moreover, none of these peptides were predicted to be HLA ligands by more common algorithms suggesting that several low-to-moderate affinity but immunodominant peptides could be neglected by more conventional methods of reverse immunology.

Reverse immunology in conjunction with the use of transgenic mice was considered the most appropriate for the purpose of this study. In order to investigate the immunogenicity of HAGE, peptides predicted to be immunogenic by web-based algorithms were synthesised and tested in HHDII (HLA-A0201) mice for their ability to generate CTL responses in a MHC class I model, and in HLA-DR0101 and HLA-DR0401 mice for their capacity to generate T helper responses in a MHC class II model. Following evaluation of their immunogenicity, peptides were further analysed for their natural processing using HAGE cDNA immunisation and *in vitro* peptide re-stimulation.

5.2 Results

5.2.1 Identification of HLA-A0201 restricted peptides from HAGE using HHDII transgenic mice

5.2.1.1 Immunogenicity of peptides predicted from HAGE sequence for HLA-A2

In order to ascertain the immunogenicity of peptides predicted from HAGE sequence for HLA-A0201, HHDII transgenic mice were immunised once with 100μg of the MHC class I peptides in 1:1 emulsion with IFA as described by Rojas *et al.* (2005). 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 pulsed with relevant peptide for five days. On day 6, the immunogenicity of the peptides was determined by measuring the capacity of T cells generated against these peptides to kill peptide-pulsed target cells in a standard four-hour chromium release assay. In this experiment, RMAS/A2 cells, pulsed with either relevant or irrelevant peptide, were used as targets to determine the specificity of the response. Results showed that after one round of immunisation, a peptide-specific cytotoxic response was achieved with four out of eight peptides tested. Peptide-specific killing of pulsed RMAS/A2 cells was detected for peptides HAGE 103, HAGE 126, HAGE 296 and HAGE 506-I with cytotoxic responses ranging from 20% to up to 80% with the highest effector to target ratio. Minimal lysis of RMAS/A2 cells pulsed with irrelevant peptides was observed confirming the specificity of the immune response (Fig. 5.1). Furthermore, the lysis was completely blocked by HLA/A2 antibody indicating that T cells generated were killing in a HLA-A2-restricted manner (Data not shown). These results were later confirmed by conducting syngeneic peptide-pulsed DC immunisation. Briefly, HHDII transgenic mice were immunised intra-dermally with $2*10^6$ BM-DC generated *in vitro* according to the method of Inaba and colleagues (1992) and loaded with 40μg of the MHC class I peptides as described by Rojas *et al.* (2005). Seven days after immunisation, splenocytes were harvested and prepared as described earlier. RMAS/A2 cells were pulsed with either relevant or irrelevant peptide and used as targets in a cytotoxicity assay. Peptide-specific killing of pulsed RMAS/A2 cells was also detected for peptides HAGE 103, HAGE 126, HAGE 296 and HAGE 506-I with similar cytotoxicity levels to peptide immunisation, whilst leading to minimal lysis of target cells pulsed with irrelevant peptides (Fig. 5.2).

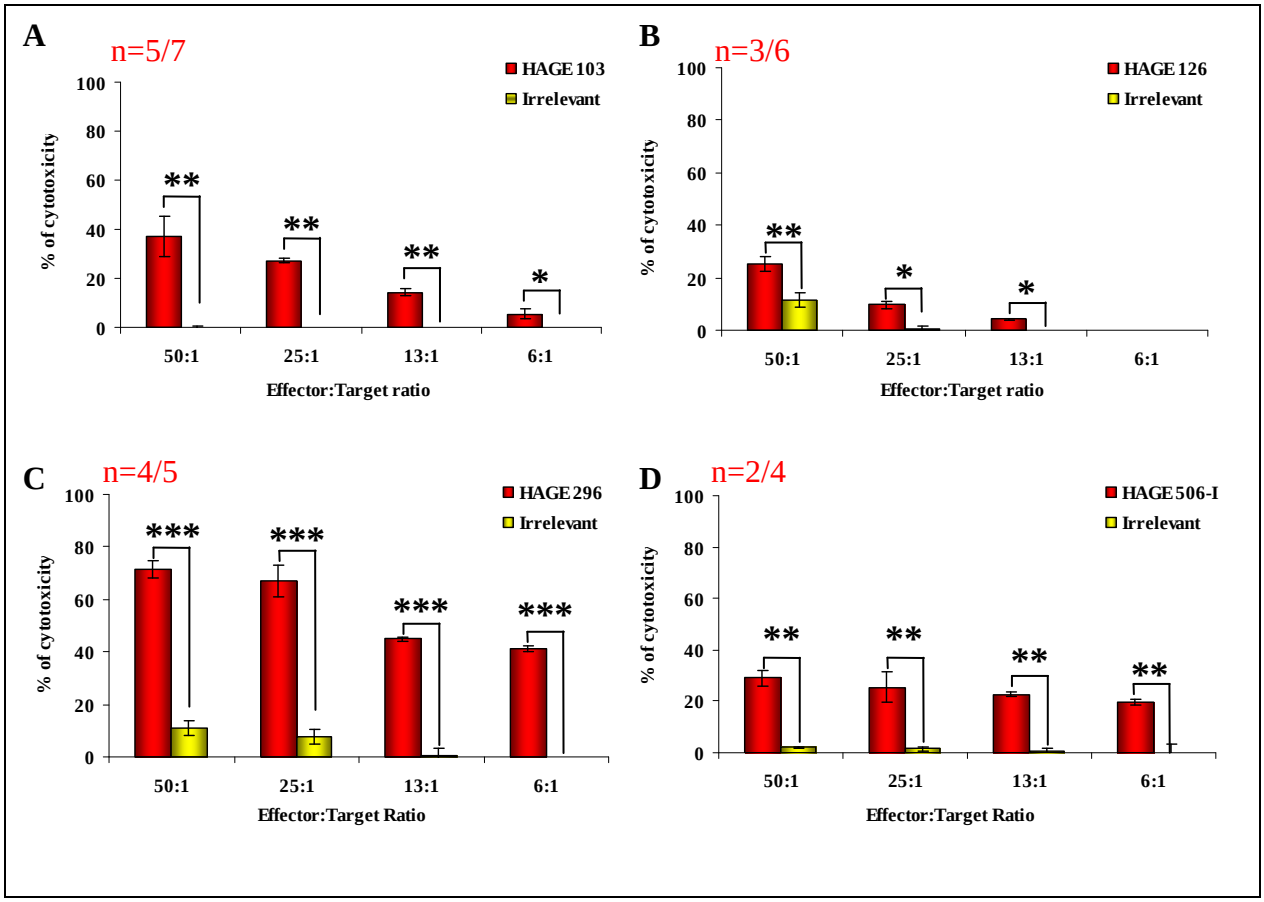

Figure 5.1: Cytotoxicity assay using T cells generated from HHDII transgenic mice immunised with HAGE-derived class I peptides. *Mice were immunised with HAGE 103 (A: IIQEQPESL), HAGE 126 (B: AVIDNFVKKL), HAGE 296 (C: YLMPGFIHLV) and HAGE 506-I (D: DLILGNISV) peptides and cytotoxicity assays were carried out after in vitro re-stimulation of immunised cells with these peptides or with an irrelevant one. *p<0.05, **p<0.01, ***p<0.001 are the statistical differences between HAGE-derived class I peptides and Irrelevant determined by unpaired Student T test n=a/b with "a" being the number of mice responding to the peptide immunisation and used to represent these graphs, and "b" being the total number of mice immunised.*

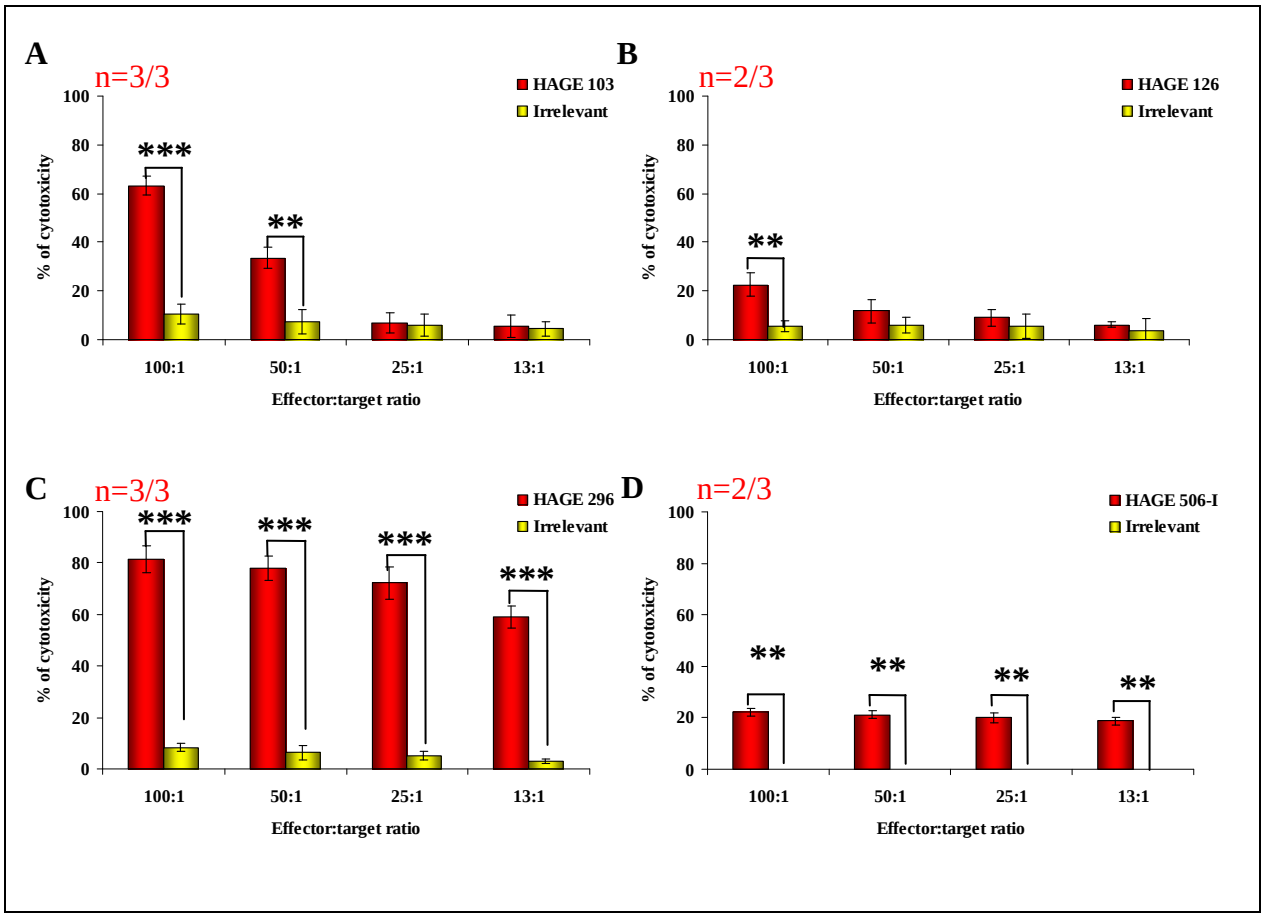

Figure 5.2: Cytotoxicity assay using T cells generated from HHDII transgenic mice immunised with DC pulsed with HAGE-derived class I peptides. *Mice were immunised with DC pulsed with HAGE 103 (A: IIQEQPESL), HAGE 126 (B: AVIDNFVKKL), HAGE 296 (C: YLMPGFIHLV) and HAGE 506-I (D: DLILGNISV) peptides, and cytotoxicity assays were carried out after in vitro re-stimulation of immunised cells with these peptides or with an irrelevant one. **p<0.01, ***p<0.001 are the statistical differences between HAGE-derived class I peptides and Irrelevant determined by unpaired Student T test. n=a/b with "a" being the number of mice responding to the peptide immunisation and used to represent these graphs, and "b" being the total number of mice immunised.*

Moreover, supernatants from the *in vitro* re-stimulation culture were harvested on day 3 and day 5 to measure IFNγ production by ELISA. Peptides HAGE 103, HAGE 126 and HAGE 506-I allowed significant levels of peptide-specific IFNγ secretion on day 5 of the culture. Peptide HAGE 296, which was found to be the most immunogenic peptide with up to 80% killing of target cells in 80% of the mice immunised, seemed to induce significant levels of peptide-specific IFNγ secretion in both day 3 and day 5 of the experiment (Fig. 5.3). On the other hand, T cells could not be generated against peptides HAGE 507, HAGE 508, HAGE 509 and HAGE 551 and hence, were defined as non-immunogenic and no further experiments were carried out with these peptides (Data not shown). Results of the immunisation of HHDII transgenic mice with HAGE-derived MHC class I peptides were summarised in table 5.1.

Table 5.1: Immunogenic HAGE-derived MHC class I peptides.

Peptides	Immunogenicity	Cytotoxicity ($p<0.05$)	IFNγ secretion ($p<0.05$)
HAGE 103	Yes	8/10	2/3
HAGE 126	Yes	5/9	1/2
HAGE 296	Yes	7/8	4/4
HAGE 506-I	Yes	4/7	1/2
HAGE 507	No	0/3	Not done
HAGE 508	No	0/6	Not done
HAGE 509	No	0/3	Not done
HAGE 551	No	0/3	Not done

5.2.1.2 Determination of natural processing of immunogenic class I peptides

In order to confirm if any of the four identified immunogenic class I peptides detailed in the table above were endogenously processed by the proteasome and naturally presented at the cell surface by HLA-A0201 molecules, two methods of immunisation were selected. They rely on the injection of gold-coated HAGE-encoding plasmid DNA by gene gun or syngeneic BM-DC prepared *ex vivo* and transfected to express HAGE in HHDII transgenic mice. In order to validate the gene gun strategy, p53 cDNA was used as a positive control to check the quality of DNA bullets, gene gun immunisation and *in vitro* experiments. This control consisted of three rounds of gene gun immunisation at seven-day intervals of a p53-encoding plasmid and *in vitro* re-stimulation with peptides p53 149 (Theobald *et al.*, 1995; Chikamatsu *et al.*, 1999), which was previously reported to be naturally endogenously processed.

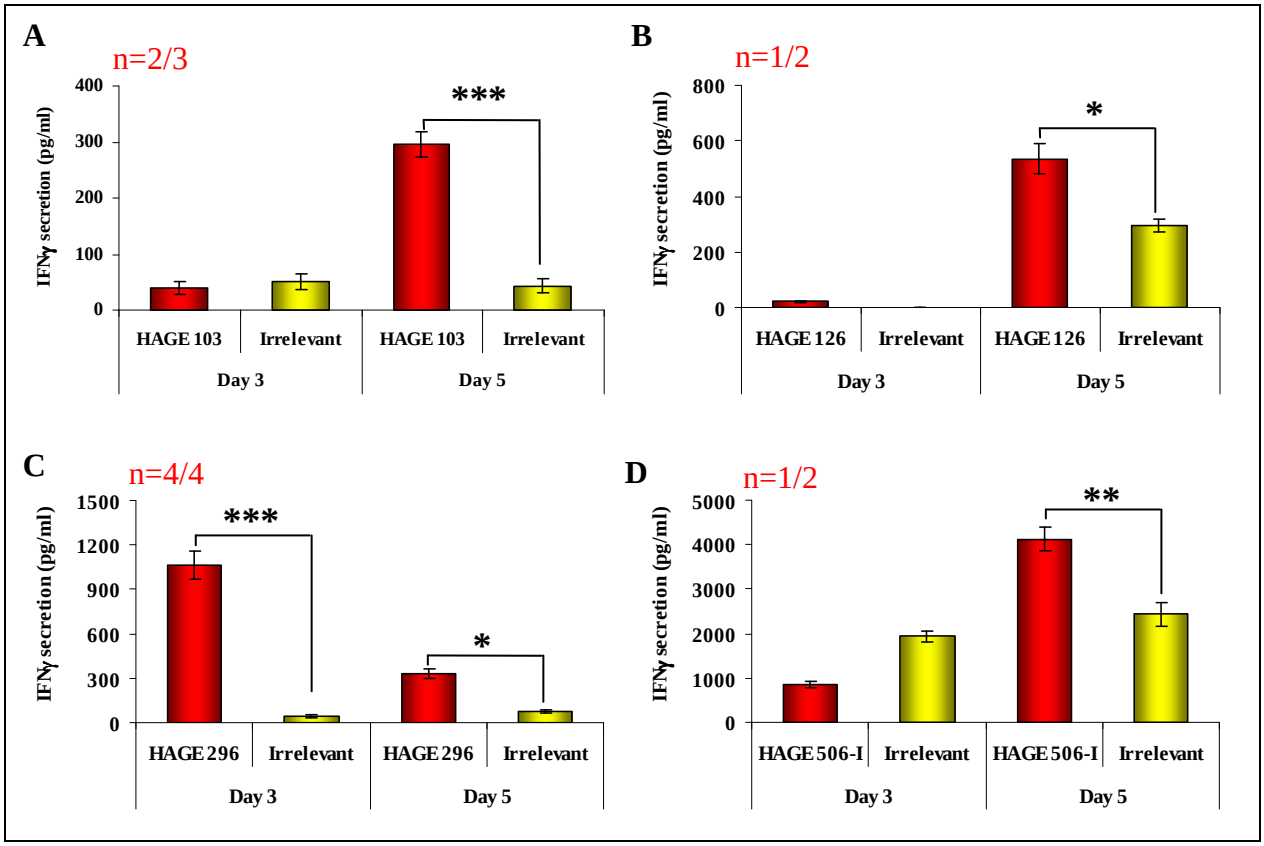

Figure 5.3: IFNγsecretion analysis by ELISA following in vitro re-stimulation with HAGE-derived class I peptides. *Cytokine analysis was carried out on supernatants harvested on day 3 and 5 of the in vitro re-stimulation with HAGE 103 (A: IIQEQPESL), HAGE 126 (B: AVIDNFVKKL), HAGE 296 (C: YLMPGFIHLV) and HAGE 506-I (D: DLILGNISV) peptides. *p<0.05, **p<0.01, ***p<0.001 are the statistical differences between HAGE-derived class I peptides and Irrelevant determined by unpaired Student T test. n=a/b with "a" being the number of mice responding to the peptide immunisation and used to represent these graphs, and "b" being the total number of mice tested.*

At the same time, another group of HHDII transgenic mice received three rounds of gene gun immunisation at seven-day intervals of a HAGE-encoding plasmid. Splenocytes were harvested and re-stimulated *in vitro* with irradiated LPS blasts pulsed with any of the four immunogenic HAGE-derived class I peptides. Following the five-day period of *in vitro* re-stimulation, T cells generated against these peptides were evaluated in a cytotoxicity assay against target cells pulsed with the p53 149 peptide for mice immunised with the p53-encoding plasmid or immunogenic HAGE-derived class I peptides for mice immunised with the HAGE-encoding plasmid. As predicted, peptide p53 149 triggered a peptide-specific cytotoxic response in every HHDII transgenic mouse tested with up to 80% of peptide-pulsed target cells killed, whereas no killing of irrelevant targets was observed. This result allowed not only to rule out any faults in the procedure, but also to confirm the production and the natural processing of the p53 protein as well as the presentation of peptide p53 149 *in vivo* to prime specific T cells, which were then expanded *in vitro* with the use of LPS blasts. Contrary to peptide p53 149, none of the four immunogenic HAGE-derived class I peptides led to statistically significant cytotoxicities, suggesting that none of these peptides were naturally processed by the proteasome and presented to T cells *via* HLA-A0201 molecules (Fig. 5.4A). Other strategies were also used to confirm these findings by evaluating the ability of T cells generated against these peptides to specifically kill HLA-A0201-positive/HAGE-positive tumour cell line such as K562-A2 and similar results were unfortunately obtained (Data not shown). Interestingly, significantly different results were obtained when HHDII transgenic mice were immunised with one round of syngeneic BM-DC transfected to express the HAGE protein. Indeed, cytotoxic responses reaching almost 40% of total cell lysis were achieved in five out of eight mice tested when splenocytes were re-stimulated *in vitro* with peptide HAGE 126. None of the other immunogenic HAGE-derived class I peptides led to significant killing of peptide-pulsed target cells (Fig. 5.4B). As a control experiment, HHDII transgenic mice received one round of syngeneic BM-DC transfected with an empty plasmid and splenocytes were re-stimulated *in vitro* with peptide HAGE 126. No cytotoxic response was achieved in both mice tested indicating that CD8+ T cell responses observed earlier were actually due to the immunisation with syngeneic BM-DC expressing HAGE and not the *in vitro* re-stimulation (Data not shown). Moreover, supernatants from the *in vitro* re-stimulation culture were harvested on day 2 and day 5 to measure IFNγ production by ELISA.

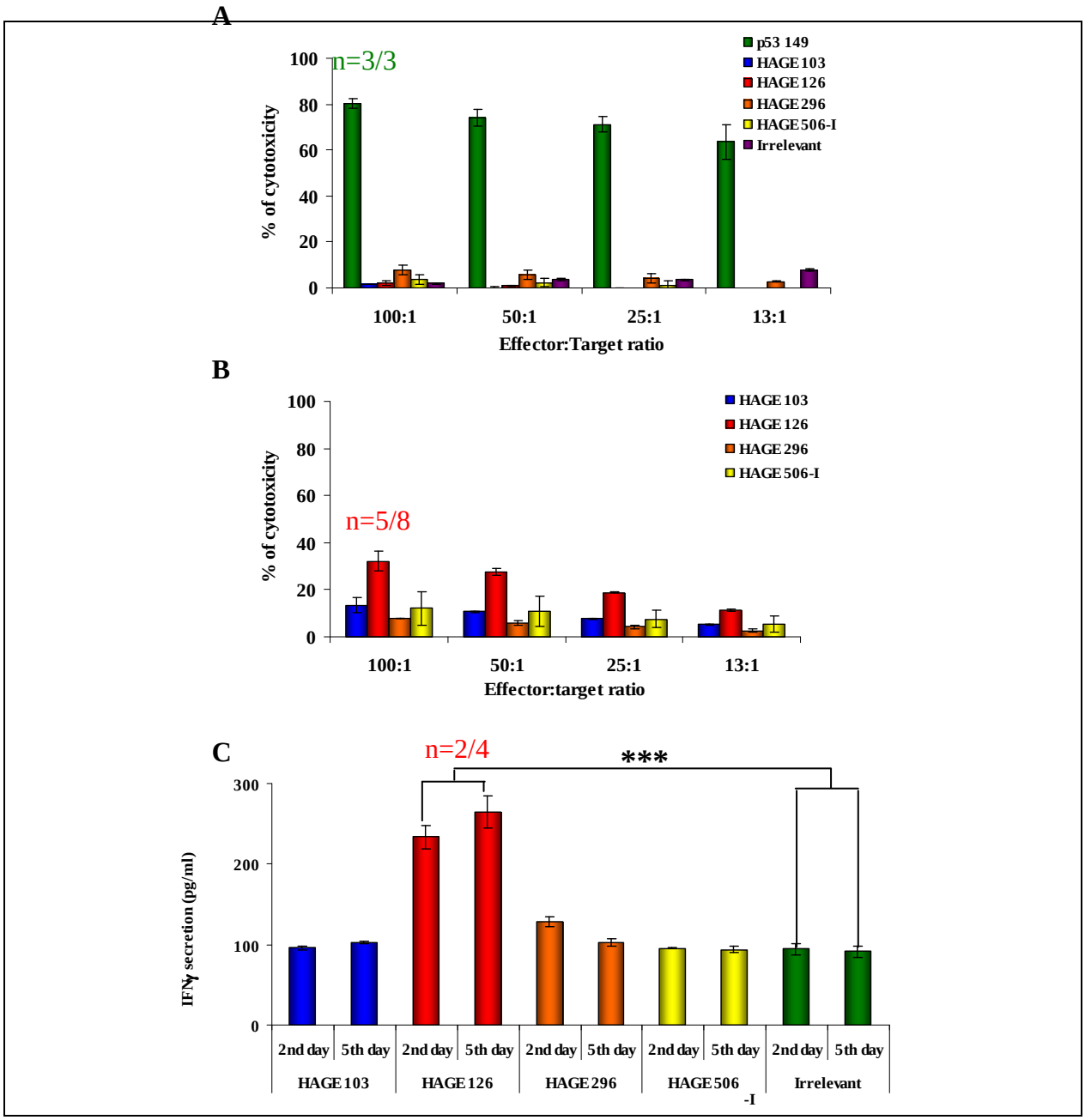

Figure 5.4: Cytotoxicity assay and IFNγsecretion using T cells generated from HHDII transgenic mice immunised with HAGE DNA. *Mice were either immunised with gold-coated pcDNA3/HAGE plasmid (A) or with syngeneic BM-DC transiently transfected with pcDNA3/HAGE (B). Cytotoxicity assays were carried out after in vitro re-stimulation of immunised cells with immunogenic HAGE-derived class I peptides. Cytokine analysis (C) was carried out on supernatants harvested on day 3 and 5 of the in vitro re-stimulation with HAGE 103 (A: IIQEQPESL), HAGE 126 (B: AVIDNFVKKL), HAGE 296 (C: YLMPGFIHLV) and HAGE 506-I (D: DLILGNISV) peptides. ***p<0.001 are the statistical differences between HAGE-derived class I peptides and Irrelevant determined by unpaired Student T test. n=a/b with "a" being the number of mice responding to the peptide immunisation and used to represent these graphs, and "b" being the total number of mice tested.*

Only peptide HAGE 126 allowed significant levels of peptide-specific IFNγ secretion on both day 2 and day 5 of the culture (Fig. 5.4C). These results were later confirmed when HHDII

mice were immunised with one round of syngeneic BM-DC pulsed with a HAGE-positive K562 cell lysate, and *in vitro* re-stimulation with HAGE 126 decamer. Indeed, peptide-specific cytotoxic response of 25% was achieved in one out of two mice (Data not shown). Altogether, these results indicate that peptide HAGE 126 is naturally processed by the proteasome, presented at the cell surface by HLA-A0201 molecules to T cells and capable of eliciting both cytotoxic and IFNγ-based responses, at least in mice.

5.2.2 Identification of HLA-DR restricted peptides from HAGE using HLA-DR0101 and HLA-DR0401 transgenic mice

5.2.2.1 Immunogenicity of peptides predicted from HAGE sequence for HLA-DR1 and HLA-DR4

In order to ascertain the immunogenicity of peptides predicted from HAGE sequence for HLA-DR0101 and HLA-DR0401, FVB/N-DR1 and C57BL/6-DR4 transgenic mice were immunised twice at a seven-day interval with 100μg of the MHC class II peptides in 1:1 emulsion with IFA as described in methods section. Seven days after the last immunisation, splenocytes were harvested and re-stimulated *in vitro* with the peptides for 6 days. Upon CD8+ T cell depletion, these cells were then co-cultured with peptide-pulsed syngeneic BM-DC in order to detect peptide-specific proliferation using tritiated thymidine incorporation assay. None of the six HAGE-derived class II peptides were able to trigger peptide-specific CD4+ T cell proliferation (Data not shown). However, when splenocytes from immunised animals were cultured for one week in presence of the peptide and then rested for another week in presence of murine IL-2 after depletion of CD8+ T cells, peptides HAGE 109, HAGE 198, HAGE 338 and HAGE 506-II were able to induce peptide-specific CD4+ T cell proliferation in both HLA-DR1 (Fig. 5.5) and HLA-DR4 (Fig. 5.6) transgenic mice. Moreover, peptide-specific proliferations to these four peptides were blocked by the addition in the culture of an anti-DR blocking antibody (L243) but not by the isotype control antibody, demonstrating the MHC class II restriction of the responses observed. Supernatants from the *in vitro* re-stimulation culture were harvested on day 3 and day 5 to measure IFNγ and IL-5 production by ELISA.

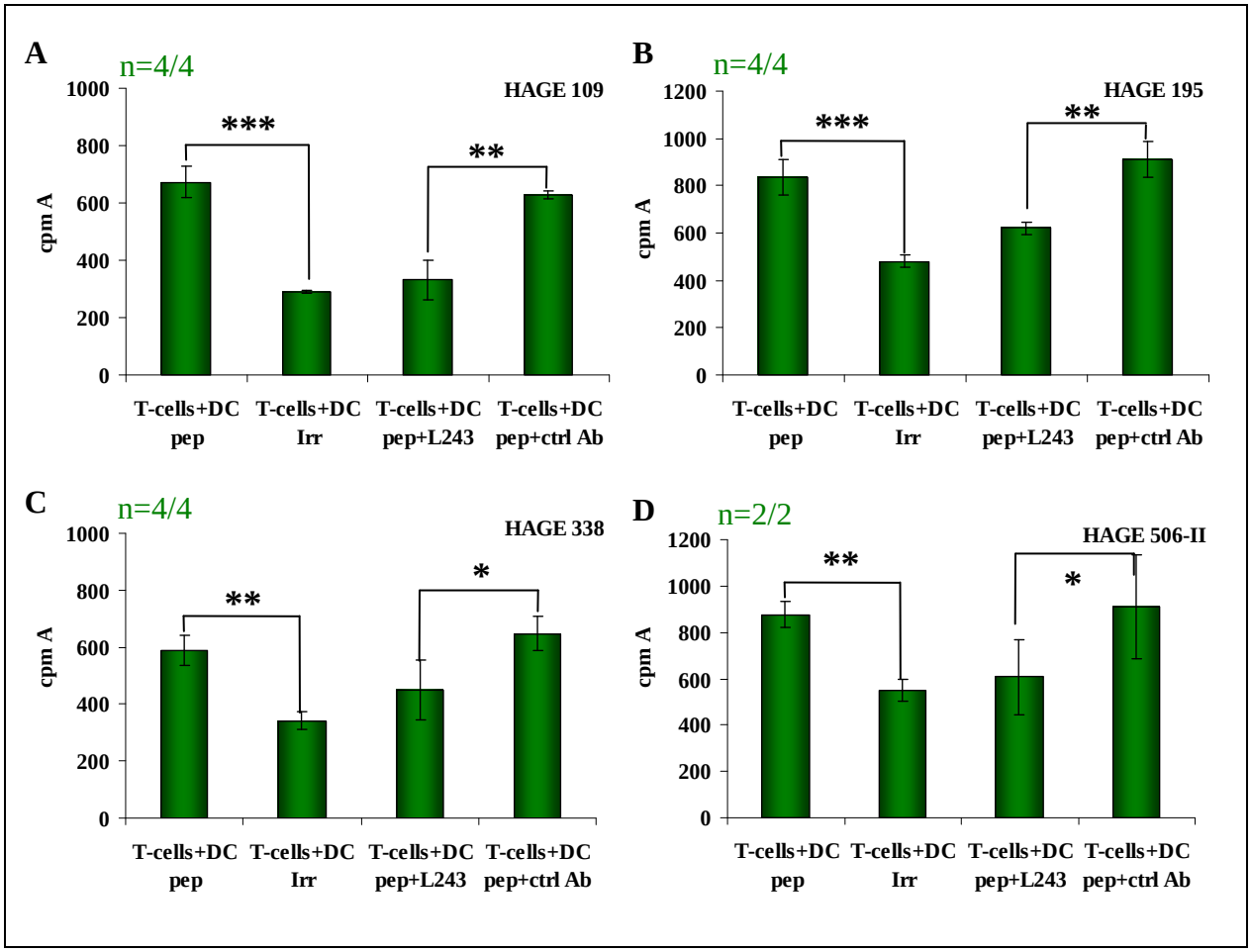

Figure 5.5: Proliferation results of HAGE-derived MHC class II peptides in HLA-DR1 transgenic mice. *Mice were immunised with 100μg of peptide in 1:1 dilution with IFA and boosted one week later. Splenocytes were harvested a week later and re-stimulated in vitro with HAGE 109 (A:* ESLVKIFGSKAMQTK*), HAGE 195 (B:* KKNFYKESTATSAMS*), HAGE 338 (C:* KYSYKGLRSVCVYGG*) and HAGE 506-II (D:* DLILGNISVESLHGD*) peptides for 7 days and then tested for proliferation by incubating them with syngeneic BM-DC pulsed with peptide. p<0.05, **p<0.01, ***p<0.001 are the statistical differences between HAGE-derived class II peptides and Irrelevant, or between L243 antibody and isotype control antibody determined by unpaired Student T test. n=a/b with "a" being the number of mice responding to the peptide immunisation and used to represent these graphs, and "b" being the total number of mice immunised.*

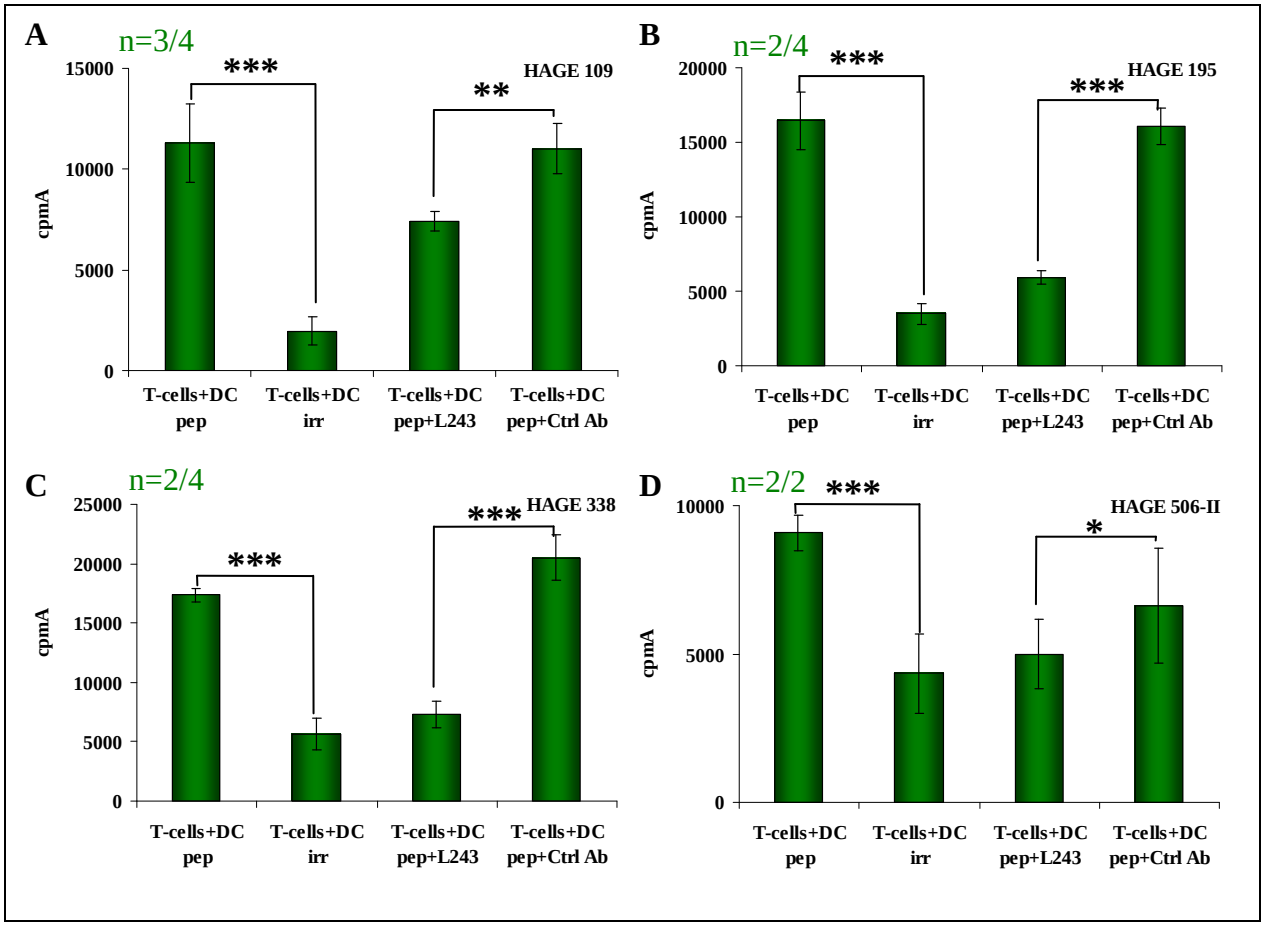

Figure 5.6: Proliferation results of HAGE-derived MHC class II peptides in HLA-DR4 transgenic mice. *Mice were immunised with 100μg of peptide in 1:1 dilution with IFA and boosted one week later. Splenocytes were harvested a week later and re-stimulated in vitro with HAGE 109 (A:* ESLVKIFGSKAMQTK*), HAGE 195 (B:* KKNFYKESTATSAMS*), HAGE 338 (C:* KYSYKGLRSVCVYGG*) and HAGE 506-II (D:* DLILGNISVESLHGD*) peptides for 7 days and then tested for proliferation by incubating them with syngeneic BM-DC pulsed with peptide. $p<0.05$, $**p<0.01$, $***p<0.001$ are the statistical differences between HAGE-derived class II peptides and Irrelevant, or between L243 antibody and isotype control antibody determined by unpaired Student T test. n=a/b with "a" being the number of mice responding to the peptide immunisation and used to represent these graphs, and "b" being the total number of mice immunised.*

Peptides HAGE 109, HAGE 195, HAGE 338 and HAGE 506-II allowed significant levels of peptide-specific IFNγ secretion on day 3 of the culture but only peptides HAGE 338 and HAGE 506-II induced peptide-specific IFNγ secretion on day 5 of the culture (Fig. 5.7A). These results suggest that not only CD4+ T cell proliferation but also Th1/IFNγ release could be achieved with these four HAGE-derived class II peptides in a peptide-specific manner. It is worth mentionning that peptides HAGE 109 and HAGE 338 also triggered secretion of IL-5 on day 3 of the culture indicating that both of these peptides may be able to induce both Th1/IFNγ and Th2/IL-5 releases in a peptide-specific fashion (Fig. 5.7B). These results confirmed the immunogenicity of these four HAGE-derived class II peptides. On the other hand, T cells could not be generated against peptides HAGE 505 and HAGE 545 and hence, were defined as non-immunogenic and no further experiments were carried out with these peptides (Data not shown). Results of the immunisation of HLA-DR1 and HLA-DR4 transgenic mice with HAGE-derived MHC class II peptides were summarised in table 5.2.

Table 5.2: Immunogenic HAGE-derived MHC class II peptides

Peptides	Immunogenicity	Proliferation in DR1 (p<0.05)	Proliferation in DR4 (p<0.05)	IFNγ in DR1/DR4 (p<0.05)	IL-5 in DR1/DR4 (p<0.05)
HAGE 109	Yes	4/4	3/4	4/4	2/4
HAGE 195	Yes	4/4	2/4	3/4	0/4
HAGE 338	Yes	4/4	2/4	3/4	1/4
HAGE 505	No	0/2	0/2	0/4	0/4
HAGE 506-II	Yes	2/2	2/2	3/4	0/4
HAGE 545	No	0/2	0/2	0/4	0/4

5.2.2.2 Determination of natural processing of immunogenic class II peptides

In order to confirm if any of the four identified immunogenic class II peptides detailed in table 5.2 were endogenously processed and naturally presented at the cell surface by HLA-DR0401 molecules, both gene gun immunisation with gold-coated HAGE-encoding DNA bullets and injection of syngeneic BM-DC prepared *ex vivo* and transfected to express HAGE were carried out in C57BL/6-DR4 transgenic mice. After three rounds of DNA immunisation, spleens of immunised animals were harvested and splenocytes were re-stimulated *in vitro* with HAGE-derived class II peptides. After a week of stimulation and a week of rest in presence of murine IL-2, proliferation assays were performed with peptide-pulsed BM-DC.

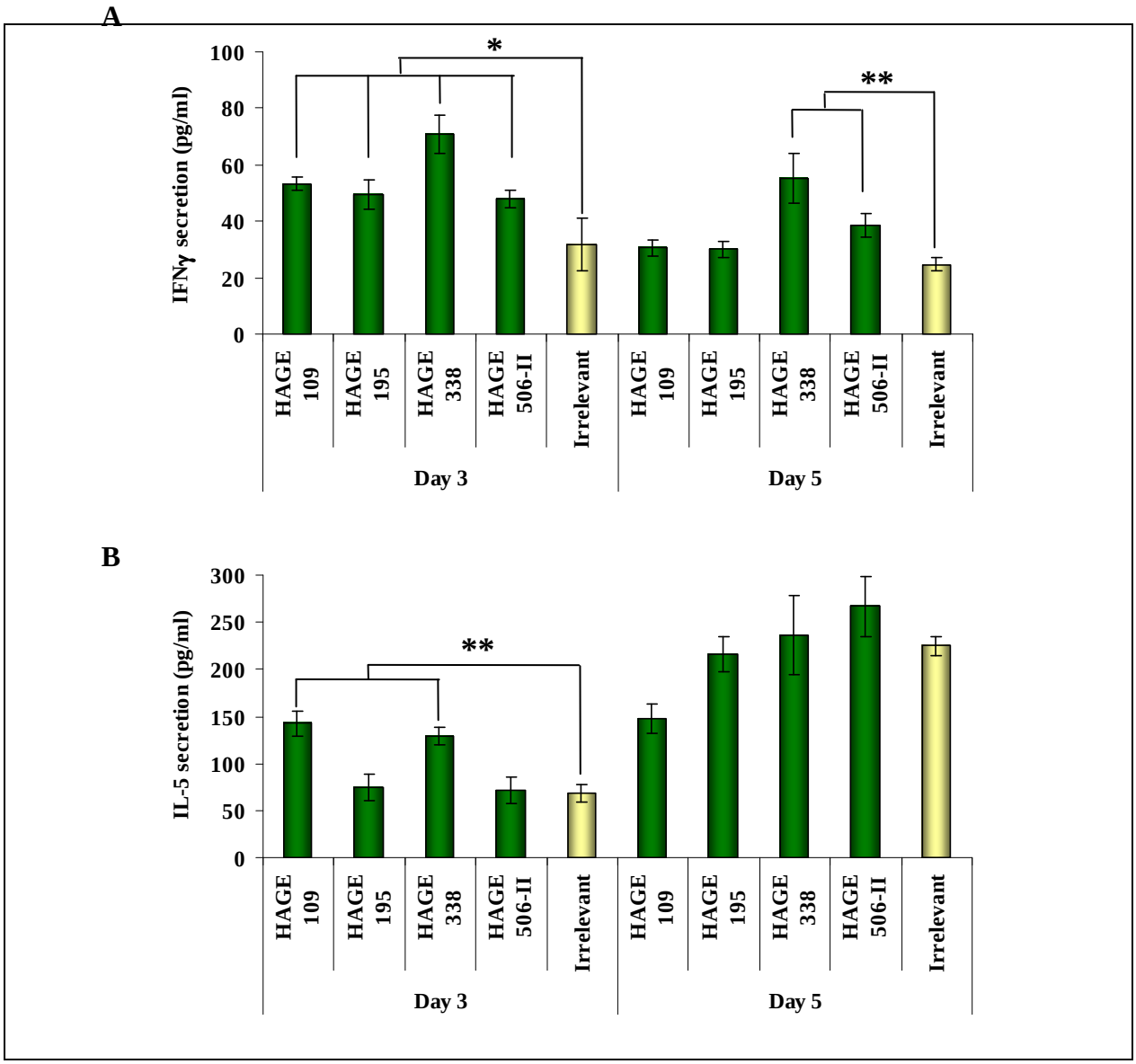

Figure 5.7: IFNγand IL-5 secretion analysis by ELISA following in vitro re-stimulation with HAGE-derived class II peptides. *IFNγ (A) and IL-5 (B) analysis was carried out on supernatants harvested on day 3 and 5 of the in vitro re-stimulation of DR4-positive splenocytes with HAGE 109 (*ESLVKIFGSKAMQTK*), HAGE 195 (*KKNFYKESTATSAMS*), HAGE 338 (*KYSYKGLRSVCVYGG*) and HAGE 506-II (*DLILGNISVESLHGD*) peptides. *p<0.05, **p<0.01 are the statistical differences between HAGE-derived class II peptides and Irrelevant determined by unpaired Student T test. n=a/b with "a" being the number of mice responding to the peptide immunisation and used to represent these graphs, and "b" being the total number of mice tested.*

Results showed that peptides HAGE 338 and HAGE 506-II, but not HAGE 109 or HAGE 195, were able to induce a peptide-specific CD4+ T cell proliferation in 50% and 100% of mice tested, respectively (Fig. 5.8). Peptide-specific proliferations to these two peptides were also

blocked by the addition in the culture of an anti-DR blocking antibody (L243) but not by the isotype control antibody, demonstrating the MHC class II restriction of the responses observed. Finally, peptide-specific IFNγ but not IL-5 production was observed for both peptides, suggesting a Th1-type response (Fig. 5.8E and 5.8F). Interestingly, after three rounds of DNA immunisation with the empty expression vector and *in vitro* re-stimulation of splenocytes from immunised animals with class II peptides, no proliferation was observed in both mice tested indicating that CD4+ T cell proliferations observed earlier are actually due to the DNA immunisation and not the *in vitro* re-stimulation (Data not shown). Moreover, similar results were obtained when C57BL/6-DR4 were immunised with one round of transfected syngeneic BM-DC. Indeed, peptide-specific and HLA-DR-dependent CD4+ T cell proliferations were achieved with identical percentages of mice responding to peptides HAGE 338 and HAGE 506-II (Fig. 5.9). Altogether, these data demonstrate that both HAGE 338 and HAGE 506-II epitopes are naturally processed, presented by HLA-DR4 molecules and capable of eliciting peptide-specific CD4+ T cell proliferation and Th1/IFNγ secretion, at least in mice.

5.2.3 Investigation of the immunogenicity and the endogenous process of HAGE-derived peptides in HHDII/HLA-DR1 double transgenic mice

In order to validate the natural processing and the immunogenicity of peptides HAGE 126, HAGE 338 and HAGE 506-II, peptide, DNA and transfected syngeneic BM-DC were injected to HHDII/DR1 double transgenic mice, which are also double knockout for murine MHC molecules. Peptide immunisation confirmed the capacity of HAGE 126, together with the helper HepB or HAGE 506-II peptides but not on its own, to trigger a peptide-specific cytotoxic response (Data not shown). Also, both peptides HAGE 338 and HAGE 506-II were shown capable of specifically priming CD4+ T cells to proliferate (Data not shown). Consequently, both DNA immunisation and syngeneic BM-DC injections were carried out in order to see if results obtained earlier in single transgenic mice were reproducible in double transgenic mice.

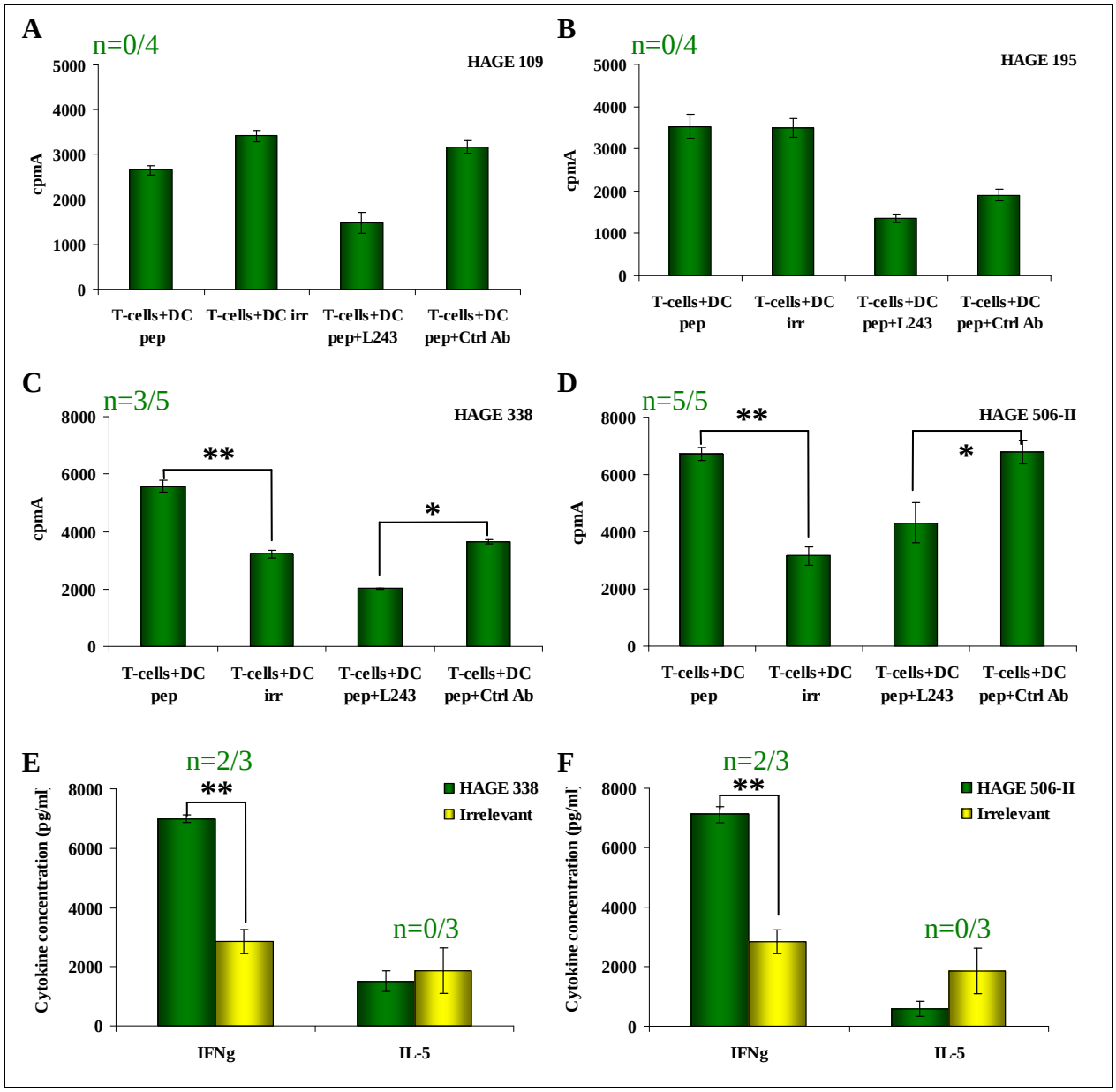

Figure 5.8: Proliferation assay and cytokine secretion using T cells generated from HLA-DR4 transgenic mice immunised with HAGE DNA. *Mice were immunised with gold-coated pcDNA3/HAGE plasmid. Proliferation assays were carried out after in vitro re-stimulation of immunised cells with HAGE 109 (A:* ESLVKIFGSKAMQTK*), HAGE 195 (B:* KKNFYKESTATSAMS*), HAGE 338 (C:* KYSYKGLRSVCVYGG*) and HAGE 506-II (D:* DLILGNISVESLHGD*) peptides. Cytokine analysis was carried out on supernatants harvested on day 3 of the in vitro re-stimulation with HAGE 338 (E) and HAGE 506-II (F). * $p<0.05$ ** $p<0.01$ are the statistical differences between HAGE-derived class II peptides and Irrelevant, or between L243 antibody and isotype control antibody determined by unpaired Student T test. n=a/b with "a" being the number of mice responding to the peptide immunisation and used to represent these graphs, and "b" being the total number of mice tested.*

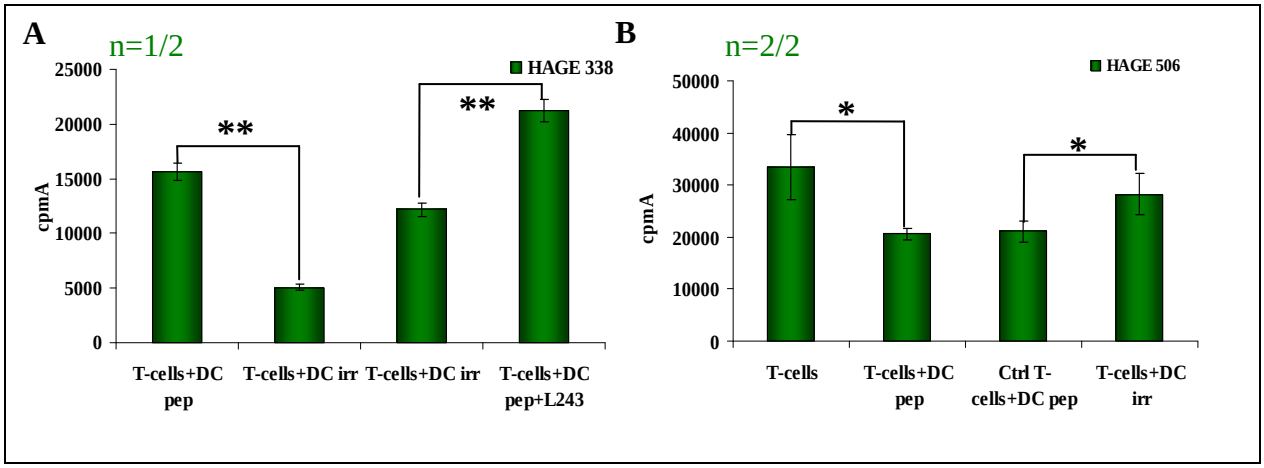

Figure 5.9: Proliferation assay using T cells generated from HLA-DR4 transgenic mice immunised with HAGE DNA. *Mice were immunised with syngeneic BM-DC transiently transfected with pSHAME2a/HAGE. Proliferation assays were carried out after in vitro re-stimulation of immunised cells with HAGE 338 (A:* KYSYKGLRSVCVYGG*) and HAGE 506-II (B:* DLILGNISVESLHGD*) peptides. * p<0.05 **p<0.01 are the statistical differences between HAGE-derived class II peptides and Irrelevant, or between L243 antibody and isotype control antibody determined by unpaired Student T test. n=a/b with "a" being the number of mice responding to the peptide immunisation and used to represent these graphs, and "b" being the total number of mice immunised.*

As seen in Fig. 5.10, DNA immunisation led to an unspecific response while syngeneic BM-DC injection followed by *in vitro* re-stimulation allowed the generation of a significant and specific cytotoxic response towards target cells presenting class I peptide HAGE 126 (up to 35% killing). Peptide HAGE 126 was therefore considered both naturally processed and immunogenic in double transgenic mice. Moreover, DNA immunisation also permitted the confirmation of the proteasomal processing of HAGE 338 and HAGE 506-II as both peptides were shown to induce peptide-specific and HLA-DR-dependent CD4+ T cell proliferations (Fig. 5.11). Results of the immunisation of HHDII, HLA-DR4 and HHDII/DR1 transgenic mice with DNA or syngeneic BM-DC were summarised in table 5.3 with highlighted in red, HAGE-derived peptides presenting natural processing and immunogenicity characteristics.

Table 5.3: Endogenously processed and immunogenic HAGE-derived MHC class I and II peptides

HLA haplotype	Peptide	Endogenously processed/immunogenic	Mice
HLA-A2	HAGE 103	No/Yes	0/4
	HAGE 126	Yes/Yes	7/12
	HAGE 296	No/Yes	0/4
	HAGE 506	No/Yes	0/4
HLA-DR1/-DR4	HAGE 109	No/Yes	0/8
	HAGE 195	No/Yes	0/8
	HAGE 338	Yes/Yes	5/10
	HAGE 506-II	Yes/Yes	10/10

Altogether, these results proved the immunogenic potential of the cancer/testis antigen HAGE with the identification of one class I and two class II peptides that could be useful for vaccination strategies of A2, DR1/DR4 patients. However, further *in vitro* work with PBMC from healthy/cancer donors are needed to confirm the results obtained in mice.

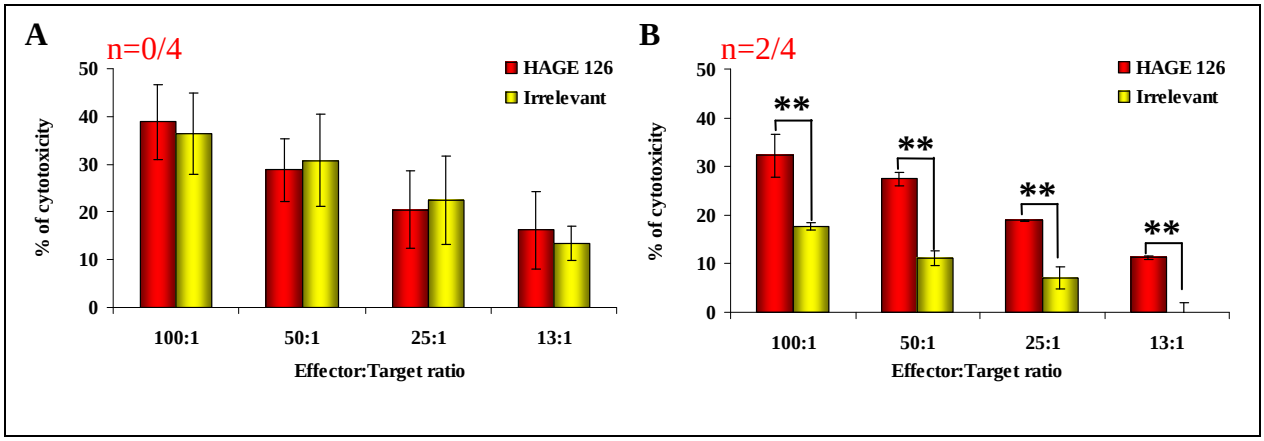

Figure 5.10: Cytotoxicity assay using T cells generated from HHDII-DR1 double transgenic mice immunised with HAGE DNA. *Mice were either immunised with gold coated pBudCE4.1/HAGE plasmid (A) or with syngeneic BM-DC transiently transfected with pBudCE4.1/HAGE (B). Cytotoxicity assay was carried out after in vitro re-stimulation of immunised cells with immunogenic HAGE 126 (AVIDNFVKKL) peptide. **p<0.01 are the statistical differences between HAGE-derived class I peptides and Irrelevant determined by unpaired Student T test. n=a/b with "a" being the number of mice responding to the peptide immunisation and used to represent these graphs, and "b" being the total number of mice immunised.*

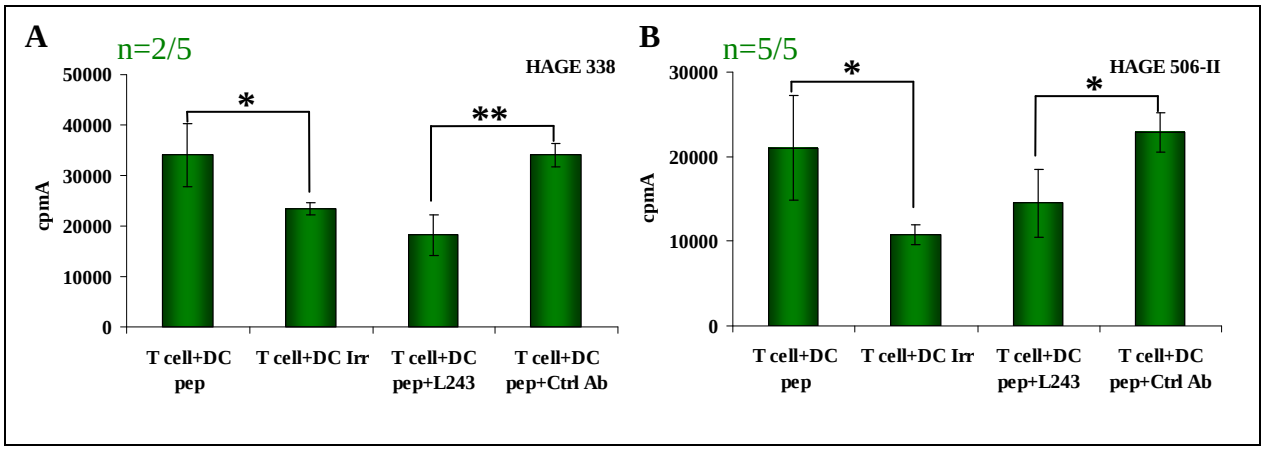

Figure 5.11: Proliferation assay using T cells generated from HHDII-DR1 double transgenic mice immunised with HAGE DNA. *Mice were immunised with gold-coated pBudCE4.1/HAGE plasmid. Splenocytes were harvested a week later after last immunisation and re-stimulated in vitro with HAGE 338 (A:* KYSYKGLRSVCVYGG*) or HAGE 506-II (B:* DLILGNISVESLHGD*) peptides for 7 days and then tested for proliferation by incubating them with syngeneic BM-DC pulsed with peptide. *p<0.05 and **p<0.01 are the statistical differences between HAGE-derived class II peptides and Irrelevant, or between L243 antibody and isotype control antibody determined by unpaired Student T test. n=a/b with "a" being the number of mice responding to the peptide immunisation and used to represent these graphs, and "b" being the total number of mice immunised.*

5.3 Discussion

Synthetic peptide-based vaccines have been shown to produce peptide-specific CD8+ T cells with protective and therapeutic abilities against tumour cells in preclinical animal models. With the help of appropriate adjuvants, they represent convenient and safe modes of immunisation easily manufactured in large quantities. For example, vaccination with CTL epitopes derived from the HER2/neu antigen was demonstrated to be effective in protective and in therapeutic experiments in HER2/neu transgenic mice (Gritzapis *et al.*, 2006), as well as being capable of activating both naive and memory CD4+ and CD8+ T cells when co-administered with GM-CSF in breast cancer patients (Hueman *et al.*, 2007). Unfortunately, the low number of peptide-specific T cells generated following peptide vaccination in patients represents the major drawback of this method. However, positive clinical responses were observed when T cells were generated *ex vivo* from TIL in the presence of antigen-loaded DC or artificial APC and re-injected back to the patients in adoptive T cell transfer strategies (Hueman *et al.*, 2007). Again, it is generally believed that the number of T cells is too low in order to have a prolonged beneficial effect. Adoptive T cell therapy was recently further improved by genetically modifying non-reactive PBMC and TIL to express a specific TCR and up to two months later, at least 10% of peripheral circulating lymphocytes were constituted of transferred T cells, often correlating with objective clinical regression in some patients (Morgan *et al.*, 2003; Johnson *et al.*, 2006; Morgan *et al.*, 2006). Furthermore, peptides have been used in DC vaccination strategies with different degrees of success as peptide-specific CTL were generated in most clinical trials (Takahashi *et al.*, 2003), but objective clinical responses in only a few (Wierecky *et al.*, 2006). TCR cloning, peptide-pulsed DC vaccination and monitoring of peptide-specific T cells in patients are only possible if the sequence of the peptide targeted is known. Therefore, peptide identification from known tumour antigens provides the platform for successful immunotherapy against several cancers.

So far, hopes that peptide vaccination could be used therapeutically have been largely undermined by results obtained in clinical trials as the numbers of antigens and MHC alleles targeted remain low. Consequently, by proposing multiple epitopes from several antigens presented by different MHC alleles to reduce risks of immune escape by antigen or MHC down-regulation, the chances of successful cancer immunotherapy would be much increased. Several studies have demonstrated the feasibility of cancer immunotherapy when objective cancer responses were obtained after immunising patients with tumour antigen-derived peptides specifically recognised by CD8+ T cells (Rosenberg *et al*, 1998)(Yee *et al*, 2000). However, the responses observed were often weak and transient (Lee *et al.*, 1999; Chen *et al.*,

2004). In pathogenic infections, bacteria, viruses or parasites provide strong signals automatically up-regulating CD40 expression on DC and thereby bypassing the need for CD4+ T cell help. Unfortunately, in the case of tumours, data about the involvement of CD4+ T cells are still contradictory. Nowadays, it is generally believed that CD4+ T cells have a pivotal role not only in the initiation and the maintenance of an anti-tumour response but also in the direct mediation of tumour regression *via* IFNγ-dependent mechanisms or apoptosis pathway engagement (Echchakir *et al.*, 2000; Egilmez *et al.*, 2002; Assudani *et al.*, 2006). CTL generation seems to depend on three distinct mechanisms: the duration of MHC-restricted peptide presentation by the APC, the affinity of TCR-MHC binding and the presence or not of CD4+ T cell help. The latter is of critical importance as high affinity MHC class I peptides do not require CD4+ T cell help (Franco *et al.*, 2000), but because low-to-moderate affinity MHC class I peptides are more likely to be efficient against tumours expressing up-regulated self-antigens, CD4+ T cell role in the anti-tumour response can be emphasised. Finally, the origin of the T helper epitope is still not clearly defined but may also have an influence on the quality/quantity of CD8+ T cells. Indeed, some researcher argue the use of irrelevant peptides derived from infectious pathogenic agent such as tetanus toxoid or hepatitis B, whereas others have suggested the incorporation of a CD4+ T cell epitope from either the same antigen as CTL epitope or a SEREX-defined self antigen in a peptide vaccine (Nishikawa *et al.*, 2001; Nishikawa *et al.*, 2005). Therefore, optimal immunisation will require the recruitment of both CD4+ and CD8+ T cells, in order to generate a long-lasting anti-tumour immune response.

High scoring peptides for HLA-A0201, HLA-DR0101 and HLA-DR0401 molecules were therefore selected and evaluated for their immunogenicity in HHDII, HLA-DR1 and HLA-DR4 transgenic mice, respectively. Of the eight peptides selected for evaluation in HHDII mice, four were moderate-to-high affinity binders as proven by stabilisation assay (Data not shown) and immunogenic as demonstrated by the development of peptide-specific cytotoxic responses and IFNγ secretion following immunisation with peptides only or DC pulsed with peptides. Since these HHDII mice are also mouse class I knockout (Firat *et al.*, 1999), class I peptides can only bind to HLA-A2 molecules and cytotoxic responses were concluded to be HLA-A2-dependent. In this study, six peptides derived from HAGE predicted to bind with high affinity to HLA-DR1 and –DR4 molecules were evaluated and four of them were found to be immunogenic in HLA-DR1 and HLA-DR4 transgenic mice with a capacity to induce both peptide-specific CD4+ T cell proliferation and IFNγ secretion. Since proliferation assays were systematically conducted with CD8+-depleted splenocytes and anti-HLA-DR blocking antibody with its

relevant isotype control antibody, proliferative responses were demonstrated to be CD4-driven and HLA-DR-restricted. Finally, it should be noticed that the binding affinity for both class I and class II peptides determined *in silico* correlated strongly with the efficiency of the peptide in generating an immune response.

Peptide immunisation of transgenic mice allowed the identification of immunogenic HLA-A2 and HLA-DR1 or –DR4 peptides derived from HAGE. However, these peptides cannot be used in future vaccine formulation if APC or tumour cells do not present them naturally on their surface as these would not be recognised by both CD4+ and CD8+ T cells and be eliminated by them. The next critical step of this study was therefore to prove whether any of these peptides are actually naturally processed by APC and/or tumour cells, and that CD4+ T cell proliferation and CD8+ cytotoxic response can still be induced. A popular way to assess the processing of a given immunogenic peptide is to immunise mice with a plasmid encoding the gene of interest using gene gun technology (Tuting, 1999). The expression of HAGE at the mRNA and protein levels by the pSHAME2a/HAGE expression vector was ascertained and described in section 4.2.3. Gene gun technology was successfully used in our laboratory to prove the processing of class I p53-derived peptides and was used here as a positive control for the procedure. No cytotoxic responses could however be generated when mice were immunised with DNA encoding HAGE and when splenocytes were re-stimulated *in vitro* with LPS blasts pulsed with one of the immunogenic HLA-A2-restricted HAGE-derived peptides. Cytotoxic T lymphocytes generated from HHDII transgenic mice were previously shown capable of lysing human tumour cells in a MHC-restricted fashion (Gritzapis *et al.*, 2006). However, CTL generated against these peptides were unable to kill previously described HAGE-positive human lymphoblastic K562-A2 cells (data not shown), although these results were later explained by very low levels of HAGE expression in these cells due to hypermethylation of the HAGE promoter and therefore low levels of HAGE-derived peptide presentation at the cell surface (Roman-Gomez *et al.*, 2007).

In order to confirm these results, cells from the bone marrow of a naive mouse were prepared *ex vivo* and transfected to express the HAGE protein. Cytotoxic responses and IFNγ production were only achieved when mice were immunised with these transfected cells and when splenocytes were re-stimulated *in vitro* with LPS blasts pulsed with the HLA-A2-restricted peptide HAGE 126. Moreover, mice immunised with DC transfected with the empty expression vector did not generate any cytotoxic response when splenocytes were re-stimulated with the same peptide indicating that the cytotoxic response was related to the immunisation

with the transfected DC and not the actual *in vitro* re-stimulation with the peptide. The same phenomenon was later confirmed when mice underwent immunisation with DC loaded with a HAGE-rich melanoma cell lysate (Data not shown). In both experiments, cytotoxic responses were peptide-specific when compared with an irrelevant peptide and suggested that HAGE 126 might also be processed. It means that the HAGE protein is indeed degraded by the proteasome into peptide fragments. Antigenic peptides such as HAGE 126 bind to the groove of MHC class I molecules, in that case HLA-A2, to be then presented at the cell surface of either dendritic cells to prime CD8+ T cells, or tumour cells to be specifically recognised and killed by primed cytotoxic T lymphocytes (Van den Eynde and Morel, 2001; Kloetzel *et al.*, 2004).

Following immunisation with DNA encoding the HAGE protein in HLA-DR1 and –DR4 transgenic mice, peptide-specific CD4+ T cell proliferations were observed with HAGE 338 and HAGE 506-II peptides. The reaction was blocked when the anti-HLA-DR antibody was added. Moreover, mice immunised with the empty expression vector did not show any proliferative response when splenocytes were re-stimulated with the same peptides (Data not shown). Altogether, these data showed that the proliferations observed were peptide- and HLA-DR-specific and consecutive to the immunisation with the expression vector encoding the HAGE protein. These experiments brought strong evidence that these two HLA-DR peptides are likely to be endogenously processed. In the same way, CD4+-enriched T cells were shown to specifically proliferate in response to mature BM-DC pulsed with peptides 338 and 506-II after immunisation with BM-DC transfected with HAGE-containing plasmid. The reaction was blocked when the anti-HLA-DR antibody was added and CD4+ T cells did not proliferate when mice had been immunised with the same BM-DC transfected with the empty plasmid. Moreover, for both types of immunisation, the same pattern of cytokines production was observed. Indeed, peptide-specific IFNγ release was observed against both HAGE 338 and HAGE 506-II. Levels of IL-5 remained low indicating that the CD4+ T cell proliferation for HAGE 338 and HAGE 506-II were mostly Th1-orientated. The processing of endogenous proteins and presentation of tumour-derived class II peptides is still not fully understood and may result from cross-presentation and/or endogenous process. Indeed, Dissanayake *et al* (2005) recently proved that processing of tumour-derived MHC class II restricted epitopes is independent of the proteasome and the TAP complex. They suggested that MHC class II-restricted endogenously synthesised epitopes might overlap with the classical endosomal pathway for presentation of exogenously synthesised molecules giving an explanation on how

peptides derived from intra-cellular proteins such as HAGE can be presented by MHC class II molecules to CD4+ T cells.

Furthermore, Dr. A. Knights (Tuebingen University, personal communication) proved that the *in vitro* generation of CD4+ T cell lines from human PBMC with HAGE 506-II showed specific HLA class II-restricted recognition of HLA-DR matched tumour cell lines, as well as DC pulsed with HAGE-positive cell lysates. These results are therefore in agreement with those obtained from DNA immunisation of HLA class II transgenic mice, suggesting that the HAGE protein is produced and cleaved to give rise to the HAGE 506-II in ways that are similar in HLA-DR4 transgenic mice and human derived DC (Mathieu *et al.*, 2007). In the future, peptides HAGE 126 and HAGE 338 will also be investigated for their ability to generate respectively CTL and T helper cells capable of specifically responding to target cells expressing the appropriate HLA molecules as well as HAGE.

In conclusion, reverse immunology combined with the use of mice transgenic for HLA molecules proved to be a successful technique in the identification of candidate peptide targets for tumour immunotherapy as one class I and two class II immunogenic and naturally processed peptides derived from the HAGE protein have been described, at least in single and double transgenic mice. However, further work is required to prove that HAGE 126 and HAGE 338 are both immunogenic and naturally processed in humans. Also, techniques such as overlapping peptides might also be envisaged in order to identify low-to-moderate affinity HAGE-derived peptide targets with immunogenic properties that might have been overlooked in this study using reverse immunology.

Chapter 6: Evaluation of HAGE as an immunotherapeutic target in an *in vivo* model

6.1 Introduction

Despite the numerous advantages offered by peptide vaccination, central and peripheral tolerance mechanisms are likely to delete high avidity T cells as well as low-to-moderate avidity T cells against tumour-shared self antigens creating a major limitation in the prospect of treating cancer. Moreover, techniques of identification of T cell epitopes such as reverse immunology often overlook peptides with low-to-moderate binding affinity as hundreds of them are predicted for a given tumour antigen and can only be tested to a limited number of HLA alleles in a transgenic mouse model. That is why DNA-based vaccination proposes an interesting alternative for cancer immunotherapy as it bypasses the need for individual peptide identification but also elicites both humoral and cellular immune responses simultaneously, which are both most likely required in order to induce tumour protection and/or regression.

Several groups have investigated the use of DNA-based vaccines to promote tumour rejection in animal models with promising results. Indeed, H-2^b and HHDII mice appeared to be protected against a lethal challenge with HPV E6/E7-expressing tumours following gene gun immunisation with DNA encoding mutated version or immunodominant epitopes of E6/E7 genes (Smahel *et al.*, 2001; Eiben *et al.*, 2002). However, these antigens are already strongly immunogenic because of their viral origin. Targeting a self antigen, such as p53, renders the task more difficult as tumour escape mechanisms such as increased mutation frequencies and MHC class I down-regulation can take place and make immunisation more detrimental than beneficial in some cases (Cicinnati *et al.*, 2005). In another study describing the use of the murine self antigen tyrosinase-related protein-1 (mTrp-1) by intra-muscular injection, mice were protected against poorly immunogenic B16 melanoma cells (Bronte *et al.*, 2000). In addition, the introduction of this gene into a vaccinia virus vector allowed the mediation of tumour regression in mice challenged with B16 cells and despite being a self antigen widely expressed in normal skin tissues, there were no signs of vitiligo, a common auto-immune reaction described in several previous studies targeting melanoma antigens. Numerous strategies have been designed to further improve DNA-based vaccines. Indeed, orientation of an antigen to a specific processing pathway, system of antigen delivery and augmentation of the immunogenicity of an antigen are all factors that should also be taken into account in the design of a DNA-based vaccine. For instance, DNA-encoded proteins can be linked to specific signal sequences such as ubiquitin and lysosome-associated membrane protein-1 (LAMP-1) for

a more efficient processing and presentation of a given antigen to CD8+ and CD4+ T lymphocytes, respectively (Ji *et al.*, 1999; Velders *et al.*, 2001). New systems of delivery have also been developed with the possibility to encapsulate the DNA into liposomes or to electroporate the DNA directly *in vivo* in order to transfect APC with improved efficacy.

Interestingly, two of the common criticised features of DNA-based vaccines are their lack of immunogenicity and their inability to break tolerance. Several strategies were developed to counteract these features. The incorporation of minigenes or linear polyepitopes encoding CTL epitopes in a vector system rather than full gene sequences was proven to be a very efficient way of eliciting CTL responses (Tine *et al.*, 2005). The use of minigenes targeting the tumour antigen gp100 was demonstrated to be even more efficient than linear polyepitopes with or without spacers, although both of them appeared to be unreliable in eliciting a balanced CTL response against all gp100 epitopes, at least in HHDII mice. In the same study, they also showed that by enhancing the epitope to increase its affinity towards specific HLA molecules, the CTL response was more likely to be balanced suggesting that epitope enhancement should be systemically envisaged in the design of vaccines containing both cryptic and immunodominant epitopes (Tine *et al.*, 2005). In spite of relying on the identification of T cell epitopes that would bypass tolerance mechanisms, leading to tumour protection and/or regression, this approach has been used for several tumour antigens such as Trp-1, Trp-2 and gp100 (Weber *et al.*, 1998; Gregor *et al.*, 2004). Another study described the existence of CD8+ and CD4+ T cell epitopes in a nested configuration in the Ras oncogene and the use of this specific sequence rather than the CTL epitope sequence in a minigene vector correlated with efficient *in vivo* processing of both epitopes and quantitative improvement of the CTL response (Lindinger *et al.*, 2003). This investigation was just one example among many emphasising the requirement of CD4+ T cell help in an anti-tumour vaccine, be it tumour-specific with CD4+ T cell epitopes derived from TAA such as Ras or foreign with CD4+ T cell epitopes derived from pathogenic agents such as tetanus toxoid (Buchan *et al.*, 2005).

Finally, the addition of cytokine or co-stimulatory molecule genes in a plasmid already encoding a tumour antigen was investigated for their capacity to enhance the potency and effectiveness of the vaccine but also to polarise the adaptive immune system towards a Th1 and/or a Th2 response. Several cytokines have been tested such as IL-2, IL-4, IL-15 and TNFα but much emphasis has been put on GM-CSF. Primary data indicated that tumour cells transfected to express GM-CSF, notably in murine acute myeloid leukaemia (AML), were potent activators of the differentiation of CD34+ bone marrow precursors into functionally

mature DC and the generation of a stronger anti-tumour immunity (Dunussi-Joannopoulos *et al.*, 1998). Because of its strong ability to attract DC, GM-CSF is now primarily used to target administered antigens to professional APC. For instance, it has been associated with the tumour antigen gp100 and demonstrated a potent ability to mediate tumour protection and regression even at very low levels of gp100 expression (Rakhmilevich *et al.*, 2001).

One way for tumour cells to escape the immune system is to anergise T cells by down-regulating co-stimulatory molecule expression on their cell surface. That is why a considerable effort has focused on the design of DNA vaccines co-administered with co-stimulatory molecules in order to thwart escape mechanisms and generate CTL responses capable of mediating protection (Corr *et al.*, 1997). Conditioning of DC following DNA injection occurs *via* MHC class II-restricted T cells in both CD40-dependent and –independent manners, which underlines the critical role of co-stimulatory molecules such as B7.1 in priming optimal CD8+ T cell activity (Chan *et al.*, 2001). Also, the administration of soluble CD28, a ligand for B7.1, B7.2 and CTLA-4 molecules, to DC *in vitro* triggered secretion of IL-6 indicating that both B7.1 and B7.2 co-stimulatory molecules were required for the generation of immunostimulatory signals and the prevention of IFNγ-driven expression of immunosuppressive tryptophan catabolism. Moreover, enhanced T cell activity to tumours and protection against tumour challenge were observed following injection of soluble CD28 *in vivo* (Orabona *et al.*, 2004). Depending on the co-stimulatory molecule picked for the stimulation of DC, polarisation of the immune response can then take place as described in a previous study showing that transduction of a viral-based vaccine allowing co-delivery of active membrane-anchored B7.1 and secreted IL-2 in AML cells led to higher levels of Th1-type cytokines and specificity towards autologous AML cells (Chan *et al.*, 2005). More recently, B7.1 and B7.2 molecules were both described as potential marker for a better survival against some forms of cancers (Chang *et al.*, 2007). A third co-stimulatory molecule from the tumour necrosis factor family, termed 4.1BBL, has brought considerable interests as it seems to overcome the need for CD4+ T cell help in the induction, amplification and persistence of cytotoxic T lymphocytes. Although 4.1BBL is as potent as B7 molecules to mediate IL-2 secretion by resting T cells (Saoulli *et al.*, 1998), intact CD28-B7.1 and CD28-B7.2 co-stimulatory pathways are still a pre-requesite for 4.1BBL to reach its full potential demonstrating its anti-tumour efficacy (Guinn *et al.*, 1999; Guinn *et al.*, 2001; Diehl *et al.*, 2002). Altogether, these data demonstrate the strong potential of co-stimulatory molecules and the advantages they can bring by combining them with tumour antigens in DNA-based vaccines. Although lack of objective

clinical response has also been the feature of DNA-based strategies, they provide an interesting and safe mode of immunotherapy still investigated in several human clinical trials.

In this study, HAGE DNA vaccination by gene gun or intra-muscular injection of HHDII-DR1 double transgenic mice was undertaken in prophylactic challenge experiments with a HAGE-expressing murine lymphoma cell line in order to investigate the immunogenic and anti-tumour potential of the cancer/testis antigen HAGE as well as determine the best mode of administration of the DNA. Challenge experiments were then repeated in a therapeutic model whereby HAGE DNA was associated or not with a murine co-stimulatory molecule in order to potentiate the anti-tumour response, thereby allowing direct comparison between each DNA vaccine. Finally, the eventual implication in the anti-tumour response of naturally processed HAGE-derived peptides described in Chapter 5 was studied.

6.2 Results

6.2.1 *In vitro* and *in vivo* HAGE expression in tumour cells

Full length HAGE cDNA was cloned into the pBudCE4.1 mammalian expression vector (See Section 4.2.1). A HLA-A2-positive murine lymphoma cell line (ALC) was then grown in a multi-chamber slide and transfected with this plasmid using lipofectamine 2000 reagent following manufacturer's instructions to confirm HAGE protein expression (See Section 2.2.2.1). Two days after transfection, cells were fixed, permeabilised and stained for HAGE expression for confocal analysis. Secondary antibody tagged with fluorescein highlights the expression and localisation of HAGE within the cells. High levels of HAGE protein expression ubiquitously found inside the cells were achieved when ALC cells were transfected with pBudCE4.1/HAGE (Fig. 6.1C). On the other hand, cells incubated with FITC-conjugated antibody showed only background staining (Fig. 6.1A and B).

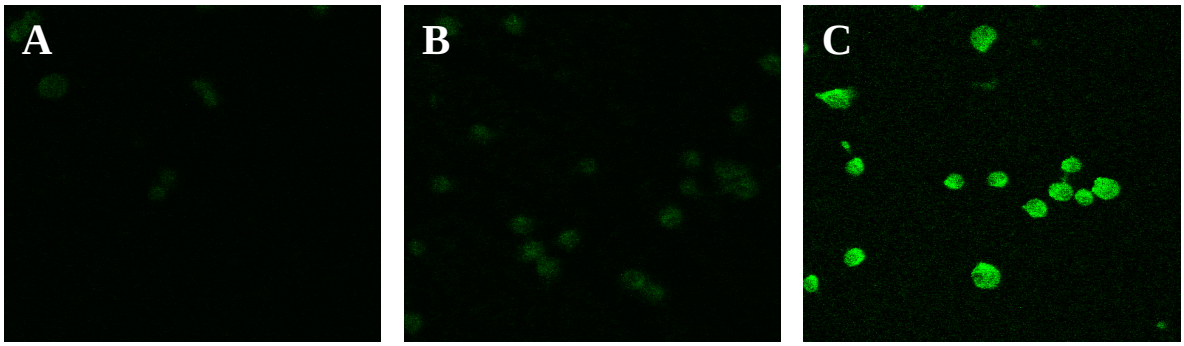

Figure 6.1: Immunofluorescence assay for HAGE protein expression analysis and localisation in ALC/HAGE cells. *Immunofluorescence was observed under a confocal microscope in ALC cells stably transfected with HAGE cDNA (C). No non-specific staining was obtained when cells were incubated in the absence of antibody (A) or with secondary antibody only (B). HAGE protein expression is mainly localised in the cytoplasm.*

ALC cells were thereafter stably transfected with pBudCE4.1/HAGE using lipofectamine 2000 as the transfection reagent and following multiple passages in a media containing the selective antibiotic zeocin, the resulting ALC/HAGE cells were used in tumour challenge experiments in HHDII-DR1 double transgenic mice. Firstly, the TD50 of these cells was determined by injecting subcutaneously different numbers of cells to the mice, varying from $2*10^4$ to $2*10^5$ cells in 100µl of serum free media. $6*10^4$ ALC/HAGE cells appeared to be the minimum number of cells allowing tumours to grow in at least 50% of the mice injected. Secondly, ten times the TD50 are commonly used in *in vivo* tumour models. Consequently, mice were injected with $6*10^5$ ALC or ALC/HAGE cells, and mice terminated and tumours excised when sizes reached about $1cm^2$. Tumours were embedded in OBC media, fixed in a cold solution of isopentane and sectioned using a cryostat. The *in vivo* expression of HAGE was then confirmed by performing immunohistochemistry and Fig. 6.2 demonstrates that whilst no significant staining in ALC tumours was obtained (Fig. 6.2C), ALC/HAGE tumours showed positive staining with the HAGE antiserum in a vast majority of cells present on the tumour sections (Fig. 6.2D). Sections incubated with biotin-conjugated secondary antibody only did not demonstrate any staining above background for both ALC and ALC/HAGE tumours (Fig. 6.2A and B).

6.2.2 Evaluation of DNA immunisation for HAGE

Firstly, the TD50 of ALC/HAGE cells in HHDII-DR1 double transgenic mice was determined (See Section 6.2.1) and found to be $6*10^4$. In agreement with other published work, it was therefore decided that $6*10^5$ cells were to be used for any further studies requiring tumour implantation. It is possible, however, that the introduction of a foreign gene will render the cells more immunogenic, which was not possible to assess using a small number of mice. So in order to confirm and expand these experiments, it was then necessary to determine whether mice injected with $6*10^5$ ALC/HAGE cells subcutaneously would truly generate tumours that continuously grow or regress at some point. Tumour growth and survival were monitored two to three times a week for at least 30 days. Results showed that 40% of injected mice survived the tumour implantation with tumours reaching an average size of $0.8cm^2$ in 15 days before completely regressing in approximately 32 days.

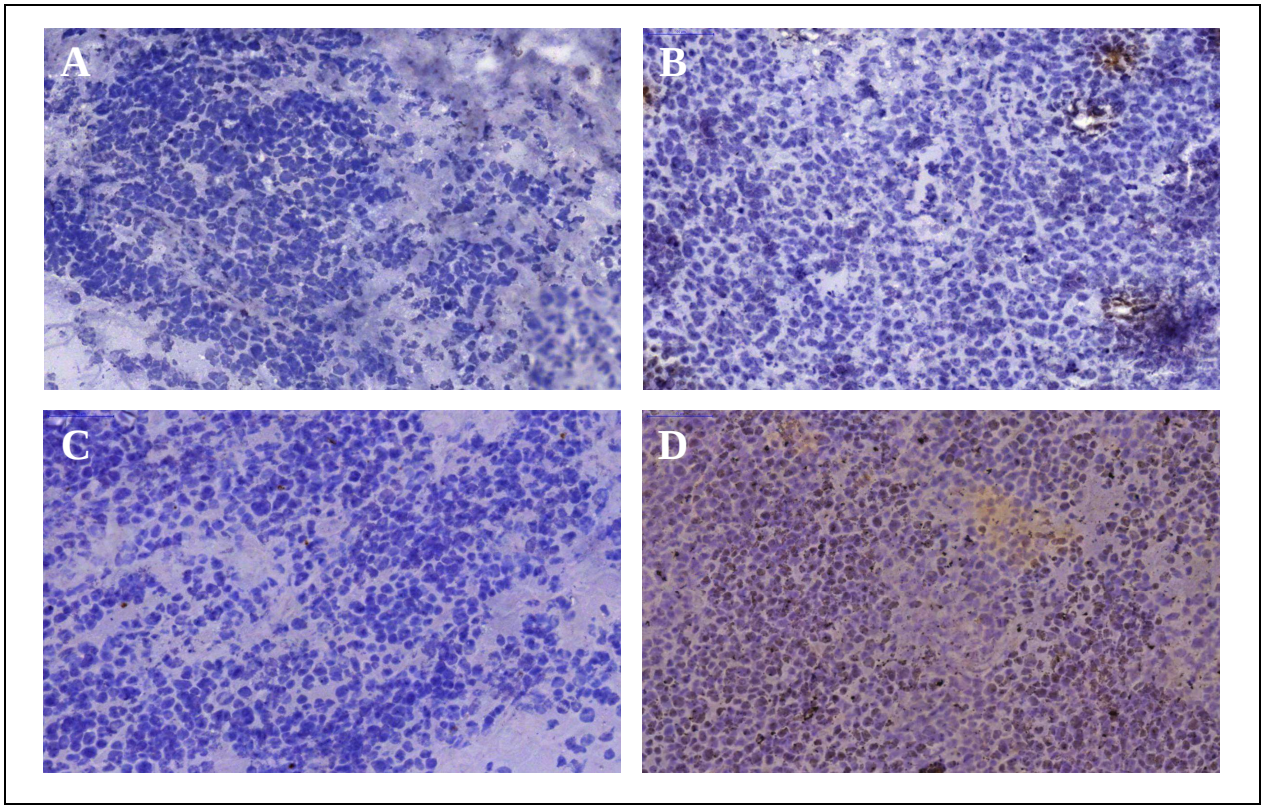

Figure 6.2: Immunohistochemistry staining for HAGE protein expression in vitro in ALC and ALC/HAGE tumours. *The immunohistochemistry demonstrates the in vivo expression of HAGE at the protein level in ALC/HAGE tumours (D), but not in ALC tumours (C) excised from DR1/HHDII mice. No non-specific secondary staining was obtained in both ALC (A) and ALC/HAGE (B) tumours. HAGE expression was found in the cytoplasm and in the nuclear and appeared to be granular. Objective magnification: x20.*

The rest of the mice injected with ALC/HAGE cells were euthanised according to the Home Office regulations as tumours attained the size limit of $1cm^2$ 20 to 25 days post-challenge (Fig. 6.3).

Gene gun immunisation has been recently used with good efficiency in several murine tumour models. Hence, pBudCE4.1/HAGE DNA was coated with 1 micron gold particles. These DNA-gold bullets were then administered intradermally three times at seven-day intervals to the shaved abdomen of HHDII-DR1 double transgenic mice using a helium gene gun. Seven days after the last immunisation, mice were challenged with $6*10^5$ ALC/HAGE cells subcutaneously and monitored as described above. A delayed growth and complete regression of ALC/HAGE tumours were observed in 95% of mice immunised with pBudCE4.1/HAGE approximately 28 days after tumour challenge, while only 50% of mice immunised with the empty pBudCE4.1 plasmid survived the challenge with ALC/HAGE cells. Also, tumour stabilisation or regression occurred much later in time in mice immunised with the control plasmid (Fig. 6.4). Several studies have also suggested that gene gun immunisation is more likely to polarise the immune response to Th2 type, a response not as efficacious as Th1 type to generate CTL. To exclude the possibility that gene gun immunisation does not provide the best mode of immunisation and protection against tumour challenge, HHDII-DR1 double transgenic mice were immunised intramuscularly twice at seven-day interval with 100µg of pBudCE4.1/HAGE or control pBudCE4.1/(-) before being challenged with $6*10^5$ ALC/HAGE cells seven days after the last immunisation. Results obtained were similar to those obtained using gene gun vaccination, where a delayed growth and complete regression of ALC/HAGE tumours were achieved in 90% of mice immunised with pBudCE4.1/HAGE DNA approximately 28 days after tumour challenge. On the other hand, only 60% of mice immunised with the empty plasmid showed signs of tumour stabilisation or regression (Fig. 6.5).

Some of the mice, which have undergone successful tumour remission, were re-challenged a second time with $6*10^5$ ALC/HAGE cells two months after total disappearance of the tumour bulks. Tumour growth and survival were recorded. Interestingly, there was no signs of tumour re-emergence over a period of 30 days in all the mice that have been re-challenged (Data not shown) eventually suggesting the existence of a memory response against HAGE and/or other tumour antigens.

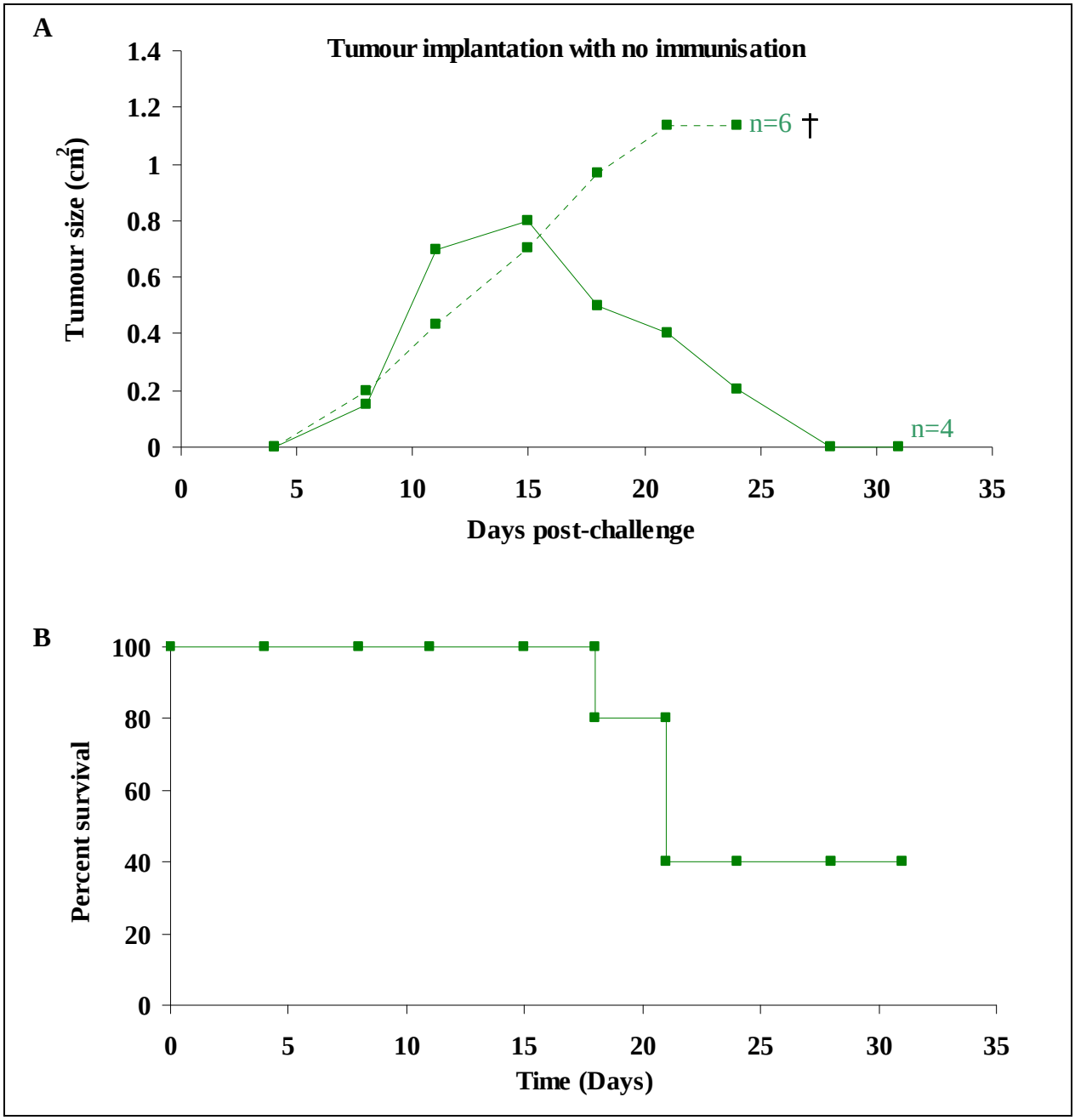

Figure 6.3: Tumour implantation experiment with ALC/HAGE in DR1/HHDII mice. *Five mice were injected with 6*10^5 ALC/HAGE cells/mouse. Tumour growth rate (A) and survival (B) were recorded. Results shown are representative of two independent experiments.* (†) *indicates termination. It seems that ALC/HAGE cells are naturally immunogenic since 4 out of 10 tumours regressed without the need for any immunotherapeutic interventions.*

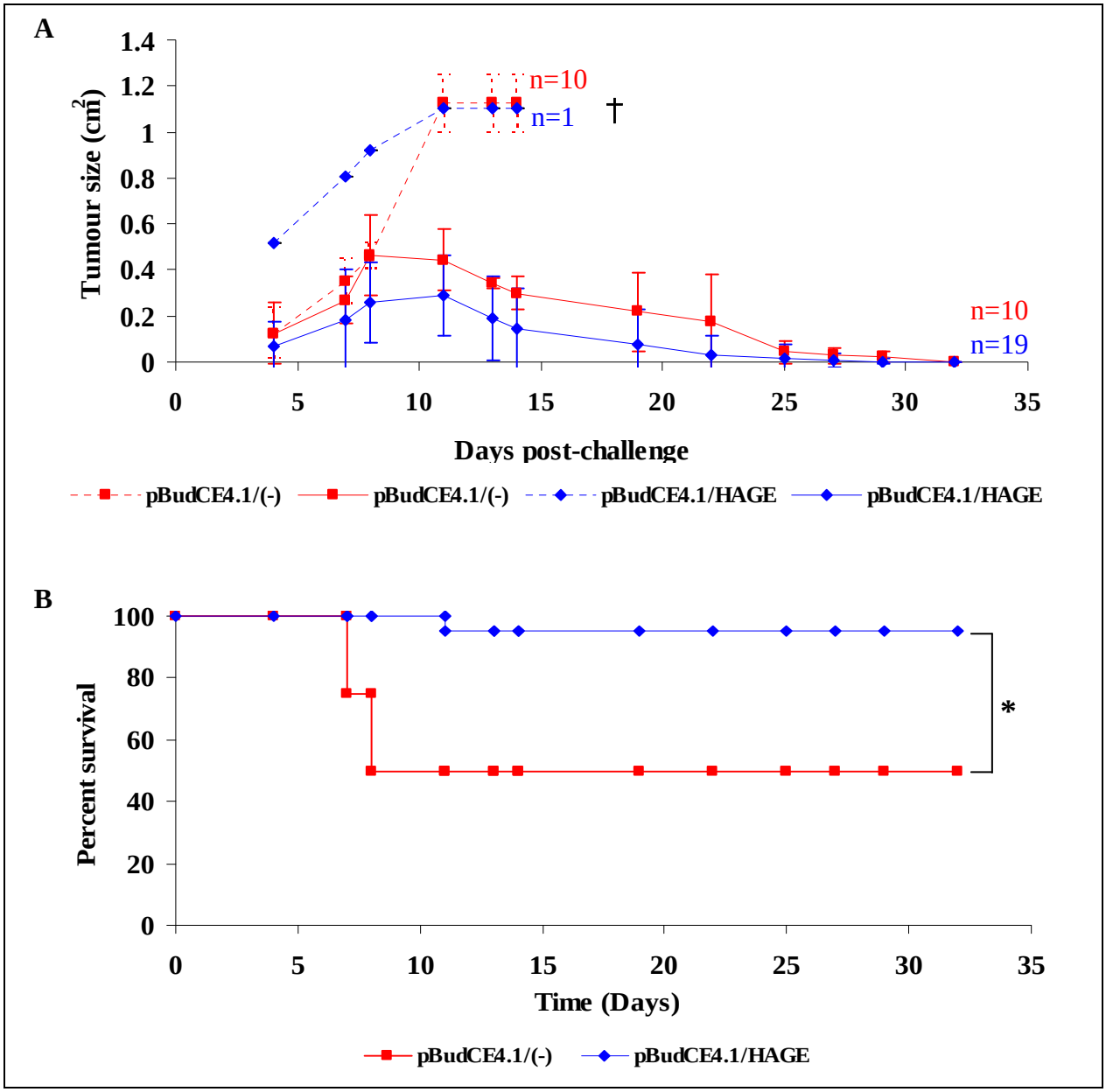

Figure 6.4: Tumour challenge experiment following gene gun immunisation in DR1/HHDII mice. *Two groups of 10 mice were immunised 3 times with gold particles coated with either pBudCE4.1/(-) or pBudCE4.1/HAGE at 7-day intervals, followed by challenge with ALC/HAGE cells. Vaccinated mice showed a slowdown of the tumour growth rate (A) as well as a statistically significant improvement of the survival (B) compared to controls. Results shown are representative of two independent experiments. *p<0.05 are the statistical differences between HAGE DNA immunisation and control DNA immunisation determined by unpaired Student T test.* (†) *indicates termination.*

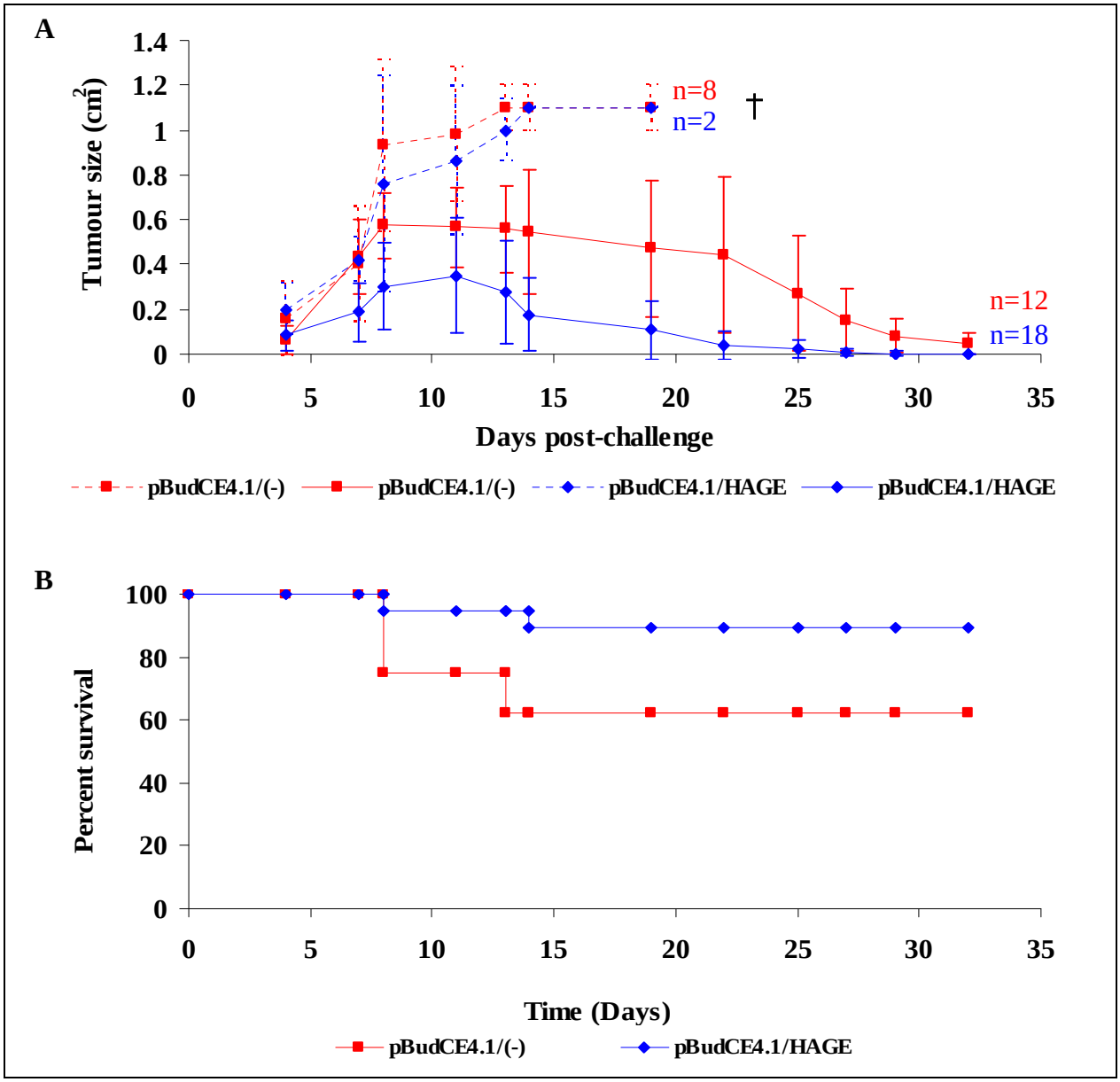

Figure 6.5: Tumour challenge experiment following intra-muscular immunisation with HAGE cDNA in DR1/HHDII mice. *Two groups of 10 mice were immunised twice with 100µg of naked pBudCE4.1/(-) or pBudCE4.1/HAGE DNA at 7-day intervals, followed by challenge with ALC/HAGE cells. Vaccinated mice showed a slowdown of the tumour growth rate (A) as well as an improvement of the survival (B) compared to controls. Results shown are representative of two independent experiments. Statistical differences between HAGE DNA immunisation and control DNA immunisation were determined by unpaired Student T test.* (†) *indicates termination.*

Amounts of DNA used in the preparation of gold particles and the immunisation procedures with gene gun are considerably lower than in intramuscular injections with naked DNA, and having established that gene gun with pBudCE4.1/HAGE DNA led to a statistically significant improvement of the percent of survival, it was next decided to assess the efficiency of gene gun immunisation with gold-coated pBudCE4.1/HAGE DNA in a therapeutic model.

HHDII-DR1 double transgenic mice were first injected subcutaneously $6*10^5$ ALC/HAGE cells and then vaccinated intradermally three days later with three rounds of DNA-gold bullets administrated at seven-day intervals. Tumour growth and survival were monitored two to three times a week for at least 30 days until the tumour size reached $1cm^2$, when mice were terminated as per the Home Office regulations. Results showed a statistically significant delayed tumour growth on day 12 in mice immunised with HAGE DNA with tumours barely reaching an average size of $0.4cm^2$ after 12 days before completely regressing after 25 days in comparison with the control group of mice, which displayed tumours of approximately $0.7cm^2$ after 12 days before completely regressing after 32 days (Fig. 6.6A). Also, only 40% of the mice survived with the latter treatment while 80% of the mice survived with multiple injections of HAGE DNA (Fig. 6.6B). It is noteworthy that immunisations were undertaken at the early stages of the tumour formation when tumour bulks were not noticeable yet.

6.2.3 Effects of co-stimulatory molecules on tumour development

Following the positive results of DNA immunisation to target the tumour antigen HAGE in a therapeutic experiment, it was next decided to wait for the presence of a tumour mass of about $0.3cm^2$ before performing the first immunisation and check whether the addition of a murine co-stimulatory molecule to the HAGE DNA vaccine improves significantly the beneficial effects already observed in the therapeutic experiment detailed above. Hence, murine B7.1, B7.2 or 4.1BBL cDNA were cloned into the only multiple cloning site available of the pBudCE4.1/HAGE mammalian vector as described previously (See Section 4.2.2).

HHDII-DR1 double transgenic mice were first injected subcutaneously $6*10^5$ ALC/HAGE cells. Tumours of the required size ($0.3cm^2$) were globally obtained seven days post-injection and on that day, mice received intradermally the first of three rounds of gold-coated DNA particles.

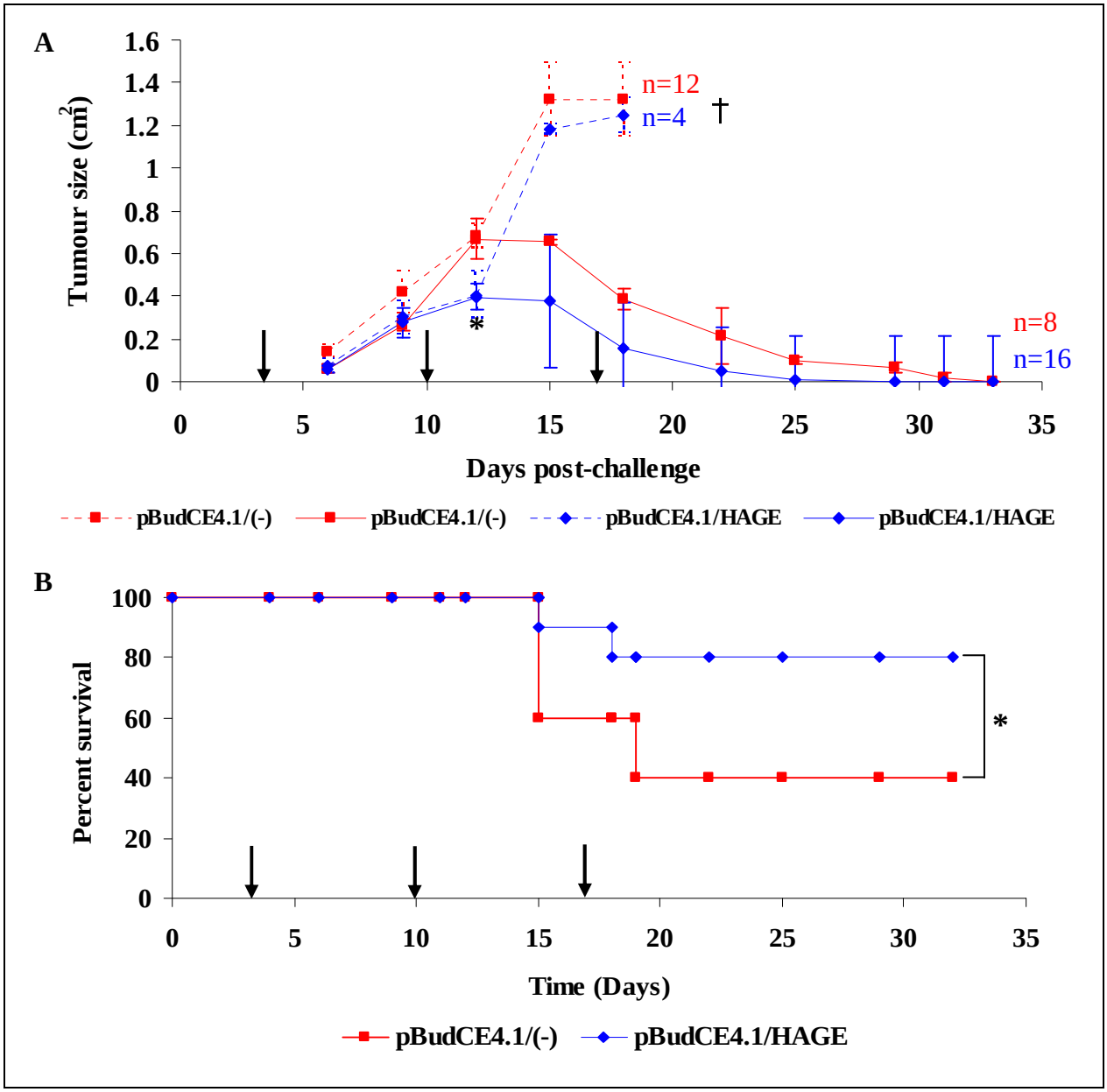

Figure 6.6: Therapy studies using gene gun immunisation in DR1/HHDII mice. *Three days after injecting 6*10^5 ALC/HAGE cells to two groups of 10 mice, mice were immunised three times with gold particles coated with either pBudCE4.1/(-) or pBudCE4.1/HAGE at 7-day intervals and monitored for tumour growth (A) and survival (B). Results shown are representative of two independent experiments. *p<0.05 are the statistical differences between HAGE DNA immunisation and control DNA immunisation determined by unpaired Student T test. (↓) indicates timepoints of immunisation, (†) indicates termination.*

The results of the immunisation with pBudCE4.1/HAGE were compared with the results of the control group immunised with the empty plasmid as well as each of the group having been injected HAGE cDNA with a murine co-stimulatory molecule, be it B7.1, B7.2 or 4.1BBL. As seen in Fig. 6.7, a statistically significant delayed tumour growth and improved survival in mice immunised with HAGE DNA was achieved, confirming the results obtained earlier but this time, with a visible and larger tumour mass before the primary immunisation emphasising the strong potential of pBudCE4.1/HAGE DNA vaccine against HAGE-positive tumour cells. Interestingly, out of the three murine co-stimulatory molecules, only B7.2 provided a statistically significant improvement of the beneficial effects observed with HAGE DNA. Indeed, complete tumour regression was achieved 17 days post-challenge in mice receiving HAGE and B7.2, opposite to 25 days post-challenge in mice receiving HAGE only. Survival curves are, on the other hand, similar for all HAGE DNA-based vaccines with or without murine co-stimulatory molecule suggesting that survival expectancy should be the same whatever the presence or not of a co-stimulatory molecule (Fig. 6.7B). However, larger groups of mice should, in the future, be challenged and vaccinated in order to confirm these observations and draw a conclusion on the different murine co-stimulatory molecules and their effects on tumour progression.

6.2.4 Involvement of identified HAGE-derived peptides in the anti-tumour response

In order to investigate whether identified HAGE-derived peptides are involved in the anti-tumour response observed in prophylactic and therapeutic experiments and could be used in monitoring experiments, splenocytes from regressing mice were harvested and re-stimulated *in vitro* with either HAGE 126 peptide-pulsed LPS blasts, or with HAGE 338 or HAGE 506-II peptides only. A cytotoxicity assay using T2 cells pulsed with peptides and ALC/HAGE cells as targets (Fig. 6.8) and a tritiated thymidine incorporation assay using peptide-pulsed BM-DC (Fig. 6.9) were carried out to confirm CD8+-dependent cell lysis to peptide HAGE 126 and CD4+ T cell proliferations to peptides HAGE 338 and HAGE 506-II, respectively. Unfortunately, none of these assays led to a peptide-specific response suggesting that these peptides might not be involved in the anti-tumour response.

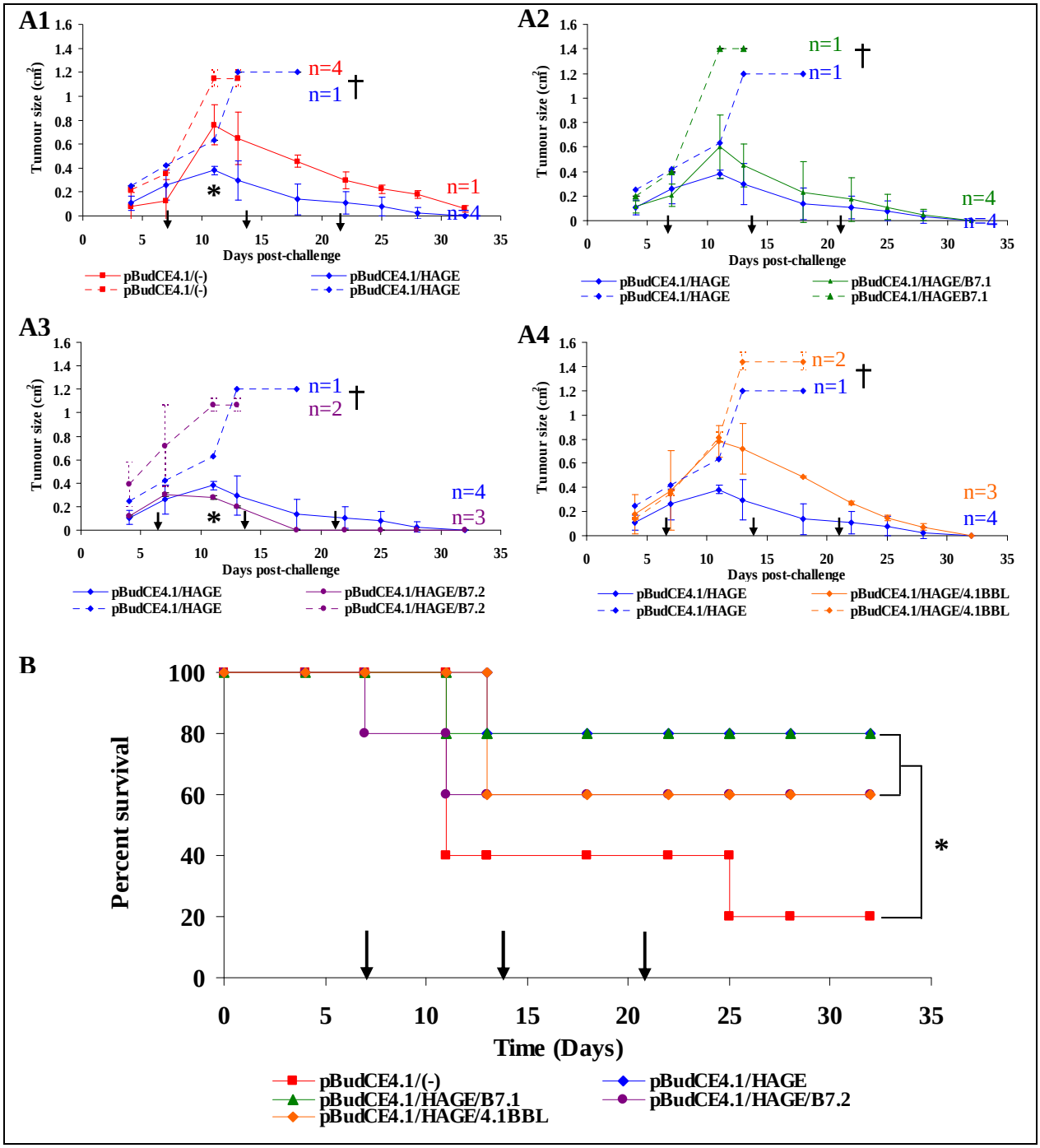

Figure 6.7: Therapy studies combining HAGE with a murine co-stimulatory molecule in DR1/HHDII mice. *Seven days after injecting 6*10^5 ALC/HAGE cells to five groups of five mice, mice were immunised three times with gold particles coated with pBudCE4.1/HAGE (A1) associated or not with mB7.1 (A2), mB7.2 (A3) or m4.1BBL (A4) at 7-day intervals and monitored for tumour growth (A) and survival (B). Results shown are representative of one experiment. *p<0.05 are the statistical differences between HAGE DNA immunisation and control DNA immunisation determined by paired Student T test. (▼) indicates timepoints of immunisation, (†) indicates termination.*

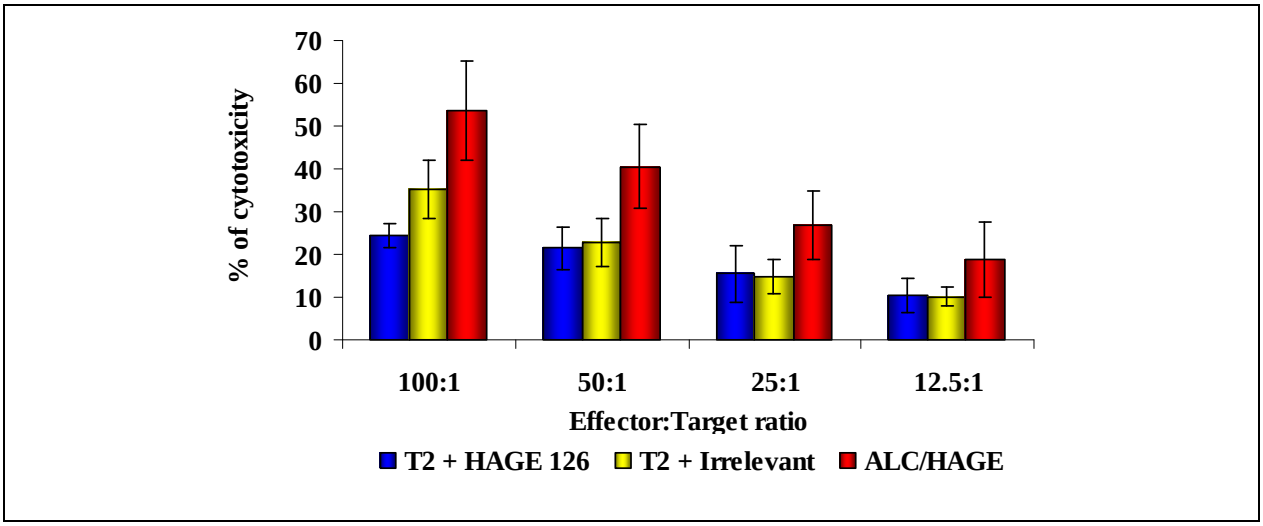

Figure 6.8: Cytotoxicity assay using T cells generated from HHDII-DR1 double transgenic mice immunised with HAGE DNA and challenged with ALC/HAGE cells. *Mice were immunised with gold coated pBudCE4.1/HAGE plasmid and challenged with ALC/HAGE cells. Splenocytes from 2 regressor mice were harvested after complete tumour regression and re-stimulated in vitro with LPS blasts pulsed with HAGE 126 peptide. Cytotoxicity assay was carried out using peptide-pulsed T2 cells or ALC/HAGE as target cells on splenocytes.*

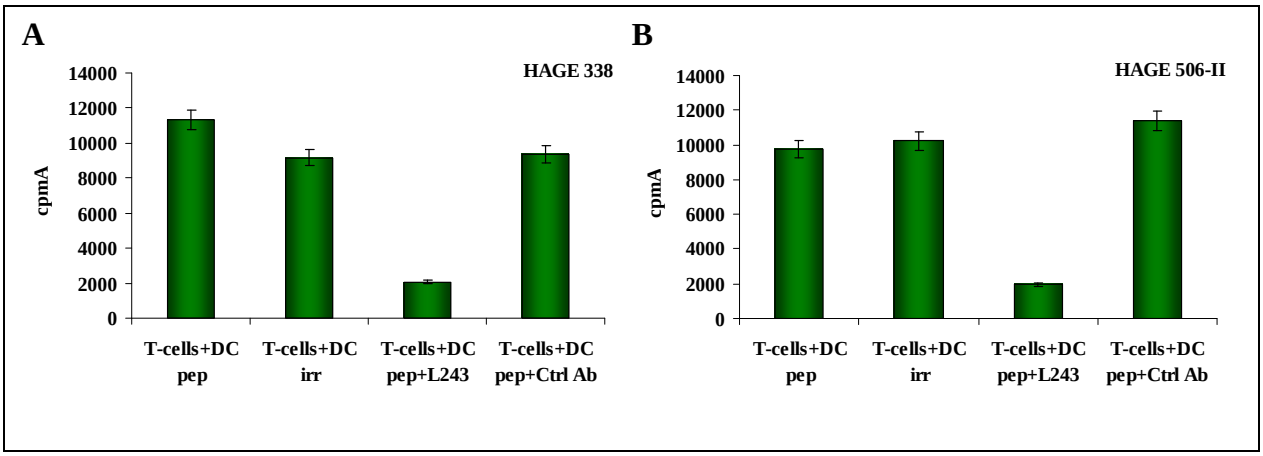

Figure 6.9: Proliferation assay using T cells generated from HHDII-DR1 double transgenic mice immunised with HAGE DNA and challenged with ALC/HAGE. *Mice were immunised with gold-coated pBudCE4.1/HAGE plasmid and challenged with ALC/HAGE cells. Splenocytes from 2 regressor mice were harvested after complete tumour regression and re-stimulated in vitro with HAGE 338 (A) or HAGE 506-II (B) for 7 days and then tested for proliferation by incubating them with syngeneic BM-DC pulsed with peptide.*

6.3 Discussion

Previous attempts to identify naturally processed epitopes from HAGE antigen were successful and could be useful for monitoring an immune response to HAGE as well as the elaboration of peptide-based vaccines, at least in HLA-A2, -DR1 or –DR4 patients. However, immunisation of cancer patients with peptides demonstrated low levels of efficiency in humans and hence, therapies targeting the full length HAGE gene were undertaken in HHDII-DR1 double transgenic mice.

In this study, HLA-A2-positive murine lymphoma ALC cells were chosen for the development of an *in vivo* tumour model in HHDII-DR1 mice. ALC cells were first transiently and then stably transfected with pBudCE4.1/HAGE to express the HAGE protein. The *in vitro* and *in vivo* expression of HAGE was confirmed by immunofluorescence of transiently transfected ALC cells and immunohistochemistry of ALC/HAGE xenografts grown in HHDII-DR1 mice to a size of $1cm^2$ before excision. Transfection of ALC cells led to a nuclear expression of HAGE *in vitro* and *in vivo* with a characteristic granular staining as shown by immunohistochemistry. Interestingly, both ALC and ALC/HAGE cells were injected for this experiment and the latter appeared to grow much faster to the required size than the former (Data not shown). Although this difference between HAGE-negative and –positive cells has been previously observed *in vitro* (See Section 3.2.3), further experiments are needed to confirm this trend and the eventual implication of the transfected gene in the proliferation rate *in vivo*.

After determination of the TD50 of ALC/HAGE, ten times this number was injected to naive HHDII-DR1 mice which had not previously encountered the HAGE antigen in order to document tumour growth and survival expectancy. Interestingly, still a very large number of mice injected (40%) showed signs of tumour regression and total regression 30 days post-challenge, suggesting that anti-tumour responses could be readily raised against ALC/HAGE cells in a proportion of mice due to epitope spreading as well as the presence of HAGE, a foreign antigen with no known murine counterpart. Despite having a strong immunogenic potential, ALC/HAGE cells allowed a margin large enough to improve the rates of tumour growth and the percent of survival. Also and contrary to other tumour cells tested in this project such as EL4/HHDII cells (Data not shown), ALC/HAGE cells allowed reproducible and constant subcutaneous tumour development.

Consequently, HHDII-DR1 mice were immunised with either gold particles coated with pBudCE4.1/HAGE using a helium gene gun or naked DNA in serum free media injected intra-muscularly. Immunisations were then followed by challenge with ALC/HAGE tumour cells

expressing HAGE at high levels. Initial experiments demonstrated a delay in tumour growth in mice vaccinated with pBudCE4.1/HAGE DNA as well as improved survival with up to 95% of the mice challenged showing signs of complete remission. This delay in tumour progression and improved survival in vaccinated animals was reproducible. However, only gene gun allowed a statistically significant improvement of the survival. The ability to protect mice and improved the slowdown of the tumour growth as well as the percent of survival was surprising, considering that several groups have doubted the limited ability of DNA vaccines, especially when using gene gun. However, as shown earlier with HER2/neu (Curcio *et al.*, 2003), HAGE is a foreign antigen and hence, holds a high immunogenic potential.

Having established the efficacy of DNA immunisation by gene gun in a prophylactic experiment, the ability of DNA-based vaccines to cure mice from tumour development in a therapeutic model was evaluated. Mice were first injected ALC/HAGE cells and then immunised with gold-coated pBudCE4.1/HAGE DNA three days later in order to demonstrate the beneficial effects of the DNA vaccination at early stages of the tumour development. Significant delay in tumour growth, shorter period of time to reach complete regression as well as significant improvement of the percent of survival from 40% to 80% in mice vaccinated with pBudCE4.1/HAGE DNA were the three main observations consecutive to this therapeutic experiment. However, because all therapeutic tumour models rely on the immunisation of animals once tumours of palpable size are obtained (0.2 to 0.3cm^2), mice were then injected with tumour cells and immunised at a later stage of tumour development seven days after injecting ALC/HAGE cells. The changes made in the immunisation schedule did not affect or modify extensively the outcome, as similar results were obtained. In the same experiment, mice were also injected with tumour cells and immunised with pBudCE4.1/HAGE DNA containing or not a murine co-stimulatory molecule (*i.e.*: B7.1, B7.2 or 4.1BBL). Although there was no significant difference between HAGE on its own and HAGE with B7.1 or 4.1BBL, a small beneficial effect was observed with the addition of the co-stimulatory molecule B7.2 in terms of maximal tumour size reached and shorter period of time leading to total tumour regression.

B7.1, B7.2 and 4.1BBL have all been described as equally efficient as each other to generate T cell proliferation and when expressed together, create a synergistic effect allowing potent co-stimulation of the immune response (Habib-Agahi *et al.*, 2007). However, contrary to its counterparts, the co-stimulatory molecule B7.2 often displays the capacity to induce higher levels of cytokine secretion such as IFNγ, GM-CSF, TNFα and IL-10 (Kronfeld *et al.*, 2005) and to promote tumour regression in animal models when incorporated in a DNA vaccine

(Yang *et al.*, 2007). Unfortunately, lack of mice did not permit this experiment to be performed on larger cohorts of animals, which would be required for the comparison between each co-stimulatory molecule and a more thorough statistical analysis.

It is worth noting that ALC cells on their own were previously described as poorly immunogenic (Gritzapis *et al.*, 2006). Indeed, in this study, animals were challenged with a number of ALC cells as low as $2*10^4$ in order to obtain palpable tumours 22-25 days after injection. Also, 100% of the mice challenged with ALC cells were sacrificed about 65 days after the start of the experiment. These results indicate that the number of cells used for challenging animals is certainly critical for the level of immunogenicity of the cells and the outcome of the study, and that smaller number of ALC/HAGE cells injected might be considered in the future in order to decrease this immunogenicity. Moreover, the immunogenicity of these cells were not affected by the transfection with a foreign antigen as similar tumour growth rate and survival expectancy were achieved following transfection of ALC cells with the human HER2/neu gene. In this project, HAGE has proved to be by nature crucial in the increase of the proliferation rate and its involvement in the process of tumorogenesis might jeopardise the immunogenicity of ALC cells even when very low number of cells are used to challenge the mice. Thus, further work is needed to develop and optimise an *in vivo* tumour model with ALC/HAGE cells or use a poorly immunogenic tumour model such as the B16 melanoma cell line (Bronte *et al.*, 2000).

It is not clear whether reduced tumour growth and improved survival is due to the development of a specific T cell response or rather a combination of innate and adaptive immune response. However, it is worth mentionning that lesions were observed on the surface of a majority of regressing tumours suggesting the presence of an inflammatory response. Histology would have been able to confirm this hypothesis. However, due to time constraints, these were not performed. Inflammation and its association with cancer evolution has been well-studied with the common understanding that inflammation and innate immune responses can exert pro-tumorogenic effects while inflammation and adaptive immune responses are more likely to generate anti-tumorogenic effects (Karin and Greten, 2005); although some discrepancies have also been reported (de Visser *et al.*, 2005). However, evidence accumulated in this study suggests the promotion of an adaptive immune response against ALC/HAGE cells, but it remains to be established whether the anti-tumour response is HAGE-specific.

As demonstrated in Chapter 5, reverse immunology and transgenic mice allowed the identification of one class I (HAGE 126) and two class II peptides (HAGE 338 and HAGE 506-II) derived from the HAGE protein. In order to confirm the hypothesis of a HAGE-specific

T cell response towards ALC/HAGE tumours in HHDII-DR1 mice, spleens of mice with regressing tumours were harvested and tested *in vitro* in cytotoxicity and proliferation assays following *in vitro* re-stimulation with the identified naturally processed and immunogenic HAGE-derived peptides. Unfortunately, non-specific responses were obtained for both class I and class II peptides. This result was expected for peptide HAGE 126 as its endogenous processing has only been shown following DC immunisation and not gene gun. However, some of the T cells from regressor mice were expected to be HAGE 338 or HAGE 506-II specific even following gene gun immunisation suggesting that other HAGE-derived epitopes could be involved in the anti-tumour responses observed. Therefore, it remains difficult to confirm at this stage whether the tumour protection observed in these experiments were HAGE-related with immune responses specifically targeting the antigen or due to epitope spreading. Challenging the mice with ALC and ALC/HAGE cells three weeks after the last immunisation instead of seven days is something to be considered in the future in order to answer that question. Also, tumour growth in immunised animals should be compared with tumour growth in non-immunised animals in order to rule non-specific effects and confirm that immunisation with the empty vector did not naturally increase in a positive way tumour growth. Unfortunately, lack of time did not permit these experiments to be conducted, which would be necessary to conclusively prove the presence of a HAGE-specific immune response towards ALC/HAGE tumour cells, at least in a prophylactic setting. Further experiments are therefore required to allow us to conclude that these three HAGE-derived peptides are not involved in the anti-tumour process and that the eventual T cell response is HAGE-specific and not due to epitope spreading.

Collectively, these data showed that gene gun gave better results than intra-muscular immunisation in this *in vivo* HAGE-positive tumour model and that the pBudCE4.1/HAGE vector seems to offer better protection to tumour challenge and better overall survival of HHDII-DR1 double transgenic mice than the empty vector in both prophylactic and therapeutic experiments. Finally, immunogenic and naturally processed HAGE-derived peptides may or may not be involved in the anti-tumour response and further work is needed to prove that these peptides are indeed responsible for the beneficial effects of the DNA vaccine on the tumour development by trying other modes of immunisation such as DC- or SFV-based vaccines.

Chapter 7: Discussion

The scope of this project was to identify potential candidate T cell epitopes derived from a novel cancer/testis antigen with immunogenic potential for cancer immmunotherapy by directly combining reverse immunology with transgenic mouse models. Furthermore, the second part of the project aimed to design DNA-based vaccine strategies and assess them in an *in vivo* tumour model in order to assess the immunotherapeutic abilities of this new antigen. But before undertaking series of experiments involving the use of several animals, the primary objective of this project was to ensure that this antigen was a valid target for cancer immunotherapy and an essential gene for cancer in order to avoid the immunoediting phenomenon and hence, tumour escape to take place.

7.1 HAGE as a potential candidate for cancer, expression and validation

Since the cloning of MAGE-1, the first gene reported to encode a tumour antigen recognised by T cells, molecular identification and characterisation of novel tumour antigens has rapidly progressed with the hidden promise of being useful in selectively targeting cancer cells expressing these antigens by immunotherapy. Using the immune response to eliminate cancer cells is a seducing idea as harmful and severe side effects can be avoided unlike chemotherapy and radiotherapy. Although successful results were achieved in animal models, very little clinical benefits were observed in humans, slowly breaking the hopes emitted in cancer immunotherapy. It is now generally admitted that immunotherapy should indeed be utilised against metastases in combination with more conventional cancer therapies such as surgery to remove the original tumour mass. Association of both modalities will allow the immune system to deal with fewer malignant cells as well as the reduction of immunosuppressive factors released by cancer cells.

The good completion of this project relied on the gathering of two determinative conditions: the validity of the tumour antigen for use in immunotherapy and the relevant animal model used to investigate it. In order to ensure the former, an ideal tumour antigen that could be useful for immunotherapy has to combine four main characteristics before pursuing with animal and human studies. The possibility of designing personalised vaccines against individual antigens has been defended by several researchers. However, this strategy appears to be time-consuming, expensive and most of all impractical, especially in developing countries. Thus, antigens with the characteristics of being totally absent from normal tissues and expressed with high frequencies in tumours represent the most interesting targets. Two of the

best and most studied tumour antigens are the tumour-specific protein Bcr/Abl expressed in CML patients and the viral antigen E7 expressed in cervical cancers, and are subject of several clinical trials (Pinilla-Ibarz *et al.*, 2005; Lin *et al.*, 2007). Tumour-specific proteins and viral antigens are very specific for certain tumours but unfortunately not all cancers have been so far characterised by such antigens. Also, cancers can over-express essential genes involved in cell growth and proliferation, and these genes, shared between different types of tumours, could represent valid targets if the risks of generating auto-immune responses were not often greater than the eventual beneficial anti-tumour effects. The last characteristic for an antigen to be an ideal target for immunotherapy is its involvement in tumorogenesis. Indeed, the down-regulation of the antigen is likely to be triggered if the latter is non-essential to the tumour transformation, leaving less immunogenic and escape variants from tumours to multiply according to the immunoediting theory (Dunn *et al.*, 2004). However, several non-essential antigens could be targeted simultaneously to avoid the generation of escape variants but ideally, targeting essential antigens with oncogenic properties would prevent the tumour cells to escape and make it a very promising immunotherapeutic modality.

Initially identified in a human sarcoma by Martelange *et al.* (2000) using representational difference analysis, several studies have reported the over-expression of the cancer/testis antigen HAGE in more than 50% of myelogenous leukaemia (Adams *et al.*, 2002), and in some cases of benign and malignant neoplasms of the salivary glands (Nagel *et al.*, 2003). HAGE was also demonstrated to be frequently expressed in about 30% of brain, colon and lung cancers among others (Scanlan *et al.*, 2004). Moreover, very little is known about HAGE but its belonging to the DEAD-box family of ATP-dependent RNA helicase and evidences of the involvement of other members of this family in tumour cell proliferation (Eberle *et al.*, 2002; Yang *et al.*, 2005) suggest a crucial role in the RNA metabolism of tumour cells. For these reasons, HAGE seems to be a good target for immunotherapy as it would be applicable for the majority of the population.

To verify HAGE expression at the mRNA and protein levels in normal tissues and cancer cells, quantitative real time PCR, immunohistochemistry and immunofluorescence were used. As expected, HAGE expression was at very low levels in normal tissues and then found to be negative using RT-Q-PCR with the exception of testis. Expression of HAGE was confirmed in several tumours of different histological origin such as blood, breast, colon, gastric, head & neck and skin malignancy. Despite the number of samples for some forms of cancer being very small and quantities of mRNA available very low, levels of HAGE expression in CML, colon carcinoma and melanoma samples were in line with those previously published. Furthermore,

neither gastric nor head & neck carcinoma have already been described in the literature. Respectively, approximately 22% and 40% of the cancer samples tested were shown to be over-expressing HAGE and the rise was at least two folds. Also, they are discrepancies between cancer cells from patients and cancer cell lines prepared *in vitro* and although, they often behave quite similarly when both types of cells are from the same cancer, they display quite a different phenotype. Indeed, in this study, we have confirmed the over-expression of HAGE in about 60% of CML samples. However, none of the CML cell lines tested expressed HAGE at any kind of detectable levels by real time PCR. This result was later explained by a study by Roman-Gomez *et al.*, (2007) describing a very high hypermethylation status of the HAGE promoter in CML cell lines and hence, preventing transcription of the HAGE gene. Low levels of HAGE expression were shown to be easily reversed in CML cell lines with the addition of a demethylating agent and synergistically enhanced with the extra addition of a deacetylase inhibitor confirming that the methylation status of the HAGE promoter was critical to the HAGE gene transcription. On the other hand, HAGE promoter in HAGE-positive melanoma cell lines might be naturally hypomethylated which would translate into high levels of HAGE expression in these cells.

Over the course of this project, relative expressions of genes of interest were normalised to two different housekeeping genes (GAPDH and rRNA), but due to very high level of expression of 18S RNA, GAPDH levels were taken into account for normalisation. However, it has been reported that GAPDH can be an unreliable housekeeping gene for normalisation in RT-PCR due to its wide variability in response to different factors (Bustin., 2002). With hindsight, other housekeeping genes such as β-glucuronidase, protein kinase cGMP-dependent type 1 or TATA-box binding protein, described as more reliable should have been utilised in this project (Lossos *et al.*, 2003).

Results obtained at the mRNA level were further confirmed at the protein level for HAGE as mRNA and protein do not necessarily always correlate. In this study, immunohistochemistry and immunofluorescence validated the results of real time PCR proving and changing the status of putative protein of the cancer/testis antigen HAGE into an actual product of translation mechanisms. Expression of HAGE at the protein level was not detected in any normal tissues, except testis and expression at the mRNA level correlated with expression at the protein level in head & neck carcinoma and melanoma samples, as well as melanoma cell lines. A previous study has demonstrated that a differentiation antigen termed metastasis-associated antigen-1 (MTA1) was not detected by immunohistochemistry because of its low

levels of sensitivity and that by concentrating the samples and performing western-blotting, this given protein could be highlighted (D. Assudani, personal communication). Here, HAGE staining appears quite intensively on normal testis sections and several other sections from different cancers suggesting high quantities of HAGE protein in these tissues. Furthermore, the use of multiple cancer tissue microarrays allowed showing up HAGE expression in cancer tissues which are very difficult to get access to such as bladder and liver carcinoma. Unfortunately, unpurified polyclonal antisera were used to highlight the expression of HAGE at the protein level hindering the quality of the staining with some residual background, even after adsorption of the antisera with rabbit liver powder. In the future, a purified monoclonal antibody customised to target a specific amino acid sequence from the HAGE protein should replace the polyclonal antisera for the staining of HAGE by immunohistochemistry and immunofluorescence.

These last two techniques also brought important additional information. Indeed, it allowed the determination of the localisation of HAGE inside the cells and interestingly enough, it seems to be expressed in different compartments of the cells depending on the culture conditions and on the cell type, be it cell lines, transfected cells or tissues. Indeed, HAGE appears to be cytoplasmic in cell lines as shown in the melanoma cell line ESTDAB-27, whilst being mainly nuclear in transfected cells and tissue sections as demonstrated in transfected murine ALC cells, BM-DC and human testis. Therefore, like other RNA helicases, HAGE could be involved in different processes of RNA metabolism depending on the cell requirements *in vitro* and *in vivo* (Charroux *et al.*, 1999; Charroux *et al.*, 2000). It remains difficult to determine at this stage what these processes and the links with the cancer phenotype are. However, it is possible to bring a few clues on what the consequences of the expression of HAGE are. Like other DEAD-box RNA helicases such as DDX1, DDX2 and DDX5, HAGE seems to be critical to the proliferation of tumour cells as shown by siRNA and cDNA transfection experiments, as well as *in vivo* in challenge experiments although this last observation will have to be repeated in the future. Moreover, cell cycle as well as cell morphology did not display any signs of being altered by the silencing of HAGE gene suggesting that HAGE is not crucial for the cells to survive but only to proliferate. Finally, the expression analysis carried out by real time PCR with CML patient cDNA showed a correlation between the expressions of Bcr/Abl and HAGE which is in line with a study published by Roman-Gomez *et al.* (2007) describing a link between high levels of HAGE expression and poor clinical outcome. Several proteins including oncogenes such as Pim1 and Jun are up-regulated following expression of the Bcr/Abl protein (Hakansson *et al.*, 2004) but the link between the latter and HAGE has been not proven yet.

Also, it is very unlikely that HAGE is produced because of the Philadelphia chromosome as it is found in other forms of cancer such as melanoma where the chromosome translocation is not found. However, it can be hypothesised that downstream of the Bcr/Abl chain of events, some mechanisms shared by a variety of cancers such as the increased expression of proto-oncogenes, growth factors, factors related to tyrosine kinase activity or factors related directly to the cell proliferation cycle (Ohba *et al.*, 2004), and production of reactive oxygen species (Kim *et al.*, 2005) could participate to the sudden transcription of numerous genes with oncogenic properties responsible for the severity of cancer and associated with poor prognosis, including HAGE.

Finally, not all tumour antigens are capable of eliciting specific immune responses and immunogenicity of an antigen would be the deciding factor in validating it as a potential immunotherapeutic target. Combined together, these data strongly suggested that should HAGE be immunogenic, it would represent the ideal immunotherapeutic target against several cancers. Although no previous evidences have been reported describing the generation of a B cell and/or T cell specific immune response towards it, HAGE immunogenicity was investigated in transgenic mouse models.

7.2 Identification of novel T-cell epitopes derived from HAGE

The modeling of human diseases such as cancer goes through the utilisation of animal models although promising results obtained with the latter rarely translate into significant clinical benefits in patients. Nonetheless, animal models remain a reliable and convenient source of great discoveries such as revolutionary drugs and other medical interventions to treat diseases in humans. The main argument against animal models is that their physiological system can differ from humans. However, failure of clinical trials in humans can often rather be explained by the choice of poor animal models in numerous studies. For instance, the use of mice in immunotherapeutic models of cancer can be explained by several reasons, among which the quick provision of answers, low costs, the facility to handle and to genetically transform, and the similarity of their physiological system with humans. But the transplantation of tumours does not represent an ideal model as tumour cells are injected at a site different from their usual anatomical location and often express foreign and highly immunogenic antigens, whereas tumours are more likely to be self in origin and less immunogenic (Dullaers *et al.*, 2006). 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 into humans. Over the last thirty years, the field of transgenic mouse models has revolutionised the research

against cancer by providing a wealth of new knowledge about mechanisms of tumorogenesis and anti-tumour immune responses. With the likes of oncomice in which tumours arise spontaneously, transgenic mice with epitope-specific human TCR or HLA transgenic mice, it is now possible to answer several questions about the roles of an antigen in tumour development, the kinetics of an antigen-specific T cell response and its regulatory mechanisms, or the immunogenicity of a given tumour antigen (Ostrand-Rosenberg., 2004). In this project, the use of mice transgenic for HLA molecules were first used to investigate the immunogenicity and the natural processing of HAGE-derived peptides selected by reverse immunology and secondly to evaluate a model of transplantable tumours over-expressing the HAGE gene in order to study different DNA-based vaccine strategies.

There are a number of different approaches that can be applied to identify new candidate class I/II-restricted target antigens for immunotherapy. In this report, the application of the reverse immunology approach to identify potential T cell epitopes from the product of the cancer/testis antigen HAGE in transgenic mice expressing human HLA molecules is described. Peptides are convenient, easy to synthesise for clinical use, easy to modify and to administer to patients. Disis *et al.* (1996) described that the administration of peptides derived from a self tumour protein rather than the whole protein was able to induce antibody production and T cell activation. Moreover, peptides can be easily administrated together with adjuvants, cytokines or dendritic cells using wild-type sequences or sequences optimised to increase their binding affinity to MHC molecules. Also, using, *ex vivo* monitoring based on tetramer technology, it was shown that T cell responses to peptide vaccines can be followed. One of the major advantages of identifying immunogenic tumour-associated peptides is the subsequent identification of T cell receptors specific for these peptides. Once identified, vectors encoding these TCR can be engineered and used to transfect lymphocytes from peripheral blood for transfer to patients (Morgan *et al.*, 2003). A recent study conducted by Morgan *et al.* (2006) indicated the presence of circulating transduced lymphocytes one year post-transfusion and metastatic regression in two of 17 treated patients. Numerous studies have demonstrated the feasibility of cancer immunotherapy using tumour antigens recognised by CD8+ T cells (Rosenberg *et al.*, 1998; Yee *et al.*, 2000). However, the responses observed were unfortunately weak and transient. CD4+ T cells play a central role in initiating and maintaining anti-tumour therapy. Thus, optimal immunisation will require the recruitment of both CD4+ and CD8+ T cells, in order to generate a long-lasting anti-tumour immune response (Assudani *et al.*, 2007).

To this end, eight HAGE-derived peptides predicted to bind strongly to HLA-A0201 were selected using the evidence-based computer algorithm SYFPEITHI. Four out of eight peptides evaluated in HHDII mice were found to be immunogenic. However, CTL generated by gene gun immunisation with HAGE cDNA and *in vitro* peptide re-stimulation were unable to kill tumour cells expressing HAGE or loaded with peptides suggesting that these peptides were not naturally processed and presented by antigen-presenting cells and tumour cells. Lack of lysis of targets could have been due to low expression of HAGE in these tumour cells, lack of expression of these specific peptides at the cell surface or low avidity of generated T cells for these peptides. However, following immunisation with DC transfected to express HAGE or loaded with HAGE-rich melanoma cell lysate, peptide HAGE 126 was proven to be naturally processed. It is difficult at this stage to explain the natural processing of peptide HAGE 126 following transfected DC immunisation but not by gene gun. The main difference between this two means of vaccination being the population of APC targeted (*i.e.*: BM-DC and Langerhans cells by DC and gene gun immunisation, respectively), it can be hypothesised that processing of HAGE by the immunoproteasome in BM-DC, but not by the proteasome in Langerhans cells, leads to the generation of peptide HAGE 126. Indeed, the proteolytic activity of these two degradation machineries differs leading to the production of two distinct panels of peptides (Kloetzel *et al.*, 2004). The exclusive processing of peptide HAGE 126 by the immunoproteasome suggest that tumour cells, which only express the proteasome, would not be presenting this peptide emphasising the importance of an adapted mode of immunisation for accurate identification of peptides naturally processed by tumour cells.

Since HAGE is an endogenous antigen, it is likely to be processed by MHC class I processing pathway. However, some of these intracellular antigens might also be presented in MHC class II-restricted context as previously described (Dissanayake *et al.*, 2005). Considering this, six HAGE-derived peptides predicted by SYFPEITHI to bind strongly to HLA-DR0101 and HLA-DR0401 molecules were synthesised. Four of them were immunogenic in both HLA-DR1 and HLA-DR4 transgenic mice. Furthermore, two of them (HAGE 338 and HAGE 506-II) were naturally processed as tested using two different methods (gene gun and transfected DC immunisation, See Chapter 5) suggesting that contrary to class I peptides, both types of APC targeted by these two means of vaccination produce both HAGE-derived class II peptides identically. With the MHC class II processing pathway being supposedly identical in both populations of APC targeted, similar results between gene and DC immunisation were therefore expected. In agreement with the results obtained following immunisation with HAGE cDNA of transgenic mice, specific HLA class II-restricted recognition of HLA-DR-matched

tumour cell lines as well as DC pulsed with HAGE-positive cell lysates were also achieved following the *in vitro* generation of CD4+ T cell lines from human PBMC with peptide HAGE 506-II indicating that this peptide was processed identically in both species (Mathieu *et al.*, 2007). Thereafter, both class I peptide HAGE 126 and class II peptide HAGE 338 will now need to be investigated for their ability to respectively generate *in vitro* CTL and T helper cells capable of specifically responding to target cells expressing the appropriate HLA molecules as well as HAGE. Also, natural processing of peptide HAGE 126 by the immunoproteasome and not the proteasome will need to be confirmed. Finally, only high binding affinity peptides were evaluated among which, three of them could be useful for the vaccination of A2, DR1 or DR4 patients but more low-to-moderate binding affinity peptides could still be valid candidate T cell epitopes and will need to be investigated.

7.3 Vaccines strategies and HAGE

The need to identify specific epitopes from antigens can be circumvented by the use of DNA-based vaccine strategies targeting whole antigens, which allow the generation of an immune response against multiple epitopes regardless of HLA haplotypes. To test this, HAGE cDNA sequence was cloned into a mammalian expression vector, which was then used to immunise HHDII-DR1 double transgenic mice using either gene gun or intra-muscular method in prophylactic and therapeutic experiments. Immunisation with HAGE by gene gun or intra-muscular injection was able to provide protection from ALC/HAGE tumour challenge. Moreover, only gene gun immunisation led to a statistically significant improvement of the percent of survival. This is in correlation with several studies defending gene gun as a method of choice to generate an anti-tumour response (Bowne *et al.*, 1999; Gold *et al.*, 2003). Also, significant delayed tumour growth and improved overall survival were achieved when mice were injected ALC/HAGE tumour cells first and then immunised three days later by gene gun in a therapeutic setup. The latter was then repeated but this time, immunisation took place when tumours were palpable. Similar delayed tumour growth and improved survival were recorded.

In spite of a number of advantages of DNA vaccines, lack of potency is often blamed as the causative factor for failure of DNA vaccines in humans and the presence in the plasmid DNA of unmethylated CpG islands, which can act as good adjuvants, is not sufficient to overcome this situation. To make up for this, attaching signalling molecules to direct antigens towards MHC class I or class II processing pathway, or co-administrating the targeted antigen with cytokine genes or co-stimulatory molecules to potentiate the immune response have all been

evaluated with different degrees of success. In order to address this issue, murine B7.1, B7.2 or 4.1BBL cDNA sequences were each cloned into a mammalian expression vector containing already HAGE cDNA. Comparisons were then made and although experiments were carried out in small groups of mice and are not statistically significant, the addition of the murine co-stimulatory molecule B7.2 to HAGE in a single mammalian expression vector seems to convincingly slow down tumour growth but unfortunately, no further improvement of the percent survival. Further experiments are required with larger cohorts to draw definitive conclusions on the beneficial effects of co-stimulatory molecules to a HAGE DNA vaccine.

The ability of HAGE DNA vaccine to generate immune response was not surprising as several studies targeting cancer/testis antigens using plasmid vaccines in mice have demonstrated similar promising results (Park *et al.*, 1999; Ma *et al.*, 2005). However, it remains difficult to determine if the response observed was HAGE-specific and not due to epitope spreading despite differences between immunisation with empty plasmid and HAGE plasmid being significant in both protection and therapeutic experiments. More work is then needed to prove the specificity of the anti-tumour response by refining protection experiments and prolonging the duration between DNA vaccination and challenge with HAGE-expressing tumour cells.

With hindsight, other modalities of vaccination should have also been attempted such as HAGE-transfected DC. Indeed, the identification of the naturally processed and immunogenic class I peptide HAGE 126 relied strictly on the immunisation of transgenic mice with syngeneic DC transfected to express the HAGE protein suggesting that BM-DC provide a different panel of antigenic peptides from Langerhans cells to T lymphocytes. Having identified this peptide, it would have allowed monitoring of the immune response as well as undertaking peptide-based vaccination in HHDII-DR1 double transgenic mice injected with ALC/HAGE tumour cells. Unfortunately, neither gene gun nor intra-muscular injection allowed confirming whether any of the naturally processed and immunogenic peptides HAGE 126, HAGE 338 and HAGE 506-II were responsible for the tumour eradications achieved.

Finally, the presence of regressor mice in the non-immunised group indicates that ALC/HAGE tumour cells are immunogenic on their own and that number and type of cells injected to the mice might be critical to the elaboration of a fatal tumour challenge. It also means that in the future, immunogenicity rather than aggressiveness should be privileged in the elaboration of an *in vivo* tumour model. This immunogenic potential of ALC/HAGE tumour cells was later confirmed when mice were challenged a second time two months after the primary challenge and showed no signs of tumour growth suggesting the existence of a strong memory response. However, it cannot be certified that by lowering the number of cells or replacing the cells with

a proven poorly immunogenic cell line such as B16, the immunogenicity of the cells will also be lowered with HAGE being a foreign antigen and therefore strongly immunogenic. This is a crucial point as it reappraises the use of transplantable tumours in tumour immunology research and emphasises the need for the creation of closer to reality *in vivo* tumour models. That is why mimicking the reality by optimising this *in vivo* model with HAGE-expressing tumour cells will turn out to be critical for the development of proper vaccine strategies in order to avoid the failure of DNA-based vaccines in future human clinical trials.

7.4 Conclusions and future work

Cancer/testis antigens represent one of the most promising groups of tumour-associated antigens identified till date because of their almost unique and specific expression in tumours, and are rightly being investigated for the development of novel immunotherapeutic strategies. However, caution had to be taken to validate HAGE, a member of this family, as target for immunotherapy in order to avoid the appearance of unwanted side-effects such as auto-immunity. Based on the results presented here and should it be immunogenic, the cancer/testis antigen HAGE represents an ideal target for immunotherapy against several cancers. It is strongly expressed at the mRNA and protein levels in a variety of cancers, totally absent from normal tissues except testis and seems to have critical oncogenic properties as cell proliferation correlates with HAGE expression.

With the clinical outcome of cancer patients often correlating with the existence of an immunological response (Mine *et al.*, 2004), the importance of CD8+ and CD4+ T cells has become apparent, which leads to the requirement for protocols to rapidly identify MHC class I and class II epitopes that could be used in the clinic. The use of HLA transgenic mice immunised with predicted peptides has provided a mean to rapidly identify these epitopes bypassing the need for time-consuming *in vitro* re-stimulation of human PBMC. A large panel of peptides can consequently be pre-screened in transgenic mice before finally assessing a reduced number of them in human T cell cultures. Put together, peptide, DNA and DC immunisations allowed the identification of several immunogenic HAGE-derived HLA-A2 and HLA-DR1/-DR4 peptides among which one class I (HAGE 126) and two class II (HAGE 338 and HAGE 506-II) peptides were also found to be naturally processed. Several studies have demonstrated that CD4+ T cells were essential for the promotion of an effector and memory CTL response (Gao *et al.*, 2002; Shedlock *et al.*, 2003) and that the administration of a heterogeneous T cell population is more efficient than transfer of CD8+ T lymphocytes alone (Dudley *et al.*, 2002; Rosenberg *et al.*, 2004). Having identified HAGE-derived class I and

class II epitopes, it remains to establish if the administration of class I and class II peptides derived from the same protein will result in increased CTL activity, long-lasting immunity and whether tumour protection and/or clearance can be achieved with these helped CTL responses, at least in mice. Also, identification of these epitopes could also be useful for the vaccination of HLA-A2, -DR1 and -DR4-positive patients and/or the monitoring of the immune response directed against HAGE in these patients.

The use of pre-clinical models allows valuable information to be obtained regarding the optimal conditions required for tumour clearance and follow-up such as mode of vaccination, use of adjuvants, system of delivery, administration schedule and immune response monitoring. Moreover, it is critical to design novel vaccine strategies that will also be suitable for clinical use. Therefore, the ideal vaccine should combine the following characteristics in order to overcome immunological barriers: low toxicity such as DNA-based vaccine, ability to stimulate all the anti-tumorogenic arms of the immune response, immunomodulatory signals such as cytokines or co-stimulatory molecules for DC and T cell stimulation and eventually, a strategy to deplete immunosuppressive cells such as Tregs and NKT cells. As part of this project, a tumour model was developed in HHDII-DR1 double transgenic mice. This tumour model relied on the creation of stable transfectants expressing HAGE, the transplantation of these genetically-modified tumour cells in mice and the assessment of several DNA-based vaccine strategies. Using a standard analysis of tumour regressions, tumour protection and/or clearance were achieved in a majority of mice immunised with HAGE DNA vaccine administrated intra-dermally by gene gun or intra-muscularly by injection indicating that HAGE as a whole antigen is strongly immunogenic, at least in mice. Moreover, tumour clearance was further improved by the addition of the murine co-stimulatory molecule B7.2 to the HAGE DNA vaccine but not B7.1 or 4.1BBL, although this result will have to be reproduced in the future to confirm the boosting of the anti-tumour response by B7.2. Transgenics have been previously used successfully in our laboratory and in others (Touloukian *et al.*, 2000; Rojas *et al.*, 2004) and are central to this study hopefully providing important information for the formulation of future cancer vaccines and an accurate insight of the immune response taking places in humans.

Future studies should aim to clarify certain aspects of this study and/or to build on some of these findings. Repeat experiments testing HAGE expression on tumour sections must be carried out with customised and purified anti-HAGE antibody to confirm previous results, to assess whether HAGE can be found at the protein level in CML PBMC, and to link expression of this antigen with tumour stage or clinical outcome in several types of cancers. Also,

experiments about the role of HAGE in tumour cells should be expanded to clarify its link with cell proliferation both upstream and downstream of its potential pathway by performing gene microarray analysis in melanoma cell lines, CML cell lines as well as in a histiocytic lymphoma cell line (U937) where the Bcr/Abl translocation characteristic of the CML phenotype can be induced. Studying the phosphorylation status of HAGE would also bring further clues on a potential correlation between HAGE and Bcr/Abl expressions.

In order to further optimise the tumour model used in this project, immunogenicity of the transplantable tumour cells has to decrease and vaccine strategies will have to be refined in order to ensure the specificity of the immune response elicited against HAGE-expressing tumours. Challenge and therapy experiments could then be setup with different modes of vaccinations such as mature DC transfected to process and present HAGE-derived peptides or the SFV/HAGE viruses generated over the course of this project. More detailed investigation of the effects of co-stimulatory molecules is also a pre-requisite in order to conclude on their ability to boost the immune response achieved against the cancer/testis antigen HAGE. Finally and before making the final link with the clinic and undertaking different immunotherapeutic approaches to target HAGE-expressing tumours in clinical trials, identified peptides will have to be tested *in vitro* in order to see their ability to generate peptide-specific CTL and T helpers using PBMC from healthy donors and patients that are A-0201-, DR-0101- and DR-0401-positive.

By completing the information gathered on HAGE and improving the transgenic tumour model, it may be then possible to develop a more efficient mode of cancer vaccination that could translate into the clinic with a more successful prognostic.

Appendix 1

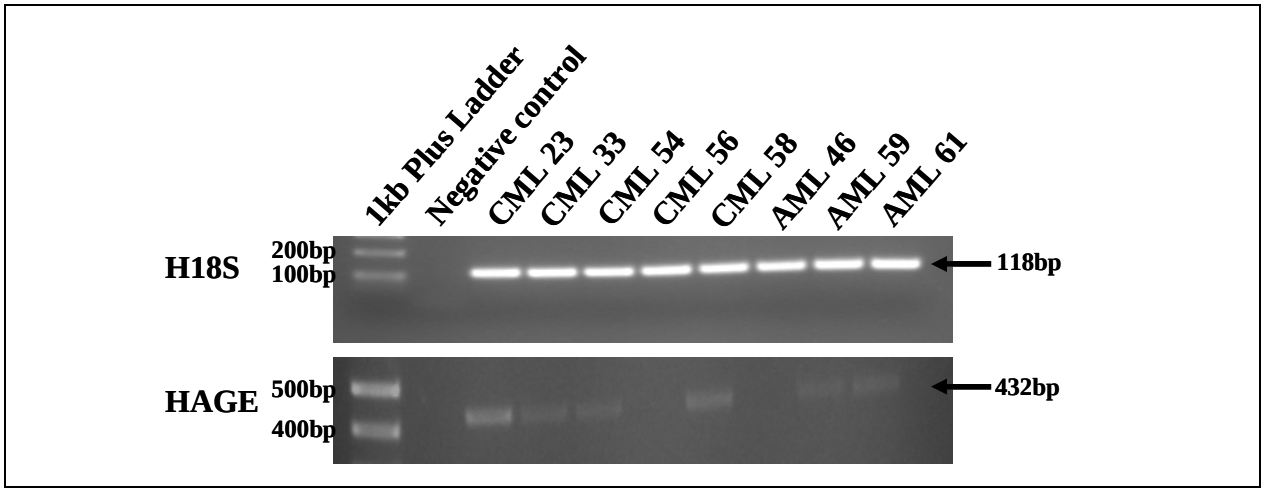

Figure A1: Expression of HAGE in AML and CML samples by RT-PCR. This figure represents the semi-quantitative analysis of expression of HAGE in AML and CML samples, using water as a negative control. PCR products for 18S ribosome and HAGE should be of 110 and 432bp, respectively.

Appendix 2

Table A1: Breast carcinoma information and their relative HAGE expression

Sample code	TNM stage	Differentiation	Histology	Relative HAGE expression
Br1T	1.0.0	Moderate	Infiltrative	-
Br1N				-
Br2T	3.1.0	Low	Infiltrative	-
Br2N				-
Br6T	2.1.0	Moderate	Infiltrative	**32.6**
Br6N				**0.14**
Br7T	2.1.0	Moderate	Infiltrative	0.00
Br7N				-
Br8T	3.1.0	Moderate	Infiltrative	0.06
Br8N				-
Br9T	-	Fibroma	N/A	**60.1**
Br9N				**0.02**
Br10T	1.0.0	Moderate	Infiltrative	0.02
Br10N				-
Br11T	2.3.0	Moderate	Infiltrative	0.41
Br11N				41.0
Br12T	4.-.-	N/A	Infiltrative	0.06
Br12N				83.1
Br13T	1.0.0	Moderate	Infiltrative	-
Br13N				65.6

-: low amount or poor quality of RNA.

Table A2: Colon carcinoma information and their relative HAGE expression

Sample code	TNM stage	Differentiation	Localisation	Relative HAGE expression
Co21T	3.1.0	Moderate	Ascendens	0.00
Co21N				0.03
Co22T	3.0.0	Low	Rectum	0.00
Co22N				0.16
Co27T	4.0.0	Moderate	Sigmoid	0.000
Co27N				0.002
Co30T	3.1.0	Moderate	Caecum	-
Co30N				-
Co33T	3.0.0	Well	Sigmoid	0.00
Co33N				0.01
Co100T	3.1.0	Moderate	Rectum/Sigmoid	**0.004**
Co100N				**0.002**
Co102T	3.0.0	Moderate	Rectum	0.000
Co102N				0.002
Co103T	3.0.0	Moderate	Rectum/Sigmoid	**0.10**
Co103N				**0.00**
Co425T	1.0.0	Moderate	Rectum/Sigmoid	-
Co425N				-
Co437T	3.1.0	Low	Sigmoid	-
Co437N				-

-: low amount or poor quality of RNA.

Table A3: Gastric carcinoma information and their relative HAGE expression

Sample code	TNM stage	Differentiation	Lauren's classification	Relative HAGE expression
Ga47T	N/A	GIII	Intestinal	0.00
Ga47N				0.04
Ga55T	4.1.0	GIV	Diffuse	-
Ga55N				0.002
Ga201T	N/A	Low	N/A	0.000
Ga201N				0.003
Ga411T	3.0.0	GIV	Intestinal	0.000
Ga411N				0.003
Ga418T	3.0.0	GIII	Diffuse	**0.009**
Ga418N				**0.004**
Ga421T	3.0.0	GIII	Diffuse	0.004
Ga421N				0.005
Ga434T	2.0.0	GII	Intestinal	-
Ga434N				-
Ga436T	2.0.0	GI	Intestinal	0.00
Ga436N				0.05
Ga438T	2.0.0	GII	Intestinal	0.01
Ga438N				0.02
Ga440T	3.1.0	GIII	Diffuse	**0.006**
Ga440N				**0.002**

-: low amount or poor quality of RNA.

Table A4: Head & neck carcinoma information and their relative HAGE expression

Sample code	Relative HAGE expression
HN28T	**0.025**
HN28N	**0.005**
HN35T	0.001
HN35N	0.004
HN36T	**0.02**
HN36N	**0.01**
HN38T	**0.002**
HN38N	**0.001**

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Communications resulting from the study

Research article:

M.G. Mathieu, A.J. Knights, G. Pawelec, C.L. Riley, D. Wernet, F.A. Lemonnier, P.T. Straten, L. Mueller, R.C. Rees, S.E.B. McArdle (2007). HAGE, a cancer/testis antigen with potential for melanoma immunotherapy: Identification of several MHC class-I/-II HAGE derived immunogenic peptides. *Cancer Immunology Immunotherapy*, **56**, 1885-1895.

Review:

D.P. Assudani, R.B.V. Horton, **M.G. Mathieu**, S.E.B. McArdle, R.C. Rees (2006). The role of CD4+ T cell help in cancer immunity and the formulation of novel cancer vaccines. *Cancer Immunology Immunotherapy*, **56**, 70-80.

Abstracts:

M.G. Mathieu, P.L.R. Bonner, R.C. Rees, S.E.B. McArdle (2006). Identification of candidate tumour antigen peptides derived from the tumour antigen HAGE for potential use in cancer immunotherapy. Poster presentation at the AACR symposium, Tumour immunology: an integrated perspective, Miami.

M.G. Mathieu, P.L.R. Bonner, R.C. Rees, S.E.B. McArdle (2006). Identification of candidate tumour antigen peptides derived from the tumour antigen HAGE for potential use in cancer immunotherapy. Poster and oral presentations at the Nottingham Trent University, BNS research conference, Nottingham.