Cellular Immune Responses against the Tumour associated Polymorphic Epithelial Mucin MUC1

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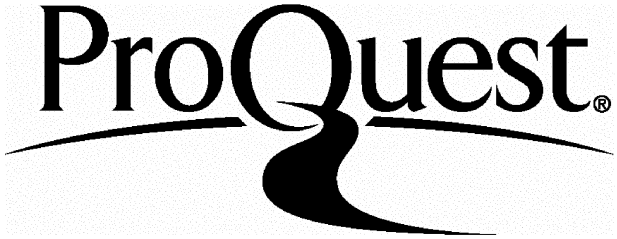

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Statement

All of the experimental work, results and ideas presented in this thesis, except where acknowledged accordingly, are entirely my own.
None of the work contained in this thesis has been submitted previously, either by myself or by any known persons, for examination within the University of London or any other awarding body.

Abstract

The human epithelial mucin MUC1 is expressed on the apical surface of a large number of normal epithelial tissues, including the ducts of the breast, ovary, pancreas, lung and colon. Adenocarcinomata that arise from these tissues can show marked over expression of MUC1, as well as aberrant glycosylation and loss of apical distribution of this mucin. These observations led to the attempt to use MUC1 a potential target for tumour immunotherapy. The work described in this thesis aims to evaluate cellular immune responses directed against the MUC1 antigen, using mouse model systems. The project addresses fundamental questions relating to the feasibility of using MUC1-based antigens for tumour immunotherapy, and has led to the identification of MHC class I-restricted epitopes both in mice and in humans; that may have clinical relevance.

Firstly, the induction of MUC1-specific CTL lines and their characterisation in a C57BL/6 mouse model is described. This includes mapping of the dominant CTL epitope recognised by MUC1-specific CTL and analysis of anti-tumour effects of these CTL *in vivo.* Adoptive transfer of CTL cultured *in vitro* into MUC1 transgenic mice, where human MUC1 is expressed as a self antigen, has allowed evaluation of immunogenicity and tumour protection in a setting in which autoimmunity may occur. The results demonstrate that the host can tolerate effective anti-tumour immune responses to MUC1 without induction of autoimmunity.

The second major part of the work aimed to identify human MHC class I-restricted MUC1-derived epitopes. The merits of the different possible approaches for epitope identification are discussed and the identification of HLA A*0201-restricted epitopes is described. Potential MHC binding peptides were identified by motif scoring. These peptides were then analysed for binding and complex stability with HLA-A*0201. The immunogenicity and physiological relevance of these peptides is shown by induction of CTL responses and tumour protection in a transgenic mouse model expressing HLA A*0201-K^b.

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Abbreviations

aa	amino acid
Ab	antibody
ag	antigen
APC	Antigen presenting cell
bp	base pairs
BSA	bovine serum albumin
C2GnT	core 2 β1,6 GlcNAc transferase
cDNA	coding deoxyribonucleic acid
CMV	human cytomegalovirus
CTL	cytotoxic T-lymphocyte
cu/ml Il-2	Cetus units of recombinant human Il-2
DC	dendritic cell
DMSO	dimethylsulfoxide
DTT	DL-Dithiothreitol
EDTA	ethylene-diamine-tetra-acetic acid
ER	endoplasmic reticulum
FACS	fluorescent activated cell scanning
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
Gal	D-(+)-galactose
Gal T	β1,4 galactosyltransferase
GalNAc	n-acetyl-D-galactosamine
Glc	D-(+)-glucose
GlcNAc	n-acetyl-D-glucosamine
HEPES	n-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonicacid]
HPLC	high performance liquid chromatography
hu	human
i.m.	intra muscular
i.p.	intraperetoneal
IFA	incomplete Freund's Adjuvant
Il-2	recombinant human interleukin- 2

IMDM	Ischove's modified Dubecco's Medium
IPTG	isopropyl β-D-Thiogalactopyranoside
kb	kilobases
LN	lymph node
mAb	monoclonal antibody
MHC	major histocompatability complex
ml	millilitre
MOPS	3-N-morpholino-propanesulfonic acid
MTT	3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
mu	murine
MuLV	murine Leukaemia virus
NeuAc	N-acetyl neuraminic acid
NK	natural killer cell
o/n	over night
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonylfluoride
rER	rough endoplasmic reticulum
RF	rat factor
rpm	revolutions per minute
s.c.	subcutaneous
SA	sialic acid
SP	Signal peptidase
SPP	Signal peptide peptidase
T	transferase
T4 PNK	T4 polynucleotide kinase
TCR	T-cell receptor
TEMED	N,N,N',N'-tetramethylethylene diamine
TFA	trifluoroacetic acid
Th	helper T Lymphocyte
TNF	tumour necrosis factor
TR	trandem repeat
VNTR	variable number tandem repeat region

VV-MUC1	recombinant Vaccinia virus encoding MUC1
WeHi Assay	TNFα release assay based on WeHi Cells 166
WeHi cells	WEHI-164 clone 13, mouse fibrosarcoma sensitive to TNFα

Chapter 1: General Introduction

Adenocarcinomata of the breast are the commonest cause of cancer death in women. Current treatments include surgery, chemo-therapy and radio-therapy; all with quite severe side effects and limited success, highlighting the need for additional therapeutic modalities. The biology of ductal epithelial cells, from which these tumours most commonly arise, has been studied, with emphasis on molecular events that take place during the malignant change. Such investigation led to the identification of the MUC1 gene that encodes a large membrane associated glycoprotein, previously termed the polymorphic epithelial mucin, but now known as MUC1 mucin. This mucin has been identified as a possible tumour associated antigen and MUC1 based immunogens are being evaluated for immunotherapy of cancer. The aim of this introduction is to provide a brief overview of the field of tumour immunology, with specific regard to how the immune system reacts to, and interacts with tumours. This interaction can range from the induction of tumours by the immune system through supply of growth factors (Pekarek et al., 1995), support of tumour growth by influencing angiogenesis, specific recognition of tumours and immune surveillance, to tumour rejection (Burnet, 1970). See review by (Sogn, 1998). This interaction will be considered in the context of how the immune system can be harnessed to treat tumours without the induction of pathological autoimmunity with MUC1 as the target tumour antigen.

The basic principles that current tumour immunology is based upon are:

- The immune system is, in the normal course of events, rarely a significant barrier to tumour growth, although lymphocytes play both a positive and a negative role in tumour growth and development (Sogn, 1998).
- Secondly, many tumours are antigenic and can be recognised by the immune system, but are not necessarily immunogneic.
- And finally, manipulation of the immune system can lead to complete tumour eradication.

Of these statements the first two are well established, but the last has only been proven in a few cases.

A more detailed discussion of these ideas will follow, after a description of some basic immunological concepts such as the antigen processing

pathways and control of CTL priming. These are relevant to the understanding of the work presented here and play a role in our current understanding on how immune responses are induced and regulated in a normal host. This is followed by an overview of previous work relating to MUC1 as a tumour antigen. The aims of this thesis will be derived from the conclusions of this chapter

1.1 Tumours and the immune system

The immune system has evolved to protect the host against foreign invaders, such as bacteria, parasites, and (tumour) viruses. For this, the immune system has two mutually interactive systems available: the evolutionary much older innate arm, and the adaptive or acquired arm. The innate immune response usually acts as a first line of defence through rapid pattern recognition of, for example, carbohydrate structures on invading pathogens or infected cells. The cellular (macrophages, natural killer cells (NK)) and soluble (complement) components of innate arm discriminate against potentially noxious substances, causing the eradication of pathogens either directly or via activation of cellular functions. Many parts of the innate immune system have been conserved across species barriers, highlighting their crucial importance to the survival of the host. Moreover, innate immunity not only activates but also shapes the quality of adaptive immune response by providing the inflammatory signals that influence lymphocyte homing, and the mobilisation and activation of dendritic cells (DC).

1.1.1 Adaptive immunity

Adaptive immunity has two advantages to innate immunity: antigen specificity and long-term memory. These characteristics make it an attractive therapeutic tool. Acquired immunity comprises humoral and cellular immune responses. Pathogens, or fragments thereof, are presented on antigen-presenting cells (APC) for recognition by antigen-specific receptors on lymphocytes. While the humoral response is able to attack native, usually extracellular antigen, the cellular arm can eliminate intra cellular pathogens or transformed cells.

B cells are activated through their B cell receptor to secrete antibodies that recognise specific conformations of native antigen. Depending on the

antibody isotype, different effector functions can be mobilised. These include viral neutralisation, bacterial agglutination, complement fixation and recruitment of effector cells expressing Fc receptors. These can result either in antibody-dependent cell-mediated cytotoxicity (ADCC) of target cells or in neutralisation of viruses and bacteria by tagging of the pathogens to be cleared from the host.

T lymphocytes, on the other hand, mediate the detection and subsequent elimination of intracellular pathogens or transformed cells. Antigen recognition mediated by T cell receptors (TCR) occurs in the context of polymorphic major histocompatibility complex (MHC) molecules at the cell surface (human leukocyte antigen (HLA) in man; H-2 in mice). The understanding of MHC-restriction (Zinkernagel and Doherty, 1974) acquired a structural basis when the crystal structures of a MHC class I molecule and TCR were solved (Bjorkman et al., 1987; Garcia et al., 1996). Since then, the structures of a number of MHC peptide TCR complexes (Hausmann et al., 1999; Jardetzky, 1997) as well as glycosylated peptides bound to MHC class I (Glithero et al., 1999; Speir et al., 1999) have been solved. Antigens in the form of immunogenic peptide are accommodated in an antigen-presenting groove on the surface of MHC molecules (Townsend and Bodmer, 1989). Interactions between a restricted number of peptide anchor side chains and pockets in the antigen-binding groove mediate allele-specific peptide binding (Madden et al., 1993; Saper et al., 1991). This holds true for both types of MHC molecules, class I and class II, which adopt strikingly similar three dimensional configurations (Brown et al., 1993). Irrespective of these structural similarities, naturally presented peptides differ substantially between MHC class I and class II molecules.

It has been shown that peptides complexed with MHC class I have a restricted length of 8-11 amino acids (aa) in length (Bjorkman and Parham, 1990; Elliott et al., 1991) and contain an allele-specific "motif" sequence of usually two conserved anchor residues (Falk et al., 1991; Rötschke et al., 1991). Most MHC class I-bound peptides result from proteasomal degradation of proteins present in the cytosol and subsequent transport to the endoplasmic reticulum (ER) by the transporter associated with antigenic peptides (TAP). (see Figure 1.1, left panel). Peptides associate with nascent class I heavy (HC) and light chain (β_2-microglobulin, β_2m) in the ER, which may be a chaperone mediated process (Solheim et al., 1997; Suto and Srivastava, 1995). The

trimeric complex travels to the cell surface (Pamer and Cresswell, 1998) while empty or unsuccessfully folded MHC class I molecules are retained in the ER (Cauley, 1995; Lewis and Elliott, 1998; Schoenhals et al., 1999).

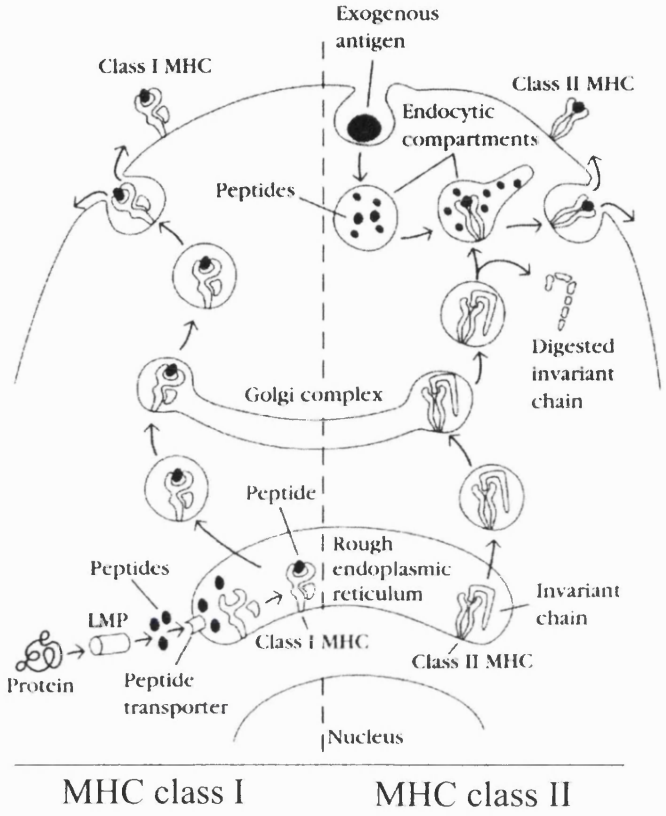


Figure 1.1: Classical MHC processing pathways.

MHC class I presented peptides are generally thought to derived from cytosolic protein that is degraded by the proteasome (LMP) in a ubiquitin dependent manner to peptides that can be transported in to the endoplamsic reticulum by TAP from where the peptide MHC complexes travel to the cell surface via the Golgi complex. Peptides presented by MHC class II molecules are usually derived from exogenous antigen, that is broken down in lysosomal compartments and loaded onto MHC class II molecules replacing the CILP fragments of the invariant chain. Figure adapted from (Kuby, 1994)

In contrast, peptides complexed with class II molecules are longer and more variable in size than class I-presented peptides, and have less well-defined anchor motifs (Chicz et al., 1993; Rudensky et al., 1991; Unanue, 1992). In general, MHC class II binding peptides are derived from extracellular proteins that are internalised via the endocytic pathway and are degraded by endosomal and lysosomal proteases. The newly synthesised MHC class II molecules are chaperoned by the invariant chain (Ii) Once the Ii has been

removed, the resulting peptide fragments bind to MHC class II and are transported to the cell surface (see Figure 1.1, right panel) (for reviews see Chapman, 1998 and Pieters, 1997).

However, there are important exceptions to these rules, dendritic cells can present peptides that are derived from exogenous proteins via the MHC class I pathway. In fact, this may be one of the major pathways for CTL priming (see below on cross-priming) (Bevan, 1976; Gordon et al., 1976; Shen et al., 1997) reviewed in (Carbone et al., 1998).

In general, MHC class I molecules present antigenic peptides to $CD8^+$ cytotoxic T lymphocytes (CTL), whereas $CD4^+$ T-helper (Th) cells recognise peptide epitopes in the context of MHC class II molecules. MHC class II is mainly expressed on cells of the immune system with a specialised antigen presenting function, such as DC, monocytes, macrophages, B cells, and thymic epithelial cells but also on some tumour cells. Upon triggering, $CD4^+$ T cells can direct the activation of B cells, macrophages, and DCs. Since virtually all nucleated cells express MHC class I molecules are on their cell surface, CTL can potentially screen most cells of the body for antigenic peptides that may be presented as a consequence of viral infection or malignant transformation. Therefore, the $CD8^+$ CTL have been assumed to be a major effector subset of specific T cells.

If one aims to manipulate the immune system to achieve complete tumour eradication, then an understanding of the mechanisms involved in CTL induction, regulation of the immune response and formation of CTL memory are of crucial importance.

1.1.2 CTL priming: Is the APC in control?

Naive T cells need to be primed in an antigen-specific manner to become fully functional effector CTL *in vivo*. In the current model of CTL priming, two signals are required. The first is antigen-specific triggering of the TCR by peptide-MHC complexes and the second involves co-stimulatory molecules binding their respective ligands, such as the CD80/86-CD28 interactions (Allison, 1994; Jenkins and Johnson, 1993; Schwartz, 1992). In the absence of appropriate co-stimulatory signals, engagement of the TCR typically leads to T cell unresponsiveness, either through anergy or apoptosis (Allison and

Krummel, 1995; Gimmi et al., 1993; Radvanyi et al., 1996). Once a CTL is primed and activated, it can perform its effector function for a limited number of cytotoxic cycles, even in the absence of costimulation, before it needs to engage its MHC-peptide ligand in the context of costimulation again (Matzinger, 1994).

Tumour cells expressing MHC class I molecules can, in principle, be targets for lysis by CTL, without the expression of co-stimulatory molecules. It had also been assumed that the tumour cell itself could induce a tumour specific response by so called direct priming of the T cell (Figure 1.2). This prompted immunisation approaches in which tumour cells were transfected with co-stimulatory molecules, such as CD80/86, to improve ability to prime CTL (Chen et al., 1992; Smith et al., 1999; Townsend and Alison, 1993). Immunisation with CD80-expressing tumour cells were supposed to induce tumour specifics CTL that would then cross-react with the untransfected tumour cells. Other approaches have been taken to favourably influence the tumour microenvironment for CTL priming by delivery of cytokines to the tumour site. Several such approaches are being tested in clinical trials. Whilst some positive results with this approach have been obtained, careful examination of the mechanisms involved in CTL priming in mice has led to focus on the role of professional APC in CTL priming.

In most physiological situations tumour cells do not directly prime CTL. In mouse models, allogeneic or MHC class I negative syngeneic tumour cells were effective in inducing CTL-mediated anti-tumour immunity in the context of the hosts own MHC class I molecules (Huang et al., 1996; Huang et al., 1994; Toes et al., 1996). This process required a professional APC derived from the host bone marrow to present antigenic determinants from the injected cells. This form of exogenous antigen presentation has been referred to as "cross-presentation" and appears to represent a general mechanism implicated in immunity to minor histocompatibility antigens, tumour antigens, viral antigens, and self-antigens (Figure 1.2) (Bennett et al., 1997; Bevan, 1976; Huang et al., 1994; Toes et al., 1996).

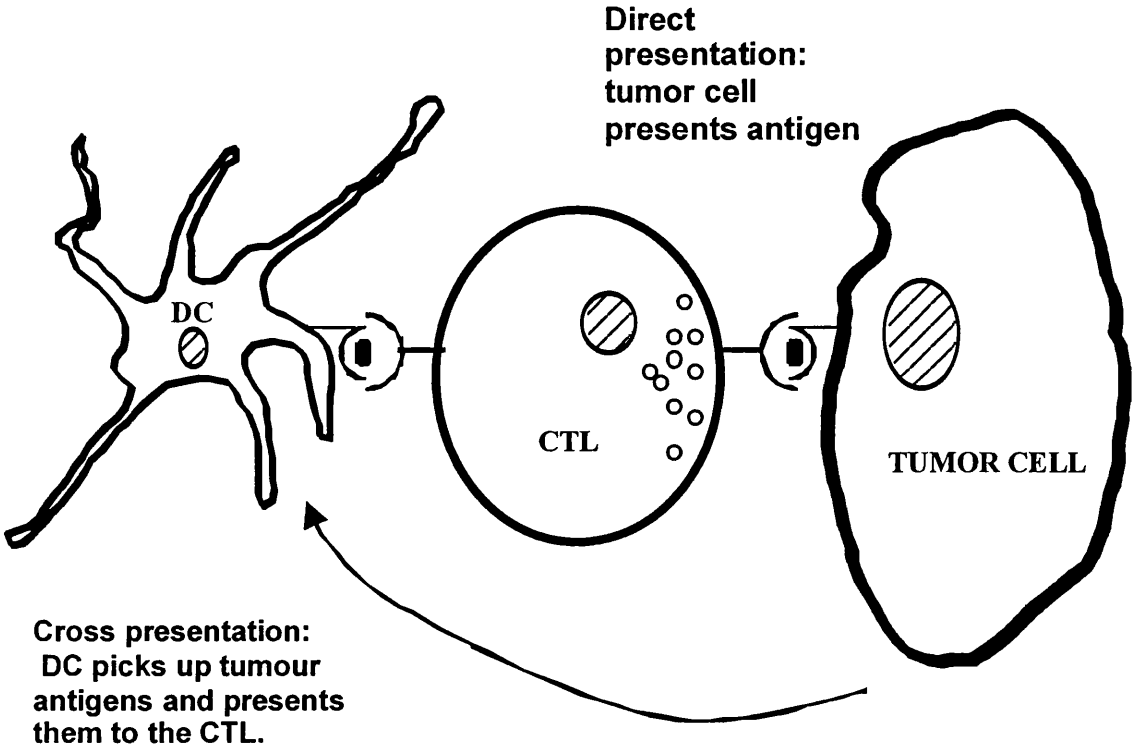


Figure 1.2: Cross priming versus direct priming of CTL.

In the classical model of direct priming the tumour cell presents its antigenic peptide via MHC class I directly to the CTL for priming while antigenic material taken up by DCs and presented to CTL in the draining lymph node is referred to as cross-presentation.

The current model of CTL induction places the APC in a central position. Naïve T-cells are not expected to enter tumour tissues efficiently, as these are non-lymphoid areas of the body, but rather recirculate between the secondary lymphoid organs, such as the spleen and lymph nodes (LN), via the blood and efferent lymphatics (Butcher and Picker, 1996; Mackay, 1991; Sallusto et al., 1998). To penetrate peripheral tissues harbouring malignant or infected cells, CTL must first be primed which usually takes place in a lymph node that drains the peripheral organ. DCs are the likely host bone marrow-derived APC responsible for cross-priming of T-cells. DCs have been demonstrated to prime $CD4^+$ helper and $CD8^+$ killer T cells *in vivo* and have a great migratory capacity (reviewed by Cella et al., 1997; Steinman, 1991).

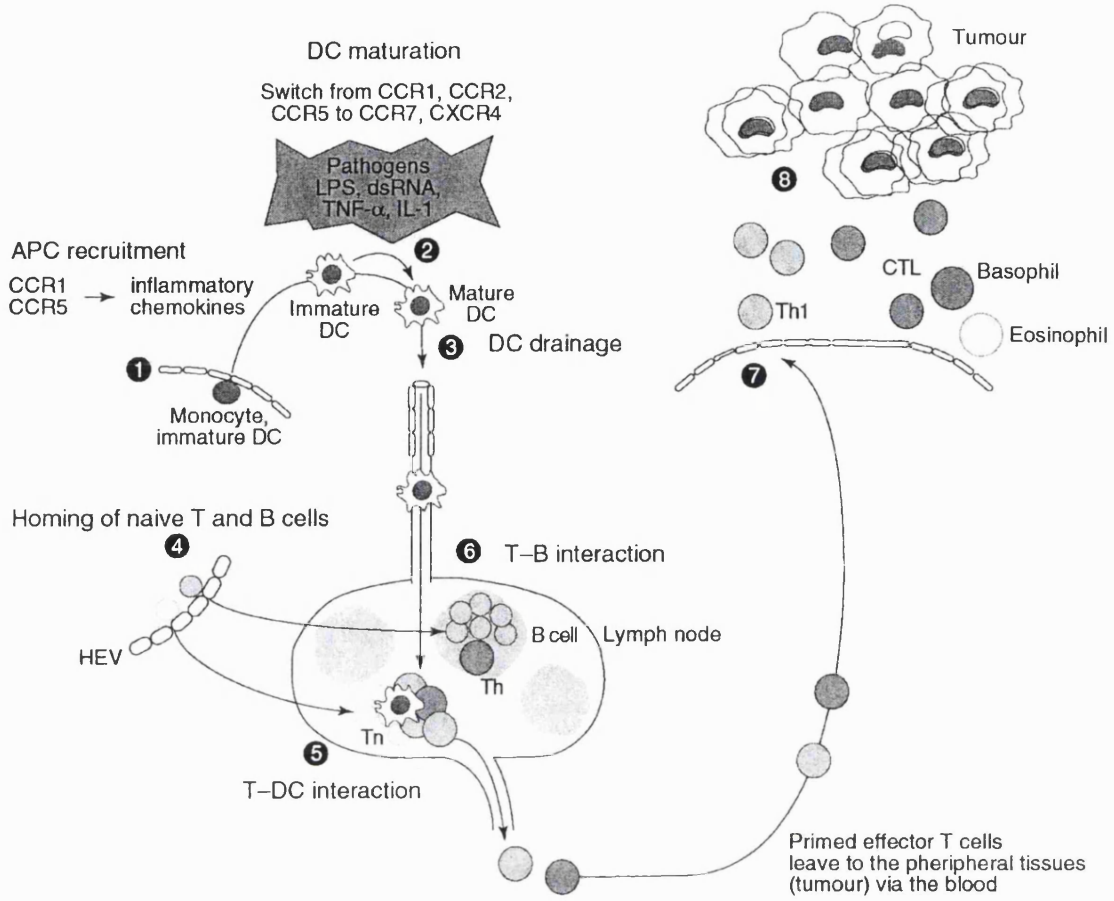


Figure 1.3: Migratory cell involved in anti tumour immune responses.

Naïve T cells express chemokine receptors that allow them to localise to secondary lymphoid organs (4,5) where they can interact with dendritic cells (DC). Immature DC home to sites of inflammation (1,2) where they take up antigen for cross presentation. Maturation signals ("danger") in the form of naked plasmid DNA, double stranded RNA (dsRNA), LPS but also TNFα and IL-1 lead to maturation. Upon maturation, the DC loses its antigen scavenging capabilities, and switches chemokine receptors that allow it to migrate to the draining lymph nodes (6). DC activated by CD40 signalling from T-helper cells are able to prime tumour-specific CTL. Primed CTL express chemokine receptors that allow circulation through peripheral tissues of the body and to interact with tumour in the periphery (7). CTL then return to local LN for restimulation. Figure adapted from (Sallusto et al., 1998)

Cross-presentation by DC can provide a mechanistic framework, explaining the apparent dichotomy between the sites where antigen is located (peripheral tissues) such as epithelia and where immune responses are predominantly induced (lymph nodes and spleen). For instance, Langerhans cells, the immature DCs in the skin, can capture antigen locally. Inflammatory

signals from the innate immune system triggered by trauma, result in DC maturation and migration to the draining lymph nodes (Figure 1.3. 1-5). Upon maturation, DC acquire the ability to effectively prime T cells by expression of high levels of MHC class I and II molecules, co-stimulatory molecules, and the production of cytokines, such as IL-12 . Primed CTL are able to home to the peripheral tissues as effector or memory cells to interact with infected or possibly malignant target cells (Figure 1.3. 7,8). The danger model of immunity (Fenton and Longo, 1997; Matzinger, 1994) gives an explanation for the requirement for an inflammatory milieu to induce effective anti-tumour immunity. This involves signals from the innate immune system to initiate the maturation of DC and their migration to the lymph nodes where they can induce adaptive immunity mediated by antigen-specific T and B cells.

1.1.3 Control of DC activation

DCs that have scavenged antigen in the peripheral tissues migrate to the lymph node (LN). Upon arrival in the LN they can induce effective CTL responses, but have been shown to require "help" from $CD4^+$ Th lymphocytes. Dissection of the cellular interactions involved in the induction of CTL revealed that a tricellular complex of the T-helper cell and CTL both recognising antigen on the same APC was required (Bennett et al., 1997; Husmann and Bevan, 1988; Keene and Forman, 1982; Mitchison and O'Malley, 1987). This was traditionally explained by a proximity requirement at the APC surface for the efficient delivery of soluble factors such as IL-2 by Th to CTL (Cassel and Forman, 1988; Keene and Forman, 1982; Mitchison and O'Malley, 1987). However, the simultaneous interaction of three rare migratory cells at the same time i.e. a DC presenting antigen and CTL and T-helper both recognising specific antigen presented on the DC is an unlikely scenario.

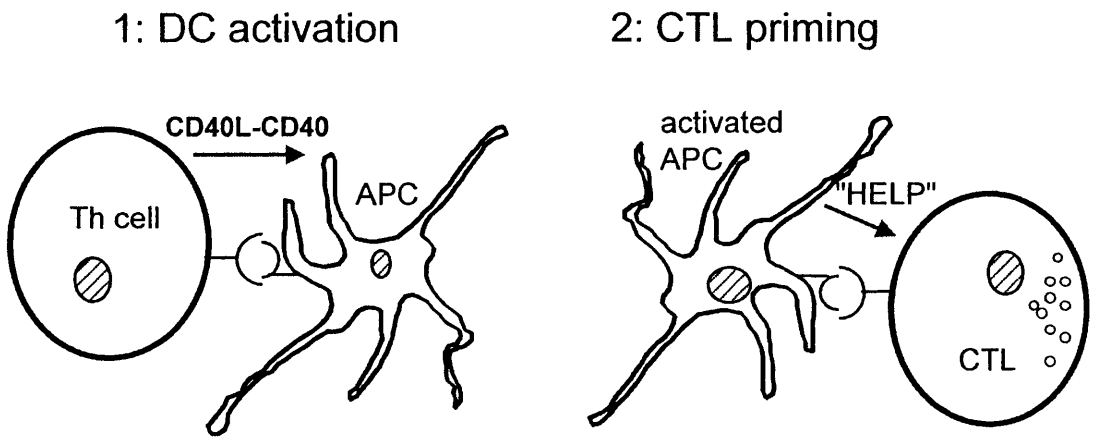


Figure 1.4:Two stage model of CTL priming

1) cognate interaction between T-helper and APC and CD40-CD40L signalling converts the APC into a "CTL-priming" state. 2) The activated APC primes the CTL.

An alternative explanation is that a cognate interaction between T-helper and APC takes place that converts the APC into a "CTL-priming" state. Therefore, instead of two simultaneous T cell-DC interactions, there are two sequential ones: between Th and the DC to activate the DC, and then between the activated DC and a CTL. Recently, it was shown that T help for CTL priming is mediated through CD40-CD40 ligand (CD154) interactions (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). A Th cell recognising antigen on the DC up-regulate expression of CD40L on the cell surface and subsequently activate the DC through interaction with CD40. The DC then expresses co-stimulatory molecules and cytokines to allow for priming of the CTL. (Figure 1.4)

1.2 Tumours and the immune system

Having discussed how the immune system handles antigen and can recognise tumours, the question whether the immune system actually protects against tumour development needs to be addressed. In 1970 Burnet proposed the concept of "immunological surveillance" (Burnet, 1970), implying that the immune system can eradicate malignant cells. A reasonable explanation can be given for virally-derived tumours, as these may evoke a virus-specific immune response. However, this concept is experimentally difficult to prove, but some

evidence exists that immuno-suppressed patients are more susceptible to virally-induced tumours including lymphomas (Klein, 1991) and HPV associated tumours (Petry et al., 1994; Shamanin et al., 1994). Surveillance also seems to play a role in non-melanoma skin cancers (Birkeland, 1997). In addition there is also some data on other solid tumours (Birkeland et al., 1995) and statistical evidence for selection against lung cancers bearing p53 mutations, that are predicted to be strongly immunogenic (Wiedenfeld et al., 1994). By inference we can propose that tumours that do arise in immuno-competent patients have successfully avoided immune detection. Several different mechanisms can account for this: Tumours can evade a CTL response by down regulating the expression of tumour antigens (Uyttenhove et al., 1983) or parts of the antigen processing machinery, including MHC (Connor and Stern, 1990; Cromme et al., 1993; Garrido et al., 1997) and TAP (Cromme et al., 1994).

TAP down-regulation was not only observed in tumour cell lines that were cultured *in vitro* but also in patient tissues after surgical excision. Interestingly, great variations between different tumour types and stages were observed and metastatic lesions were affected to a larger extend than primary tumours (Seliger et al., 1997). In breast cancer 33% of the primary lesions and 44% of metastatic lesions showed down-regulation of MHC class I antigens and TAP (Kaklamanis et al., 1995).

A molecular mechanism for loss of TAP1 expression has only been identified in a small cell lung cancer cells line, in which a point mutation affected an ATP binding site (Chen et al., 1996). In many cases other regulatory mechanisms may play a role, including secretion of IL-10 leading to down regulation of MHC class I and TAP expression by the tumour (Petersson et al., 1998).

Down-regulation of TAP leads to a defect in peptide transport into the ER and results in reduced levels of MHC class I molecules on the cell surface. Thus, TAP abnormalities provide malignant cells with a mechanism to escape from CTL mediated cytotoxicity. However, a number of TAP-independent, signal sequence derived epitopes have been described, including one HLA*0201-restricted tyrosinase epitope that maps to the putative tyrosinase leader sequence and may mediate TAP independent lysis of melanoma cells (Wolfel et al., 1994).

TAP-deficient tumour cells exhibiting MHC down-regulation may provide targets for immune cells other than $CD8^+$ CTLs. TAP abnormalities can enhance the susceptibility of target cells to lysis by NK cells (Salcedo et al., 1994). Also TAP-independent antigen presentation, including MHC class II restricted cytotoxicity, CD1 molecules presenting tumour derived non-peptide antigens, including lipid and glycolipid antigens and minor histo-compatability antigens, such as TL, may offer alternative routes.

However, the effect of tumours on the immune system is further reaching than just the alterations at the target-cell level. Tumours can actively suppress the immune system by secreting cytokines such as TGFβ (Schoenberger et al.; Young et al., 1996) or by the expression of Fas ligand killing Fas-positive T cells (Villunger et al., 1997). In the early stages of tumour development these effects appear to be localised, but there is no doubt that over time tumours can induce a more extensive generalised immune suppression in patients (reviewed in Finke et al., 1999).

Effective immunotherapy hinges on two areas. Firstly, the induction of effective tumour-specific immune responses and secondly the identification of tumour-specific antigens, against which these responses should be targeted. Successful immunisation protocols will have to be able to mobilise professional APC, once it has taken up the antigen for processing. This will require some form of an inflammatory response and the choice of adjuvant or viral vector will be important. Secondly, there is need to activate the APC via $CD4^+$ T-helper cells or by artificial means similar to CD40 triggering (Diehl et al., 1999). Thirdly, the immune response will need to induce CTL memory. Therefore, before human cancers can be successfully treated by immunotherapy, fundamental questions need to be answered which are important in tumour rejection.

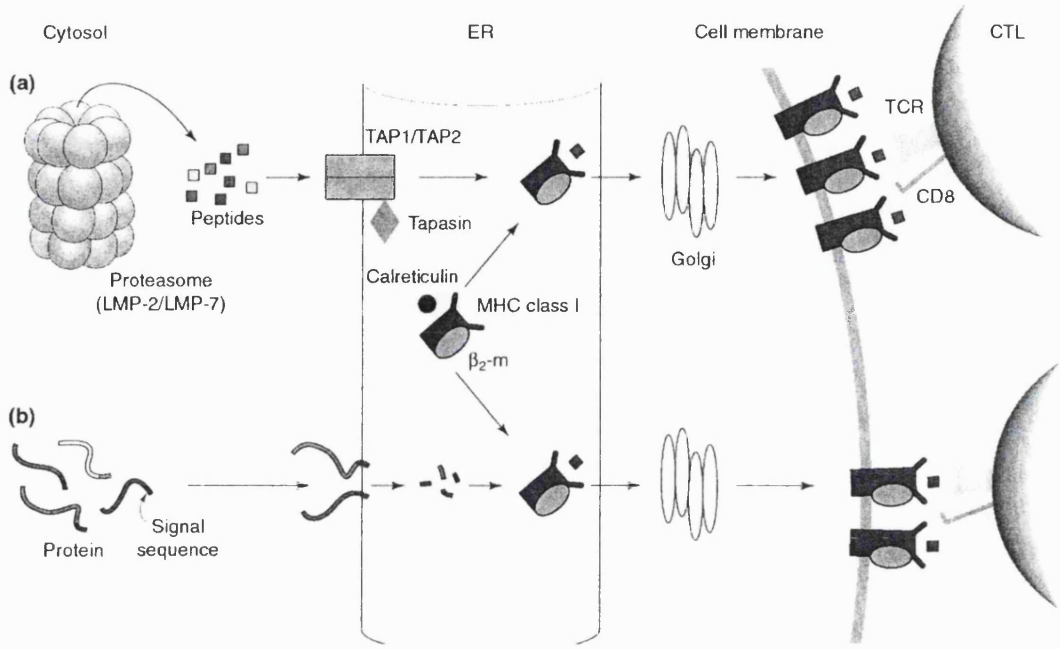


Figure 1.5: Immune escape of tumours.

Tumours can loose TAP expression thus preventing antigenic peptide presentation by the MHC class I pathway (a). However, signal sequence derived epitopes can be presented in a TAP independent manner and may still function as tumour rejection-antigens (b).

Many tumour antigens have been identified by defining the specificity of tumour infiltrating lymphocytes (TIL). The obvious candidates are virally derived tumour associated antigens, for example E6 and E7 proteins from human papiloma virus HPV 16 in cervical carcinoma (Feltkamp et al., 1993; Ressing et al., 1995) and mutated genes that are involved in malignant change such as p53 mutants (Hollstein et al., 1991). However, surprisingly most of the antigens identified in human tumours are non-mutated differentiation antigens that are inappropriately expressed or over-expressed in tumours. Examples include lineage specific *MART* (Kawakami et al., 1994; Rivoltini et al., 1995; Sensi et al., 1995; van Elsas et al., 1996), *tyrosinase* (Brichard et al., 1993) and *gp100* (Bakker et al., 1994; Bakker et al., 1995; Salgaller et al., 1995; Zhai et al., 1996) genes in melanoma.

For MUC1, the reverse approach was taken: MUC1 was postulated as a tumour antigen after its gene had been cloned and its basic biology studied. The following section will elaborate how MUC1 may be able to confer tumour

specificity as a target antigen, and the feasibility of this approach will be addressed as part of the thesis.

1.3 MUC1 protein

MUC1 was the first mucin to be cloned (Gendler et al., 1990) after antibodies were raised against MUC1 secreted as part of human milk fat globules (Ormerod et al., 1984). MUC1 is normally expressed at the apical surface of glandular epithelial cells, including the breast from where it is shed during lactation (Pemberton et al., 1996). Mucins generally are heavily *O*-glycosylated proteins that have a mechanical or protective function. MUC1 has an integral membrane structure and the transmembrane and cytoplasmic tail show a high degree of homology between species suggesting that it may have a basic signal transduction function.

As shown from cloning studies, the large extracellular domain of the core protein of MUC1 is mainly made up of exact tandem repeats (TR) 20 amino acids in length, containing a large number of prolines, as well as several threonines and serines that function as *O*-glycosylation sites (Gendler et al., 1990). The number of tandem repeats is subject to allelic polymorphism, with a range of variation between 20 to 125 repeats. Consequently, the length of the extracellular domain can vary between ~600 and 2200 amino acids (Swallow et al., 1987; Swallow et al., 1987). Each tandem repeat has five potential *O*-glycosylation sites, which may be differentially utilised in different tissues and tumours (see below). The glycosylation of the serines and threonines, and the large number of helix-breaking prolines of the VNTR, are thought to give MUC1 a long and extended structure. An *O*-glycosylated VNTR peptide of 28 amino acids is thought to be 7nm in length. The extracellular domain of MUC1 may thus extend 150-500 nm above the cell membrane.

1.3.1 Synthesis and transport of MUC1 to the cell surface

Several targeting domains have been identified that play a role in controlling targeting of MUC1 to the apical surface in polarised cells (Pemberton et al., 1996). MUC1 has a putative signal sequence that directs its translocation into the ER. Two different splice variants of MUC1 were identified that differ in

their signal sequences and in the extreme amino-terminal parts of the mature proteins (Ligtenberg et al., 1990). Once MUC1 is translocated into the ER, it is cleaved between 71 and 53 amino acids upstream of the transmembrane domain. Both cleavage products remain associated (Ligtenberg et al., 1992). MUC1 is further processed by the *O*-glycosylation machinery in the cis and trans Golgi apparatus and then travels to the cell surface.

1.3.2 Signal sequence function and further processing

A detailed description of the cellular machinery that is involved in signal peptide recognition and translocation into the ER is crucial to understand some of the data presented in this thesis, and will be discussed here. Transmembrane proteins like MUC1, which normally reside at least partially in a hydrophilic environment, attain a conformation that normally prohibits their transit through hydrophobic membranes such as that of the rER. The proteins must therefore be prevented from folding into a translocation- incompetent conformation before it is translocated into the ER. It is the role of the signal peptide recognition particle (SRP) to perform this function. The SRP mediates the attachment of ribosomes translating mRNAs for such proteins to the rough ER (rER) membrane before the majority of the molecule has been synthesised. For this, the hydrophobic h-region of the signal peptide is important, as it is recognised and bound by SRP as soon as it emerges from the ribosome. The length of the signal sequence and an additional 35-40 amino acids are believed to transverse the ribosome before SRP can bind to the signal sequence (Lutcke, 1995). Upon binding to the signal sequence, SRP inhibits the further elongation of the nascent polypeptide chain until its complex with ribosome and nascent polypeptide chain binds to a SRP-receptor that is anchored on the rER-membrane. As a consequence of a GTP-dependent interaction with the SRP receptor in the rER membrane, SRP releases the signal sequence into the translocation apparatus and detaches from the ribosome. The elongation proceeds, and the newly synthesised polypeptide is co-translationally translocated across the ER membrane. It is important to note that most signal sequences that are cleaved from the native protein are inserted into the ER membrane in a loop-like fashion, leaving the N-terminal end on the cytoplasmic side while the C-terminal part is translocated into the ER lumen. It is this

luminal, or trans side, of the membrane where the signal peptide cleavage occurs. Signal sequences that have been cleaved from precursor proteins are further processed by a signal peptide peptidases (SPP), most likely to release signal sequences from the ER membranesince signal peptides not cleaved by SPP would otherwise be 'stuck' in the membrane, held in place by polar C- and N-termini that cannot transverse the membrane. (see Figure 5.21) MUC1 is thought to translocated into the ER lumen by this mechanism before it passes through the Golgi apparatus where it is *O*-glycosylated on its way to the apical cell membrane.

1.3.3 Tissue distribution and possible function of MUC1

MUC1 is expressed on the apical surface of most glandular epithelial tissues like the breast, pancreas, ovary, colon and salivary gland. To further assess the role of MUC1, a Muc1-/- mouse has been useful (Spicer et al., 1995) At first these mice appeared to develop normally and were healthy and fertile. During organogenesis in the mouse, initiation of Muc1 (NB: human *MUC1* and murine *Muc1*) expression coincides with lumen formation in various organs, but a functional role is not clear (Braga et al., 1992) since Muc1-deficient mice (Muc1 -/-) have no abnormal lumen formation (Spicer et al., 1995).. Only, the growth rate of primary breast tumours induced by polyoma middle T antigen was found to be significantly slower in Muc1-/- mice. This suggests that MUC1 may play a role in the progression of mammary carcinoma. Recently, however it became apparent that the MUC1-/- mice may also have an immune deficiency in that T cells derived from these mice were not able to proliferate to stimulation with anti CD3 mAb (S. Gendler, personal communication) and MUC1-/- were found to be predisposed to developing bacterial conjunctivitis when compared to wild-type animals with an intact Muc1 gene (Kardon et al., 1999).

MUC1 expression is increased in the adult breast during pregnancy and lactation, at which time it is shed from the cell surface into the milk. Recently, it has been shown that MUC1 is also expressed on activated T-cells after stimulation with PHA (Agrawal et al., 1998) or anti-CD3 mAb and on T-cells from synovial infiltrates of rheumatoid arthritis patients (I. Correa, personal communication), but is absent in resting T-cells. The role of MUC1 expressed on these immune cells awaits clarification.

1.4 MUC1 in malignancy

1.4.1 Over expression of MUC1

In breast, ovarian and pancreatic cancers MUC1 expression is upregulated (Chen et al., 1995; Ho et al., 1995). Certainly high MUC1 expression levels have been found in epithelial tumours at the protein and mRNA level. Furthermore, breast cancer cell lines express higher MUC1 levels than normal luminal breast cells immortalised with the SV40 large-T antigen (Bartek et al., 1991). However, data on MUC1 expression levels in MUC1 expressing tumours is difficult. Most MUC1 mRNA quantification has been carried out by blotting of steady-state RNA followed by hybridisation with labelled MUC1 probes. The increase in MUC1 expression has then been calculated based on equal amounts of total RNA per sample. Although these studies have all shown an increase in MUC1 expression this may only reflect the increased number of epithelial (tumour) cells contributing to the RNA sample over other cell types that do not express MUC1. In other words the increase in the proportion of MUC1 expressing cells in the sample, rather than an increase in MUC1 expression levels per cell may account for the RNA data. There is a need to quantify MUC1 expression levels that takes the altered amounts of MUC1 expressing cells (luminal epithelial or tumour cells) between normal and tumour samples into account.

Overexpression of MUC1 has been thought to give malignant cells an advantage in several ways. Firstly, over expression of MUC1 decreases E-cadherin-mediated cell-cell and integrin-mediated cell-matrix interactions, especially when its normal apical distribution is lost. As a result, the overexpression of MUC1 may increase cellular metastatic potential (Nakamori et al., 1994). It has also been shown that transfection of MUC1 into tumour cells affects efficient lysis of these cells by allospecific CTL (van de Wiel-van Kemenade et al., 1993).

1.4.2 Aberrant glycosylation of *O*-linked glycoproteins in breast cancer

MUC1 expressed by breast carcinomas contains shorter sugar-side chains than the mucin expressed by normal mammary epithelial cells (Lloyd et al.,

1996), resulting in the exposure of normally cryptic peptide epitopes, such as that recognised by the monoclonal antibody SM3 (Burchell et al., 1987; Girling et al., 1989), and the creation of novel carbohydrate epitopes. This change in glycans attached to MUC1 has been correlated with changes in the expression of key glycosyltransferases (Brockhausen et al., 1995; Whitehouse et al., 1997). O-glycosylation is initiated in the Golgi by the addition of N-acetylgalactosamine (GalNAc) forming Tn antigen and sugars are then added individually and sequentially. In normal mammary epithelium, galactose is added to form the core 1 structure Galß1-3GalNAc or T antigen. This structure can then be elongated by the enzyme ß1-6-N-acetylglucosamine transferase (ß1-6 GlcNAc-T) adding N-acetylglucosamine (GlcNAc) to the GalNAc to form the core 2 structure. It can then be further extended with polylactosamine side chains. See Figure 1.6 left half. In adenocarcinomata aberrant truncated side chains are formed on the MUC1 VNTR. The enzyme α2-3-sialyl transferase (α2-3SA-T) competes with ß1-6 GlcNAc-T for the core 1 structure as substrate (Whitehouse et al., 1997). The chains are then terminated by the addition of sialic acid to the Gal of core 1 to form Sialyl T rather than extending by addition of GlcNAc to form core 2. (SeeFigure 1.6)

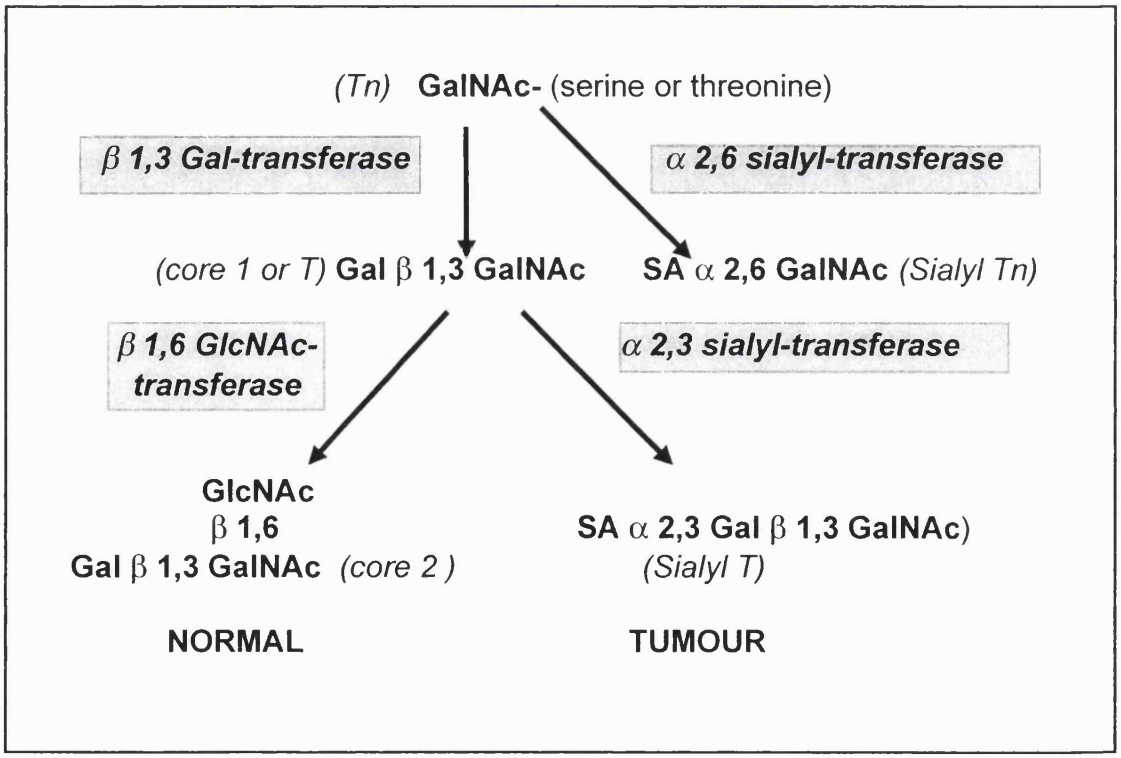


Figure 1.6: Glycosylation pathways in normal and tumour cells

The increase in sialyltransferase activity, which terminates chain extension by adding sialic acid to core 1, was first seen in breast cancer cell lines (Brockhausen et al., 1995) and this has been confirmed in primary breast carcinomas. A corresponding decrease in the activity of the enzyme (C2GnT) which initiates chain extension, by catalysing the synthesis of core 2 from core 1 has also been noted in the cell lines. In addition breast tumours also express Sialyl Tn as well as unsialylated structures such as GalNac (Tn) and core 1.

1.5 MUC1 as a target for immunotherapy

Suggestions that MUC1 might be useful as a tumour-associated antigen came from the spectrum of reactivity of mouse monoclonal antibodies, directed against the tandem repeat structure of MUC1(reviewed in (Price et al., 1998)). One such antibody, SM3, reacts with MUC1 expressed by breast carcinomata but shows little or no reactivity with normal resting or lactating breast. This is due to masking of the SM3 epitopes in normal breast epithelial cells by carbohydrate side chains (Burchell and Taylor Papadimitriou, 1993). SM3 binding to a naked or glycopeptide has very recently been crystallised (Dokurno et al., 1998) and yielded insight into the structural interaction of the Ab with its epitope, confirming these earlier assumptions.

1.5.1 HLA-unrestricted human responses against the MUC1 VNTR

As a result of the Ab studies, the tandem repeat has been the focus of several groups investigating cellular immune responses to MUC1. The rationale for this was that the repetitive structure of the VNTR would provide high levels of protein entering antigen processing and presentation pathway for recognition by CTL. It was also suggested that the altered glycosylation pattern of the VNTR in cancer cells could lead to tumour specific T-cell epitopes being presented. However, naturally processed and presented MHC-restricted glycopeptide epitopes from MUC1 have not been described so far and little is known on how glycosylation may affect antigen processing.

The notion that glycosylation could play a role was supported by the finding that MHC-unrestricted CTL responses lysed aberrantly glycosylated MUC1 expressing target cells (Jerome et al., 1991). The authors proposed a model in which peptide epitopes, within the tandem repeat were exposed by the

shortened oligosaccaride side chains. These epitopes are then thought to interact directly with the TCR of the CTL. The highly repetitive nature of the mucin supposedly allows cross-linking of the T-cell receptor on mucin-specific T-cells and therefore accounts for the lack of MHC restriction seen in this system (Finn et al., 1995). They further suggest that this mucin core epitope recognised on tumour cells is not expressed on normal epithelial cells in a manner that can be recognised by tumour-reactive CTLs. However, attempts by several groups, including the original authors, to clone the effector cells to further characterise the response have not met with success. Furthermore, such a MHC-unrestricted response has not been found in mouse models.

1.5.2 HLA restricted human responses against the VNTR

Attempts have been made to identify more conventional MHC-restricted CTL and proliferative responses against MUC1. Again emphasis was put on the VNTR as altered glycosylation has been studied in this region, peptide epitopes derived from the VNTR are expressed at high levels, and the number of possible overlapping peptides to test for MHC binding is relatively small.

Using MHC-peptide binding studies, a tandem repeat derived 9-mer peptide (STAPPAHGV p9-17) that binds to several HLA class I alleles, including HLA-A*01, A*0201 and A*11 was identified (Domenech et al., 1995). Tumour infiltrating lymphocytes from an HLA-A*11 positive donor as well as PBL from healthy donors, were restimulated for several weeks with peptide loaded APC. The CTL were peptide-specific, but did not recognise endogenously presented antigen. Since peptide concentrations of up to 100µg/ml were used to induce and read out CTL activity, low affinity CTL may have been induced that lysed peptide loaded target cells only and this may explain the lack of tumour cell lysis.

Further recent work using either MUC1 VNTR peptides linked to a lipid tail or formulated in liposomes, failed to show convincing lysis of tumour cell lines endogenously expressing MUC1 (Agrawal et al., 1998). The authors stimulated PBL from three healthy donors with MUC1 VNTR peptide containing liposomes for two weeks and assessed immune responses against 9-mer peptide loaded T2 cells. Since no recognition of endogenously processed MUC1 antigen was seen, and very high doses of peptide were used to induce and test the CTL bulk

cultures it is likely that the responses observed where of low affinity and peptide specific only.

1.6 Mouse model systems for human MUC1 responses.

One of the drawbacks of immunology research in humans is the difficulty of obtaining specific T-cells. *In vitro* priming protocols for human cells are still very difficult to implement and material from immunised patients is not readily available. Therefore mouse models have been developed that can overcome some of these difficulties.

1.6.1 A2/K^b restricted responses against the VNTR

A2/K^b transgenic mice are a useful tools to identify HLA-A*0201-restricted responses. These mice express the product of the HLA-A*0201/K^b chimeric gene in which the α_3 domain of the heavy chain is replaced by the corresponding murine H-2 K^b domain while leaving the HLA-A*0102 α_1 and α_2 domains unaffected (Vitiello et al., 1991).

A2/K^b transgenic mice have been immunised with a MUC1 VNTR mannan fusion protein examine for HLA-A*0201-restricted CTL responses (Apostolopoulos et al., 1997). Primary bulk cultures showed cytotoxic activity against overlapping VNTR peptides pulsed on syngeneic PHA-induced blasts. However, these cells not only express the A2/K^b transgene, but also the H-2^b mouse alleles K^b and D^b at higher expression levels. To confirm that the responses were HLA-A*0201- rather than K^b- and D^b- restricted, longer peptides were pulsed onto HLA-A*0201-expressing EBV-transformed B cells and shown to be recognised by the polyclonal CTL populations. The bulk cultures were also able to lyse the human MUC1+ and HLA*0201+ adenocarcinoma cell line MCF7 while BT20, a MUC1-positive and HLA-mismatched cell line, was not recognised. These data indicate that the CTL were HLA*0201-restricted and probably MUC1-specific.

It would be of interest to know whether CTL clones derived from these bulk cultures specific for the described SAPDTRPAP peptide were able to recognise endogenously processed and presented MUC1 in a HLA*0201 restricted context.

1.6.2 Mouse MHC class I-restricted CTL responses against the VNTR.

Several wild type mouse strains also make MHC-restricted responses against MUC1 VNTR peptides. However, only in H-2^d mice could bulk cultures recognise naturally processed and presented MUC1 (Apostolopoulos et al., 1997; Apostolopoulos et al., 1995). All others peptide specific responses against the VNTR in different mouse strains did not show cross reactivity against naturally processed and presented MUC1 and these peptides should not be considered functional CTL epitopes at this stage.

1.6.3 Mouse MHC class I-restricted CTL against full length MUC1

It has previously been demonstrated that MUC1-specific immune responses could be induced by immunisation with the whole MUC1 antigen either in the form of a recombinant (rec) Vaccinia virus (VV-MUC1) expressing the full length MUC1 (VV-MUC1) or by immunisation with MUC1 cDNA i.m. Immunisation with VV-MUC1 gave tumour protection in 30% of vaccinated DBA/2 mice against MUC1 expressing P815 tumours (Acres et al., 1993). VV-MUC1 could also induce CTL responses in C57BL/6 mice that lysed a MUC1 transfectant of the Rauscher Murine Leukaemia Virus (MuLV) induced thymoma RMA, called RMA-MUC1 (Bruce Acres, personal communication)

A syngeneic mouse tumour model in C57BL/6 mice using RMA-MUC1 has been established. In this system, immunisation with MUC1 cDNA was shown to protect C57BL/10 mice against subsequent challenge with RMA-MUC1 (Graham et al., 1996; Graham et al., 1995). MUC1-specific CTL responses could only be found in mice that survived the RMA-MUC1 tumour challenge, or in other words had been boosted with a MUC1 expressing tumour cell *in vivo*. These CTL lysed RMA-MUC1-specifically but a control transfectant carrying only the hygromycin resistance gene, was not recognised. This tumour model is currently being used to evaluate MUC1 vaccination strategies including several different MUC1 cDNA constructs. For example MUC1 cDNA vaccination with a constructs lacking the VNTR region is as efficient as the full-length construct (Graham, personal communication) which implies that the dominant CTL response is not directed against the VNTR region.

Obtaining CTL lines or clones from and defining the dominant epitope would determine which part of the molecule is immunodominant and whether the current focus on the VNTR is justified.

1.6.4 MUC1 transgenic mice

It is perhaps not surprising that MUC1-specific CTL responses can be induced in wild type mice that do not express MUC1 as a self antigen, since T-cell precursors specific for MUC1 would neither be deleted during thymic education nor tolerised by peripheral tolerance mechanisms. Whether anti MUC1 immune responses can be induced in MUC1 transgenic animals and whether a tumour protective MUC1-specific immune response would lead to autoimmunity are important questions. To address these issues a MUC1 transgenic mouse strain (SacII) has been made, expressing human MUC1 under it own tissue specific promoter. These mice express human MUC1 in a similar pattern to the human (Peat et al., 1992).

Whether MUC1-specific immune responses could be induced in MUC1 transgenic mice was addressed by a number of investigators:

In MUC1-transgenic mice there is some indication that an immune response to the MUC1 antigen can be induced. Immunisation with the mammary epithelial cell tumour line (410.4) expressing B7.1 and MUC1 resulted in inhibition of tumour growth of MUC1 expressing tumours compared to control transfected 410.4 cells. This effect was dependent on both the CD4+ and CD8+ T cell compartment. The transgenic mice that reject the B7.1, MUC1-expressing tumours showed no evidence of autoimmunity (Smith et al., 1999).

In another study, MUC1 transgenic mice immunised with live MUC1 expressing syngeneic mouse melanoma B16 cells were not protected from these tumours. In contrast to wild type mice showed a delay in tumour growth (Rowse et al., 1998). The authors concluded that wildtype mice could mount anti MUC1 immune responses, while the MUC1 transgenic mice where tolerant. To overcome this tolerance to MUC1, Gong et al. immunised MUC1 transgenic mice with fusions of dendritic cells and MUC1-expressing carcinoma cells M38/MUC1 (Gong et al., 1998). Mice immunised with the M-38/MUC1-DC fusion cells were protected against tumour challenge by untransfected M-38

and MUC1 expressing M-38/MUC1. To confirm that immunity to MUC1 played a role in the protective effect, an unrelated mouse bladder tumour cell line (MB49) was also transfected with the same human MUC1 expression vector. Mice challenged with MB49 were not protected against tumour outgrowth while MB49-MUC1 challenged mice were protected. These results indicate that immunisation with M-38/MUC1-DC induced immune responses against M-38 derived epitopes as well as MUC1 plasmid derived antigens. The authors concluded that immunisation with the M-38/MUC1-DC fusion could break MUC1-specific tolerance in MUC1 transgenic mice. The authors did not define that MUC1, rather than other vector derived sequences, conferred tumour protection in this model. Vector-derived sequences can lead to unexpected CTL reactivity in tumour model systems in which the peptide epitope is not defined has been demonstrated recently (van Hall et al., 1998). This is of particular importance in this study since the MUC1 transgenic mice may be tolerant to MUC1 to a greater degree than to cryptic epitopes derived from for example the vector coded ampicillin gene that could account for these observations. Since the MUC1 specificity of the immune responses in the MUC1 transgenic mice is not certain, the conclusion that MUC1-specific immune responses do not lead to autoimmunity in MUC1 transgenic mice has to be treated with caution.

1.7 Aim and scope of this thesis

The introduction above has summarised how immune responses against MUC1 have been induced in previous studies. However, several questions remain unanswered or only partially answered and the aim of this thesis was to readdress:

First of all, whether the focus on the VNTR region for cellular responses is justified. The characteristics of MUC1 that make it a potential target for immunotherapy mediated by antibodies, namely the loss of apical restricted expression and its altered glycosylation pattern in tumours, should not have a bearing on T-cell mediated immunity, as far as our current understanding of antigen processing and presentation machinery stands to date. A great deal of

data on glycopeptide-specific T-cell responses exists but no known naturally presented epitope has been identified so far.

Neither is loss of apical polarisation of MUC1 expected to confer tumour specificity to a MUC1 specific CTL response since the basolateral surfaces of normal epithelial cells also express MHC class I molecules for presentation of MUC1 derived epitopes. Upregulation and over expression of a tumour antigen alone can be sufficient for specific cytotoxicity directed against a tumour (Stauss et al., 1997), as has been shown in the case of murine p53 (Vierboom et al., 1997). A cellular tumour specific immune response could be directed against the peptide backbone of MUC1 via classical MHC restriction, with tumour specificity being conferred purely by MUC1 overexpression and this may apply to the whole of the MUC1 sequence and not only the VNTR region.

Secondly, this thesis will consider whether a MUC1-antigen-specific CTL response can confer tumour protection without induces autoimmunity against normal MUC1 expressing tissues. To examine the effect of a tumour antigen specific CTL response on a tumour bearing host, CTL lines are invaluable tools, as these can be used for adoptive transfer experiments into transgenic mice expressing MUC1 protein as a self antigen comparable to the human situation. Therefore an attempt was made to generate clonal MUC1 specific CTL.

An thirdly, this thesis aimed to identify HLA*0201 restricted CTL epitopes outside the TR of MUC1 for use clinical use in humans.

1.7.1 Approach to aims

To test whether the non-VNTR of MUC1 plays an important contribution to an anti-MUC1 CTL response the dominant CTL response against the full length MUC1 was identified. Chapter 3 describes the induction of MUC1-specific CTL lines in C57BL/6 mice by immunisation protocols using the whole MUC1 antigen as cDNA and/or VV-MUC1.

The epitope recognised by the MUC1-specific H-2^b restricted CTL lines maps outside the VNTR region. Further mapping of the epitope and the relevance for MUC1 vaccination strategies are discussed in chapter 5.

The CTL generated in chapter 3, and their further characterisation in chapter 5, provided a well-defined effector cell population that was then used to address the second aim: namely, to determine whether a MUC1-specific CTL can confer tumour protection without the induction of autoimmunity against MUC1

expressing tissues in MUC1 transgenic mice. The MUC1-specific CTL were used in an adoptive transfer model into MUC1 transgenic mice to achieve tumour protection without autoimmune pathology. These results are presented in chapter 4.

Having shown that MUC1 specific CTL directed against an non-VNTR epitope can protect MUC1 transgenic mice from tumour challenge, chapter 6 describes the identification of clinically more relevant CTL epitopes restricted by human HLA-A*0201. Since human responses are difficult to elicit from non-immunised donors, transgenic mice that express the human HLA*0201 ($A2/K^b$ transgenic mice) where used. However, the immunisation of $A2/K^b$ mice with the whole MUC1 antigen in a similar way to the experiments in wildtype mice is not likely to yield HLA-A*0201 restricted CTL. This is because expression levels of the $A2/K^b$ transgene are considerably lower than the endogenous $H\text{-}2^b$ alleles, making the latter immunodominant. For this reason a reverse immunology approach was taken to identify HLA-A*0201 restricted CTL epitopes. This involved the prediction of MHC binding peptides, determination of their affinity, and subsequent analysis of their immunogenicity in $A2/K^b$ mice.

In the light of the results obtained in this thesis, the feasibility of MUC1 as a tumour antigen for T cell based immunotherapy will be discussed.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Miscellaneous

Culture disposables

Standard tissue culture disposables	NUNC or Costar
Filtration units (0.2 and 0.45 µm)	Millipore

Molecular biology reagents

Agarose	FMC Bioproducts, Rockland, USA
DNA-purification Kit	Qiagen
pCR3.1 uni	Invitrogen
pCDNA3.1	Invitrogen
Agarose gel DNA extraction kit	Boehringer Mannheim

Enzymes

All restriction enzymes and buffers were purchased from New England Biolabs

T4-DNA ligase and buffer	Life technologies
Polynucleotide kinase	Boehringer Mannheim

Antibiotics and cytokines and tissue culture media supplements

Geneticin (G418)	Life technologies
Puromycine	Life technologies
Hygromycine B	Life technologies
Ampicillin	Boehringer Mannheim
Kanamycin	Boehringer Mannheim
Zeocin	Invitrogen
Mitomycine C	Kyowa pharm.
Interleukin-2, human recombinant	Cetus
Interferon-γ, human recombinant	SanverTech
TGFβ	SanverTech
ß-2Microglobuline	Nuclilab
Brefaldin A	Sigma
MTT	Sigma
DMSO	Sigma
$Na^{51}CrO_4$	Amersham

Antibodies

The following antibodies were obtained from ATCC or by laboratories within the Imperial Cancer Research Fund (ICRF) or from a commercial source as indicated.

Antigen	Name	Species	Label	Origin
Biotin	N/A, Steptavidin		HRP	PharMingen
CD8b.2	Ly-3.2	Rat IgG1		PharMingen
H-2 D^b	28.14.85		FITC	ICRF
H-2 K^b	B8.24.3	mouse IgG1	FITC	ICRF
HLA-A*0201	BB7.2	mouse	FITC	ICRF
human MUC1	BC2	mouse	-	(Xing et al., 1992)
human MUC1	12C10	mouse	-	ICRF
human MUC1	HMFG-1	mouse	-	ICRF[1]
human MUC1	HMFG-2	mouse	-	ICRF[1]
human MUC1	SM3	mouse	-	ICRF[1]
mouse IFNγ	R4-6-A2	rat	-	ITK
mouse IFNγ	XMG1.2	rat	biotin	ITK
mouse IgG	polyclonal	goat	FITC	DAKO
murine CD3	145-2C11	hamster IgG		PharMingen
murine CD4	GK4.5	rat		ATCC
murine CD8	2.43	rat	biotin	ATCC
murine TCRα β	H57-597	hamster IgG	FITC	PharMingen
murine TCRγδ	GL3	hamster IgG	FITC	PharMingen

Table 2.1: Monoclonal antibodies

[1]Developed in the laboratory of Dr. J. Taylor-Papadimitriou, (Arklie et al., 1981; Taylor Papadimitriou et al., 1981) and (Burchell et al., 1987). Supernatants were used for FACScan analysis.

Buffers and solutions

All solutions were prepared using sterile de-ionised water and stored at room temperature unless otherwise stated. Solutions were sterilised when necessary by autoclaving, or by filtering through a 0.22 µm filter unit as appropriate.

For DNA methods

Tfb1 buffer:
30 mM potassium acetate
100 mM $RbCl_2$
10 mM $CaCl_2$
50 mM $MnCl_2$
15% glycerol

Adjusted to pH 5.8 with 0.2 M acetic acid and sterilised by filtration.

Tfb2 buffer:
10 mM MOPS
75 mM $CaCl_2$
10 mM $RbCl_2$
15% glycerol

Adjust to pH 6.5 with KOH and sterilised by filtration.

L-Broth:
10 g/l Bacto Tryptone
5 g/l Bacto yeast extract
10 g/l NaCl
(produced by media production unit at the ICRF)

L-Agar:
L-Broth with 15 g/l Bacto Agar

TENS lysis buffer:
10 mM Trizma base
1 mM EDTA
0.1 M NaOH
0.5% SDS

10x TBE:
0.89 M Trizma base
0.89 M Boric acid
10 mM EDTA pH 8

5x DNA sample buffer:	33% glycerol 3.75 X TBE 125 mM EDTA pH 8 0.275% SDS 0.008% bromophenol blue
TE:	10 mM Trizma base 1 mM EDTA (adjusted to the appropriate pH with HCl)
10x PNK buffer:	0.5 M Tris-HCl pH 7.6 0.1 M $MgCl_2$ 50 mM DTT 1 mM spermidine 1 mM EDTA Aliquots were stored at -20°C.

For Cell Culture

Trypsin/Versene mix for detaching cells:	Trypsin (0.25% in Tris Saline) and Versene (EDTA, 0.2 g/l in PBSA) stocks were produced by the media production unit at the ICRF.

4 ml of Trypsin stock were mixed with 16 ml of Versene stock to give the trypsin/versene mix with which adherent cells were detached. This gives a final concentration of 0.05% trypsin and 0.16 g/l EDTA/versene.

Cell Culture Media

Iscove's modified Dubbecco's medium (IMDM, GibCo) supplemented with 10% heat inactivated foetal calf serum (FCS) was used through out all experiments

unless otherwise stated. The growth of murine and human T-cells was supported by supplementing the culture media with 1-10 Cetus units of recombinant human Il-2. (Cetus Ltd.) Where 6 Cetus units is equivalent to one international Unit.

IFA

Incomplete Freund's Adjuvant was used to make peptide emulsions and Il-2 for injections of mice (Difco Laboratories, Detroit, Michigan, USA)

Mitomycine C

a stock solution was prepared at 2 mg/ml in PBS and stored at 4°C in the dark. (Kyowa pharm.)

Other buffers and solutions

PBS: 137 mM NaCl
3.4 mM KCl
20 mM Na_2HPO_4
1.8 mM KH_2PO_4
pH 7.2

PBS was produced by media production unit at the ICRF.

PBA: PBS
1% BSA fraction V w/v
0.02% sodium Azide

1M HEPES buffered 1M HEPES pH7.5 (GibcoBRL)

Cell lines

Cell line	SPECIES	Source	Growth conditions	Reference
B16- A2/K^b-MUC1	mouse	mouse melanoma cell transfected with A2K^b and pMUC1	IMDM 8% FCS, 2µg/ml puromycine, 500µg/ml G418	
MULV CTL	mouse C57BL/6	CTL clone, H-2D^b restricted, specific for MuLV gag gene, LCCL	IMDM 8% FCS, 10cu/ml IL-2 irradiated, mit C, RMA	T. van Hall
COS7	monkey	kidney fibroblast	IMDM 8% FCS	ATCC
COS7-D^b	monkey	kidney fibroblast transfected with H-2 D^b	IMDM 8% FCS 500µg/ml G418	T. van Hall
COS7-K^b	monkey	kidney fibroblast transfected with H-2 K^b	IMDM 8% FCS 500µg/ml G418	T. van Hall
Hela D^b	human	human cervical carcinoma transfected with H-2 D^b	IMDM 8% FCS 500µg/ml G418	T. van Hall
Hela K^b	human	human cervical carcinoma transfected with H-2 K^b	IMDM 8% FCS 500µg/ml G418	T. van Hall
L8	mouse C57BL/6	CTL clone, H-2K^b restricted, specific for MUC1	IMDM 8% FCS, 10cu/ml IL-2 irradiated, mit C, RMA-MUC1	
MCF-7	human	metastatic breast carcinoma isolated from a pleural effusion	RPMI 10% FCS 10 µg/ml insulin	(Soule et al., 1973)
RK13	rabbit	rabbit kidney cell line used to propagate rec. vaccinia viruses	IMDM 8% FCS	ATCC
RMA	mouse C57BL/6	Rauscher MuLV transformed thymoma	IMDM 8% FCS	ATCC
RMA-Hygro	mouse C57BL/6	control transfectant of RMA	IMDM 8% FCS 500µg/ml of hygromycine	(Graham et al., 1995)
RMA-MUC1	mouse C57BL/6	MUC1 transfectant of RMA	IMDM 8% FCS 500µg/ml of hygromycine	(Graham et al., 1995)
T47D	human	metastatic breast carcinoma isolated from a pleural effusion	E4 10% FCS	(Keydar et al., 1979)
T47D A2/K^b	human	Clone of T47D transfected with A2/K^b gene	IMDM 8% FCS 350µg/ml G418	
T47D neo	human	Clone of T47D transfected with pcDNA1 neo vector	IMDM 8% FCS 350µg/ml G418	
TAP -/-	murine	TAKO, embryo fibroblast from TAP -/- mouse	IMDM 8% FCS	IMDM 8% FCS 350µg/ml G418
TAP +/+	murine	Xho C3, embryo fibroblast from C57BL/6 mouse	IMDM 8% FCS	IMDM 8% FCS 350µg/ml G418
WeHi	mouse	WeHi-164 clone 13, fibrosarcoma sensitive to TNFα	RPM1 8% FCS, L-arginine (116mg/l), L-Asparagine (36mg/l) and L-glutamine (216mg/l)	(Traversari et al., 1992)

Table 2.2: Cell lines

Recombinant viruses

In this study two MUC1 expressing recombinant viruses were used both supplied as a generous gift by Bruce Acres, TRANSGENE, France.

VV-MUC1: a recombinant vaccinia virus encoding human MUC1 with ca. 3 TR and the human Il-2 gene.
VV-4843: the corresponding empty vector control only encoding human IL-2

Adeno MUC1: a recombinant adenovirus encoding MUC1

2.2 Methods

2.2.1 Molecular biology methods involving DNA

All plasmids obtained from external sources were transformed into competent bacteria to make large-scale preparations for further use as described below.

Bacterial strains used

The *Escherichia coli* strain XL-1 Blue (Stratagene) was used to propagate and amplify all plasmid DNA described, except constructs based on the pDNA3.1 neo and pCR3.1uni plasmids (Invitrogen). Transformed XL-1 colonies were grown on under the selection marker appropriate to the vector, usually 100 µg/ml ampicillin. The *Escherichia coli* strain TOPF10 (Invitrogen) was used to propagate constructs based on pDNA3.1 neo and pCR3.1uni, allowed ampicillin resistance in transformed colonies. These were selected on 50 µg/ml ampicillin

Preparation of competent bacteria

Bacteria were streaked out from a frozen stock onto L-Agar plates and incubated overnight at 37°C. A single colony was picked, inoculated into 5ml of L-Broth and grown in a shaking incubator (>200 rpm) at 37°C overnight. The following morning the bacteria were sub-cultured 1:20 into 100 ml of pre-warmed L-Broth and grown at 37°C until an OD_{550} of 0.48 was reached. Cells were then chilled on wet ice for 5 min before recovery by centrifugation in pre-chilled corex tubes at 6 000 rpm at 4°C for 5 min. The cell pellet was resuspended in 0.4 volumes of Tfb1 buffer and left to rest on wet ice for 5 min. Cells were centrifuged as described before, and the cell pellet was resuspended in 0.04 times the original volume in Tfb2 buffer. The cell suspension was left on wet ice for 15 min before aliquoting into freezing vials (200 µl of cells suspension per vial), and snap frozen in an dry ice ethanol bath. For long term storage, cells were stored under liquid nitrogen or -70^{0}C freezer.

Transformation of competent bacteria

Competent bacterial cells were left on wet ice for approximately 15 min to thaw. For transformation of supercoiled plasmid, 40 µl of cell suspension was added to 5-20 ng of DNA dissolved in an equal volume of water. When the products of a ligation reaction were used usually half of the reaction was used but no more than 40µl in total. The tubes were placed on wet ice. After approximately 30 min, mixing gently every 10 min., the cells were heat shocked at 42°C for 90 seconds and immediately returned to ice. 160 µl of L-Broth was then added to the cell-DNA mixture and incubated at 37°C for 30 min to allow expression of the drug resistance gene. Transformed cells were then spread onto pre-dried L-Agar plates containing appropriate concentrations of antibiotics and incubated upside o/n at 37°C.

Small scale preparation (mini prep) of plasmid DNA for restriction digest.

Before large scale preparation of plasmid DNA, transformed colonies were analysed to ensure that the correct plasmid or ligation product was present. Single colonies were picked from a fresh plate, inoculated into 5 ml of L-Broth and grown o/n at 37°C in a shaking incubator. 1.5 ml of culture was transferred to an Eppendorf tube and spun in a microcentrifuge at 15,000 rpm for 20 sec to collect the cell pellet. The L-Broth was decanted, leaving 50-100 µl behind and then vortexed briefly to resuspend the cell pellet. 300 µl of TENS buffer was added, briefly vortexed followed by the addition of 150 µl 3 M sodium acetate pH 5.2 and vortexed again. After centrifugation at top speed in a microcentrifuge for 2 min to remove the cell debris, the supernatant was removed to a fresh tube and nucleic acid precipitated by the addition of 0.9 ml ice cold absolute ethanol. This was then centrifuged as before, the supernatant removed, and the nucleic acid pellet was washed with cold 70% ethanol. After a further centrifugation step the 70% ethanol was poured off and the nucleic acid pellet air-dried before dissolving in 30 µl water. This 'miniprep' DNA was then analysed by restriction digest confirm that the DNA was correct before a large scale preparation was made.

Small scale preparation (mini prep) of plasmid DNA for sequencing.

The same protocol was used for obtaining 5 ml cultures. However, to achieve a higher degree of purity for direct sequencing mini prep DNA was made using a Quiagen miniprep kit according to the manufactures instructions or alternatively the mini prep robot (Quiagen) at the service laboratories at ICRF was employed when more than 60 minipreps had to be made.

Large scale preparation of plasmid DNA

Large scale DNA preparations (Maxi prep) were made using Qiagen maxiprep kits according to the manufacturer's instructions. The resultant DNA pellet was washed with 70% ethanol, air-dried and dissolved in 500 µl distilled water. The concentration and purity of the plasmid DNA was determined by optical density measurement before storage at -20°C.

Restriction enzyme digestion of plasmid DNA

Restriction endonuclease digestion of plasmid DNA was typically carried with a DNA concentration of 0.1µg/µl. Per µg of DNA 1 to 3 units of restriction enzyme were used. 10x reaction buffers were supplied with the individual restriction enzymes and used at a final 1x concentration. The optimal buffer for digests involving two restriction endonucleases was determined according to the supplier's instructions. If no optimal buffer could be used in which simultaneous digestion by both enzymes was possible, the respective digests were carried out sequentially. Plasmid DNA was digested for a minimum of 2 hr at the optimal temperature, but where star activity was expected 0.1mg/ml of BSA was added and the incubation time limited to two hours.

Agarose gel electrophoresis of DNA

Agarose gels were prepared by dissolving agarose at 0.8 -3.0% (w/v) in 1x TBE or TAE buffer in a microwave oven. The gel was allowed to cool to 50°C just above the indicated gelling temperature in a water bath before ethidium bromide (sigma)

was added to a final concentration of 0.1 µg/ml. Then the poured gels were allowed to set at room temperature in a gel try. DNA samples were prepared by the addition of DNA sample buffer to 1/5th final volume, and loaded into the wells of the gel submerged in 1x TBE or TAE buffer. Gels were electrophoresed in 1x TBE or TAE using a horizontal gel electrophoresis apparatus (BRL) at 5-7 V/cm at room temperature until the desired range of separation of the DNA fragments was achieved. DNA was visualised using a long wave UV light box, and photographed with Polaroid film or a digitised video camera. The sizes of fragments were estimated by comparison of their mobility relative to molecular weight markers of known size (New England Biolabs). Molecular weight markers utilised were as follows:

a) HindIII restriction endonuclease digestion of bacteriophage λ DNA.

b) HaeIII restriction endonuclease digestion of bacteriophage ϕX174 DNA.

c) 1 kb DNA ladder containing bands from 1-12 repeats of a 1018 bp DNA fragment.

For preparative gels from which DNA was later isolated TAE rather than TBE was used as borate ions can interfere with this process.

Isolation of DNA fragments from TBE-agarose gels.

Unless otherwise stated, the purification of DNA fragments from TAE-agarose gels was performed using a gel extraction kit (Quiagen). After the appropriate restriction digest, reactions were run on an agarose gel and the band of interest excised from the gel under long wave UV illumination as a thin slice. This was transferred to a pre-weighed Eppendorf tube and the weight of the gel slice noted. Elution of the DNA from the agarose gel slice was carried out as described in the manufacturer's instructions.

Spectrophometric determination of nucleic acid concentration

For quantification of DNA and nucleic acid concentration, OD readings were taken at 260 nm and 280 nm of an appropriate dilution of nucleic acid stock. An OD of

1.0 at 260 nm corresponds to approximately 50 µg/ml DNA, 40 µg/ml RNA and 20 µg/ml single stranded oligonucleotides. Estimates of purity were obtained by the ratio of OD_{260}/OD_{280}, where for pure preparations, the ratios were 1.8 (DNA) and 2.0 (RNA).

Polymerase chain reaction (PCR)

PCR reactions were used to amplify sequences from plasmid DNA and RNA by RT-PCR. All oligonucleotides used for PCR reactions were synthesised as single stranded primers by the ICRF Oligonucleotide Synthesis Laboratory.

50 ng plasmid DNA were amplified in typically 50 µl reaction: 2% deionised formamide, 0.2 mM dNTP mix of dATP, dCTP, dGTP and dTTP, 1x PCR buffer (Promega), 25µM each of 5' and 3' oligonucleotides and 0.5-2.5 mM $MgCl_2$. PCR reactions were overlaid with 1-2 drops of mineral oil to prevent sample evaporation. Typical cycling conditions included a 'hot start' of 10 min at 94°C before 1 µl (5 units/µl) Taq DNA polymerase was added and cycling according to the following schedule:

Denature	1 min at 94°C
Anneal	1 min at oligonucleotide melting temperature - 5°C
Extend	1 min at 72°C.

25 cycles

Incubate at 72°C for 10 min.

Store at 0°C

TA cloning

When PCR products needed to be cloned into mammalian expression vectors a TA cloning kit based on pcDNA3.1 was used according to the manufactures instructions (pCR3.1 TA kloning kit. Invitorgen).

After transformation colonies were picked, resuspended in 100 ml of water of which a fraction was used to inoculate 5 ml cultures while the rest was boiled for 5 min to release plasmid DNA from the bacteria. Cellular debris was removed by centrifugation at 14.000 rpm in a bench top centrifuge and typically 5µl were used as a template for an analytical PCR. Sequencing primers complementary to vector

sequences in conjunction with the appropriate PCR primer that mapped to the original PCR product were used. The products of the reaction were examined by agarose gel electrophoresis and colonies giving rise to the expected bands were grown up overnight for plasmid DNA preparation.

DNA sequencing

DNA sequencing was performed either using the Sequenase™ Version 2.0 DNA sequencing kit (United States Biochemical) or PRISM™ ready reaction dyedeoxy™ terminator cycle sequencing kit, both as directe by the manufacturers.

DNA ligation reaction

Vector and insert plasmid DNA were digested with the appropriate restriction endonucleases and gel-purified using the Gel extraction kit as described previously. Approximately 100 ng vector DNA was incubated with insert DNA in molar ratios of 1:1, 1:3 and 1:5 in the presence of 1x T4 DNA ligase buffer (Gibco, BRL) and 1 µl (1 U/µl) T4 DNA ligase in a 10 µl reaction volume. Sticky ligations were incubated at 16°C for at least 8 hrs while blunt ended ligations were incubated at 12°C for at least 8 hrs. Ligation reactions were made up to a 40 µl volume with water before transformation into competent bacteria.

Annealing of complementary oligonucleotides and TA cloning

This method was used to prepare expression constructs encoding very small fragments of the MUC1 gene when purification of a small PCR product would have been difficult. Double stranded oligos encoding the region of interest including a start codon and stop codon were synthesised. The sequence was prepared in a way that one base-pair overhangs compatible with the pCR3.1 (invitrogen) TA cloning vector would form after annealing of the oligos.

400 pmol of each oligo with 0.4 µl of 5M NaCl were mixed in a final volume of 20 µl of water.

The tube was then placed in a beaker of boiling water, the beaker was then allowed to cool to room temperature. Then 80 µl of TE were added resulting in a

final concentration of 20mM of NaCl which was compatible with most enzyme reaction.

The double stranded oligo was then treated like a PCR product and directly cloned into the TA vector following the manufactures instructions.

2.2.2 Immunological techniques

Fluorescence activated cell scanning analysis (FACScan)

Monolayer cells were trypsinised, washed once in growth medium and a minimum of 1 x 10^4 cells were resuspended in 50 µl of the appropriate specific antibody diluted PBA, neat tissue culture supernatant, or in 100 µl PBA as a negative control in the wells of a V bottom 96 well plate. All incubations were carried out in the presence of 0.02% sodium azide and at 4°C. After 30 min incubation, cells were washed three times in PBA by spinning the plates for 2min. at 1300 rpm and then flicking of the supernatant carefully. Cells were resuspended in 50 µl of fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse diluted 1:40 in PBA and incubated for 30 min in the dark. After washing three times in PBA, cells were resuspended in 80 µl PBSA with 0.5 % paraformaldehyde and transferred to micronic tubes using a multichannel pipette. 10000 live gated cells were analysed by a Becton-Dickinson FACScan flow cytometer equipped with an argon ion laser tuned to 488 nm. FITC fluorescence was collected at 530 nm +/- 15 nm and cells were gated on scatter profiles to exclude dead cells. When analysis was not possible immediately, cells were stored at 4°C until analysis could be performed.

Fluorescence activated cell sorting (FACSort)

For FACSorting cells were stained as for FACSanning but kept under sterile conditions at 4^0C without sodium azide where possible. The cells were sorted by the ICRF Service FACS Lab and collected in normal culture medium.

Immunofluorescence staining of adherent cells

Adherent cells were grown on 9 mm acid washed, sterile cover-slips in the wells of a 24 well tissue culture plate. Cells were washed once in PBS, and fixed either in 3% paraformaldehyde for 15 min (fixative used when staining with 9E10 alone) or 1:1 methanol:acetone prechilled to -20°C (used for all other antibodies and double staining) for 5 min. After fixation, methanol:acetone fixed cells were allowed to air dry before proceeding to antibody incubation. Cells fixed with 3% paraformaldehyde were further processed by washing 3 times with PBS, followed by a 10 min incubation with 0.1 M ammonium chloride to quench free aldehyde groups. After washing 3 times with PBS, cells were permeablelised with 0.1% Triton X-100 for 5 min, and then washed 3 times with PBS. All cells were then incubated with 200 µl of the appropriate antibody for 1 hr at room temperature. Cells were washed 3 times with PBSA 10% FCS before incubation with the secondary antibody (FITC conjugated goat anti-mouse). Secondary antibodies was obtained from DAKO and diluted 1:40 in PBSA 10% FCS. Incubation with secondary antibodies were carried out at room temperature in the dark for 30-60 min. Cells were then washed twice in PBSA 10% FCS and once in PBSA, before the cover-slips were mounted on glass microscope slides in Citifluor (glycerol/PBS). Slides were viewed under a Zeiss axiophot fluorescence microscope and photographed.

2.2.3 Peptide binding assays

Direct competition assay

Peptide binding to HLA-A*0201 was analysed using HLA-A*0201$^+$ B lymphoblastoid JY cells in a semi-quantitative competition assay (van der Burg et al., 1995).

The assay is based on competitive binding of two peptides to acid stripped MHC class I molecules on a B cell line (JY). A test peptide competes with a fluorescently labelled reference peptide for the empty class I molecules on the cell surface. Mild-acid-treated JY cells were incubated with 150nM fluorescein (FL)-labelled

reference peptide FLPSDC(-FL)FPSV and with several concentrations of competitor peptide for 24 hours at 4^0C in the presence of 1.0µg/ml β_2-microglobulin. Subsequently, the cells were washed, fixed with paraformaldehyde and analysed by flow cytometry. The mean fluorescence (MF) obtained in the absence of competitor peptide was regarded as maximal binding and equated to 0%; the MF obtained without reference peptide was equated to 100% inhibition. The percentage inhibition was calculated using the formula:

$$\{1-(\text{MF 150nM referance and competitor peptide - MF no reference peptide}) \over (\text{MF 150nM reference peptide - MF no reference peptide})\} \times 100\%$$

The binding capacity of competitor peptides is expressed as the concentration needed to inhibit 50% of binding of the FL-labelled reference peptide (IC_{50}).

2.2.4 Tissue culture and immunology

Cell culture

Cell culture was performed according to standard aseptic culture protocols. Most cell lines were grown in IMDM N consisting of Ischove's modified Dubecco's Medium supplemented with 10% heat inactivated foetal calf serum (FCS), glutamine, ß-mercaptoethanol, penicillin G and a selection drug as indicated.

Established CTL lines and clones where grown in IMDM N further supplemented with 5-10 Cu/Il-2. CTL were restimulated on a weekly basis (day 0) and recovered over a Ficoll cushion on day 3.

Harvesting of T cells:

T cells were harvested by vigorously pipetting the cells several times. Then 1mM EDTA in PBS pH7 was added to detach the adherent cells. (2ml per T75 flask or

200µl per 24 well). And incubated at 37°C for 5 min. The adherent fraction was then collected with a larger volume of culture medium.

Washing of cell:

Cells were resuspended in a large volume of culture medium of PBS and centrifuged at 1500 rpm for 5 min. with a low brake setting.

Ficolling of cells.

To remove debris and dead cells, usually several days after restimulation the cells were collected in a 15 or 50 ml tube and underplayed with 2 or 10 ml of Ficoll respectively. The tubes were then centrifuged for 12 min. at 2100 rpm without brake. Life cells with a lower density forming a 'buffy coat' on top of the Ficoll cushion were collected using a pipette. The cell pellet was discarded and the collected cells washed once and resuspended in culture medium.

Immunisation protocols

Mice used to derive T cell cultures for in vitro work were immunised using a variety of protocols. An out line of the different protocols will be given here and more details provided in the results section.

Peptide immunisation:

Mice were injected with 100 µg of peptide in a homogenous suspension of 200 µl of peptide in IFA subcutaneously on the flank.
Peptides were synthesised using fmoc chemistry and provided as lyophilised dry powder. A known amount of dry powder was then dissolved in DMSO at a starting concentration of 100 mg/ml. Some peptides that did not dissolve at this concentration were further diluted down to a final concentration of 20 mg/ml.
These peptide stocks were keep at –20 °C and thawed when needed.
For immunisations peptides were further diluted in PBS to the appropriate concentration.

Usually 50% more peptide homogenate was made than needed to account to loss during syringe loading.
The required amount of peptide was dissolved in half the final volume of PBS and mixed with an equal volume of IFA. A homogenate was then made by extensive vortexing until a thick paste was formed.

DNA vaccination

Animals were injected with 80 µl of 10 mM Cardiotoxin in PBS intra muscularly in the thigh. Four days later they were injected at the same site with 100µg of plasmid DNA dissolved in 100µl of PBS using a insulin hypodermic needle.
Boosters were given two weeks later by the same protocol.

Vaccinia immunisations:

Mice were immunised i. p. with $2x10^7$ pfu of recombinant vaccinia virus in a total volume of 200µl of Tris pH 8 (see Section on Vaccinia)

Primary bulks

Mouse splenocytes were taken into in vitro culture and restimulated in vitro by the following protocol

Stimulator cells (RMA-MUC1 or EL4-MUC1) growing in log phase were harvested and 10^7 cells per spleen resuspended in a small volume of culture medium. Mitomycin C to a final conc. of 50µg/ml (stock of 1 mg/ml) was added and the cells incubate for 1 hr in the shaking water bath at 37°C. The cells were then wash four times and irradiated for 8 min. at 2000 Rad and wash once more.

Responder cells:
At least 14 days after the last immunisation spleens were removed aseptically from immunised mice and single cell suspensions were made from each individual spleen, washed once and counted. (Usually 60-100.10^6/spleen). 10% Mitomycin C

treated, irradiated and washed RMA-MUC1 or EL4-MUC1 stimulator cells were added and the volume made up to 50 ml.
The cells were then divided over 24 wells of a 24 well-plate. (2ml/well=2-4.10^6 splenocytes/well) and incubated at 37^0C.
On day seven of the bulk culture the cells were collected using EDTA and ficolled to remove, cellular debris.
The responder cells were then placed in a 24 well plate (2.10^6 cells/well) in culture medium containing 3-5% Rat Factor. (Rat Factor (RF) is conditioned medium from ConA and PMA-stimulated rat splenocytes. See separate protocol). The cultures were then closely watched and split if necessary.
After one week the cells were harvested, ficolled if needed and tested in a CTL assay.

Long term culture of T lymphocytes

Primary bulks that were shown to be antigen specific were taken into long term culture to derive T cell lines and clones.
For this the cells were restimulated in U-bottomed 96-well plates containing in a total volume of 100 µl medium 80.000 responder cells, 5.000 stimulator cells prepared as for primary bulks and 50.000 irradiated feeder cells (naive spleen) supplemented with 2-3% RF.
As before the plates were incubate for 3-4 days then harvest and ficolled and reseeded into U-bottomed 96-well plates at 50.000 cells per well containing 2-3% RF.
The cells were then very closely watched for the next 3-4 days splitting the cells if necessary. Specificity was tested on days 5-7 following restimulation.
This procedure was repeated weekly slowly replacing the RF by 5 CU/ml rIL-2 and leaving the feeders out.

Initiation of limiting dilutions and generation of CTL clones

The protocol used for generation of CTL clones is given below:

- When restimulating bulks for the 3rd time 45.10^3 cells to start limiting dilution (LD) on the day of the restimulation.
- For each LD set up seven 50ml tubes marked 300,100,30,10,3,1,0.3 cells/well and each containing 10ml medium.
- Add 45.10^3 responder cells to tube '300'. Adjust the final volume to 15 ml. gently and serially dilute cell suspension by transferring 5ml to the next tube.
- Mark seven 96 well U-bottomed plated 300,100, etc. and transfer 100µl of appropriate cell suspension to each well.
- To each well add 50µl of 'stimulator mix' consisting of:
  -5.000 Mitomycine-C, irradiated stimulator cells
  -50.000 irradiated feeders
  -6-9% RF
  After one week add to each well 25µl of 10% RF
- After the following week take 50µl medium out off each well and add 100µl of fresh medium containing:
  -3.000 Mitomycin-C, irradiated stimulator cells
  -25.000 irradiated feeders
  -4% RF
- During the following week monitor T cell growth in the plates and decide on whether to add fresh stimulator cells and/or feeder cells. If a particular well shows a strong expansion of T cells, transfer this culture to 2-4 wells on a new plate and add restimulation mix containing fresh medium, RF, stimulator cells and feeder cells.

Established clones can be restimulated as follows in 96 well plates, -100 µl medium

-5.000 stimulator cells

-20.000 CTL

-5-10 CU rIL-2

-(30.000 feeders)

Cell storage in liquid nitrogen and recovery into culture medium at 37°C

Cells to be stored long-term in liquid nitrogen were trypsinised from a subconfluent flask, washed in complete medium to inactivate the trypsin and pelleted by centrifugation at 1,200 rpm for 5 min. Cells were resuspended at an approximate concentration of 1 x 10^6/ml in E4, 20% FCS and 10% DMSO and 1 ml aliquots stored in cryovials (Grainer). Vials were wrapped in four layers of tissue paper to prevent rapid freezing and transferred to dry ice. Vials were placed at -70°C overnight and subsequently transferred to liquid nitrogen for long term storage.

Cells to be recovered from liquid nitrogen were thawed rapidly at 37°C and washed once in growth medium to remove DMSO present in the freezing medium. Cells were pelleted as described above and resuspended in an appropriate volume of fresh growth medium before seeding in a tissue culture flask. Growth medium was replaced with fresh medium 24 hr later to remove any dead cells from the culture.

CTL assays

To test for Cytotoxic activity of T cell cultures standard $Na^{51}CrO_4$ release assays were performed. For this, effectors were incubated with $Na^{51}CrO_4$ loaded target cells at varying Effector to Target ratios (E:T) for 4-6 hours and the released Cr was measured using a gamma radiation counter.

Preparations of the Targets

Usually 2000 labelled targets were used per experimental well and a total of 10^6 cells were labelled. Target cells growing in log phase were harvested and washed once counted and 10^6 cells were transferred in to a 1.5 ml Eppendorf tube. These cells were the centrifuged at 4000 rpm for 1 min. in a benchtop microcentrifuge (Eppendorf). The supernatant was then taken off leaving a dry pellet with was resuspended in 100µl of $Na^{51}CrO_4$ solution. Immediately 5µl of 1M Hepes pH 7 was added and mixed by gently pipetting the cell suspension. The tubes were then

transferred to a 37°C water bath for 1h. The cells were resuspended once during this time by flicking the tube.
The cells were then washed four times in IMDM N by centrifuged at 4000 rpm for 1 min. in a benchtop microcentrifuge. After the last wash the cells were resuspended in a 50 ml Falcon tube in 25 ml of IMDM N. 50µl of this suspension containing 2000 cells was then added to the plates containing the effector cells or controls (see below).

Preparation of effectors:

Effectors were harvested and ficolled unless the cells had already been ficolled since the last restimulation, washed and resuspended in IMDM N at the appropriate concentration and added to the wells of a round bottom 96 well plate. Two fold dilutions were made in the plate resulting in triplicates of 100µl of effector cells at each E:T ratio. For bulk cultures E:T ratios going down form 100:1 and for established lines clones E:T rations of 30:1 or less were used. For each target cell line six wells containing IMDM N or PBS 2% triton were prepared as controls for spontaneous and maximum release of ^{51}Cr.

Then the labelled target cells were added to the plates which were then centrifuged for 2 min at 1200 rpm and incubated in a Tissue culture incubator at 37°C for 4-6 hours.
After the incubation period the supernatants were harvested using Skaton Harvesting frames according to the manufactures instructions and counted on a Wallac gamma counter.

The date was presented a %specific ^{51}Cr release, defined as 100x({experimental cpm-spontaneous cpm}/{total cpm - spontaneous cpm}) where the experimental value was the average of the experimental triplicate, the spontaneous value the average of six wells with IMDM N and targets and the total value the average of six wells containing 2% triton and target cells.

TNFα release assay (WeHi Assay)

Specific TCR triggering leads to TNFα release by T-Cells which can be bioassayed using WEHI-164 clone 13 cells. W13 is a mouse fibrosarcoma cell line sensitive to TNF.(Traversari et al., 1992). The culture medium for W13 was RPMI-1640 (Gibco) containing 10% FCS supplemented with L-arginine (116mg/l), L-asparagine (36mg/l) and L-glutamine (216mg/l). M13 cells were culture subconfluent and passaged regularly at ratios up to 1:30 twice per week. Cells were harvested using trypsin and washed one in culture medium before reculture or use.

Resting CTL were used at least five days after the last restimulation. They were incubated with target or stimulator cells in 96 well plates for 24 hours.
In order to maximise the sensitivity of the assay and minimise background TNFα release only resting CTL were used. Since mouse splenocytes produce TNFα, WeHi assays were not performed in the first three weeks of bulk culture when residual cell were still present.

DAY1:
Stimulator cell were washed in culture medium and brought to a concentration of $4x10^5$ cells per ml in IMDM 8%FCS while the effectors were washed 3-4 times to remove any residual TNFα and resuspended at 3-10x 10^4 cells per ml in IMDM 8% FCS with 25 Cu/ml rIl-2.
To each experimental well of a round bottom 96 well plate 50 µl of either medium alone as control or stimulator cells were added. Subsequently 50 µl of either medium with 25 Cu/ml rIL-2 (control) or effector cells (1500-1000/well).
Triplicates of the following combinations were included on each experimental plate:

1st 50µl	2nd 50µl	
medium	medium + IL-2	medium control
medium	effector cells + IL-2	TNFα background of effectors
targets	medium + IL-2	TNFα background of targets
Targets	effector cells + IL-2	specific TNFα release

The plates were then wrapped in seran wrap and incubated at 37^{0}C for 24 hrs.

DAY2:

After 24 hours the 96 well assay plates were spun at 1600 rpm for 5 min. and 75µl of supernatant transferred to a new flat bottom 96 well micoplate. To each well $3x10^4$ W13 cells in 25µl of W13 culture medium supplemented with 2µg/ml of actinomycin D (Stock 200µg/ml) were added.

On each plate dilutions of a known standard of rTNFα where added to W13 cells as a control for their sensitivity.

The plates were then incubated at 37° C over night.

Day 3:

The percentage of dead cells was measured by a colorimetric assay which is based on the activity of living cells to produce a coloured tetrazolium product when incubated in the presence of MTT.

50µl of MTT (3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Merck) at 2.5 mg/ml in PBS was added to the plates which were then incubated for 2h at 37°C. The dark forazan crystals were dissolved by adding 100µl of lysis buffer.

1 vol. of N,N-dimethylformamide was mixed at 37°C with 2 vols. 30%SDS. The pH was then adjusted to 4.7 by adding a small volume of 40% acitic acid/0.5N HCl. The plates were then incubated for at least four hours or o/n at 37°C. The optical densities (OD) were then measured at 550nm.

Percentage specific W13 death was determined as:

IFNγ ELISA

The assays where arranged in a similar way to the TNFαrelease assay and supernatants were analyes for IFNγ using a sandwich ELISA.

Maxisorb ELISA plates were coated with 1µg/ml of IFN mAb R4-6-A2 in 10mM Tris at pH 10 o/n. The plates where then washed three times in PBS with 0.5% Tween

20 (PBS-T20) and blocked for 1 hr at 37oC with PBS-T20 supplemented with 1% BSA.

The plates were then washed three times in PBS-T20 and the supernatants from the CTL cultures added in triplicate and incubated for 4 hrs at 37°C. In each plate a dilution series of rec. INFγ was added. After three washes with PBS-T20, 1µg/ml of biotinylated mouse IFNγmAb XMG1.2 was added in PBS-T20 and incubated for After three washes with PBS-T20 1µg/ml HRP conjugated strptavadin was added for 1 hr at 37°C again washed three times and developed with ABTS (sigma) according to the manufactures instructions. Plates were read on an ELISA plate reader at 415nm.

Transient transfection of targets for WeHi assay

To determine the specificity and restriction element of the described CTL recognition of transiently transfected target cells for the WeHi assay were used. For this adherent cell lines were transfected transiently in flat bottom 96 well plates and used as targets.

Reagents:
Serum free medium
NU serum IV Culture Supplement (Collaborative Biomedical Products cat #51004
DEAE-Dextran sulphate (Pharmacia, MW 500000) 10mg/ml in serum free medium filtered 0.22m
Chloroquine (Sigma ref C-6628), 10mM in water, filtered
For this approx. 10.000 adherent cells of interest in 100µl growth medium were seeded into each 96 well of a flat bottom tissue culture plate and incubated o/n or until 80% confluency.
A DEAE DNA complex was prepared by mixing DNA 50ng of DNA in 15µl of IMDM 10% NU per well with an equal volume of IMDM 10% NU supplemented with 200 µM chloroquine and 800µM DEAE. This was done to avoid contact between DEAE-Dextran and concentrated DNA solution.

The 96 well plates were then sucked dry and incubated with 30µl of the DNA-DEAE mix per well added.
After 4 hours the cells were DMSO shocked for 1.5 min. with 100 µl of sterile PBS supplemented with 10% DMSO at room temperature. The DMSO was then removed by carefully flicking the plated onto tissue paper. And washing the cells twice with PBS each time removing the fluid by flicking the plates onto fresh tissue paper.
Then 100µl of growth medium were added and the cell incubated for another 2 days.
On the day of the WeHi assay the cells were sucked dry and fresh IMDM N (50µl/ well) was added. The CTL were then added directly to the plated following the standard WeHi protocol (see above)

Cell transfection

Transient and stable transfection of cells by electroporation

Electroporation of cells growing in monolayers was used for transient transfection of COS7 cells to check expression of DNA constructs and for the generation of stable transfectants of RMA.

Subconfluent cells were trypsinised, washed in growth medium and resuspended in PBSA. Cells were counted and resuspended at a concentration of 4 x 10^5 cell/ml. 0.8 ml of cells was mixed with 15 µg plasmid DNA and transferred to a 0.4 cm electroporation cuvette. The cuvette was left on wet ice for 5-10 min before cell electroporation using a Biorad Gene Pulser. For transient transfection of COS7 cells, the electroporator was set to 450 V/250 µF, where the time constant measured should be 3-4 s. For electroporation and stable selection of T47D clones, the electroporator was set to 450 V/960 µF. After electroporation, cuvettes were returned to wet ice for 10 min before plating out in fresh growth medium.

COS7 cells, transiently transfected to check expression of cDNA constructs were immunolabelled three days post transfection, T47D cells transfected by electroporation were split 1:10 three days post transfection into selectable medium.

Selection and expansion of cell clones showing stable expression of the transfected plasmid.

After trypsinisation 3 days post transfection, medium containing the appropriate selection marker was changed every three days until all the cells had died on the control mock transfected plates. Individual clones were isolated as follows. Plates were washed with growth medium in the absence of FCS and sterile Whatman 3MM paper squares soaked in trypsin/versene were placed over the isolated colonies. Cells detaching from the plate and stuck to the paper were transferred to individual wells of a 24 well plate and 1 ml fresh medium added. Three days later, or when cells were visible in the well, the paper was removed. Cell clones were subsequently expanded by trypsinization.

2.2.5 Preparation of recombinant viruses

Solution for growth and purification of Vaccinia virus recombinants.

Host cells: RK13 a Rabbit kidney cell line

Culture medium IMDM N

Buffers for virus purification:

RBS: Tris 10mM pH7.6
$MgCl_2$ 1 mM
LCl 1mM

Tris 10mM pH8

Sucrose 36% in sterile water

RK 13 cells were grown as adherent cells and split regularly using Trypsin.

Infection of RK13 with recombinant Vaccinia virus.

Cells were cultures in T175 flasks until confluent. Then the medium was removed and $5x10^6$ pfu of virus added in 5 ml of IMDM with 1% FCS per flask. The flask was the rocked to ensure even distribution of the virus and allowed to attach at room temperature for one hour. Then 10 ml of IMDM N were added to the flasks which were then incubated at 37°C.

Harvesting of virus.

After 48 hours the RK13 cells started to detach and remaining cells were detached by hitting the side of the flask. The cells were harvested and centrifuged to pellet the cells at 2000rpm for 5 min. the supernatant was discarded and the cells were resuspended in 5ml of RSB per flask.
The virus in the supernatant was recovered by pelleting the cells which were resuspended and sonicated as before.
The supernatants from both were pooled and stored at -70°C until concentration of the virus.

Concentration of the virus bulks

For concentration and removal of cellular debris the virus supernatants were thawed and briefly sonicated. 25ml of virus supernatant were underlayed with 10 ml of 36% sucrose in a Beckman SW28 tube and centrifuged for 2 hrs at 14.000 rpm at 5°C in an ultra centrifuge.
The supernatant and intermediate layers were then discarded and the virus pellet resuspended in 2-3 ml 10mM Tris pH 8.

Titration of viral stocks

Virus titer was determined by a standard plaque assay. $2.5 x10^5$ RK13 cells were plated in each well of a 6 well plates and grown until confluency. Then the medium was removed and recombinant Vaccinia virus in IMDM with 1% FCS diluted in ten fold serial dilutions starting with a 1000X dilutions. Each dilution series was

included in duplicate. 200µ of virus was added to each well, rocked to allow even distribution and incubated at room temperature. After one hour 2 ml of growth medium was added and the plates were then incubated over night at 37°C.
The next day the medium was removed and the cells were fixed by adding 2 ml of 7% paraformaldehyde solution.

The plaques were then stained using brilliant violet by removing the paraformaldehyde and washing the plates 2x with PBS. All PBS was then removed and 3ml of brilliant violet stock was added for 10min, after which the plates were washed extensively iin tab water. Plaques were then visible as clear spots in stained lawn of cells and counted to calculate the final viral titer.

Preparation of virus for injection

For injection of rec. vaccinia virus into mice the viral stocks were thawed, briefly sonicated and diluted on the day of injections appropriately in 10mM Tris pH8.

Chapter 3: Induction of MUC1-specific MHC H-2^b restricted CTL responses in C57BL/6 mice

3.1 Introduction

The ability of the cellular arm of the immune system to specifically recognise and eliminate cells harbouring foreign invaders such as viruses and parasites has been studied in great detail. This body of work has led to the assumption that the immune system can, in principle, recognise and eliminate cells that have been virally transformed. The identification of epitopes recognised by tumour infiltrating lymphocytes has shown that this principle can also be extended to include non-viral tumour antigens such as the melanoma antigens. This implies that the immune system can also recognise cells that have undergone malignant-change and thus differ from normal healthy cells. In the case of MUC1 as tumour antigen, a number of changes that take place during the malignant change of epithelial cells have been discussed in Chapter 1. These include not only its aberrant glycosylation pattern but also overexpression of MUC1 protein by tumours. To address whether MUC1 overexpression is sufficient for specific recognition of tumour cells the aim was to induce CTL responses against MUC1 in C57Bl/6 mice. This chapter will address whether human MUC1 antigen specific CTL responses can be induced in a C57BL/6 mouse model. In wild type C57BL/6 mice, human MUC1 is not a self antigen, as it is not germ line encoded and the homology between murine Muc1 and human MUC1 is low (Spicer et al., 1991). See Figure 3.1

```
HS  MTPGTQSPFFLLLLLTVLTVVTGSGHASSTPGGEKETSATQRSSVPSSTEKNAVSMTSSV
MM  MTPGIRAPFFLLLLLASLKGFL-----A-LPSEENSVTSSQ---DTSS--S-LASTTTPV
    **** ::********: *. .      :  *. *:..:::*    .**  .  .* *:.*

HS  LSSHSPGSGSSTTQGQDVTLAPATEPASGSAATWGQDVTSVPVTRPALGSTTPPAHDVTS
MM  HSSNS-----------D----PATRPPGDSTSSPVQSSTSSPATRAPEDSTSTAVLSGTS
     **:*           *    ***.*...*:::  *. ** *.**.. .**:... . **

HS  APDN-KPAPGSTAPPAHGVTSAPDTRPAPGS-TAPPAHGVTSAPDTRPAPGSTAPPAHGV
MM  SPATTAPVNSASSPVAHGDTSSPATSLSKDSNSSPVVHSGTSS-----APATTAP-----
    :* .  *. .:::* *** **:* *  : .* ::* .*. **:     **.:***

HS  TSAPDTRPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAHGV
MM  --------VDSTSSPVVHGGTSSPATSPPGDSTSSPDHSSTSSPATRAPEDSTSTAVLSG
          . .:::* .** **:* * *. .**:.* *. **:* **.. .**:... .

HS  TSAPDT-RPAPGSTAPPAHGVTSAPDTRPAPG-STAPPAHGVTSAPDTRPAPGSTAPPAH
MM  TSSPATTAPVDSTSSPVAHDDTSSPATSLSEDSASSPVAHGGTSSPATSPLRDSTSSPVH
    **:* *  *. .:::* **. **:* *  : . :::* *** **:* * *  .**:.*.*

HS  GVTSAPDTRPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAH
MM  SSASIQNIKTTSDLASTPDHNGTSVTTTSSALGSATSPDHSGTSTTTNSSESVLATTPVY
    . :*  : :.:.. ::.* *. **.. * .* **::.* *. **:. . . .  ::.*.:

HS  GVTSAPDTRPAPGSTAPPAHGVTSAPDNRPALGSTAPPVHNVTSASGSASGSASTLVHNG
MM  SSMPFSTTKVTSGSAIIPDH-N----------GSSVLPT----S---SVLGSATSLVYN-
    .  . . *: :.**:  * *           **:. *.    *   *. ***::**:*

HS  TSARATTTPASKSTPFSIPSHHSDTPTTLASHSTKTDASSTHHSTVPPLTSSNHSTSPQL
MM  TSAIATT-PVSNGTQPSVPSQYPVSPTMATTSSHSTIASSSYYSTVPFSTFSSNS-SPQL
    *** *** *.*:.*  *:**::. :**  :: * .* ***:::****  * *.:* ****

HS  STGVSFFFLSFHISNLQFNSSLEDPSTDYYQELQRDISEMFLQIYKQGGFLGLSNIKFRP
MM  SVGVSFFFLSFYIQNHPFNSSLEDPSSNYYQELKRNISGLFLQIFN-GDFLGISSIKFRS
    *.*********:*.*  *********::*****:*:** :****:: *.***:*.****.

HS  GSVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAGV
MM  GSVVVESTVVFREGTFSASDVKSQLIQHKKEADD-YNLTISEVKVNEMQFPPSAQSRPGV
    *****: *:.*****:.. **::*: *:*.** . ******:*.*.::  ** **** .**

HS  PGWGIALLVLVCVLVALAIVYLIALAVCQCRRKNYGQLDIFPARDTYHPMSEYPTYHTHG
MM  PGWGIALLVLVCILVALAIVYFLALAVCQCRRKSYGQLDIFPTQDTYHPMSEYPTYHTHG
    ***********:********::**********.********::****************

HS  RYVPPSSTDRSPYEKVSAGNGGSSLSYTNPAVAATSANL
MM  RYVPPGSTKRSPYEEVSAGNGSSSLSYTNPAVVTTSANL
    *****.**.*****:******.**********.:*****.
```

Figure 3.1: Protein sequence comparison of human MUC1 and murine Muc1.
for legend see next page

Past experience has shown that recombinant MUC1 vaccinia virus (VV-MUC1) or by MUC1 cDNA vaccination MUC1-specific immune responses could be protected against tumour challenge (Acres et al., 1993); (Graham et al., 1995) We therefore used this approach to generate MUC1 specific T cell clones. The first part of this chapter describes the tools used to derive the MUC1 specific T cell clones, including immunogens used for immunisation of the mice and the MUC1 expressing tumour cells used for restimulation of the CTL bulk cultures. This is followed by the analysis of the CTL cultures for MUC1 specificity. In the following chapters these CTL will be further characterised (Chapter 5) and adoptive transfer experiments of these MUC1 specific CTL into MUC1 transgenic mice to confer tumour protection without inducing autoimmunity will be described in Chapter 4.

Figure 3.1: Protein sequence comparison of human MUC1 and murine Muc1.

*Figure on the privious page. Identical amino acids in both human MUC1 (HS) and murine Muc1(MM) are indicated with a * while conservative substitutions are indicated by a : or .. Gaps inserted into the sequences for alignment are shown by-. This alignment was made using the GAP programme of the GCG analysis suite*

3.2 Results

3.2.1 Immunogens

An expression vector using the house keeping β-actin promoter to drive expression of the full length human MUC1 cDNA with 24 TRs (pMUC1) that expresses constitutive high levels of MUC1 mucin in most mammalian cells was available. This construct has previously been used for MUC1 cDNA vaccination in C57BL/10 mice to confer immunity against tumour challenge with RMA-MUC1 but did not protect mice against the control transfectant RMA-Hygro (Graham et al., 1996). The relevant vector control plasmid identical to pMUC1, but lacking the MUC1 cDNA insert will be referred to as pVO[1].

A recombinant vaccinia virus encoding human MUC1, estimated to express three TRs, as well the human IL-2 cDNA (VV-MUC1) was kindly provided by Bruce Acres, TRANSGENE. This virus can confer MUC1-specific tumour protection in syngeneic tumour models in DBA-2 and Balb/c mice, but no CTL were detected *in vitro* (Acres et al., 1993; Balloul et al., 1994; Bizouarne et al., 1996). In C57BL/6 mice CTL could be induced following two injections with $2x10^8$ pfu of VV-MUC1 and one week of *in vitro* restimulation (Bruce Acres, personal communication). A viral stock was prepared according to standard techniques, titered and stored for further use(for details see Materials and Methods).

3.2.2 Tumour cell lines

The mouse tumour cell line RMA is a Rauscher Murine Leukaemia virus (MuLV) induced thymoma, syngeneic to C57BL/6 mice. It expresses high levels of the MHC class I alleles H-2 K^b and D^b (Figure 3.2) and no detectable levels of MHC class II (data not shown) on the cell surface.

[1] For details on all plasmid vectors used in this study please refer to the appendix: List of DNA vectors.

Permanent transfectants of RMA expressing the human MUC1 cDNA, called RMA-MUC1, and control transfectant expressing only the hygromycin B drug resistance gene, called RMA-hygro were available in the lab (Graham et al., 1996; Graham et al., 1995). RMA-MUC1 expresses high levels of human MUC1 protein that is not detectable on RMA-Hygro as shown by staining with the MUC1-specific mAb 12C10 (Figure 3.2).

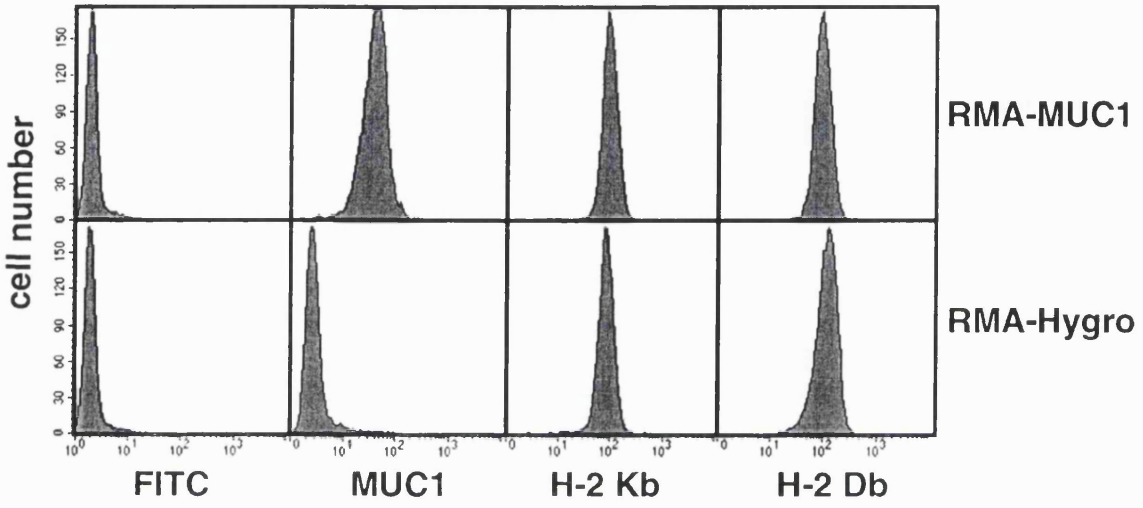


Figure 3.2: High expression levels of MUC1 mucin on RMA-MUC1.

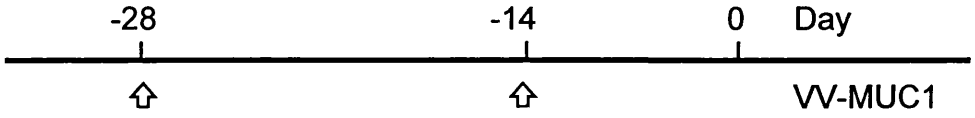
RMA-MUC1 and control transfectant RMA-Hygro were stained with MUC1-specific mAb 12C10, and mAbs against mouse MHC class I alleles H-2 K^b and D^b. Background staining with secondary FITC labelled rabbit anti mouse mAb alone is shown (FITC). Both RMA-MUC1 and RMA-Hygro endogenously express high levels of MHC class I; RMA-MUC1 also expresses high levels of MUC1 protein.

3.2.3 Immunisation protocols

To generate MUC1-specific splenocyte bulk cultures female C57BL/6 mice aged 6-8 weeks were immunised in groups of 10 mice with one of the following immunisation protocols:

Vaccinia immunisation (VV-MUC1)

Mice were immunised twice with rec. Vaccinia virus encoding MUC1 and IL-2.

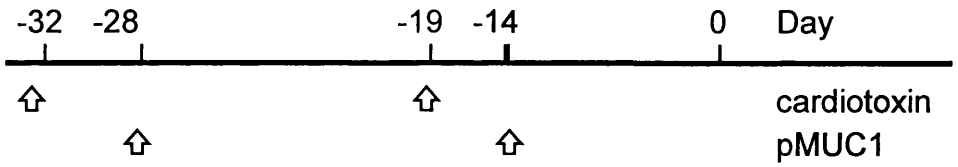


Day –28: Mice were injected with 1 x10^8 pfu of VV-MUC1 in 200µl 10mM Tris pH8 i.p.

Day –14: To boost the immune response mice were injected for a second time with VV-MUC1 by the same protocol.

MUC1 cDNA vaccination (pMUC1 or pVO)

Mice were pre-treated by the injection with cardiotoxin i.m. before plasmid DNA vaccination. Cardiotoxin damages the myofibres and increases DNA uptake while during the regeneration phase (Davis et al., 1993).



Day –32: Mice were pre-treated with 75µl of 10mM cardiotoxin i.m. in the hind leg 5 days prior to cDNA administration

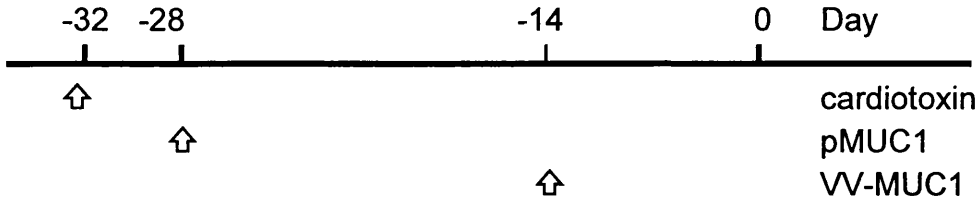
Day –28: Immunisation with 100µg of full length MUC1 cDNA (pMUC1) or vector control (pVO) in PBS at the same site as the cardiotoxin

Day –19: same as Day –32

Day –14: same as day –28

Prime-boost protocol (pMUC1:VV-MUC1)

Mice were primed by cDNA vaccination followed by a boost immunisation with VV-MUC1.



Day –32: For cDNA vaccination, mice were pre-treated with 75µl of 10mM Cardiotoxin i.m. (5 days prior to plasmid DNA vaccination)

Day –28: Immunisation with 100µg of pMUC1 full length MUC1 cDNA in PBS

Day –14: Mice were immunisation with 1 $x10^8$ pfu of rec. Vaccinia MUC1/IL-2 in 200µl 10mM Tris pH8 i.p.

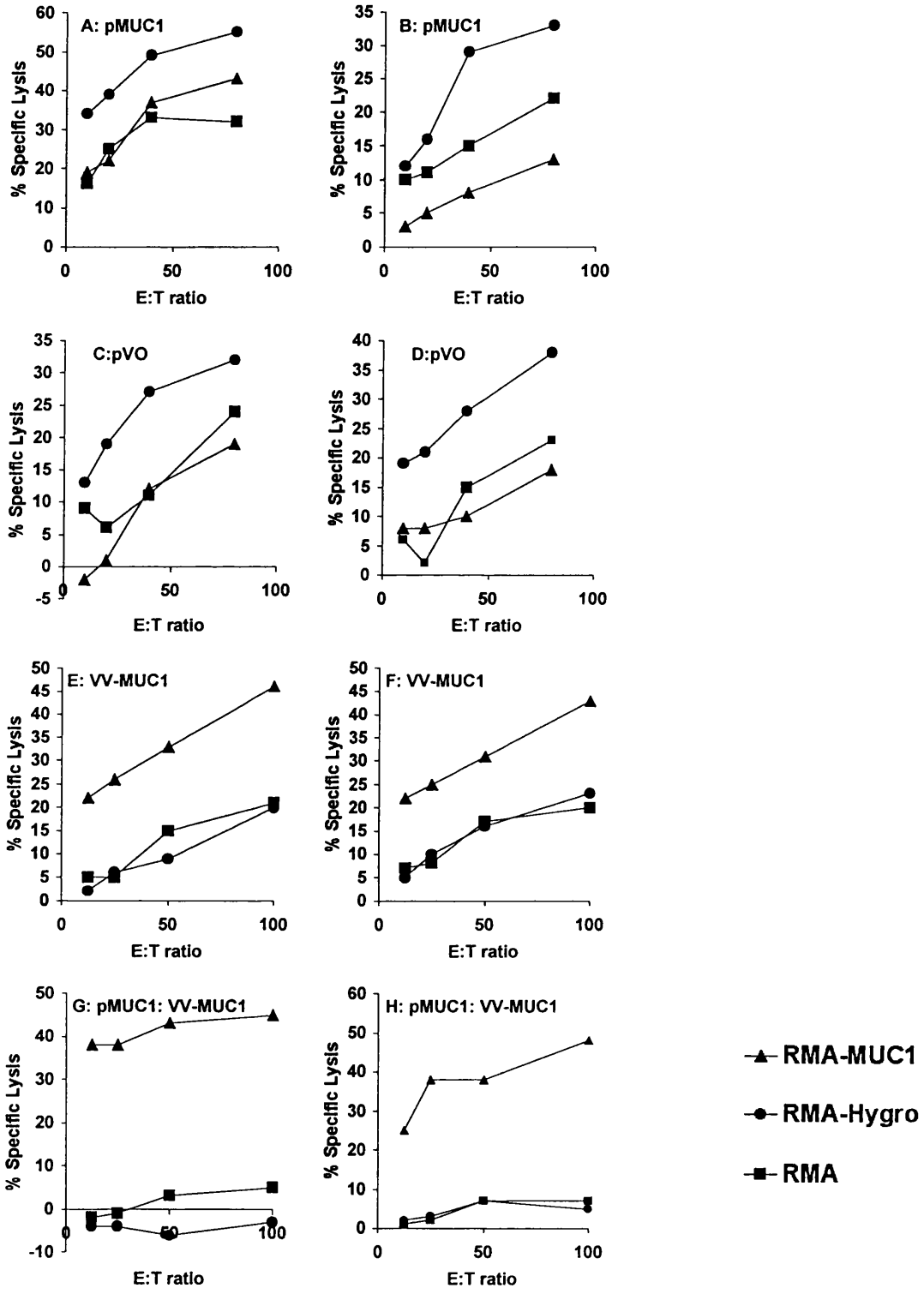
On day 0 (14 days after the last immunisation) single cell splenocyte suspensions were made and restimulated with Mitomycine C treated RMA-MUC1 cells as described in detail in Material and Methods.

3.2.4 Primary CTL bulks

After one week of *in vitro* restimulation, CTL assays were performed to test the bulk cultures for reactivity against MUC1 expressing tumour cells. The remaining cells were restimulated weekly with Mitomycin C treated RMA-MUC1 tumour cells and kept in culture for further analysis and generation of CTL clones and lines (see Material and Methods) . In Figure 3.3 a standard ^{51}Cr release assay is shown with representative CTL bulks generated by the different immunisation protocols.

Figure 3.3/4. Induction of MUC1-specific CTL

Mice were immunised and boosted with MUC1 cDNA (A,B), Vector control DNA (C,D) or primed with MUC1 cDNA and boosted with $2x10^8$ pfu of VV-MUC1 (G,H) or immunised with vaccinia alone (E,F). 14 days after the last immunisation splenocytes were restimulated for one week with irradiated Mitomycine C treated RMA-MUC1 cells, passed over a Ficoll gradient and analysed in a 6h ^{51}Cr release assay. Only mice immunised with VV-MUC1 alone or after priming with MUC1 cDNA showed specific lysis of RMA-MUC1 greater than of control transfectant RMA-Hygro.



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Immunisation protocols with cDNA alone do not induce strong MUC1-specific CTL responses that are able to lyse RMA-MUC1 cells to a greater extent than RMA-Hygro or RMA. Importantly, mice immunised with the rec. Vaccinia alone (Figure 3.3,F) or by the prime/boost protocol with MUC1 cDNA followed by VV-MUC1 (Figure 3.3,E) show specific lysis of RMA-MUC1 that is significantly greater than lysis of the control transfectants. The prime/boost protocol appeared to give the best results and therefore was chosen for further generation of CTL bulks.

3.2.5 Confirmation of MUC1-specificity of CTL

CTL produce TNFα and IFNγ upon specific recognition of MHC peptide complexes. Most CTL lines cultured *in vitro* will produce these cytokines after recognition of specific antigen, which can be used as a very sensitive read out for CTL reactivity. However, since fresh splenocyte cultures produce large amounts of these cytokines short-term lines (3-4 weeks) have to be established before this technique can be used. To define the MHC restriction element of the MUC1-specific CTL and to further map the epitope, TNFα secretion was used as a read out of CTL activation. TNFα released into the culture supernatant of MUC1-specific CTL lines was quantified by a bio-assay using a mouse fibrosarcoma cell line sensitive to TNFα (WeHi-164 clone 13 cells) (Traversari et al., 1992). WeHi cell death after o/n culture in the presence of TNFα containing CTL culture supernatant was measured and recorded as %specific WeHi death. Figure 3.5 shows a TNFα release assay of two MUC1 CTL lines stimulated with several different targets. MUC1 CTL lines 1 and 3 co-cultured with RMA-MUC1 produce high levels of TNFα, whereas control transfectant RMA-Hygro or RMA was not recognised. These results are in line with the cytotoxicity data presented in (Figure 3.3). Both, direct cytotoxicity as measured by ^{51}Cr release and TNFα release show MUC1-specificity of the CTL lines bulk cultures.

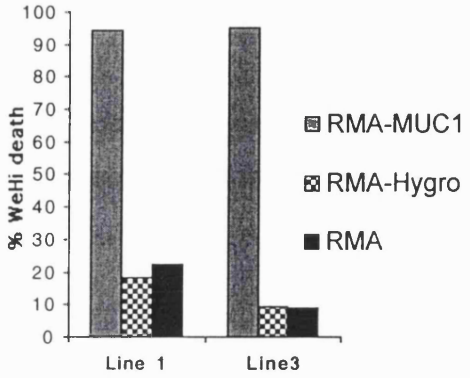


Figure 3.5: TNFα release assay of two independent CTL lines raised against RMA-MUC1.

CTL were incubated with target cells overnight and culture supernatants were harvested. TNFα production was assayed using the TNFα sensitive WeHi-164 cell line. WeHi death was measured using a standard MTT viability assay after overnight exposure to the supernatants and % specific WeHi death calculated as a measure of CTL activity (see methods for details).

3.2.6 Identification of the MHC restriction element of the MUC1-specific CTL

Cytokine release assays have the advantage over standard cytotoxicity assays in that they do not require a large number of target cells. Small number of target cells can easily be transfected in 96 flat bottom well plates with a number of different cDNA constructs and tested for recognition of the MUC1-specific CTL. Since the MUC1-specific CTL lines produce TNFα upon specific stimulation, the TNFα release assay was used to define the MHC restriction element and to confirm MUC1-specificity. For this, the monkey kidney cell line COS7 which expresses neither human MUC1 nor MHC H-2^b alleles, was transiently transfected with full length MUC1 cDNA (pMUC1) in combination with cDNAs for the restriction elements K^b and D^b. Three days after transfection, the cells were washed in the wells and 1500 CTL were added per well. Then supernatants were harvested and analysed in a standard TNFα Figure 3.6).

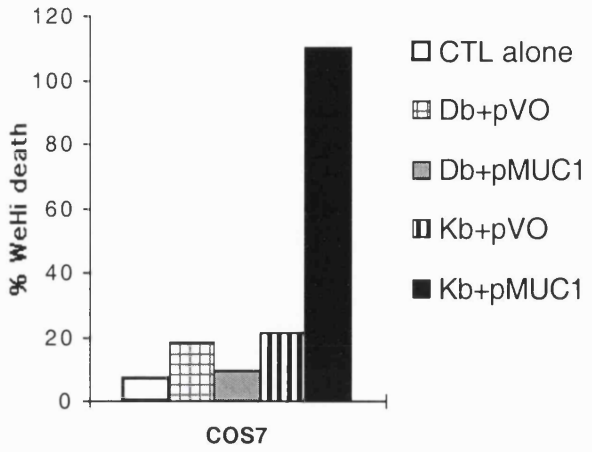


Figure 3.5: MUC1 CTL are MHC H-2 K^b restricted.

MUC1 CTL recognise COS7 cells transiently transfected with pMUC1 in the context of MHC H-2 K^b. COS7 cells were transiently transfected with full length MUC1 cDNA (pMUC1) or vector control (pVO) in combination with the murine MHC class I alleles K^b or D^b. The background TNFα production of MUC1 CTL alone is shown.

COS7 cells transfected with the empty vector (pVO) in combination with either MHC K^b or D^b cDNA were not recognised by the CTL lines while COS7 cells transfected with the vector encoding full length MUC1 cDNA (pMUC1) were recognised in combination with K^b. This strongly suggests that the CTL lines recognise MUC1 in the context of MHC H-2 K^b.

3.2.7 MUC1 CTL recognise endogenously expressed human MUC1

Target cells either permanently (RMA-MUC1) or transiently transfected (COS7) with the full length MUC1 cDNA are recognised. To show that the MUC1-specific CTL cross react with endogenously expressed and processed MUC1, human breast carcinoma cell lines MCF7 and T47D, as well as the cervical carcinoma line HeLa were used. These cells express MUC1 endogenously as shown in

Figure 3.8. HeLa, MCF7 and T47D were transiently transfected with the mouse MHC H-2 K^b cDNA alone, or in combination with pMUC1 and used as targets in a TNFα release assay (Figure 3.6). All three cell lines were recognised by the CTL

line. Super-transfection with a MUC1 plasmid increased the TNFα release in the case of MCF7 that had lower endogenous MUC1 expression by FACS analysis .

To show that the MUC1 expression level in these cells were high enough to sensitise them for recognition by the MUC1-specific CTL lines in a standard ^{51}Cr release assay, a permanent transfectant of HeLa with the mouse MHC H-2 K^b cDNA (HeLa K^b) was used. In a CTL assay HeLa-K^b is lysed (Figure 3.7) while the control transfectant HeLa-D^b is not. This indicates that the epitope is processed in sufficient amounts both in mouse and human tumour cell lines to lead to target cell sensitisation

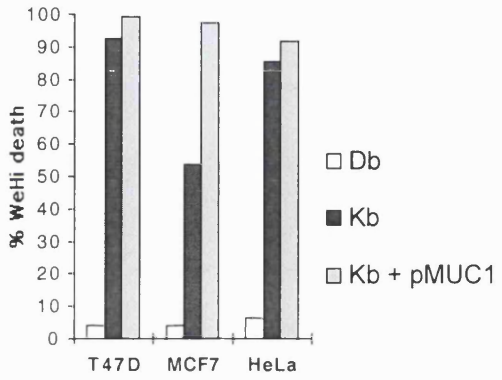


Figure 3.6: Recognition of endogenously expressed MUC1

Recognition of endogenous MUC1 expressed in T47D, MCF7 and HeLa after transient transfection with H-2 K^b in a WeHi assay by MUC1-specific CTL lines (week 4).

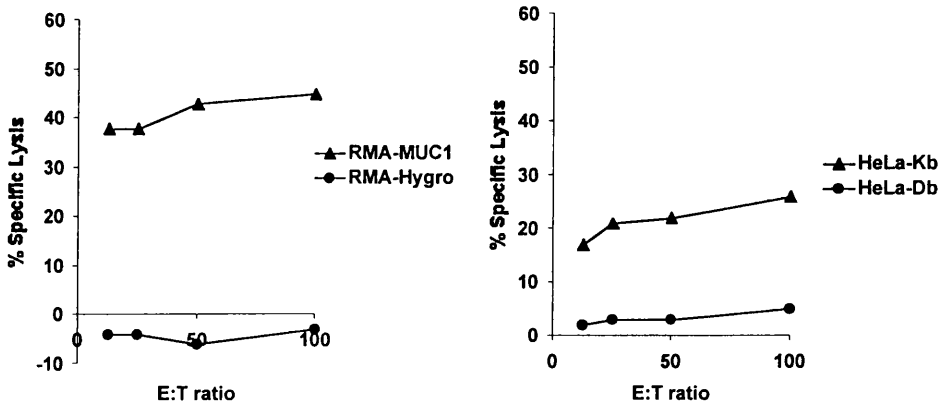


Figure 3.8: Lysis of human carcinoma cell line.

MUC1-specific CTL bulk lyses target cells expressing endogenously expressed MUC1 HeLa-K^b, control transfectant HeLa-D^b and were tested for recognition by CTL bulks in a Cr^{51} release assay. For comparison lysis of RMA-MUC1 and HeLa-K^b in the same experiment are shown in adjacent panels.

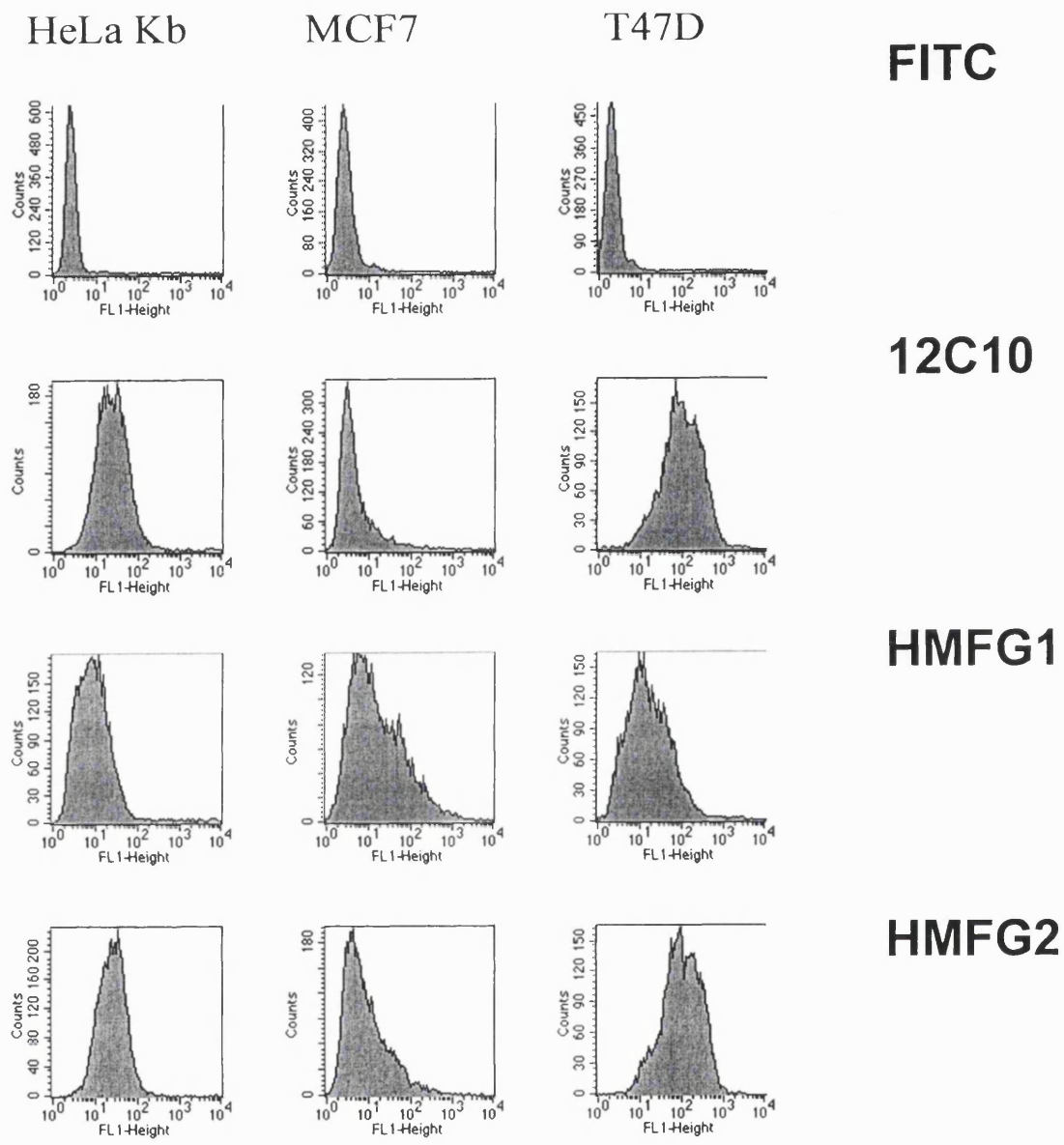


Figure 3.9: HeLa, MCF7 and T47D express MUC1 mucin.

HeLa-K^b, MCF7 and T47D were harvested and stained with MUC1-specific mAb hybridoma supernatants. A secondary rat anti mouse FITC mAb was used for detection.

3.2.8 Phenotypic analysis of CTL Lines

The MUC1-specific CTL lines are MHC class I restricted suggesting that they were classical CD8, alpha/beta TCR expressing T lymphocytes. Once several lines and clones had been established, expression of these surface markers was checked by FACS analysis. This confirmed that phenotypically the MUC1-specific CTL are CD3 and CD8 positive and express the TCR α and β chains (see Figure 3.10).

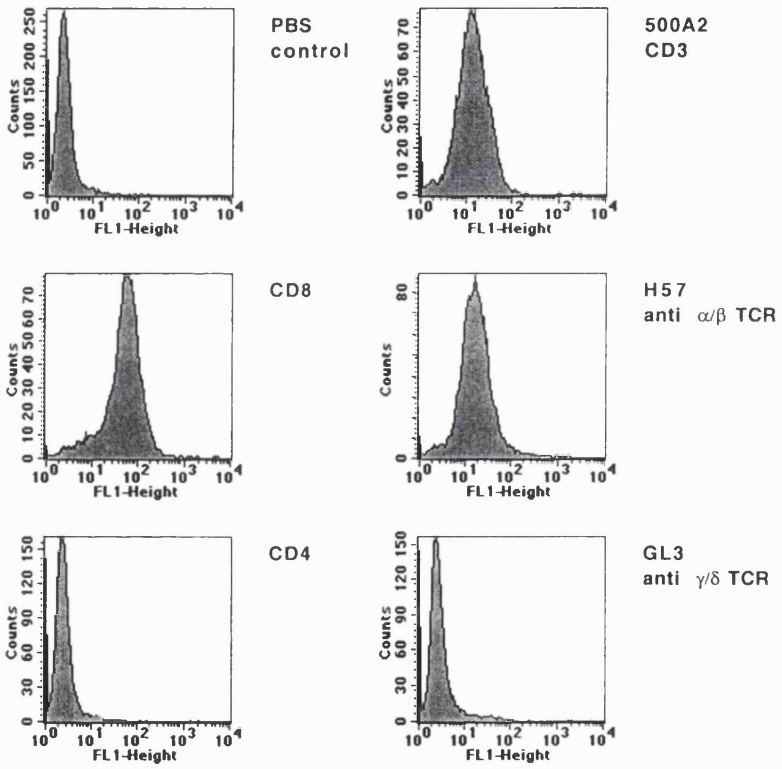


Figure 3.10: Phenotypic analysis of MUC1-specific CTL.

Resting MUC1-specific CTL L8 cells were stained on day 7 after the last restimulation with FITC conjugated mAb against CD8, CD4, CD3, αβTCR, and γδTCR. The MUC1-specific CTL are $CD8^+$, $CD3^+$ and also express TCR α and β chains.

3.2.9 In summary:

The data presented in this chapter demonstrates that MUC1-specific MHC H-2 K^b restricted CTL can be induced by immunisation with full length MUC1 cDNA in combination with rec. VV-MUC1 or VV-MUC1 alone. The CTL recognise both murine and human targets expressing MUC1 protein in the context of H-2 K^b in a chromium release and in a TNFα release assays. Several bulks that showed MUC1-specificity were selected and kept in culture for the generation of CTL lines and clones. Unless otherwise indicated any further data presented was generated using a CTL line designated L8 that was derived from a mouse immunised with MUC1 cDNA and boosted with VV-MUC1.

3.3 Discussion

For the induction of MUC1-specific CTL responses three different immunisation protocols were used. While cDNA vaccination combined with VV-MUC1 gave the best results, VV-MUC1 alone was able to generate bulks that showed convincing MUC1-specific cytotoxicity in *in vitro* assays. However, cDNA vaccination alone could not generate MUC1-specific responses that were detectable *in vitro* after one week of restimulation. This is consistent with the previous findings that MUC1 cDNA vaccination could partially protect mice from tumour challenge, but did not induce CTL responses that were detectable *in vitro* after one round of restimulation. Mice that survived the tumour challenge with RMA-MUC1 showed strong CTL responses *in vitro* (Graham et al., 1996). This can be explained by cDNA vaccination successfully priming a CTL response *in vivo* that requires a second immunisation or booster to lead to detectable responses *in vitro*. In the case of the tumour challenge experiments by Graham et al. the live tumour cells provided the second immunisation. To activate naïve T cells, foreign antigens must traffic from the site of immunisation to the draining lymph nodes, where they can be presented by professional APC via the cross-presentation pathway (Kurts et al., 1996). A recent study has shown that the level of antigen expressed by the peripheral tissues must be relatively high for cross-presentation

to naïve $CD8^+$ T cells to occur (Kurts et al., 1998). This is consistent in that DNA vaccination only leads to expression of the antigen in a relatively small number of cells while VV-MUC1 provides larger amounts of antigen.

cDNA vaccination alone, both with MUC1 cDNA (pMUC1) or empty vector control (pVO) generated CTL bulk cultures that preferentially lyse the control transfectant RMA-hygro over both RMA or RMA-MUC1. This unexpected finding can be explained in several ways. These bulks may show some specific reactivity for epitopes encoded by cryptic open reading frames in the plasmid vector backbone present in the vectors used for immunisation and to transfect RMA-MUC1 and RMA-Hygro. Recently a cryptic open reading frame in the ampicillin resistance gene has been found to encode a highly immunogenic CTL epitope that could induce cytotoxic T-lymphocyte epitopes (van Hall et al., 1998). RMA-MUC1 and RMA-hygro both to express this particular epitope and are sensitive to lysis by CTL directed against it, while the untransfected parental cell line RMA was not lysed (data not shown).

The preferential lysis of the RMA-Hygro over RMA-MUC1 which both express the same vector derived sequences can be explained by reduced susceptibility of MUC1 expressing targets to lysis by CTL in general. This could be due to stearic effects of MUC1 on the cell surface which is thought to protrude some 500 nm off the cell surface. Furthermore, MUC1 is strongly negatively charged and can inhibit cell-cell interactions aspecifically. It has previously been shown that melanoma cells could be rendered less susceptible to lysis by IL-2 activated killer cells (LAK) or allospecific T cells upon transfection with MUC1 (van de Wiel-van Kemenade et al., 1993).

Importantly, strong MUC1-specific CTL responses can be induced by immunisation with VV-MUC1 that are detectable at an early bulk level. VV-MUC1 had previously been shown to induce MUC1-specific immunity by protecting Balb/c mice from syngeneic tumour challenge, but without any CTL being observed *in vitro* assays (Acres et al., 1993; Balloul et al., 1994; Bizouarne et al., 1996). In C57Bl/6 mice CTL could be induced following two injections with $2x10^8$ pfu of VV-MUC1 (Bruce Acres personal communication). Consistent with these finding mice

immunised twice with rec. VV-MUC1 mount MUC1-specific CTL responses (Figure 3.3,E,F). However the heterologous prime/boost protocol using first MUC1 cDNA, followed by recombinant vaccinia virus consistently gave bulks with the greatest MUC1-specific lysis (Figure 3.3,G,H) that were maintained more easily in long term culture. Homologous prime/boost immunisations in which either cDNA or vaccinia virus are used for both immunisations probably induce vector specific responses or neutralising Ab, respectively, thus limiting the effectiveness of the immunisation. Initial priming with cDNA presumably delivers antigen in a very immunogenic context, in that it provides for activation of the DC involved in cross priming. Since the activation state of the APC is crucial to the outcome of the antigen presentation it is interesting that bacterial DNA contains CpG motifs that have been shown to be strongly immunogenic, while DNA that lacks these sequences can fail to induce immune responses (Sato et al., 1996). This may be the molecular basis for bacterial DNA itself functioning as a 'danger signal' able to activate professional APC (Matzinger, 1994). Furthermore, CpG containing DNA activates the innate arm of the immune system by inducing NK cells to produce IFNγ and macrophages to produce IL12 (Chu et al., 1999).

The population of MUC1-specific CTL primed by the DNA vaccination can then be expanded by immunisation with VV-MUC1 that produces a relatively large amount of MUC1 antigen but not other plasmid backbone derived sequences present in the priming phase. Similar results have been obtained in parasitic and HIV models where prime boost protocol work best for response induction in pre-clinical models (Hanke et al., 1998; Plebanski et al., 1998). For HIV similar approaches are being used in clinical trials (Hanke et al., 1999) with similar effects.

In the light of unrestricted CTL responses that have been described in humans (Jerome et al., 1991; Magarian-Blander et al., 1998) it is important to note that the CTL lines described here are classically MHC class I restricted and express TCR α and β chains in conjunction with CD8.

The specificity of the CTL lines and clones was further confirmed by transient transfection studies showing, that both H-2K^b and endogenously expressed or transiently transfected MUC1 on the target cells are required for recognition by the

MUC1-specific CTL. Human tumour cell lines that express MUC1 endogenously are recognised both in TNFα release assays as well as ^{51}Cr release assays when transfected with H-2 K^b (Figure 3.7 Figure 3.8).

Chapter 4: *In vivo* analysis of MUC1-specific CTL lines in wild type and MUC1 transgenic mice

4.1 Introduction

As discussed in the introduction, most tumour-antigens are tumour-selective rather than entirely tumour-specific, since their physiological expression is often also detected in non transformed tissues. Tumour antigens can be divided into three groups: Firstly, tumour specific antigens that are only expressed in tumours, but not in healthy tissue, with few exceptions like the testis. This group includes the MAGE family of genes. The second group includes cell lineage specific antigens that have been used as tumour antigens like the melanoma associated antigens tyrosinase, gp100 and MART. These are not only expressed in melanomas but also in normal melanocytes. The third group of antigens are merely tumour selective an example of which is wildtype p53 as it is over expressed in 50% of all malignancies but also expressed at a low level in nearly all nucleated cells. In the case of MUC1, expression is detectable in a number of normal epithelial tissues, while adenocarcinomas of the breast and several tumour cell lines, derived from these tissues show upregulation of expression. Tumour specificity thus relies on the over expression of MUC1 by the tumour, since normal epithelial tissues expressing MUC1 at low levels may also be accessible to MUC1 specific lymphocytes. Therefore there is a possible risk that autoimmune disease may occur, once CTLs are activated for therapeutic purposes. To evaluate whether MUC1 specific CTL can differentiate between MUC1 over-expressing tumours and normal epithelial tissues without autoimmune damage to healthy tissues is relevant if one is to use MUC1 as a tumour antigen in a clinical setting. Having established MUC1 specific CTL as described in the previous chapter, adoptive transfer of these CTL into an animal which expresses human MUC1 in its normal tissue distribution, allows one to assess tumour specificity and potential for auto immunity induction using defined effector cells.

T-lymphocytes have been used in adoptive transfer experiments in a number of different model systems since the early 1980's (Greenberg and Cheever, 1984; Greenberg et al., 1981). Work by Greenberg and his lab showed that adoptively transferred immune cells could protect mice against subsequent viral or even tumour challenge if IL-2 was co-administered to allow the CTL to survive *in vivo* .

The aim of the work described in this chapter was to convincingly address whether adoptive transfer of well defined CD8+ $\alpha\beta^+$, MHC class I restricted MUC1-specific CTL into MUC1 transgenic mice could prevent tumour growth without autoimmune destruction of MUC1 expressing tissues.

4.2 Results

4.2.1 The model system:

The Rauscher Murine Leukaemia virus (MuLV) transformed thymoma RMA was chosen as a tumour model for several reasons:

- RMA is a syngeneic tumour of C57BL/6 mice that grows rapidly when given i.p. and has a good reproducible tumour take in C57BL/6 wild type mice as well as in MUC1 transgenic animals.
- RMA expresses the Rauscher Murine Leukaemia virus genome. The MuLV model is widely used to develop strategies to induce immunity against (virally induced) tumour. As part of this work several CTL and T-helper epitopes are known. In our collaborators lab (C. Melief, Leiden) a CTL clone directed against the H-2 D^b restricted gag leader epitope with the sequence CCLCLTVFL (MuLV CTL) was available.
- It has previously been shown that adoptive transfer of MuLV specific CTL could protect nude mice against subsequent tumour challenge with RMA. In these experiments 10^7 CTL were given i.v. while 10^3 RMA cells were given i.p. on the same day, so that the CTL were required to home to the tumour site.
- *In vitro* cytotoxicity assays had shown, that RMA-MUC1 was lysed by MuLV CTL to a similar extend as untransfected RMA, therefore this CTL clone could be used as a positive control in the adoptive transfer experiments since they were expected to clear RMA-MUC1 tumour cells without auto immune side effects on MUC1 expressing tissues.

The adoptive transfer experiments were set up in a Winn type assay: CTL were given i.v. while the tumour cells were injected at the same time i.p. For tumour clearance the CTL not only have to overcome the regulatory mechanisms of the host immune system but also have to home to the tumour site in the peritoneal cavity. As discussed in the introduction, memory or effector CTL are able to home to peripheral tissues. Therefore one can assume that CTL that are able to home to, and clear the tumour cells would also be able to reach the epithelial tissues in

these mice, where they could potentially induce autoimmune destruction when given to MUC1 transgenic mice. Therefore, tumour clearance was used as a read out for effective CTL *in vivo.*

4.2.2 Adoptive transfer experiment in nu/nu mice

To assess the *in vivo* functionality of the MUC1-specific CTL Line (MUC1 CTL L8) that was described in the previous chapter a Winn type assay set up to see if MUC1-CTL could eradicate RMA-MUC1 tumour cells in nude mice. For the initial experiments C57BL/6 nu/nu mice were chosen as these mice lack a functional T cell compartment and suppressive effects of the host immune system on the adoptively transferred CTL were expected to me minimal.

Figure 4.1 shows two representative adoptive transfer experiments in nude mice. In both experiments 80% of the mice were protected against RMA-MUC1 tumour challenge, when adoptively transferred with either the MUC1 CTL or MuLV CTL. Mice that received a RMA tumour challenge and the MUC1-CTL were not protected, confirming that the MUC1-CTL are antigen specific. These experiments clearly show that the MUC1-CTL L8 is functional *in vivo* and can confer MUC1-specific tumour protection in this model.

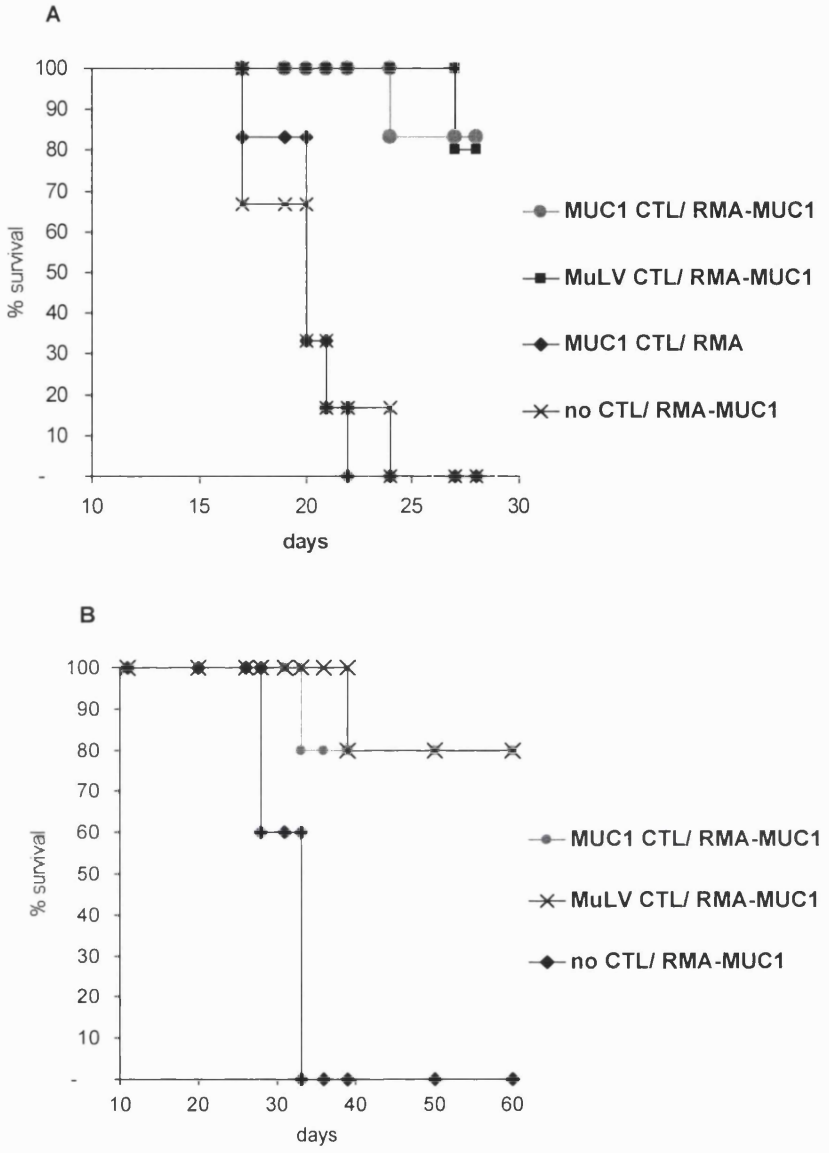


Figure 4.1: Protection of C57BL/6 nu/nu mice against RMA-MUC1 tumour challenge by adoptive transfer of MuLV specific CTL and MUC1-specific CTL.

On day 0, groups of 6-8 week old C57BL/6 nu/nu mice received 10 ^{5} Cu rhu Il-2 in IFA s.c on the flank and 1-2x10^{7} CTL i.v. in 200µl PBA specific for either MUC1 (MUC1 CTL) or the Rauscher MuLV gag leader epitope (MuLV CTL) as indicated in the diagram. All mice also received 1000 RMA or RMA-MUC1 cells in 200µl PBA i.p. Mice were sacrificed when pronounced acites developed or their body weight increased by more than 15% of starting weight.

4.2.3 Adoptive transfer in C57BL/6

The next question was whether the MUC1-specific CTL could protect wild type C57BL/6 mice from tumour challenge in the presence of a functional host immune system. Figure 4.2 shows that adoptive transfer of MuLV specific CTL gave full protection in 100% of the mice. MUC1-CTL gave a 10 day delay in tumour growth and completely protected 60% of the mice against tumour challenge.

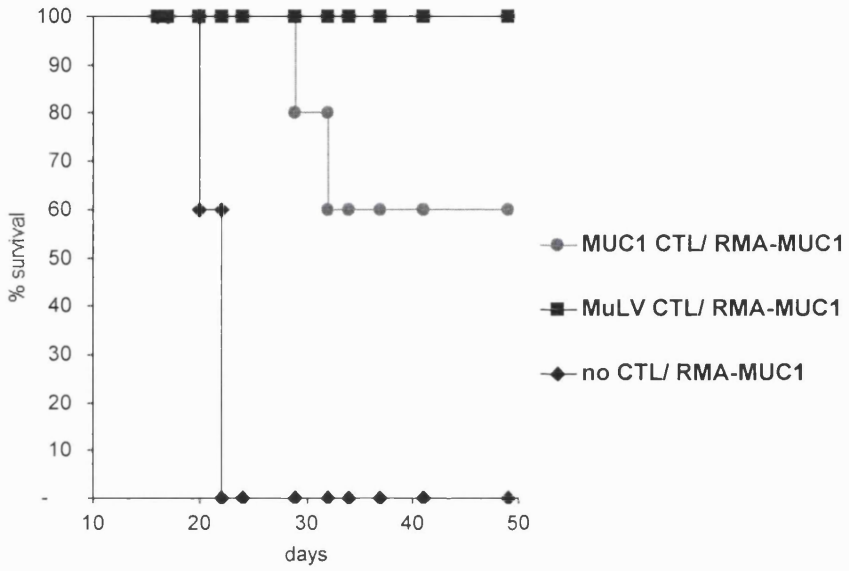


Figure 4.2: Protection of C57BL/6 wild type mice against RMA-MUC1 tumour challenge by adoptive transfer of MuLV specific CTL and MUC1-specific CTL).

On day 0, groups of 6-8 week old C57BL/6 mice received 10 ^{5} Cu rhu Il-2 in IFA s.c on the flank and 1-2x10^{7} MUC1- or MULV-specific CTL i.v. in 200µl PBA as well as 1000 RMA-MUC1 cells in 200µl PBA i.p. Mice were sacrificed when pronounced acites developed or their body weight increased by more than 15% of starting weight.

4.2.4 Adoptive transfer in MUC1 transgenic mice

Having shown that the MUC1-specific CTL could protect nude and wild type mice from tumour challenge, an adoptive transfer in MUC1 transgenic mice was carried out to observe whether effective tumour protection can be mediated by large numbers of MUC1-specific CTL without the induction of MUC1-specific auto immune responses. The MUC1 homozygote transgenic mice (SacII) were

developed in the Lab (Peat et al., 1992) and the homozygotes had an H-2^k background. Since the back cross regime into C57BL/6 was not complete at the time of these experiments F1(Sac II x C57BL/6) mice were used. These mice were expected to support growth of RMA-MUC1 cells that are derived from a C57BL/6 background.

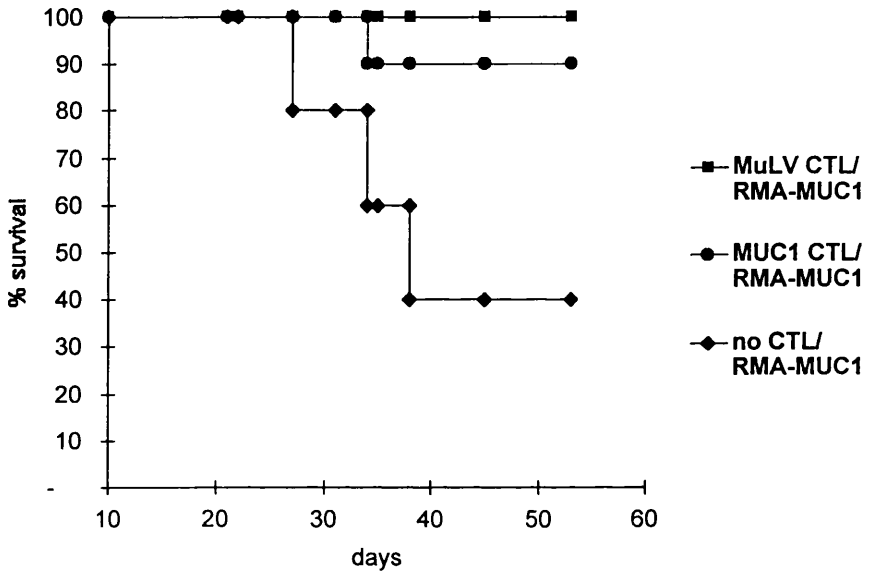


Figure 4.3: Protection of MUC1 transgenic F1(SacII x C57BL/6) mice against RMA-MUC1 tumour challenge by adoptive transfer of MuLV-specific and MUC1-specific CTL.

On day 0, groups of 6-8 week old F1(SacII x C57Bl/6) MUC1 transgenic mice received 10^5 Cu rhu Il-2 in IFA s.c on the flank and 1-2x10^7 MUC1 or MuLv CTL i.v. in 200µl PBA as well as 1000 RMA-MUC1 cells in 200µl PBA i.p. Mice were monitored as before.

Figure 4.3 shows that both MUC1 and MuLV specific CTL protect the MUC1 transgenic mice against RMA-MUC1 tumour challenge to 90% and 100% respectively. While only 60% of the mice that received no CTL developed tumours, there was a clear difference between the groups receiving CTL of either specificity, and the group that only received tumour cells alone. Since the CTL were able to home to the tumour site to control tumour growth it is likely that the CTL should also be able to reach the MUC1 expressing epithelial tissues.

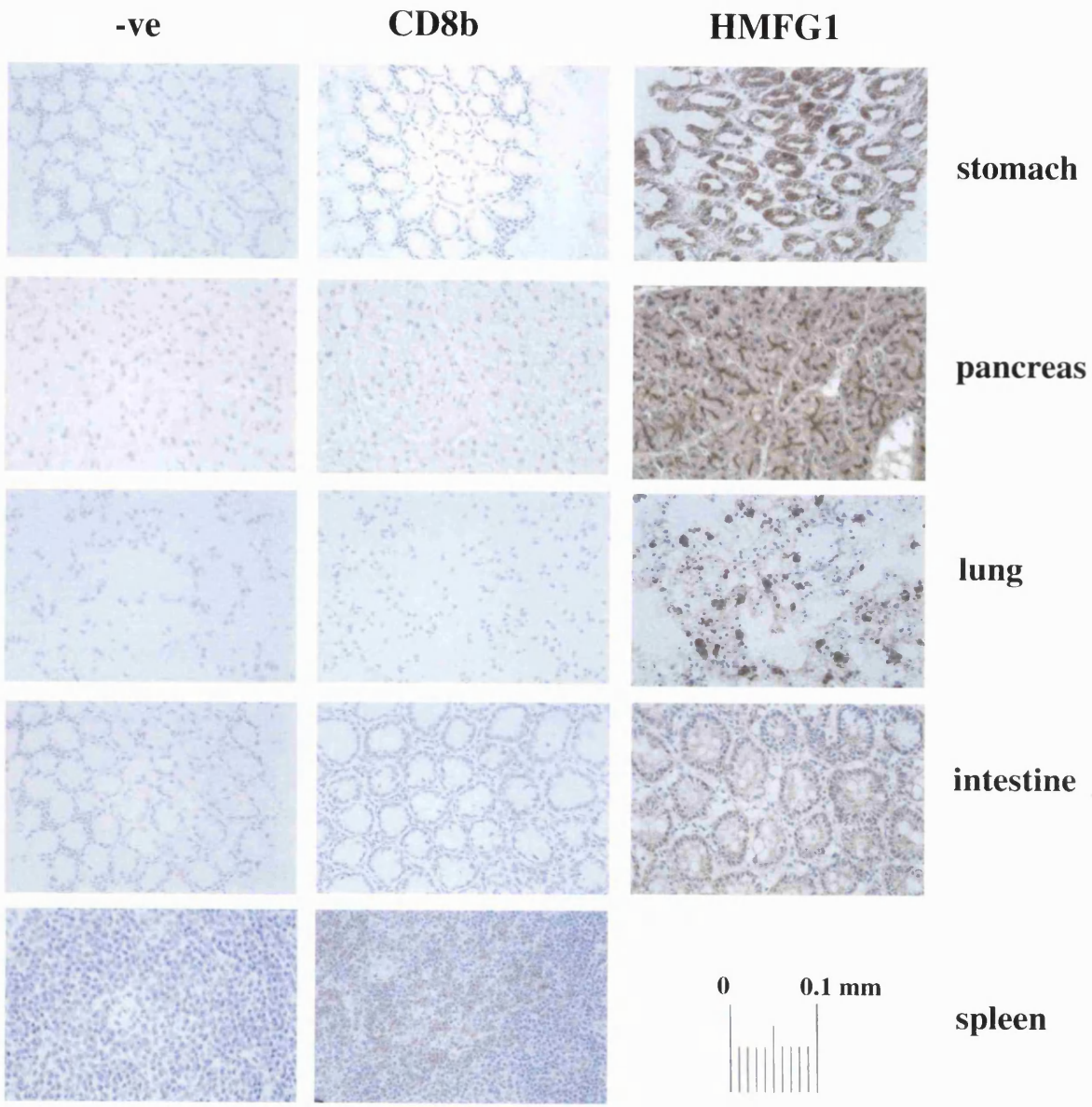


Figure 4.4: Immuno-histochemistry of MUC1 transgenic mice following adoptive transfer of MUC1 specific CTL.

Frozen sections of organs from MUC1 transgenic mice adoptively transferred with MUC1 specific CTL were stained for CD8b and MUC1 (HMFG1) after tumour rejection. Negative control staining omitting the primary antibody is shown in the left column. MUC1 staining with HMFG1 in the right column shows strong MUC1 staining of epithelial tissues. CD8b staining shows no staining in the MUC1 expressing tissues. As a positive control a section of normal spleen is shown in which 13 % of cells are expected to show positive staining with the anti CD8b antibody.

However, none of the treated mice showed any symptoms of autoimmunity. Tissue samples were taken of stomach, intestine, pancreas, lung and spleen. Frozen section were stained by H&E (data not shown) and immuno- histochemistry for $CD8b^+$ cells and MUC1 (Figure 4.4). H&E staining showed a normal histological pattern without signs of inflammation or mononuclar cell infiltrate. MUC1 immuno-histochemistry confirmed that all the mice were expressing high levels of the MUC1 transgene. The immuno-histochemistry of tissues of mice adoptively transferred with neither MuLV nor MUC1-specific CTL showed any macroscopic or microscopic immune pathology. More detailed immunohistochemical staining with anti CD8b mAb showed no infiltrate of the adoptively transferred CTL in MUC1 expressing tissues while sections of normal spleen stained strongly positive.
These findings indicate that adoptive immunotherapy with MUC1-specific CTL is feasible without the induction of MUC1-specific autoimmunity.

4.3 Discussion

In this chapter the MUC1-specific CTL were used for adoptive transfer experiments which showed that that MUC1-specific CTL L8 exhibit the same recognition pattern *in vivo* as in vitro assays, in that they were protective against RMA-MUC1 but not against control transfectant RMA. Furthermore, adoptive transfer of MUC1-specific or the MuLV-specific CTL into MUC1 transgenic mice showed that both CTL clones the can protect the mice against tumour growth in the absence of obvious macroscopic autoimmunity. In a more detailed analysis by immuno-histochemical staining of MUC1 expressing tissues from MUC1 transgenic mice that had received MUC1 specific CTL, no $CD8^+$ T-cell infiltrate was detectable. The sparing of normal epithelial cells from possible autoimmune damage can not be explained by inaccessibility of MUC1 due to luminal expression of MUC1 since MHC class I molecules are expressed on the basal membrane of normal epithelial cells and should be able to present MUC1 derived epitopes to CTL.

There is a good indication that the adoptively transferred effector CTL were able to recirculate outside the secondary lymphoid organs and had access to the MUC1 expressing tissues, since the MUC1-specific and MuLV-specific CTL that where administered intravenously, could prevent intra-peritoneal RMA-MUC1 tumours. This implies that the CTL could home to the tumour and traffic through out the animal and were also able to traffic to MUC1 expressing epithelial tissues. Therefore overexpression of MUC1 by the tumour cells, compared to the normal epithelial tissues may account for an increased antigen density on the tumour cells that allows the CTL to discriminate between the normal epithelial tissues and the tumours. These findings are consistent with the studies on wildtype murine p53 briefly discussed in the introduction: Murine p53-specific CTL which were generated in p53 KO mice could protect p53 wild type mice against a p53 over-expressing tumours, when adoptively transferred without induction of auto-immunity. Also in this case overexpression of p53 by the tumour cells was though to be sufficient to mediate tumour specificity of the CTL (Vierboom et al., 1997).

From a clinical point of view adoptive transfer of *in vitro* expanded CTL is possible and has been performed by Greenberg and co-workers (Brodie et al., 1999) but is logistically very difficult even with advanced tetramer technology to enrich for specific T cells(Yee et al., 1999). For this reason, approaches inducing tumour specific immunity in a patient by immunisation are favourable. Having shown that MUC1-specific CTL derived from wild type mouse can protect MUC1 transgenic mice against MUC1 over-expressing tumours, it is of interest whether these CTL responses can be induced in the transgenic animals. This question will be addressed in more detail in chapter 7.

Chapter 5: Mapping of the MUC1 derived CTL epitope.

5.1 Introduction

Chapter 3 has described the establishment of MHC H-2 K^b restricted, $CD8^+$ MUC1-specific CTL lines and chapter 4 showed that these CTL could protect wild type and MUC1-transgenic mice against tumour challenge when adoptively transferred. The next aim was to identify the minimal CTL epitope recognised by these CTL. Several different strategies have been developed to map and identify MHC class I restricted epitopes in the past. In this project a combination of several methods was use: Primarily, cDNA constructs encoding length variants of the MUC1 cDNA were screened for recognition by the MUC1-specific CTL to quickly map the epitope to a smaller region of the molecule. To do this, COS7 cells permanently transfected with H-2 K^b (COS7-K^b) were transiently transfected with MUC1 cDNA length variants and tested for CTL recognition. Once the epitope had been mapped to a small region of MUC1 overlapping synthetic peptides were made to determine the minimal recognised epitope.

5.2 Results

5.2.1 Schematic view of constructs

Deletion constructs of MUC1 were used to quickly map the CTL epitope to a defined region of the MUC1 cDNA. An overview of the MUC1 mucin structure and MUC1 cDNA deletion constructs is shown in Figure 5.1.

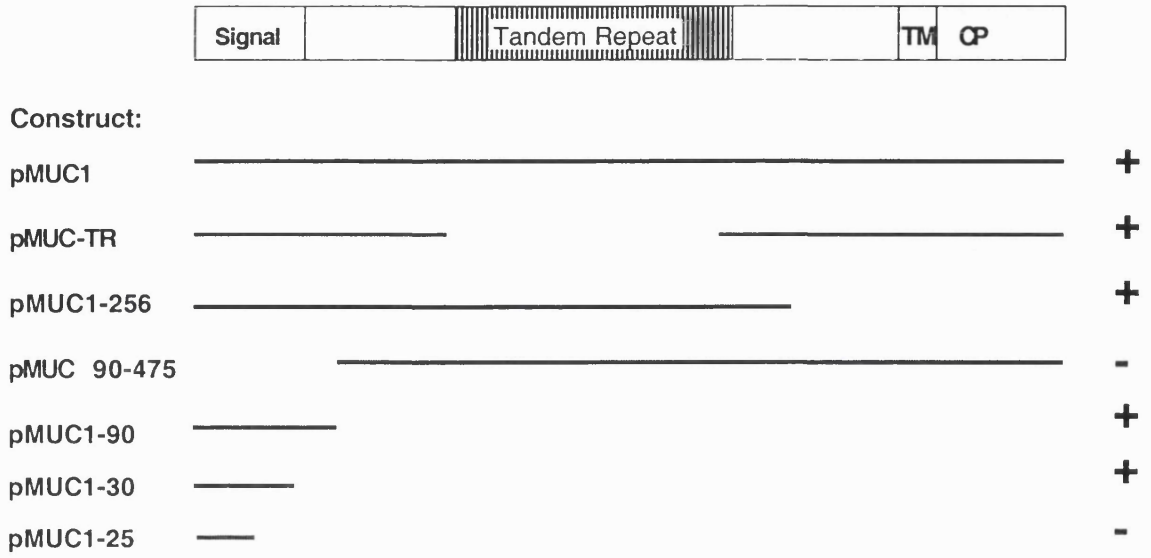


Figure 5.1: Schematic representation of MUC1 cDNA constructs.

The full length MUC1 construct (pMUC1) encodes the signal sequence, N-terminal degenerate VNTR region, 42 TR, C terminal non VNTR sequence, the transmembrane domain (TM) and the cytoplasmic domain (CP). Construct pMUC-TR lacks the tandem repeat region, while pMUC1-256 misses C-terminal degenerate VNTR region TM and CP. Construct pMUC90-475 lacks the sequence encoding the first 90 amino acids including the signal sequence. All these constructs are based on a vector under the control of the β-actin promoter. The last three constructs pMUC1-90, pMUC1-30 and pMUC1-25 are based on derivatives of pCDNA3.1 (INVITROGEN). Expression of these constructs is directed by the CMV promoter. Constructs recognised by MUC1-specific CTL are marked with a +; while those not recognised are marked with a -.

Constructs encoding full length MUC1(pMUC1) and deletion constructs pMUC-TR and pMUC1-256 were already available (Aurelia Rugatti, personal communication). All other DNA constructs were made and a comprehensive list of all the constructs and cloning strategy can be found in appendix 1.

5.2.2 Gross mapping of the MUC1 CTL epitope

Since the experiments in chapter 3 showed that the MUC1-specific CTL response was restricted by H-2 K^b, transient transfection assays with MUC1 length variant constructs into COS7-K^b was used for further mapping of the epitope. COS7-K^b cells transfected with pMUC1, pMUC-TR and pMUC1-256 are strongly

recognised by MUC1-specific CTL while only construct pMUC90-475, lacking the first 90 amino acids of the native MUC1 amino acid sequence was not recognised. (Figure 5.2).

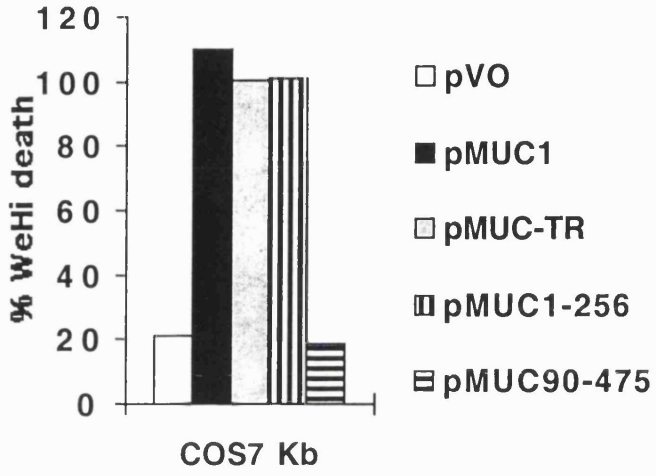


Figure 5.2: COS7-K^b cells transiently transfected with pMUC1 encoding amino acids 90 to 475 are not recognised.

COS7-K^b cells were transiently transfected with MUC1 deletion constructs and after two days MUC1-specific CTL were added. Supernatants were assayed for TNFα the following day using a WeHi bio-assay. Specific TNFα secretion is given as %specific WeHi death.

This suggested that the epitope maps to the N-terminal part of MUC1 protein, but several alternative explanations for lack of recognition of this construct by MUC1 specific CTL need to be considered. pMUC90-475 was made by removing the cDNA sequence coding for the N-terminal 90 amino acids of MUC1, including N-terminal upstream and Kozak sequence, start codon and the putative signal sequence (Gendler et al., 1991). The native MUC1 Kozak sequence (Kozak, 1984), start codon and several amino acids were regenerated (Figure 5.3). The truncated MUC1 protein lacked the ER targeting signal and was therefore expected to be expressed in the cytoplasm.

```
pMUC1:       MTPGTQSPFFLLLLLTVLTVVTGSGHASSTPGGEKETSAT
             |||||
pMUC90-475:  MTPGT-----------------------------------

pMUC1:       QRSSVPSSTEKNAVSMTSSVLSSHSPGSGSSTTQGQDVTL

pMUC90-475:  ------------------------------------

pMUC1:       APATEPASGSAATWGQDVTSV...
                   ||||  ||||||||||
pMUC90-475:  ------ASGSPATWGQDVTSV...
```

Figure 5.3: An alignment of pMUC1 and pMUC90-475.

The native MUC1 N-terminal sequence encoded by pMUC1 and pMUC90-475 is shown in alignment. Aminoacids that are preserved are indicated by a |, while amino acids lacking in pMUC90-475 are shown as --. For details on how these constructs were made refer to appendix 1.

To confirm that construct pMUC90-475 was able to direct cytoplasmic MUC1 expression, COS7 cells were transiently transfected with pMUC90-475 or vector contol pVO and stained with MUC1-specific mAbs. Figure 5.4 shows strong cytoplasmic expression of pMUC190-475 in COS7 cells. This implies that the lack of recognition of pMUC90-475 by the MUc1 specific CTL can not be accounted for by lack of expression go this construct. Furthermore, cytoplasmic expression of pMUC90-475 suggests that the resulting protein should have access to the MHC class I processing pathway.

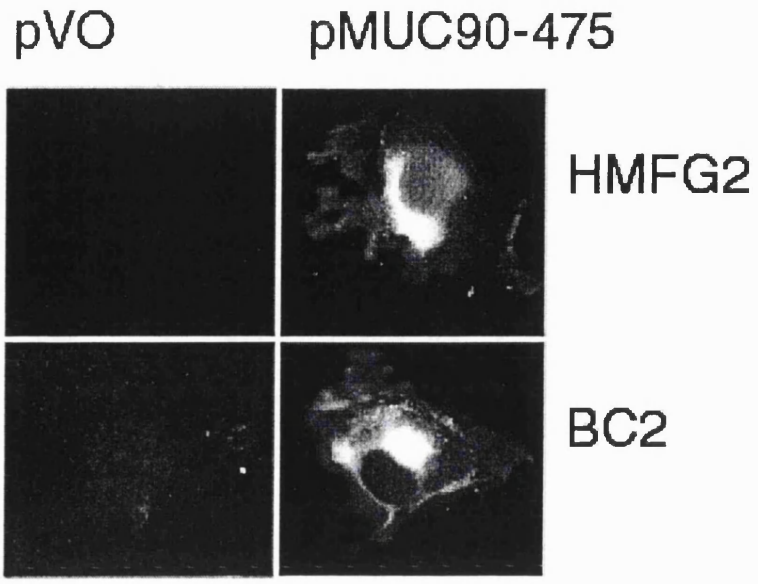


Figure 5.4: Expression of MUC1 by transiently transfected COS7 cells.
COS7 cells were electroporated in the presence of either vector alone (pVO) or deletion construct (pMUC90-475), and stained with MUC1-specific mAbs HMFG2 and BC2 and FITC labelled secondary rabbit anti mouse mAb. The mAbs HMFG2 and BC2 are both directed against the peptide backbone of the VNTR region of MUC1 and show no staining in the cells transfected with pVO. Cells transfected with pMUC90-475 show strong cytoplasmic staining with both MUC1-specific mAbs.

These data suggest that the epitope recognised by the MUC1 specific CTL maps to the N-terminal part of MUC1 that is missing from pMUC90-475. However the possibility remains that the epitope may be encoded by pMUC90-475, but is not presented via the MHC class I pathway due to lack of a functional signal sequence.

5.2.3 The epitope maps to the N-terminal sequence of MUC1

To confirm that the epitope recognised by the MUC1 specific CTL mapped to the 90 amino acids of the N-terminus of MUC1, a construct expressing these amino acids, called pMUC1-90, was made (Figure 5.5) and tested for recognition in a TNFα release assay after transfection into COS7-K^b cells (Figure 5.6).

pMUC1-90: **MTPGTQSPFFLLLLLTVLTVVTGSGHASSTPGGEKETSA TQRSSVPSSTEKNAVSMTSSVLSSHSPGSGSSTTQGQDVTLAPATEPASGS**DP AQWRPLESRGPV

Figure 5.5: Amino acid sequence encoded by pMUC1-90.

pMUC1-9 is predicted to express the amino acid sequence shown above. MUC1 derived amino acids are shown in bold and vector derived amino acids are shown in normal letter type. For details on cloning strategy refer to Appendix 1.

Figure 5.6 shows that pMUC1-90 as well as full length MUC1 (pMUC1) are recognised by MUC1-specific CTL in the TNFα release assays. This strongly suggests that the MUC1 epitope maps to the N-terminal 90 amino acids of the MUC1 sequence.

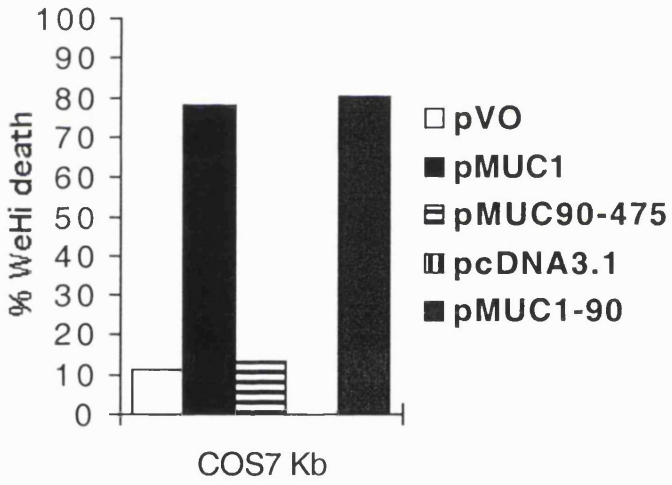


Figure 5.6: The epitope maps to the N-terminal sequence of MUC1.

COS7-K^b cells were transiently transfected with full length MUC1 (pMUC1), pMUC90-475, or pMUC1-90 and the corresponding empty vector controls pVO and pcDNA3.1 and tested for recognition by MUC1-specific CTL. TNFα release as shown in %WeHi death.

Furthermore, it is very unlikely that the epitope recognised by these MUC1-specific CTL stems from vector derived sequences rather than MUC1 itself, as the empty vectors from which these constructs were derived are not recognised (Figure 5.6.).

5.2.4 Further mapping of the K^b-restricted CTL epitope to the N-terminus of MUC1

To further map the epitope within the first 90 amino acids, MUC1 cDNA length variants were made by cloning PCR products of appropriate length into a commercial TA cloning vector (pCR3.1, Invitrogen). The PCR primers were designed so that the resulting PCR products included the native MUC1 Kozak sequence and a 3' STOP codon, to ensure a gene product of a defined length. Figure 5.7 shows the amino acid sequences encoded by the constructs that were made are.

pMUC1-39	MTPGTQSPFFLLLLLTVLTVVTGSGHASSTPGGEKETSA	+
pMUC1-30	MTPGTQSPFFLLLLLT**VLTVVTGSGHASST**	+
pMUC1-25	MTPGTQSPFFLLLLLTVLTVVTGSG	–
pMUC1-21	MTPGTQSPFFLLLLLTVLTVV	–

Figure 5.7: Constructs pMUC1-21, pMUC1-25, pMUC1-30 and pMUC1-39
code for the given number of N-terminal amino acids of MUC1. Recognition by MUC1 CTL is indicated by +/-. The putative epitope is shown bold.

When COS7-K^b cells were transiently transfected with these constructs along side appropriate controls, only those transfected with pMUC1-30 and pMUC1-39 induced specific TNFα secretion by MUC1-specific CTL that was comparable to the full length MUC1 construct (pMUC1)(Figure 5.8). This indicates that the epitope recognised by L8 maps to the first 30 amino acids of the MUC1 protein.

Since most known H-2 K^b restricted epitopes are eight or nine amino acids long (Rammensee et al., 1995) and if one assumes that the shorter construct pMUC1-25 is not recognised because it does not contain the full length epitope, then the epitope should map to the overlapping sequence TVVTGSGHASST (Figure 5.7). On the other hand, it is conceivable that the epitope is encoded by pMUC1-25, but is not recognised because the resulting protein was too short for proper antigen processing.

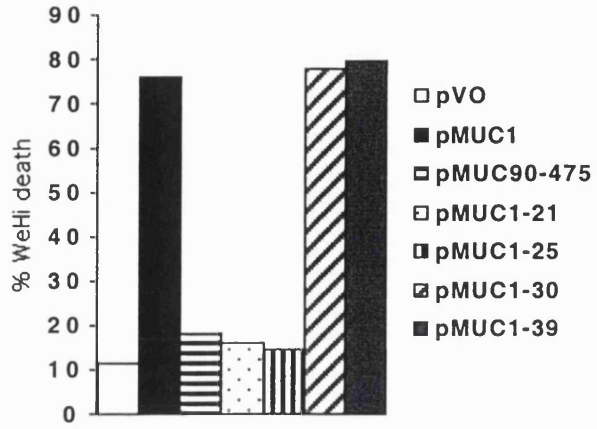


Figure 5.8: The epitope recognised by MUC1-specific CTL maps to the first 30 amino acids.

MUC1 cDNA length variants encoding 21, 25, 30 and 39 N-terminal amino acids were made by TA cloning of PCR products (pMUC1-21 etc) and transiently transfected into COS7-K^b cells as targets for MUC1-specific CTL in a TNFα release assay. Other constructs included as controls were: Vector only (pVO), full length MUC1 (pMUC1) and MUC1 lacking the first 90 amino acids pMUC90-475.

5.2.5 Epitope mapping using an Adenovirus E3/19K signal sequence

Since the putative epitope TVVTGSGHASST needed to be confirmed further, a cassette vector encoding a non related signal sequence was used to direct expression of MUC1 fragments directly into the ER of a target cell. The vector pSPCV which encodes the signal sequence of the adenovirus E3/19K protein followed by a unique NotI restriction site, a short stuffer sequence and a unique SwaI restriction site was available (Schoenberger et al., 1998). Double stranded oligonucleotides that encode an epitope of choice and have the appropriate nucleotide overhangs can be cloned into pSPCV that has been appropriately digested with NotI and SwaI creating an in frame termination codon at the 3' end of the oligonucleotide upon ligation (Figure 5.9). This yields a minigene which encodes an epitope of choice whose expression is targeted into the ER by the adeno E3/19K signal sequence in a TAP independent manner.

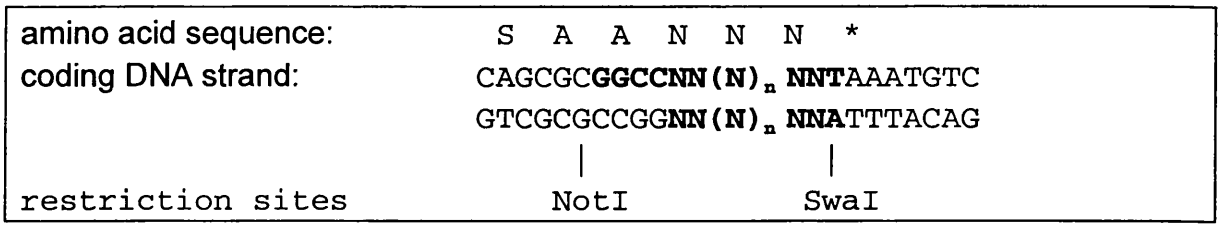


Figure 5.9: pSPCV adenovirus E3/19K signal sequence cassette vector.

The double stranded oligonucleotide encoding a CTL epitope of choice is shown in bold. Vector derived sequences are shown in normal letter type. The restriction sites for NotI and SwaI are indicated below. The amino acid sequence encoded by the vector and the 3' Stop codon are also indicated.

The final construct encodes the signal sequence followed by the insert of interest (here shown as N): MILGLLALAAVCSA*ANNNNNNNNN

In most settings the predicted signal cleavage for this particular construct takes place between the two alanine residues in position 15 as indicated by A *. See Figure 5.10 for signal peptidase cleavage site prediction of one such construct).

Two MUC1 constructs encoding amino acids 18-30 and 19-27 were made and construct with an unrelated MCF epitope named M8 was kindly provided by Marcel Champs and used as a specificity control. The following inserts were made (shown in italics):

MUC1 pSig 18-30 MILGLLALAAVCSA*ALTVVTGSGHASST

MUC1 pSig19-27 MILGLLALAAVCSA*ATVVTGSGHA

MCF pSigM8 MILGLLALAAVCSA*AKSPWFTTL

It is important to note that the peptide fragment that is released into the ER by this method will include the vector encoded N-terminal Alanine. i.e. pSig19-27 will encode MILGLLALAAVCSA*ATVVTGSGHA which will result in ATVVTGSGHA being released into the ER.

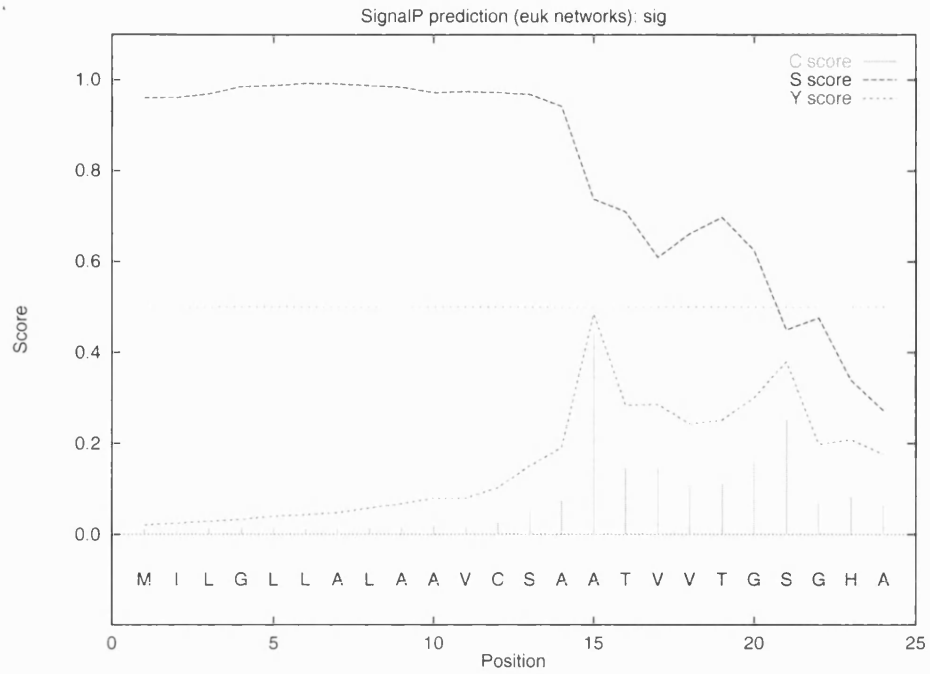


Figure 5.10: Signal peptide cleavage site prediction for MUC1 pSig 19-27.

The S score shows the likelihood of this part of the molecule to be part of the signal sequence according to a number of factors including hydrophobicity and peptide length. The C scores for individual amino acids positions indicate the likelihood of cleavage taking place at a particular site and is based or signal peptidase recognition patterns. i.e. +charged residue at position +3. For a more detailed discussion see (Nielsen et al., 1997; von Heijne, 1990). The two likely sites for signal peptidase cleavage are in position 24 or 28 with the highes C-scores.

To test the MUC1 pSig constructs for recognition by MUC1-specific CTL L8, they were transiently transfected into COS7-K^b cells and used as targets in an IFNγ release assay.

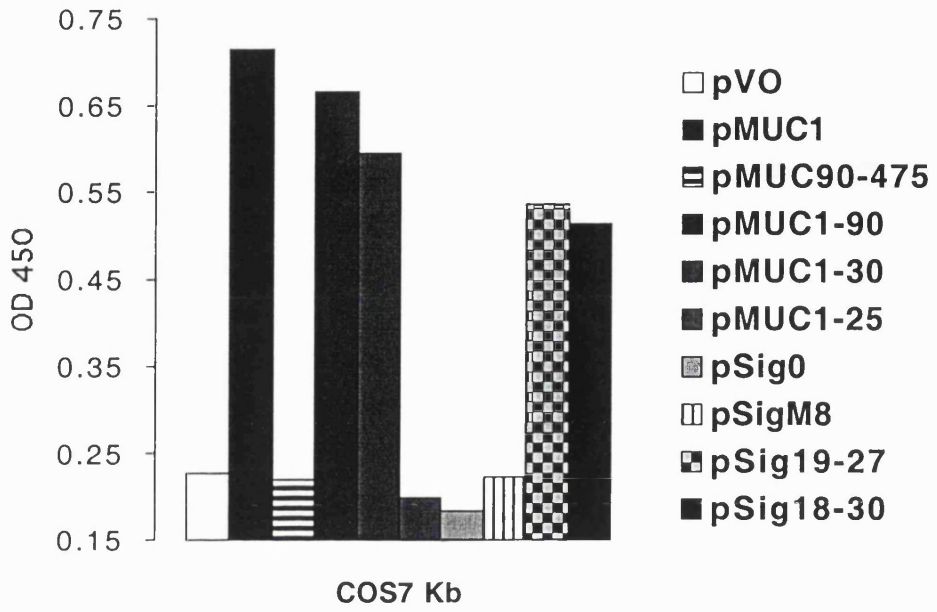


Figure 5.11: MUC1-specific CTL recognise the MUC1 fragment TVVTGSGHA *in the context of the Adeno E5/19K signal sequence. Overnight culture supernatants of CTL stimulated with transiently transfected COS7-K^b cells were assayed for IFNγ production by ELISA.*

The results in Figure 5.11 show that pSig19-27 and pSig18-30 are recognised while the empty vector control pSigO and a construct encoding the MCF epitope M8 (pSigM8) are not recognised. This strongly suggests that the epitope maps to the 9-mer sequence TVVTGSGHA.

5.2.6 Overlapping synthetic peptides are not recognised by MUC1-specific CTL

Once the epitope was mapped to the 30 N-terminal amino acids of MUC1 a set of overlapping synthetic peptides was tested for recognition (Figure 5.12).

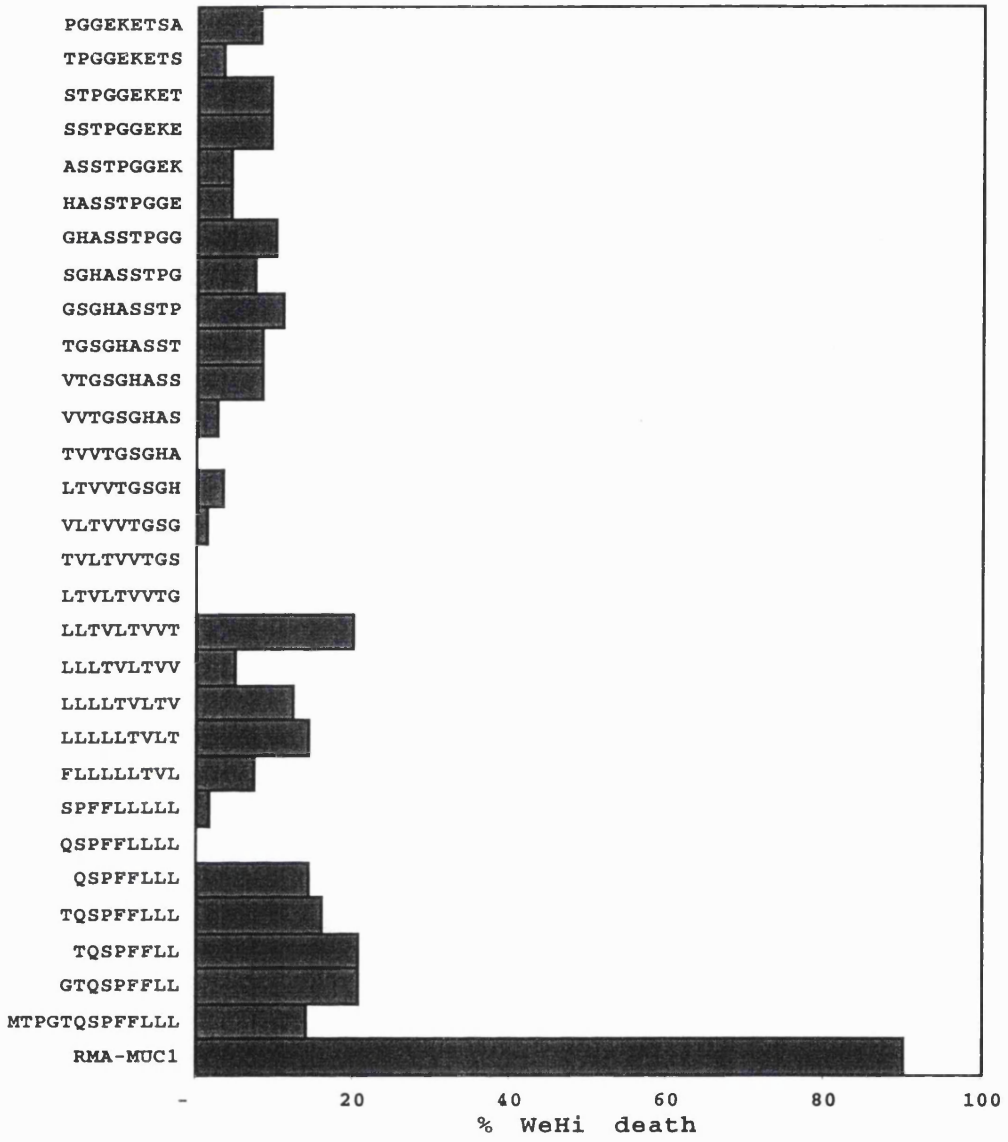


Figure 5.12: MUC1-derived synthetic peptides are not recognised.

MUC1-derived synthetic peptides were pulsed on RMA cells at final concentration of 10µg/ml and tested for recognition by MUC1-specific CTL in a TNFα release assay. The peptide sequence is indicated and recognition of RMA-MUC1 is shown as a positive control.

Most peptides were synthesised with a length of nine amino acids and it was assumed that even if the minimal CTL epitope was represented by an 8-mer sequence, there would be sufficient contamination of the synthetic peptide prep to allow for a specific response to be detected (Schumacher et al., 1991). However,

none of the peptides tested was recognised by MUC1-specific CTL which showed strong reactivity with RMA-MUC1 in the same assay (Figure 5.12). Similar experiment were repeated several times with a number of different CTL lines and APC with similar results, in that no convincing recognition of any of the synthetic peptides could be found (data not shown).

5.2.7 Naked synthetic peptide epitope does not protect mice against tumour challenge

Figure 5.12 shows that the synthetic peptide TVVTGSGHA was not recognised by the MUC1-specific CTL *in vitro.* To evaluate whether this peptide was a functional CTL epitope an *in vivo* tumour protection model was used. Mice immunised with minimal CTL epitopes can be protected against tumour challenge in a number of models. In C57BL/6 mice RMA-MUC1 is a syngeneic tumour that grows when inoculated i.p. to eventually kill the mouse. To further confirm that the naked synthetic peptide was not recognised by L8, C57BL/6 mice were immunised with peptide in IFA subcutaneously. RMA expresses the Rauscher murine leukaemia virus for which a dominant CTL epitope has been identified, that maps to the gag leader sequence *gag*L LCCLCLTVFL (Chen et al., 1996). This peptide has previously been shown to partially protect mice form tumour challenge (Th. Van Hall personal communication) and was used as a positive control in this experiment (Figure 5.13). The data showed that 100% of the control mice immunised with the dominant MuLV gag leader epitope *gag*L were protected against RMA-MUC1 tumour challenge. All mice immunised with the MUC1 peptides died at a comparable rate to control animals vaccinated with IFA alone (see Figure 5.13). Non of the synthetic MUC1 peptides were able to protect mice against RMA-MUC1 tumour challenge. This further indicates that the K^b restricted MUC1 epitope is not represented by a naked synthetic peptide.

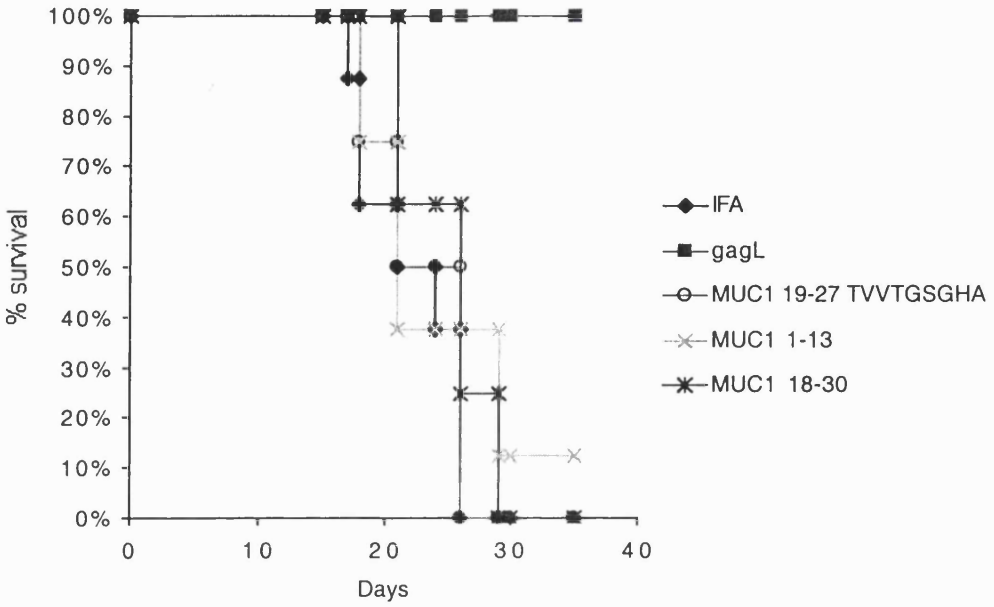


Figure 5.13: Peptide immunisation with TVVTGSGHA does not protect C57BL/6 mice *against RMA-MUC1 challenge Groups of 8 female C57BL/6 mice were immunised with 100µg of peptide in IFA, boosted after two weeks and tumour challenged with 10^3 RMA-MUC1 cells in PBS i.p 14 days after the last immunisation. Mice immunised with IFA alone or MUC1 peptides MUC1 1-13, MUC1 18-30 or MUC1 19-27 were not protected against tumour outgrowth while gagL LCCLCLTVFL protects the mice fully against tumour challenge.*

5.2.8 MUC1 CTL epitope may be posttranslationally modified

The data presented so far shows that it is possible to map the MUC1 CTL epitope by a number of different cDNA constructs to a minimal sequence of around 9 amino acids (TVVTGSGHA). However synthetic peptides corresponding to the N-terminal MUC1 sequence are not recognized in *in vitro* assays nor are they able to protect mice against MUC1 expressing tumour challenge. The main difference between the two epitope mapping approaches lies in the possibility for post-translational modifications to take place: The cDNA based constructs rely on gene expression, protein synthesis and the antigen processing machinery of the cell allowing for physiological postranslational modifcations to take place, while peptides pulsed onto the target cells exogenously may be trimmed in length but will not be subject to this machinery in a similar way. One possible explanation for the

lack of recognition of the synthetic material may thus be a post-translational modification present in the naturally processed epitope. The sequence identified by DNA constructs encodes one serine and two threonines which can be sites for a number of post-translational modifications, including cytosolic and mucin type O-glycosylation, phosphorylation and sulphation amongst others. To test whether the MUC1 epitope could be posttranslationally modified two approaches were taken. Firstly synthetic peptides carrying modified serine and threonine residues were synthesised and secondly a longer peptide (L**TVVTGSGHA**SST) containing additional N- and C- terminal sequence was used as a substrate for recombinant GalNAc-T1,-T2 and -T3 transferase (Wandall et al., 1997).

This work was done by Helle Hassan in Henrik Clausens Lab in Copenhagen. She concluded that only GalNAc-T3 can glycosylate the above peptide *in vitro* and incorporated 2 mol of GalNAc per mol of peptide. The mapping of the glycosylation sites indicates that the threonine in VVT*G was glycosylated by GalNac-T3 as well as one of the amino acids is the C-terminal SST sequence (Helle Hassan, personal communication). This glycosylated 13-mer peptide was not recognised by the MUC1-specific CTL nor in a TNFα release assay (Figure 5.14) in a CTL assay (data not shown). The usual length requirement of MHC class I binding peptides is 8-10 amino acids in length due to the conformation of the MHC class I binding grove (Bjorkman and Parham, 1990) and C-terminal trimming may be required for the 13-mer peptide to bind to H-2 K^b. This in turn may be inhibited if one of the C-terminal amino acids is glycosylated. Therefore, an attempt was also made to glycosylate the 9-mer peptide (TVVTGSGHA) with the recombinant enzymes but the GalNAc-transferases were found not to function on substrates of this length. (Helle Hassan, personal communication).

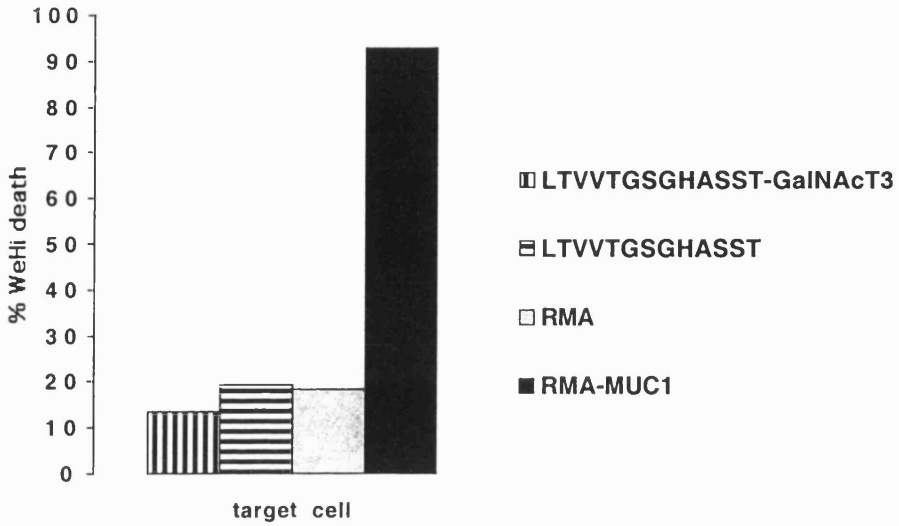


Figure 5.14: 13-mer peptide glycosylated by GalNAc-T3 is not recognised.
The 13-mer peptide LTVVTGSGHASST was glycosylated by GalNAc-T3 and pulsed on to RMA cells as targets for MUC1-specific CTL. RMA cells alone and pulsed with unglycosylated peptide are also shown.

To test modified peptides based on TVVTGSGHA, a number of synthetic peptides were made carrying GalNAc or phosphate groups on the serine and threonine residues since fmoc building blocks were available (Figure 5.15). None of these peptides were recognised, however a number of likely candidate modifications were not available and thus could not be tested so far. These are discussed in more detail at the end of the chapter. (see discussion on modification of the epitope).

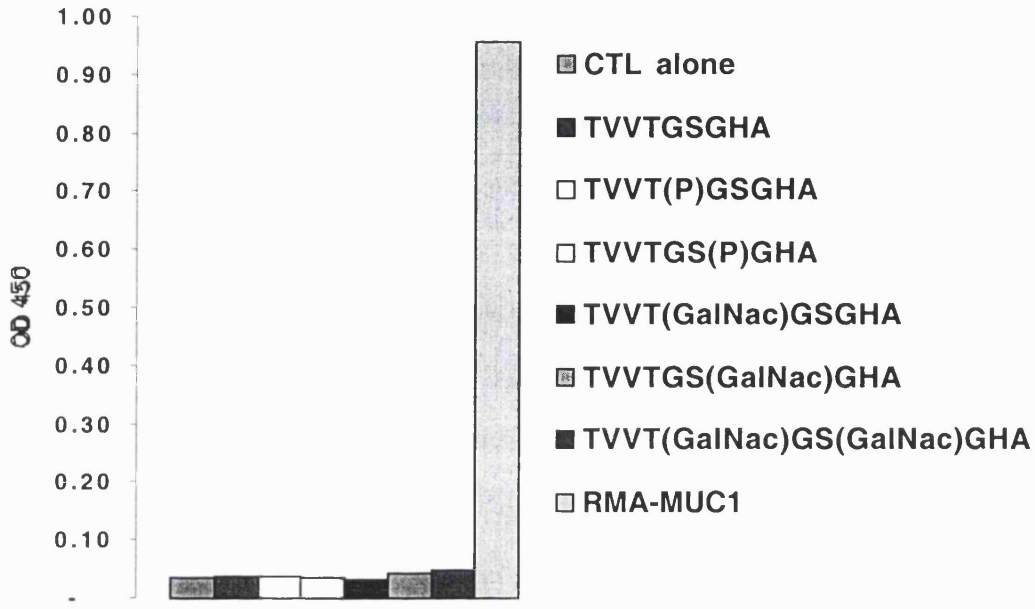


Figure 5.15: Analysis of synthetic modified peptides.

Synthetic peptides either unmodifed or with amino acids carrying either GalNac, or a phosphate (P) were made by fmoc chemistry and pulsed onto RMA cells (10μg/ml) and tested for recognition by MUC1-specific CTL in an IFNγ release assay.

5.2.9 The K^b-restricted MUC1 epitope is part of the signal sequence:

To understand how the MUC1 epitope is generated and how it might be post-translationally modified its location close to the N-terminus is important. The signal cleavage site for MUC1 had originally been predicted to take place at position 24 as indicated by the star VLTVVTG*SGHASST (Gendler et al., 1990) (see Figure 5.16 and Figure 5.17).

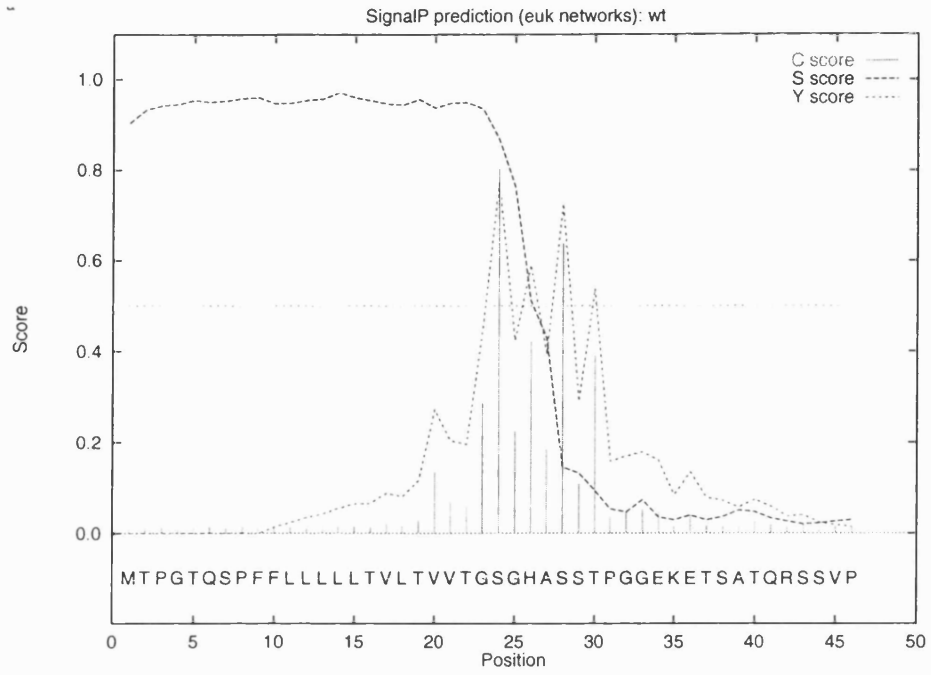


Figure 5.16: Signal sequence cleavage site prediction of the wild type MUC1 signal sequence.

The S score shows the likelihood of this part of the molecule to be part of the signal sequence according to a number of factors including hydrophobicity and peptide length. The C scores for individual amino acids positions indicate the likelihood of cleavage taking place at a particular site and is based or signal peptidase recognition patterns. i.e. +charged residue at position +3. For a more detailed discussion see (Nielsen et al., 1997; von Heijne, 1990). The two likely sites for signal peptidase cleavage are in position 24 or 28 with the highes C-scores.

Reanalysis and prediction of the signal cleavage site using a recent prediction algorithm based on a neural network server on the WWW predicts two likely cleavage sites: In position 24 as before or alternatively a cleavage in position 28 as indicated by a backslash (/) in the following sequence: VLTVVTG*SGHA/SST. This implies that the CTL epitope is part of the MUC1 signal sequence and may not be part of the final native MUC1 protein.

```
Amino acid:  1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17
ACC ACC ACC ATG ACA CCG GGC ACC CAG TCT CCT TTC TTC CTG CTG CTG CTC CTC ACA GTG
TGG TGG TGG TAC TGT GGC CCG TGG GTC AGA GGA AAG AAG GAC GAC GAC GAG GAG TGT CAC
             M   T   P   G   T   Q   S   P   F   F   L   L   L   L   L   T   V

 18  19  20  21  22  23  24  25  26  27  28  29  30  31  32  33  34  35  36  37
CTT ACA GTT GTT ACA GGT TCT GGT CAT GCA AGC TCT ACC CCA GGT GGA GAA AAG GAG ACT
GAA TGT CAA CAA TGT CCA AGA CCA GTA CGT TCG AGA TGG GGT CCA CCT CTT TTC CTC TGA
 L   T   V   V   T   G   S   G   H   A   S   S   T   P   G   G   E   K   E   T

 38  39  40  41  42  43
TCG GCT ACC CAG AGA AGT
AGC CGA TGG GTC TCT TCA
 S   A   T   Q   R   S
```

Figure 5.17: cDNA Sequence of MUC1 encoding for amino acids 1-50

The predicted CTL epitopes is shown in bold

5.2.10 The MUC1 CTL epitope is processed in a proteosome independent manner:

Since the naked peptide was not recognised, the processing of the epitope was becoming more central to our investigation, to explain how the epitope lying in the signal peptide was processed and potentially post-translationally modified.

The model for processing of MHC class I restricted peptides assumes that peptides are generated by the ubiquitin-proteasome pathway that degrades proteins in an ATP and ubiquitin dependant manner, by targeting proteins to the multicatalytic proteinase complex, the 26S proteasome. The peptides generated can then be translocated into the ER for MHC class I loading by TAP. However, a few examples of signal sequence derived MHC class I peptides are known that can be presented independently from these mechanisms (Wei and Cresswell, 1992). Signal sequences are usually N-terminal extensions that target newly synthesised proteins to translocation sites in the plasma membrane of the endoplasmic reticulum of eukaryotic cells. Following membrane insertion, the signal sequences can be cleaved from the precursor protein by membrane bound signal peptidase

(SP) and further processed by signal peptide peptidases (SPP) (for review see (Martoglio and Dobberstein, 1998)). The characteristic feature of signal sequences is the hydrophobic core (h-) region containing usually 6-15 amino acids which is N-terminally flanked by a polar n-region while the C-terminal side is flanked by a polar c-region (von Heijne, 1990). Depending on which of these domains the epitope is derived from, it will either be released directly into the ER-lumen or into the cytosol. In the former case antigen presentation is TAP and proteasome independent and in the latter the processing can be dependent on both TAP and the proteasome. (Henderson et al., 1992; Hombach et al., 1995; Lehner and Cresswell, 1996; Uger and Barber, 1997). So see also Figure 5.22 and discussion below.

To further analyse the processing of the MUC1 epitope and to determine whether the epitope was processed independently of the proteasome, several well defined proteasome inhibitors were used. Lactacystin (Fenteany et al., 1994), is a product of Streptomyces and covalently binds to ß-subunits of the proteasome, preferentially inhibiting their chymotryptic-like and tryptic-like activities (Fenteany and Schreiber, 1996). Lactacystin is thought to only interfere with the proteosome, unlike other peptide aldehyde inhibitors like LLNL which inhibits numerous other cellular proteases including the signal peptide peptidase (Martoglio, B. personal communication) and possibly other ER resident proteases (Hughes et al., 1996). The other inhibitor that was used is the potent tripeptide vinyl sulfone proteasome inhibitor 4-hydroxy-3-iodo-2- nitrophenyl-leucinyl-leucinyl-leucine vinyl sulfone (NLSS) (Bogyo et al., 1998).

In the experiments presented here the proteasome inhibitors were used to treat RMA-MUC1 cells which were then analysed to see whether recognition by the MUC1-specific CTL could be abrogated in ^{51}Cr release assays. As a control, to show that the proteasome inhibitors were functional, a CTL clones directed against a MuLV epitope was used. The control CTL clone (RMA CTL) is directed against a H-2 D^b restricted Rauscher MuLV derived epitope that has recently been identified.(T. van Hall, unpublished data). This CTL lyses RMA and RMA-MUC1 in Cr^{51} release assays to a comparable degree (Figure 5.18 A). It can be inhibited by all three proteasome inhibitors used in these experiments and thus served as an internal control. (Figure 5.18 D)

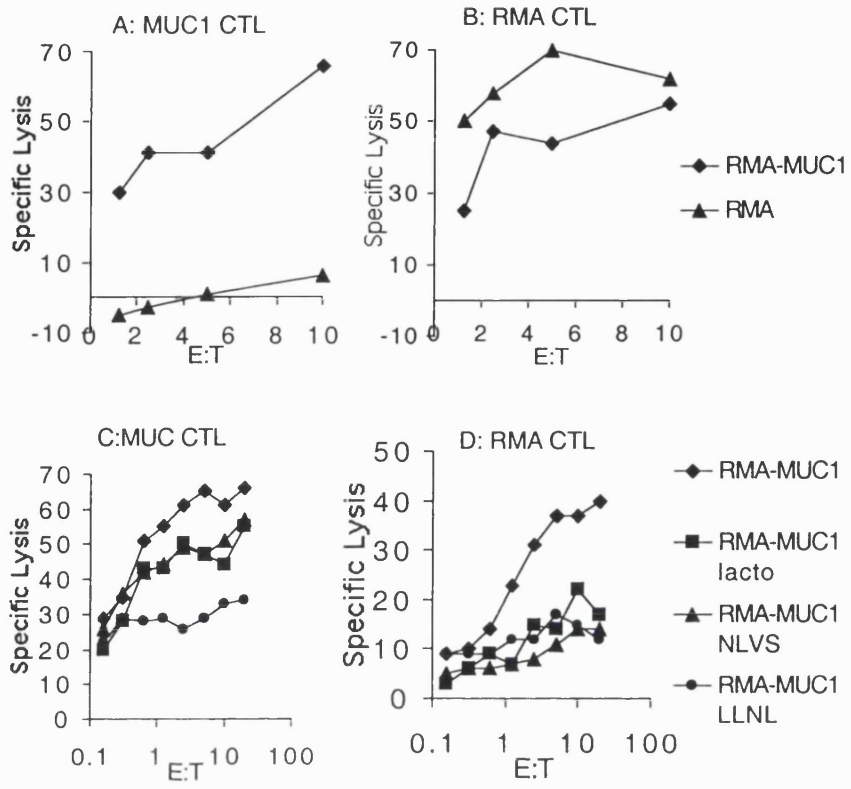


Figure 5.18: MUC1-specific CTL are not inhibitable by the proteasome inhibitors lactacystein or NLVS.

C,D: Target cells were treated with proteasome inhibitors over night before labelling with ^{51}Cr for a standard CTL assay. Target cells were treated with 10μM Lactacystin, 2.5μM NLVS or 10 μM LLnL as indicated in the legend. Lysis of RMA and RMA-MUC1 by MUC1-specific CTL (A) and RMA-specific CTL (B) are shown.

While the lysis of RMA-MUC1 by RMA-specific CTL is inhibitable by all three inhibitors (Figure 5.18 D), the recognition of the MUC1 epitope by MUC1-specific CTL was not significantly inhibited by the more specific proteasome inhibitors Lactacystin or NLVS but was at least partially inhibited by the broad spectrum protease inhibitor LLNL(Figure 5.18 C).

5.2.11 The MUC1 CTL epitope is processed in a TAP-independent manner:

Even though the processing of the MUC1 epitope appears to be proteasome independent it is conceivable that signal peptide fragments released into the cytosol could be transported back into the ER for trimming and loading into MHC class I molecules without the need for further proteasomal degradation. To test whether the processing of this epitope was dependent on TAP, mouse embryo fibroblasts from TAP-deficient mice (TAP-/-) were used as targets (Schoenberger et al., 1998). To avoid having to make permanent transfectants of these TAP-/- cells, Bruce Acres, TRANSGENE, kindly provided a MUC1-expressing recombinant adenovirus (AdMUC). For infection TAP-/- cells were harvested and resuspended in PBS with 0.5% BSA and incubated with adenovirus at an MOI of 100 for 1 hour at 37^{0}C. The cells where then plated out in normal culture medium and used after three days.

Figure 5.19 shows a FACS analysis of TAP-/- cells infected with AdMUC three days after infection. The same batch of cell were used in a TNFα release assay to test for recognition by MUC1-specific CTL which showed that the epitope was processed and presented in both TAP-/- and TAP+/+ cells. (Figure 5.20).

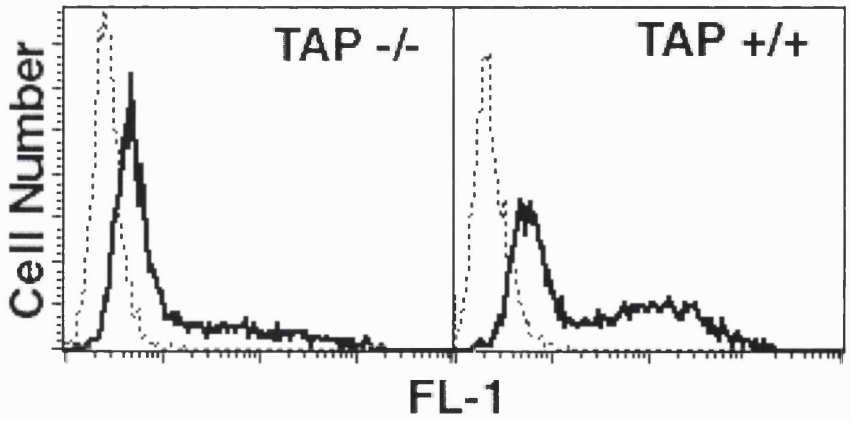


Figure 5.19: Expression levels of MUC1 in TAP-/- cells.

FACS data showing expression levels of MUC1 by staining with 12C10 FITC on AdMUC infected (black line) and un-infected (broken line) mouse embryo cell lines derived from TAP -/- and TAP +/+ mice.

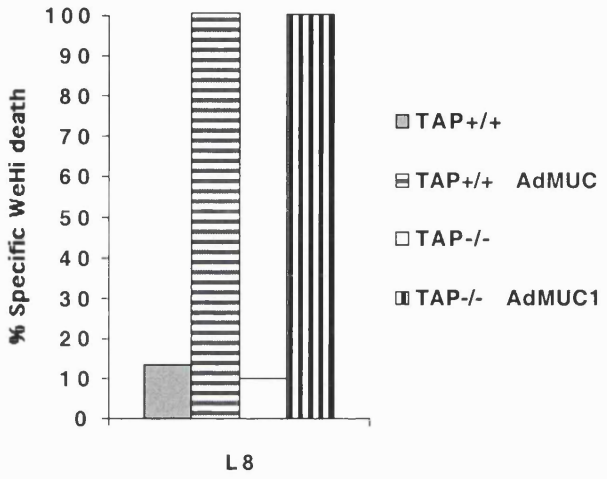


Figure 5.20: Processing of MUC1 epitope is TAP-independent.

TAP -/- cells and wild type control (TAP+/+) cells were infected in suspension with AdMUC with a MOI of 100 for 1h. at 37C and tested in FACS three days later for MUC1 expression and recognition by the CTL line.

5.3 Summary

1) MUC1-specific C57BL/6 derived CTL have been generated that recognise a H-2 K^b restricted epitope
2) The CTL recognise a minimal MUC1 cDNA construct that encodes the MUC1 9-mer TVVTGSGHA in the context of a non-related signal sequence
3) The CTL do not recognise the corresponding naked synthetic peptide
4) Only the proteasome inhibitor LLNL and not lactacystin or NLVS can partially inhibit the processing of the MUC1 epitope.
5) The epitope is processed and presented independently of proteasome cleavage and TAP translocation and is probably located within the MUC1 signal sequence.

5.4 Discussion

5.4.1 MUC1 CTL Epitope lies outside the VNTR:

For historical reasons work describing MUC1 as a tumour antigen has focused so far on the tandem repeat region as the main immunogenic part of the molecule. Mouse monoclonal antibodies raised against human milk fat globules were directed against this immunodominant part of the molecule (Berry et al., 1985; Ormerod et al., 1984; Taylor Papadimitriou et al., 1981). Furthermore, the gene dosage effect of having 20-120 copies of the VNTR suggested that it would be a good target for the induction of cellular immune responses. Several CTL epitopes in the VNTR have been mapped, all from experiments where the VNTR rather than the whole MUC1 was used as an immunogen. Apostolopoulos et al. used VNTR peptides fused to mannan to induce responses both in mice and humans (Apostolopoulos et al., 1997; Apostolopoulos et al., 1997), while others used naked synthetic peptides to induce CTL in healthy donors and patients (Domenech et al., 1995). In this study mice were immunised with the whole MUC1 sequence in the form of a recombinant vaccinia virus and again restimulated with tumour cells expressing endogenously processed epitopes derived from the whole MUC1 sequence. Several MUC1-specific CTL clones and lines were derived from a number of different mice and all appeared to recognised the same region of MUC1.

Our own experiments show that in C57BL/6 mice the dominant CTL response to MUC1 however is not directed against the VNTR but to the N-terminal signal sequence of the MUC1 molecule and is MHC restricted.
These findings underline the importance of using the whole of MUC1 protein as an immunogen.

5.4.2 Mapping of the epitope

In this work two different approaches were used to map the epitope: cDNA constructs were used to map the epitope to the first 90 amino acids of MUC1 and

eventually to the 9-mer sequence TVVTGSGHA using an Adeno E5/19K signal sequence construct. Synthetic peptides were made to confirm that the minimal epitope was represented by this sequence. Since naked synthetic peptides corresponding to this sequence were not recognised, it appears likely that the epitope may be post-translationally modified. The two threonines (T) and the serine (S) are potential sites for a number of post-translational modifications. These included cytosolic O-type glycosylation that attaches a single O-linked N-acetylglucosamine (GlcNAc) residue reversibly to serine or a thereonine residues on cytosolic and nuclear proteins and is controlled by glycosyl-transferases located in the cytosol (Haltiwanger et al., 1992; Holt et al., 1987). O-phosphorylation of serine and threonines can use the similar attachment sites and there appears to be a reciprocity between O-phosphorylation and O-glycosylation (Hart et al., 1996). Mucin type O-linked glycosylation on the other hand is initiated by the GalNac-transferases located throughout the cis- medial- and trans- Golgi-pathway (Nilsson et al., 1993) but may also be functional in the ER (Carraway and Hull, 1989).
Since these modifications take place in different subcellular compartments it is important to consider how MUC1 is expressed and processed to understand what post-translational modifications are likely.

5.4.3 Processing of signal peptide derived epitopes

Signal sequences are usually N-terminal extensions that target newly synthesised proteins to translocation sites in the plasma membrane of the ER of eukaryotic cells. Following membrane insertion, the signal sequences can be cleaved from the precursor protein by a membrane bound signal peptidase (SP) and even further processed by signal peptide peptidases (SPP) (for review see (Martoglio and Dobberstein, 1998)). Because several features of the entire signal sequence seem to influence the signal cleavage site, it is not always possible to predict whether and where cleavage will occur and data from prediction algorithms have to be treated with caution.

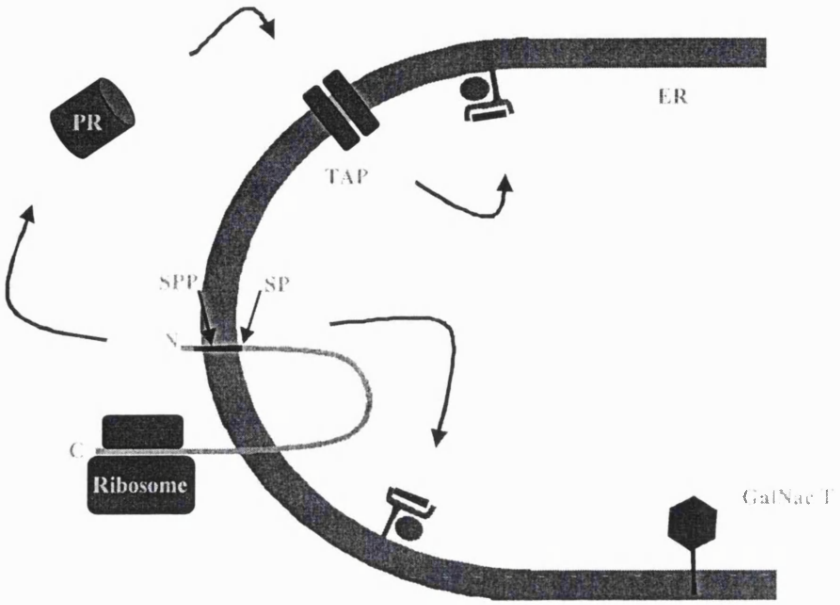


Figure 5.21: Signal sequence processing in the ER.

The signal sequence inserts in to the ER membrane in a loop like fashion where it can be cleaved by the signal peptidase (SP) and signal peptide peptidase (SPP). Signal sequence derived peptides (black) can either enter the MHC class I processing pathway via the cytosol in a proteasome (PR) and TAP dependant manner or by direct loading on to class I molecules.

It is important to note that most cleaved signal sequences are inserted into the ER membrane in a loop-like fashion, leaving the N-terminal end on the cytoplasmic side while the C-terminal part is translocated into the ER lumen.(See figure Figure 5.21) It is this luminal or trans side of the membrane where the signal peptide cleavage may occur. Signal sequences that have been cleaved from precursor proteins are further processed by signal peptide peptidases, probably to release signal sequences from the ER membrane that would otherwise be 'stuck' in the membrane held in place by polar C- and N-termini that can not transverse the membrane. The further processing is mediated by a so far unidentified signal peptide peptidase that appears to be a membrane bound endo-peptidase that cleaves somewhere inside the h-region of the signal peptide. The fate of signal sequences has been investigated in a synchronised cell-free system using ER-derived rough microsomes (Lyko et al., 1995; Martoglio et al., 1997). In this system the signal sequences of bovine pre-prolactin, which consists of 30 amino acids, is

cleaved by signal peptidase and then further processed by a signal peptide peptidase. Cleavage within the h-region results in an N-terminal signal peptide fragment of about 20 amino acids corresponding to the n-region and about half the h-region. This signal peptide fragment resides initially in the membrane and is eventually released into the cytosol. The signal sequence fragment made up of the other half of the h-region and the c-region is presumably released into the ER-lumen.

Both peptide fragments could become accessible for loading in the MHC class I pathway for antigen presentation. The fragments released into the cytosol are subject to proteosome degradation and transported by TAP whereas the fragment released directly into the ER, are presented in a TAP and proteosome independent way. This included C-terminal fragments of long and some N-terminal sequences of short signal peptides. In TAP -/- cells peptides associated with MHC class I are derived from the h- and c-region of long signal sequences such as interferon-inducible protein, and ip30 (MUC1 also falls in this category) and from the n- and h-regions of short signal sequences such as calreticulin and ER-resident protein (Figure 5.22) (Henderson et al., 1992). (Hombach et al., 1995; Lehner and Cresswell, 1996; Uger and Barber, 1997)

A)

MKWVTFISLLFLFSSAYS- Serum albumin

MRVKEKYQHLWRWGWRWGTMLLGMLMICSA- HIV-1 gp 160

MTPGTQSPFFLLLLLTVLTVVTGSGHA- MUC1

B)

TAP-independent:

MDSRHTFpMUC1AAMTLSPLLLFLPPLLLLLDVPTAAVQA- IP-30

MLLSVPLLLGLLGLAVA- CR

TAP-dependant:

MGQIVTMFEALPHIIDEVINIVIIVLIIITSIKAVYNFATCGILALVSFLFLAGRSCG-LCMV

MASNENMETMLLLLLAAALAPTQTRA- NP-HC

MGVMpMUC1RTLLLLLAAALpMUC1TQTRA- HLA-A

Figure 5.22: Examples of signal sequence derived epitopes.

Signal sequences have a tripartite structure: the central hydrophobic h-region (red) and a hydrophilic n- (green) and C-terminal (blue) flanking regions. A) shows the MUC1 signal peptide along with two other signal sequences with n-regions of different length. B) Epitopes known to be TAP independent map to the c-region of long signal sequences that transverse the ER membrane. The signal peptide stays inserted into the ER membrane, after signal peptidase (SP) cleavage with the n-region towards the cytosol and the c-region exposed to the ER lumen. The signal sequence can then be released by means of the signal peptide peptidase (SPP) cleaving the h region, after that the c-region and part of the h-region attached to it are released into the ER-lumen and can be loaded onto MHC class I. Short signal sequences derived epitopes mapping to the h-region can be TAP-independent since this part of the molecule is likely to pass from the ER membrane into the ER lumen. By the same rationale epitopes derived from the n-region tend to be released into the cytosol where they can be proteasome degraded and TAP translocated for MHC class I loading.

The MUC1 epitope identified in this thesis maps to what can be presumed to be the C-terminal part of the h-region and c-region of the signal sequence. This is consistent with the finding that the processing of this epitope is functional in wildtype and TAP -/- cells. Furthermore, the inability to inhibit presentation by

blocking the proteosome with several inhibitors is also consistent with this model. Significantly, the only proteosome inhibitor that had a partial effect is LLNL which is also know to inhibit the SPP. Therefore the most compelling model for the processing of the MUC1 epitope involves the C-terminal anchor residue being generated by SP cleavage after the A in TVVTGSGHA and that SPP cuts the MUC1 sequence N-terminally to the epitope generating either an exact N-terminus or generating a longer peptide that can then further trimmed by other carboxy-peptidases present in the ER (Figure 5.21)(Yewdell et al., 1998).

5.4.4 Immuno-dominance of the MUC1 CTL epitope

Independent CTL cultures, derived form animals immunised with the full length MUC1 antigen, recognise an epitope that maps to the N-terminus of MUC1 rather than the previously published epitopes located inside the VNTR region, identified in mice immunised only with the VNTR(Apostolopoulos et al., 1995). Since none of the CTL cultures described here recognised any of the VNTR epitopes it appears that the N-terminal MUC1 derived epitope is immunodominant in C57BL/6 mice.

The processing of the native MUC1 epitope is similar to the synthetic constructs based on the adenovirus E5/19K signal sequence (pSigMUC1 constructs) that were used in this chapter to target the MUC1 epitope directly into the ER by use of a signal sequence. This type of peptide delivery has been shown to be about 2,000 times more efficient in presenting model antigens for MHC class I presentation than their natural presentation in a TAP- and proteosome-dependent way (Yewdell et al., 1998). If one extrapolates these findings, then the MUC1 epitope that is part of the natural MUC1 signal sequence may be immunodominat due to the large amount of the peptide competing for MHC class I molecules for binding in the ER.

5.4.5 Possible modifications of the MUC1 epitope TVVTGSGHA

Since the MUC1 epitope appears to be directly targeted to the ER modifications by cytosolic O-linked glycosylation are unlikely, however it is conceivable that potential O-glycosylation sites in the region of the epitope are modified by mucin type O-liked glycosylation. The proposed 9-mer peptide epitope

has three potential attachment sites and modification of one or several of these amino acids could explain the lack of recognition of the naked synthetic peptide by the MUC1-specifc CTL. The initiation of mucin-type O-linked glycosylation is controlled by a family of UDP-GalNAc: polypeptide N-acetyl-galactosaminyl-transferases (GalNAc-T) (Clausen and Bennett, 1996) and these enzymes have been found to be present throughout the Golgi pathway. Several members of this gene family have been cloned and expressed (Bennett et al., 1998; Rottger et al., 1996; Schwientek et al., 1998; Sorensen et al., 1995) and can be used *in vitro to* glycosylate synthetic peptides bearing threonines and serines. So far the glycosylation pattern of recombinant enzymes used *in vitro* has correlated well with the glycosylation pattern found *in vivo.* Glycopeptides can also be produced synthetically using fmoc building blocks of serine and threonines carrying different O-glycans (Kihlberg et al., 1997).

However, for the MUC1 epitope to be glycosylated several conceptual hurdles have to be overcome. Once the peptide is generated in the ER, it would presumably be loaded onto class I MHC molecules rather than being passed though the Golgi network for glycosylation, prior to retrieval to the MHC class I loading compartment. Since short peptides would probably be degraded too quickly, this MUC1 peptide could be glycosylated in several other ways: For example the peptide which binds the MHC class I molecule in the ER could be glycosylated later while the MHC class I peptide complex passes though the Golgi network. This would imply that only the glycosylation sites facing out of the MHC groove could be glycosylated and therefore an oligosaccaride is unlikely to form an anchor residues. Another possibility is that the peptide is glycosylated in the ER prior to MHC class I loading by GalNAc transferases being recycled though this compartment as has been recently shown (Storrie et al., 1998).

5.4.6 Glycosylated CTL epitopes

Glycosylation is a common post translational modification of proteins which are secreted or transported to the cell surface (Varki, 1993). Nevertheless, evidence that glycosylation plays a role in the natural repertoire of peptide MHC complexes presented to T cells is scarce. One example where glycosylation plays

a role in a naturally processed and presented epitope involved N-glycosylation of the tyrosinase antigen. A naturally processed and presented epitope sequence of the melanoma associated tyrosinase gene was found to have an aspartic acid in place of an expected asparagine when eluted from HLA-A*0201 molecules. The genomically encoded asparagine is N-glycosylated in the native protein(Skipper et al., 1996), but prior to antigen presentation the N-glycan is removed by a deglycanase that leads to a post-translational deamination of asparagine to aspartic acid(Mosse et al., 1996). This amino acid change has no impact on peptide binding to the MHC molecule, but is of central importance for peptide recognition by melanoma-specific T cells.

There are very few examples in the literature for the endogenous processing of glycoproteins to glycopeptides and subsequent presentation by MHC molecules and the data that is available concerns glycopeptides presented via the MHC class II pathway. These include the immunodominant epitope in type II collagen involved in the initiation of collagen-induced arthritis in mice. In this peptide (aa 256-270) lysines at positions 264 and 270 are post-translationally modified by hydroxylation and subsequent O- linked glycosylation (Michaelsson et al., 1996; Michaelsson et al., 1994). This group was able to generate a panel of T-cell hybridomas that reacted specifically with different synthetic glycoforms of the peptide and concluded that the glycosylation specific responses over the unglycosylated peptide were immunodominant (Corthay et al., 1998).

Dudler et al. were able to generate several human T-helper cell clones specific for bee venom phospolipase A2 from allergic human individuals, which proliferate in response to the glycoprotein but not to its non-glycosylated variants (Dudler et al., 1995). However, in both of these cases the authors fail to convincingly show that the MHC bound peptide presented on the cell surface is glycosylated.

For the MHC class I pathway, data on naturally processed and presented glycopeptides is even more thinly spread and for a long time it has been questionable whether glycopeptides could enter the class I processing pathway at all. For entry into the class II pathway, shedding of glycoprotein from cells that

could then be endocytosed and degraded in the classical antigen presentation pathway for class II was conceivable. Particularly since the TAP activities and the proteasome are not involved in this pathway.

For the classical MHC class I pathway, which has been outlined in chapter 1, it is less clear whether and if so how glycoproteins are degraded by the proteosome. At least for N-glycosylation a deglycanase is known that can mediate the deamination reaction that is involved in the tyrosinase epitope modification describe above. However how mucin type O-glycosylated proteins, taking place in the Golgi apparatus are broken down and whether this happens via the proteosome prior or after sugar removal is not known. For stearic reasons one can probably assume that extended mucin type sugars will not be able to pass through the proteasome directly. There is some evidence that glycosylated peptides with single sugars can be translocated into the ER by TAP1 (Haurum, 1996). Furthermore, mucin type O-glycosylated proteins would have to be accessible to the processing machinery after having passed through the glycosylation machinery mainly located in the Golgi apparatus. This is at least conceivable in a setting where an APC takes up the antigen, to present it to the immune system via cross presentation but less clear for somatic cells where antigen access to the Class I pathway is usually via the cytosol. In this light it is interesting to note that the MUC1 epitope described in this chapter is neither TAP nor proteasome dependent and may get around this hurdle.

5.4.7 Recognition of glycopeptides *in vitro* .

Since specific protection groups for oligosaccaride carrying amino acids have been developed, glycopeptides can be made by relatively standard fmoc chemistry (Kihlberg et al., 1997). This has been instrumental for probing the T-cell repertoire with glycopeptides with a wide range of oligosccharides (Carbone and Gleeson, 1997; Corthay et al., 1998; Deck et al., 1995; Deck et al., 1999; Galli-Stampino et al., 1997; Jensen et al., 1996; Jensen et al., 1997; Michaelsson et al., 1996; Michaelsson et al., 1994) The possibility of glycopeptides binding to MHC has been explored in great depth both for MHC class I and II (Haurum et al., 1994; Haurum et al., 1995; Jensen et al., 1997). Recently crystal structures of three

different glycopeptide- MHC class I complexes have been solved (Glithero et al., 1999; Speir et al., 1999) and this allowed modelling and prediction of how CTL recognition and cross-reactivity to other oligosaccarides or even unrestricted CTL responses (Abdel-Motal et al., 1996) can be explained.

This data collectively indicates that MHC ligands can be modified to carry a number of different oligosaccharides provided affinity of the peptide to the MHC is not lost. However, only a subset of the glycopeptides tested did induce immune responses. The general trend seems to be that the size of the O-glycan that can be recognised is limited to a di- and possibly a tri-saccharide and that certain structures are more immunogenic than others. For example, Tn (GalNAc) and T(Gal.GalNac) are recognised when presented on the artificial epitope of haemoglobin, while core 2 a trisaccaride is not immunogenic (Galli-Stampino et al., 1997; Jensen et al., 1996). The most likely explanation for this is that the combination of size, shape and charge is limiting for interaction with the TCR. Two recent papers describing the crystal structures of glycopeptides bound to H-2 K^b and D^b show that the oligosaccaride faces away from the MHC molecule towards the T-cell receptor and propose that K^b, which has a deeper MHC groove than D^b, is able to accommodate sugars up to a tri-saccaride attached to one of the central amino acids of the epitope without loosing CTL recognition. Since the MUC1 CTL epitope is restricted by H-2 K^b is possible that it may carry a more complex sugars than just a simple GalNAc tested for recognition in this chapter.

Chapter 6: Mouse HLA-A*0201/K^b restricted responses

6.1 Introduction:

The previous two Chapters have made use of H-2 restricted mouse models to assess whether MUC1-specific CTL mediated immunotherapy is in principle feasible. The role of overexpression and altered glycosylation in generating tumour specific responses, as well as the risk of MUC1-specific autoimmunity was examined. It was shown that the immunodominant epitope in H-2^b mice is K^b restricted and maps to the MUC1 signal sequence when the animals were immunised with VV-MUC1. Immunisation with the virus (Acres et al., 1993) or adoptive transfer of MUC1 specific CTL can protect MUC1 transgenic mice from tumour challenge without induction of detectable autoimmunity. The epitope probably bears a post-translational modification that may be tumour specific. To extrapolate these findings to the human situation where the same recombinant vaccina virus is used in clinical trials (Balloul et al., 1994; Bizouarne et al., 1996), the identification of human CTL epitopes was under taken.

Until recently the search for anti-MUC1 immune responses has focused on the VNTR part of the molecule. It has been argued that this area is immunodominant due to its high copy number (30-90 copies per molecule). This is certainly supported with a large body of data describing antibodies directed against the VNTR that are specific for the tumour associated altered glycosylation pattern (Reis et al., 1998). In addition, non-classical MHC-unrestricted T cell responses have been described that depend on aberrant glycosylation of the VNTR (Agrawal et al., 1998; Domenech et al., 1995; Jerome et al., 1991; Noto et al., 1997). Also classical MHC class I restricted responses have been described. The VNTR-derived peptide sequence STAPPAHGV was shown to constitute a target for both HLA-A*11 and HLA-A*0201-restricted CTL (Apostolopoulos et al., 1997; Domenech et al., 1995). Of note, this peptide does not comply to the binding-motifs for these HLA-molecules and shows relatively poor binding as compared to many other known epitopes, suggesting that it interacts with these HLA-molecules in a non-conventional manner (Apostolopoulos et al., 1997).

Although on the basis of its high copy number and apparent immunogenicity it may seem attractive to focus a search for HLA-restricted CTL epitopes on the VNTR region, the fact that MUC1 is expressed in considerable levels by normal epithelia has to be taken into account. Since the VNTR constitutes a relatively abundant source for antigen, even in normal epithelial cells, it is conceivable that peptides derived from this sequence may be presented by such normal cells, as well as by professional antigen presenting cells that have taken up significant quantities of antigen derived from MUC1 expressing tissues (Heath et al., 1998). This may result in T cell tolerance, as was shown with several transgenic mouse strains expressing artificial auto-antigens in a tissue specific manner. Depending on the pattern and level of expression, these mice developed a certain level of tolerance, either at a thymic and/or peripheral level, against the auto-antigens. In two of these models a direct relation between the expression level of these artificial auto-antigens and the degree of the tolerance was demonstrated (Kurts et al., 1998; Morgan et al., 1998). In other cases, expression of such tissue specific auto-antigens was shown to be ignored by the T cell immune system, leading neither to a T cell immune response nor to tolerance. In such cases, breaking of this T cell ignorance by a strong antigenic stimulus can however result in T cell-mediated destruction of the tissues expressing the auto-antigen (Ohashi et al., 1991; Oldstone et al., 1991).

The sequences outside the MUC1 VNTR constitute a less abundant source of antigen than those derived from the VNTR. Consequently, the T cell immune system is less likely to have become tolerised for epitopes mapping in the non-VNTR part of the MUC1 protein. Furthermore, such epitopes are more likely to provide a window for discrimination by effector T cells between the MUC1-over-expressing tumour and normal MUC1-expressing tissue. Finally, the VNTR does not harbour peptide sequences that comply well with the binding motifs for the most common HLA-A-molecules, whereas these motifs predict a vast quantity of potential epitopes in other areas of the MUC1 sequence. Therefore the aim was to identify HLA-A*0201-restricted epitopes in the entire MUC1 sequence with emphasis on the non-VNTR areas.

The approach that was taken involves measurement of HLA-A*0201-binding and proteasome-mediated processing using several in vitro assays, followed by analysis of the capacity of selected peptides to elicit specific anti-tumour immunity in HLA-A2/Kb mice. These experiments resulted in the identification of three MUC1-derived HLA-A*0201-binding epitopes that are distinct from two recently described peptides (Brossart et al., 1999).

6.2 Results:

6.2.1 Selection of MUC1 peptides for their HLA-A*0201 binding capacity.

The identification of epitopes presented by MHC class I alleles, pool sequence data of naturally processed and presented peptides that have been eluted from a number of MHC class I alleles (Engelhard, 1994) and peptide binding studies (Drijfhout et al., 1995) have lead to the derivation of anchor motifs. These motifs can be used to predict peptides that will bind to specific MHC alleles for any given protein sequence(Rammensee et al., 1995).

Computer programmes (D'Amaro et al., 1995 ; Parker et al., 1994) were employed to scan the entire MUC1 protein sequence for peptides that match the motifs for HLA-A*0201 to select peptides for biochemical synthesis. The MUC1 sequence comprising two tandem repeats (TR) was searched for nine amino acid long peptides complying with the anchor residue motifs for HLA-A*0201. A full set of 9-mers with an eight amino acid overlap covering the VNTR, as well as 9-mers in the top 10% of the scoring data for HLA-A*0201, were synthesised and tested for binding to HLA-A*0201.

Peptide binding to HLA-A*0201 was analysed using HLA-A*0201^{+} B lymphoblastoid JY cells in a semi-quantitative competition assay (van der Burg et al., 1995). Briefly, the assay is based on competitive binding of two peptides to acid stripped MHC class I molecules on a B cell line (JY). A test peptide competes with a fluorescently labelled reference peptide for the empty class I molecules on the cell surface. The mean fluorescence (MF) obtained in the absence of competitor

peptide was regarded as maximal binding and equated to 0%; the MF obtained without reference peptide was equated to 100% inhibition. The percentage inhibition was calculated using the formula:

$$\frac{\{1-(\text{MF 150nM referance and competitor peptide} - \text{MF no reference peptide})}{(\text{MF 150nM reference peptide} - \text{MF no reference peptide})\}} \times 100\%$$

The binding capacity of competitor peptides is expressed as the concentration needed to inhibit 50% of binding of the FL-labelled reference peptide (IC_{50}).

All peptides were tested several times is two fold dilutions starting with a concentration of 100μM. Out of 90 peptides tested, 5 were able to compete for HLA-A*0201 binding with the fluorescently labelled reference peptide to 50% at concentrations lower than 15 μM (Table 6.1B). This range of binding capacity at concentrations below 15μM has been observed for known naturally processed epitopes (see Flu-$M1^{58-66}$ in Table 6.1A) (van der Burg et al., 1995). In addition, we identified six peptides that showed measurable but weaker binding to HLA-A*0201 (Table 6.1,C). None of the remaining peptides exhibited any detectable binding to HLA-A*0201 (data not shown).

	Peptide	Sequence	Motif[a]	Motif[b]	IC_{50} [c]	Stability	CTL response [d]	Tumour-protection [e]
A	FLU-M1 $^{58-66}$	GILGFVFTL	54	55 [a]	3 [b]	+	N.T.	N.T.[e]
	HPV16 E6	KLPQLCTEL			9	-		
B	$MUC^{264-272}$	FLSFHISNL	59	226	7	+	3/7	+
	$MUC^{460-468}$	SLSYTNPAV	62	69	8	+	0/6	-
	MUC^{13-21}	LLLTVLTVV	63	412	6	+	0/4	-
	$MUC^{167-175}$	ALGSTAPPV	64	69	10	+	4/6	+
	MUC^{79-87}	TLAPATEPA	58	2	11	+	4/6	+
C	$MUC^{107-115}$	ALGSTTPPA	56	5	25	+/-	N.T.	N.T.
	$MUC^{257-265}$	STGVSFFFL	45	17	50	-	N.T.	N.T.
	$MUC^{353-361}$	NLTISDVSV	57	69	85	-	N.T.	N.T.
	$MUC^{130-138}$	STAPPAHGV	51	1	>100	-	N.T.	N.T.
	$MUC^{170-179}$	STAPPVHNV	53	2	>100	-	N.T.	N.T.
	MUC^{12-20}	LLLLTVLTV	64	1006	>100	-	N.T.	N.T.

Table 6.1: Summary table peptide analysis.

Peptide sequences of +ve control peptides (A), strong binders (B) and week binders (C) are shown with amino acid sequence.

a) Motif score derived using (D'Amaro et al., 1995). b) Motif score derived using http://bimas.cit.nih.gov/molbio/hla_bind/index.html (Parker et al., 1994)

*c)Average binding affinity was measured as described (van der Burg et al., 1995). IC_{50} represents the amount of peptide required for 50% inhibition of binding of the fluorescein-labeled reference peptide to HLA-A*0201*

d) Immunogenicity is given as the fraction of mice that mounted a peptide-specific responses. Mice where considered positive with 20% specific lysis over control targets in two E:T ratios.

e) Tumour protection against MUC1 expressing tumours after peptide vaccination.

+,Peptides that protect mice significantly against tumours compared to control vaccination;

-, peptides that fail to show tumour protection; N.T. not tested.

Since the peptide-binding assay is performed at 4^0C, it measures the capacity of peptides to bind MHC while ignoring the influence of higher physiological temperature on the stability of MHC peptide complexes. The latter parameter was shown to correlate more accurately with immunogenicity (van der Burg et al., 1996; Van der Burg et al., 1997; van Elsas et al., 1996; Visseren et al., 1997). We therefore tested the stability of the good and intermediate binding peptides at 24°C as well as 4°C after 3 and 24 hours. (Table 6.2BC). All five peptides that exhibited strong binding also formed stable MHC peptide complexes (Table 6.1B). Of the six peptides with lower binding capacity, only $MUC^{107-115}$ bound well enough to permit

stability-analysis. As shown in Table 6.2C, this peptide formed moderately stable complexes with HLA-A*0201. Peptide HPV16 E6 is included as an examples of a peptide that binds well but does not form stable complexes with HLA-A*0201 and is not immunogenic (van der Burg et al., 1996). Based on these results, the five best binding peptides shown in Table 6.1B were selected for further experiments.

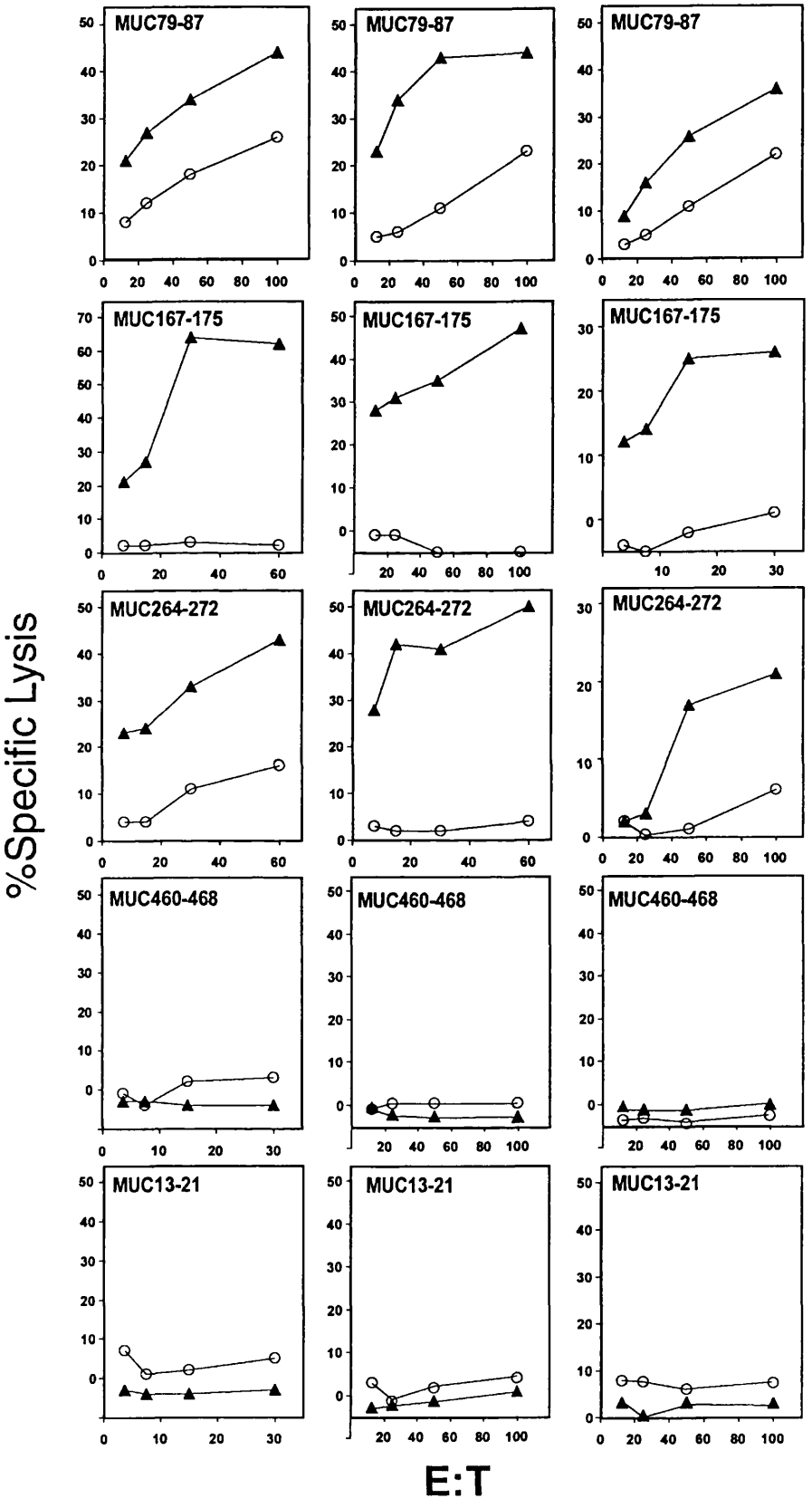
	Peptide stability	IC_{50}			
		4°C		24°C	
		3hr	24hr	3hr	24hr
A	FLU-M1 $^{58-66}$	1	3[b]	2	3
	HPV16 E6	26	9	27	55
B	MUC$^{264-272}$	6	7	4	35
	MUC$^{460-468}$	8	8	7	17
	MUC$^{13-21}$	20	6	6	25
	MUC$^{167-175}$	27	10	13	25
	MUC$^{79-87}$	20	11	7	12
C	MUC$^{107-115}$	20	25	24	45
	MUC$^{257-265}$	100	50	20	>100
	MUC$^{353-361}$	40	85	40	>100
	MUC$^{130-138}$	80	>100	>100	>100
	MUC$^{170-179}$	35	>100	45	>100
	MUC$^{12-20}$	>100	>100	>100	>100

Table 6.2: Table of stability data

*Stability of the peptides was tested by the HLA-A*0201 binding assay incubating the assay at 4°C or 24°C for 3 or 24 hours.*

6.2.2 Immunogenicity of MUC1 derived peptides in A2/K^b transgenic mice

The five peptides that were selected by the binding assays (Table 6.2B) were tested for their capacity to induce peptide-specific CTL immunity *in vivo*. Mouse transgenic for a chimeric HLA-A*0201/H-K^b molecule (A2/K^b) (Vitiello et al., 1991), which have successfully been used by many researchers including ourselves to identify HLA-A*0201-restricted CTL epitopes derived from tumour antigens (e.g.: (Apostolopoulos et al., 1997; Butterfield et al., 1999; Correale et al., 1998; Irvine et al., 1999; Ressing et al., 1995; Wentworth et al., 1996)), were immunised with the synthetic peptides emulsified in IFA. Bulk splenocyte cultures taken from these mice two weeks after the last immunisation were tested for cytotoxic activity after one week of *in vitro* restimulation against peptide loaded JA2/K^b cells.


%Specific Lysis
E:T
MUC79-87
MUC167-175
MUC264-272
MUC460-468
MUC13-21

Three of the five peptides, namely MUC^{79-87}, $MUC^{167-175}$ and $MUC^{264-272}$ are immunogenic in $A2/K^b$ mice, in that they reproducibly induced strong peptide specific CTL responses. In contrast peptides MUC^{13-21} and $MUC^{460-468}$ did not induce peptide specific responses, even though their binding to HLA-A*0201 is comparable to that of the other peptides. (Table 6.1; Figure 6.1)

Figure 6.1: MUC1 derived HLA-A*0201 binding peptides induce peptide specific cytotoxic CTL responses.

$A2/K^b$ mice were immunised twice with 100µg of MUC1 peptide in IFA and 140µg of the pan-DR-binding T helper peptide PADRE on day –28 and –14, as described previously (Ressing et al. 1995). On day 0 single cell splenocytes suspensions were restimulated in vitro for one week with peptide loaded syngeneic LPS-elicited lymphoblasts and tested for cytotoxicity of peptide loaded Jurkat-$A2/K^b$. Groups of $A2/K^b$ mice were immunised with MUC1 peptides $MUC1^{265-272}$, $MUC1^{460-468}$, $MUC1^{13-21}$, $MUC1^{167-175}$ or $MUC1^{79-87}$. CTL bulk cultures were tested against Jurkat-$A2/K^b$ cells loaded with the cognate peptide (filled triangles) or irrelevant influenza matrix control peptide (open circles). For each peptide data from three mice are shown. A summary of all results is provided in Table 6.1.

In further experiments we analysed whether the *in vivo* induced peptide-specific CTL responses would be capable of killing MUC1-overexpressing tumours. A tumour model in the A2/K^b transgenic mice was developed by transfection of the mouse melanoma B16 F1 with the MUC1 cDNA and the HLA-A2/K^b gene. From these transfections clone B16-MUC1-HLA-A2/K^b was derived which showed stable MUC1 and IFNγ-inducible A2/K^b expression (Figure 6.2).

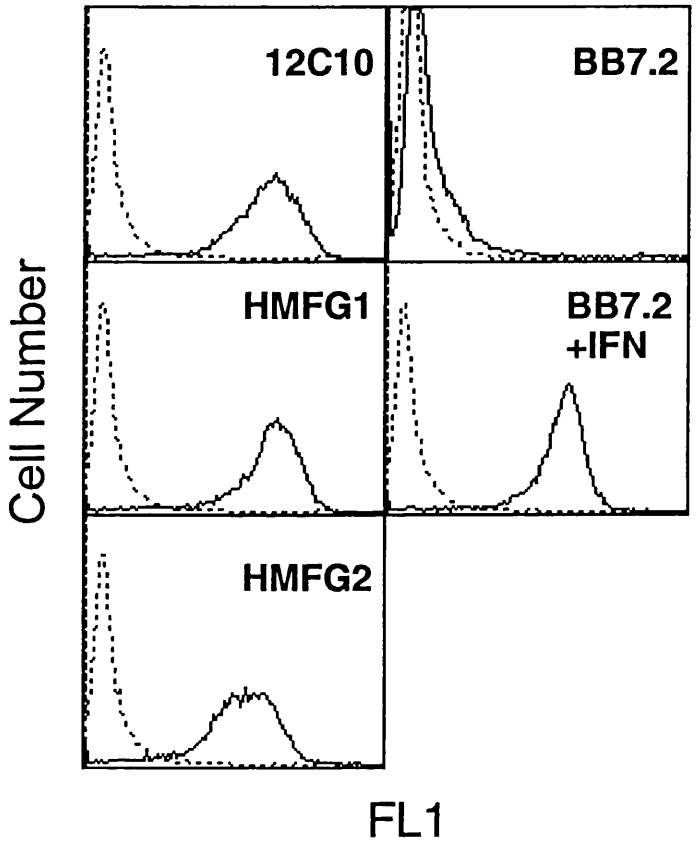


Figure 6.2: Surface expression of MUC1 and A2/K^b on B16 F1 transfectants.
*A stable transfectant of B16 F1 with the MUC1 cDNA and the chimeric A2/K^b cDNA construct expresses high levels of MUC1 and A2/K^b on the cell surface as detected by flow cytometry. B16-MUC1-A2/K^b was stained with mAb hybridoma supernatants specific for MUC1 (12C10, HMFG1 and HMFG2) and specific for HLA-A*0201 (BB7.2). Background staining with secondary FITC labelled mAb alone is shown with a dotted line. The A2/K^b expression of B16-MUC1-A2/K^b was increased by treatment of the cell culture with 10u/ml murine IFNγ for three days prior to staining (+IFNγ).*

In tumour take experiments s.c. administration of 10^5 B16-MUC1-A2/K^b cells resulted in 80-100% tumour take in A2/K^b transgenic mice (data not shown). This tumour dose was used for all further experiments (Figure 6.3).

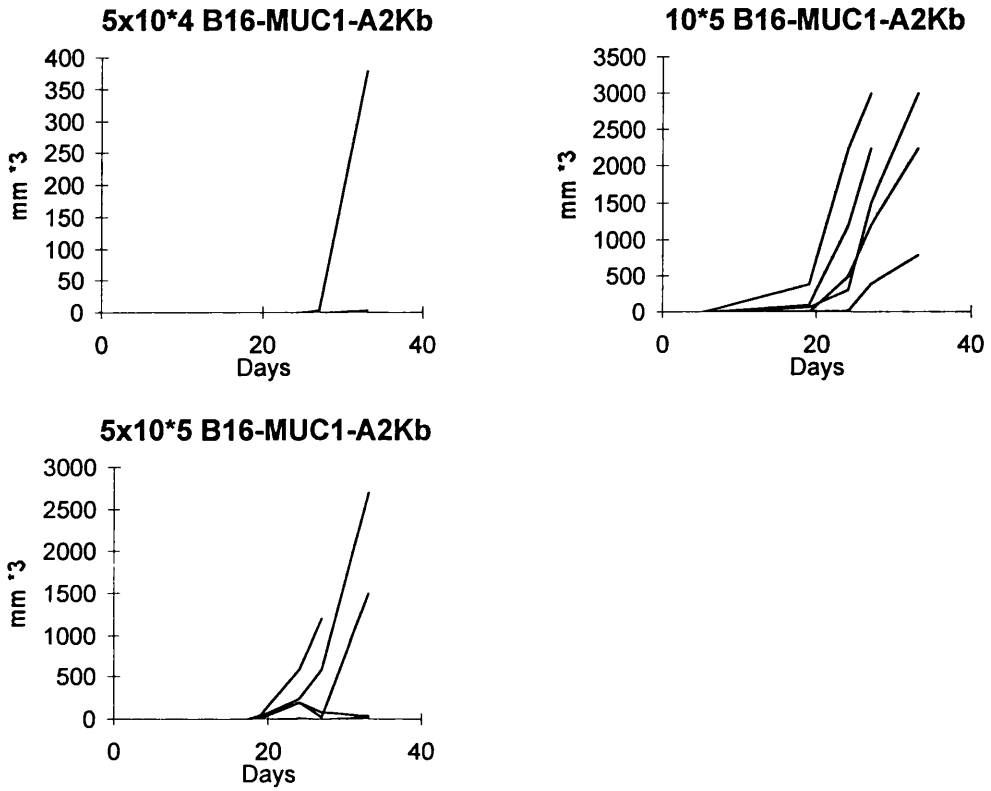


Figure 6.3: Tumour take of B16-MUC1-A2K^b cells in in A2/K^b transgenic mice.
Groups of 6 A2/K^b mice where tumour challenged with 5x10^4, 10^5 and 5x10^5 B16-MUC1-A2/K^b cells in 200µl of PBS subcutaneously on the flank. Tumour growth was monitored by measurements in three dimensions to calculate tumour volume. Mice challenged with 10^5 B16-MUC1-A2/K^b cells gave consistent tumour take.

To test whether the MUC1 derived HLA-A*0201 binding peptides could protect A2/K^b transgenic mice against tumour challenge, groups of 8 mice were immunised with MUC1 peptides in IFA and challenged with B16-MUC1-A2/K^b subsequently. As a positive control for this experiment one group of mice was immunised i.p with 10^8 pfu of a recombinant Vaccinia virus expressing MUC1 (VV-MUC1), a mode of antigen delivery which was previously shown to protect mice against MUC-1 positive tumours (Acres et al., 1993).

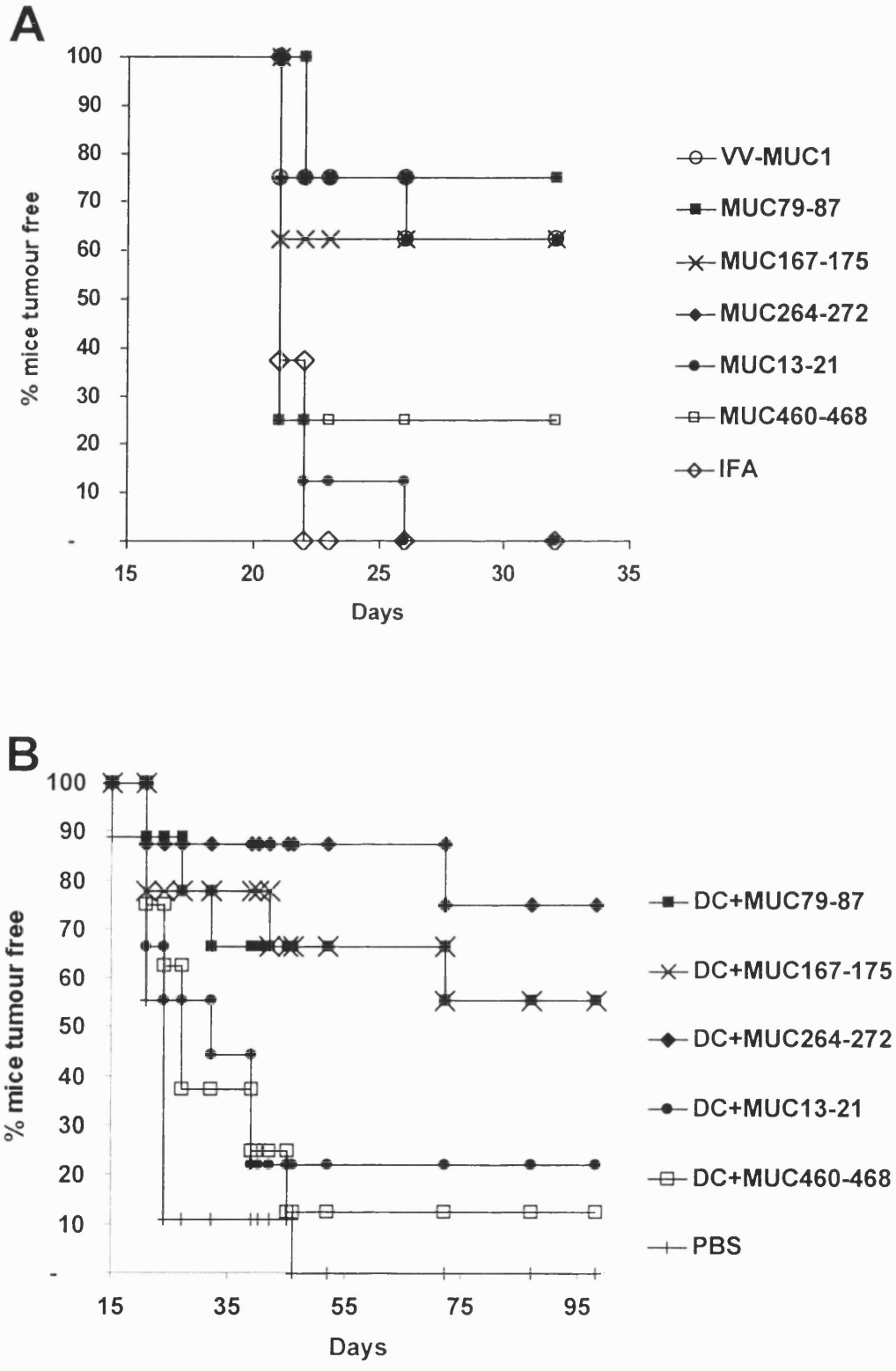


Figure 6.4: see next page for legend

Immunisation with peptides MUC^{79-87}, $MUC^{167-175}$ and $MUC^{264-272}$ as well as with VV-MUC1 induced significant protection of the mice against tumour growth compared to mice immunised with IFA alone (p=0.0008, p=0.005 and p=0.02 respectively). Peptides MUC^{13-21} and $MUC^{460-468}$, which failed to induce peptide specific CTL responses (Figure 6.1), were also not able to protect mice against tumour challenge. Mice immunised with these peptides did not survive significantly better than mice that had received IFA alone (p=0.536 and p=0.926 respectively) (Figure 6.4A) and tumour growth was recorded by measuring three dimensions. No changes in tumour status were recorded after the last day shown.

Figure 6.4: MUC1 peptide vaccination protects A2/K^b mice against tumour challenge.

A: Groups of 8 A2/K^b mice were immunised s.c. on day –28 and –14 with 100µg of MUC1 peptide in IFA, IFA alone or with 10^8 pfu rec. VV-MUC1 i.p. B: Alternatively, mice were immunised i.v. with $5x10^5$ DC per mouse loaded with 20µg/ml of MUC1 peptide, or with PBS alone i.v. 14 days after the last immunisation, on day 0 all mice were challenged s.c with 10^5 B16-MUC1-A2/K^b

Vaccination with peptides emulsified in IFA has in certain cases been shown to induce epitope specific tolerance (Nieland et al., 1999; Toes et al., 1996). On the other hand, presentation of these peptides in an appropriate costimulatory context, for instance by loading of the peptides on dendritic cells, was shown to prevent tolerance induction and instead result in CTL-priming (Toes et al., 1997). To exclude the possibility that we might overlook the immunogenic potential of one or more of the MUC1-derived peptides under investigation, in particular peptides MUC^{13-21} and $MUC^{460-468}$, $A2/K^b$ mice were immunised, prior to tumour challenge, with $5x10^5$ peptide loaded spleen derived DC (Figure 6.4B). This experiment confirmed that peptides $MUC^{264-272}$, MUC^{79-87}, and $MUC^{167-175}$ induced significant protection against tumour challenge ($p=0.006$, $p=0.002$ and $p=0.001$ respectively). Peptides MUC^{13-21} and $MUC^{460-468}$, even when loaded on DC, failed to result in significant protection of the mice. ($p=0.122$ and $p=0.172$ respectively) (Figure 6.4B).

6.3 Discussion:

The human polymorphic epithelial mucin MUC1 is over-expressed on a number of epithelial and haematological malignancies and therefore is a potential target for T-cell meditated immunity. We have identified three non-VNTR MUC1-derived HLA-A*0201 restricted CTL epitopes. In contrast to most previously reported MUC1-derived CTL-epitopes (Apostolopoulos et al., 1997; Jerome et al., 1991; Noto et al., 1997), the epitopes identified in this study comply to the HLA-A*0201 motif, show strong binding to this molecule and map outside the TR region of MUC1. In addition, the data demonstrates that vaccination of A2/K^b mice with peptides MUC^{79-87}, $MUC^{167-175}$ and $MUC^{264-272}$ elicits peptide-specific CTL immunity capable of protecting mice against a challenge with MUC1-expressing tumour cells. The latter experiment suggests that peptides MUC^{79-87}, $MUC^{167-175}$ and $MUC^{264-272}$ are not merely immunogenic, but that they may represent naturally processed CTL epitopes. Moreover, the tumour-challenge experiments in A2/K^b mice indicate that these peptides can indeed serve as targets in a CTL-mediated immune response against MUC-1 over-expressing, HLA-A*0201-positive tumours *in vivo.*

In a recent report by Brossart and coworkers (Brossart et al., 1999) two other MUC1-derived HLA-A*0201-restricted CTL epitopes have been described: peptides MUC^{12-20} and $MUC^{170-179}$ (see Table 6.1 for sequences). Like the epitopes identified in this thesis, these map outside the VNTR sequence and are in compliance with the binding motif for HLA-A*0201. CTL raised against these peptides recognise MUC1-expressing tumour cells, indicating that also these peptides represent naturally processed epitopes (Brossart et al., 1999). The fact that independent attempts to identify non-VNTR MUC1-derived HLA-A*0201-restricted T cell epitopes resulted in the identification of complementary sets of epitopes can best be explained by differences in experimental approach. Brossart and coworkers have screened the MUC1 sequence for peptides matching the motif for HLA-A*0201-binding, after which they have selected two peptides for induction of CTL responses without prior analysis of their capacity to bind to HLA-A*0201. Although this publication does not provide an elaborate description of the basis on

which these two peptides were selected for further studies, it is conceivable that these peptides were selected because of their high score in the HLA-A*0201 motif as made available by Rammensee et al. (Rammensee et al., 1993) http://www.uni-tuebingen.de/uni/kxi/immunol.html. Accordingly, peptides MUC^{12-20} and $MUC^{170-179}$ were found to score high in computer scoring available in the Lab (D'Amaro et al., 1995). These two peptides were in fact among the set of 90 MUC1-derived peptides tested for HLA-A*0201 binding. Although these peptides indeed showed weak binding in our assays, the binding data did not encourage further experiments concerning immunogenicity of these peptides. Instead, the experiments presented here led to the identification of three other MUC-derived, HLA-A*0201-restricted epitopes: MUC^{79-87}, $MUC^{167-175}$ and $MUC^{264-272}$.

In conclusion, three independent and complementary research projects aimed at the identification of HLA-A*0201-restricted MUC1 epitopes have resulted in the identification of six distinct immunogenic peptides, one derived from the VNTR sequence (Apostolopoulos et al., 1997) and five derived from the non-VNTR areas of the MUC1 protein ((Brossart et al., 1999), this chapter). Analysis of T cell immunity against these peptides in cancer patients with MUC1-positive tumours will be required to show which of these peptides constitute targets of the natural and/or vaccine-induced CTL response against MUC1.

Chapter 7: Summary and Discussion of the Thesis

7.1 Summary:

The previous three chapters have dealt with different aspects of immune responses to the human epithelial mucin MUC1. The questions that were addressed experimentally in this thesis concerned:

- Whether MUC1-specific immunotherapy mediated by MHC class I-restricted CTL could confer tumour protection without induction of autoimmunity. This was addressed in a mouse model system in chapters 3 and 4.
- Whether over-expression of MUC1 was sufficient to confer tumour specificity was addressed in chapter 4.
- Whether the immune response against MUC1 in this tumour model was directed against the TR or non VNTR region. The mapping of the epitope to a potentially modified peptide was described in Chapter 5.
- Which MUC1-derived epitopes could be useful in MHC class I-restricted response against MUC1 in humans. Putative HLA-A*0201 restricted CTL epitopes were identified by work described in chapter 6.

This chapter will review some of these data and will indicate the importance and implications of these findings for MUC1-specific immunotherapy in humans. It will also address where further work and focus is needed.

The work in this thesis set out to generate MUC1-specific CTL lines in a murine C57BL/6 mouse model and to address whether MHC-restricted CTL could be used for tumour specific immunotherapy against MUC1 expressing tumours. MHC-unrestricted CTL responses had been observed in humans and were postulated to be tumour-specific due to aberrant glycosylation of the MUC1 TR in cancer cell lines (Jerome et al., 1991). MHC-restricted CTL responses, in contrast, may rely on differential expression levels of MUC1 between normal and transformed cells to confer tumour specificity, as has been reported for other antigens. The MUC1 specific immune response in C57BL/6 mice immunised with VV-MUC1 encoding

the full length MUC1 protein was found to be MHC class I-restricted and the MUC1 specific CTL lines that were generated are classical $CD8^+$ CTL that express the TCR α and β chains.

Once MUC1-specific CTL lines had been generated (Chapter 3), it was of interest to deterimine whether the CTL responses in mice immunised with the full length MUC1 antigen mapped to the VNTR region of MUC1 or to the non-repetitive part of the molecule. Mapping approaches using length variants of the MUC1 cDNA showed that the dominant epitope maps to the N-terminal signal sequence of MUC1.

The CTL were use in an adoptive transfer model and could protect wildtype as well as MUC1-transgenic mice from tumour challenge, while no autoimmunity against MUC1-expressing epithelial tissues was observed in the MUC1-transgenic mice. This implies that MUC1 specific CTL that recognise a non-VNTR derived epitope, can differentiate between tumour cells expressing high levels of MUC1 and endogenous MUC1 expression on normal epithelial tissues of the transgenic mouse (Chapter 4). Furthermore, from a clinical point of view these results indicate that MUC1 specific immunotherapy directed against non-VNTR derived epitopes may be able to differentiate between normal MUC1-expressing tissues and MUC1 over-expressing tumours in humans, and emphasises that the whole MUC1 sequence should be regarded as a potential target for immunotherapy. In line with these findings, work described in chapter 6 aimed to identify HLA-A*0201 restricted CTL from the whole MUC1 sequence in humans.

7.2 Discussion:

7.2.1 Cross-presentation of signal sequence derived epitopes

Chapter 5 showed that the immunodominant epitope recognised by the MUC1-specific CTL that were induced by immunisation with VV-MUC1, mapped to the signal sequence of MUC1. This epitope is processed and presented in a TAP- and proteasome-independent manner, which is not unusual for signal peptide derived epitopes. In chapter 5, the processing and presentation of such epitopes for the endogenous MHC class I pathway was discussed at length. It is likely that the signal sequence containing the epitope is cleaved by a signal peptidase after the signal sequence has inserted into the membrane in a loop like fashion. The epitope may then be released from the C-terminal part of the signal sequence directly into the ER by a signal peptide peptidase (SPP) which cleaves the signal sequence in the hydrophobic H-region. Having gained access to the ER, the epitope can enter the MHC class I pathway for direct presentation by MUC1-expressing epithelial and tumour cells. It is interesting to consider how CTL are primed against such an epitope. In Chapter 1 the role of a cross-presenting professional APC in priming of CTL was emphasised and it is likely that the MUC1 specific CTL recognising this signal sequence derived epitope are also primed by a professional APC. For this to take place, the antigen has to be either expressed by the APC or transferred from the MUC1-expressing cells to the professional APC.

Epitopes derived from the native MUC1 protein could be cross-presented by professional APC, that take up MUC1 protein shed from the cell surface of VV-MUC1 infected or CDNA transfected myocytes. However, the short signal sequence-derived epitope, which is not part of the native MUC1 protein, is probably not shed from the cell and may very well be degraded before it reaches the MHC class I processing pathway of the cross-presenting APC. One mechanism that could account for how this epitope is transferred to the APC, involves heat shock proteins. Heat shock proteins chaperoning short antigenic peptides derived from the ER have been shown to be transferred to cross-priming APC thus

allowing peptide transfer from a somatic cell to the MHC class I processing pathway of the APC (Suto and Srivastava, 1995).

Alternatively, VV-MUC1 may infect the professional APC directly which could then present the signal sequence derived epitope for CTL priming. This explanation is consistent with the finding that VV-MUC1 could induce MUC1 specific CTL response while i.m. DNA vaccination alone was not successful. I.m. DNA vaccination is known to direct antigen expression by myocytes and is less efficient in transfecting APC directly.

7.2.2 Tolerance to MUC1

As part of this thesis, MUC1-specific CTL responses were induced in mice that do not express MUC1 as a self antigen. This is quite different from the situation in humans, were MUC1 is expressed widely on glandular epithelial cells of the breast, stomach, intestine, ovary, pancreas, kidney and salivary gland but also on activated T-cells and a large proportion of EBV transformed B cell lines *in vitro*. Immuno-histochemistry of adult human thymus did not show any MUC1 staining, while a small number of large cells located in the thymic medulla of MUC1 transgenic mice stained positive for MUC1 (Isabel Correa, personal communication). These cell may represent medullary DC that are involved in inducing central tolerance. Clarification of this matter both in MUC1 transgenic mice and humans is important. Questions that were not addressed in this thesis and will need further work are whether MUC1-specific immune responses can also be induced in a host where MUC1 is expressed as a self antigen similar to the human situation, This is likely to depend on the degree of T-cell tolerance to MUC1.

Mechanisms of tolerance

MUC1-specific tolerance in MUC1 transgenic mice has been reported previously (Gong et al., 1998; Rowse et al., 1998), and several mechanisms may be responsible:

- Firstly, MUC1 expression in the thymic medulla of the could lead to deletion of MUC1-specific T cells during thymic selection.
- Secondly, there may be peripheral tolerance to MUC1 due to presentation of MUC1 epitopes by non-priming APC in the periphery.
- Thirdly, there may be tumour-induced immuno-suppression.

Tolerance in MUC1 transgenic mice could be overcome in some experimental settings in which immune responses against MUC1-expressing tumours could be induced by using immunisation protocols with B7.1 transfected MUC1 positive tumour cells (Smith et al., 1999) or fusions of DC and MUC1 expressing tumours (Gong et al., 1998).

Differentiating between central and peripheral tolerance in MUC1 TG mice

Mouse models may prove helpful for a more direct to differentiation between the impact of central versus peripheral tolerance. For example, spleen- and lymphnode-derived lymphocytes from MUC1 transgenic mice could be transferred into wild type mice that have been sub-lethally irradiated. This would reconstitute a mouse that does not express MUC1 in its peripheral tissues and will thus not induce peripheral tolerance to MUC1. If such an animal is immunised with MUC1 cDNA and VV-MUC1, a MUC1 specific CTL response should only develop if MUC1 specific T cells were not previously deleted in the MUC1 transgenic mice.

To exclude that MUC1-specific CTL induced in such a mouse where derived from the T-cell repertoire of the wild type animal, rather than the MUC1 transgenic mouse, congenic mouse strains could be used that only differ in one allele for a lymphocyte marker such as Thy1.1 and Thy1.2 against which detection mAb are available.

Peripheral tolerance mechanisms: The decision between cross-tolerance and cross-priming

The introduction already described the paradox of the tumour antigen being located in the periphery, while naïve T cells recirculate throughout the secondary lymphoid organs. In the current model that overcomes this dilemma, peripheral antigen is taken up by a bone marrow-derived APC (Figure 7.1;1) that migrates (Figure 7.1;2) to the secondary lymphoid organs (Figure 7.1;3,4) to cross present antigen to naïve T-cells (Tn) that have emigrated from the thymus (Figure 7.1;9) and recirculate via the secondary lymphoid (Figure 7.1;10). Depending on the context of this presentation either CTL-priming (Figure 7.1;3) or CTL tolerisation (Figure 7.1;4) takes place.

Most of these data were obtained in experimental models using self-antigens. If cross-presentation of antigen by a professional APC automatically lead to activation of CTL (Figure 7.1;3), then the presentation of self-antigens would automatically lead to autoimmunity. This was addressed useing a transgenic mice expressing a membrane bound form of OVA under the rat insulin promoter in the pancreatic islets and in the kidney proximal tubular cells, thymus and testis (OVA-rip mice). It was demonstrated, that when OVA specific TCR transgenic $CD8^+$ cells were adoptively transferred to these animals OVA was presented on BM-derived APC in the local draining lymph nodes. After initial proliferation of the OVA specific TCR transgenic $CD8^+$ cells in the lymph node these cell were deleted from the T cell pool (Kurts et al., 1996) (Figure 7.1;4). This deletion was overcome and auto immunity was induced when antigen-specific $CD4^+$ T helper cells were co-administered. This suggests that by provision of $CD4^+$ T-cell help, the environment in which the CTL engages with its antigen is changed from a tolerising one into a CTL priming one (Kurts et al., 1997). Similar results were obtained with non-self antigens using the H-Y model, i.e response to Q1a and H-Y were tolerogenic in the absence of $CD4^+$ T cell help (Guerder and Matzinger, 1992) H-Y specific transgenic T cells which normally proliferate and then die in response to priming by

male spleen cells was increased by providing a stimulus of CD4 cells (Kirberg et al., 1993).

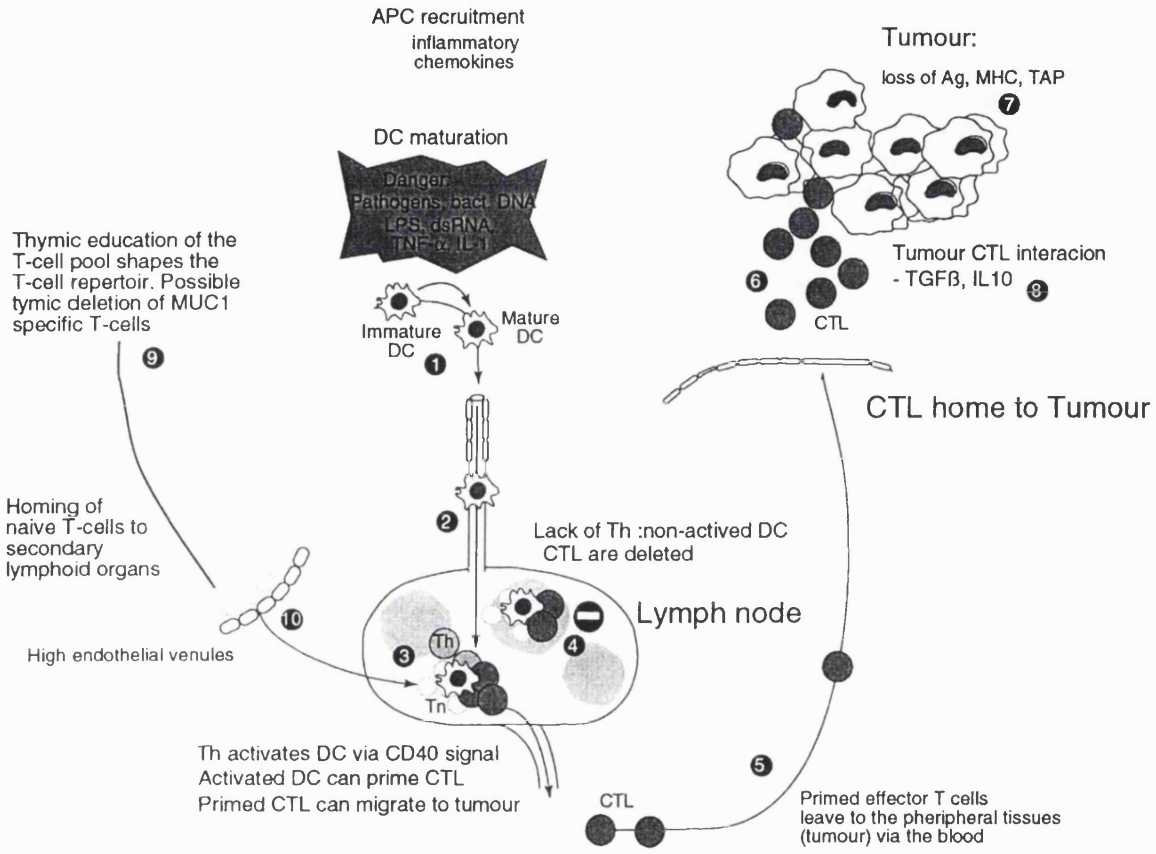


Figure 7.1: Overview of cellular immune responses against tumours.

Naïve T cells emigrate from the thymus having escaped central tolerance mechanisms (9) and localise to secondary lymphoid organs (10) where they can interact with dendritic cells (DC). Immature DC home to sites of inflammation (1,2) where they take up antigen for cross presentation. Maturation signals ("danger") in the form of naked plasmid DNA, double stranded RNA (dsRNA), LPS but also TNFαand IL-1 lead to maturation. Upon maturation, the DC migrate to the draining lymph nodes (2).In the lymophnode CTL are either primed (3) or deleted (4). Primed CTL recirculate through peripheral tissues of the body and to interact with tumour in the periphery (6) where tumour esape mechanisms may allow tumour escape from the CTL response (7,8). adapted from (Sallusto et al., 1998)

Most tumour antigens should be handled in a similar manner to these model self antigens. Recently a similar result was obtained in a tumour model in which heamaglutinin (HA) expressing tumours could be controlled by large numbers of $CD8^+$ T-cells form HA specific TCR transgenic mice (Marzo et al., 1999). However, when CTL numbers were lower, tumour-specific $CD4^+$ T cells greatly enhance the eradication of tumour cells, stressing the importance of antigen presentation to MHC class II restricted cells (Marzo et al., 1999).

Similarly APC from MUC1- transgenic mice may present MUC1 derived epitopes in the draining lymph nodes. This may result in tolerisation of these CTL unless the context in which the antigen is presented can be altered into a CTL priming one. This should be the aim of immunisation protocols using MUC1 as the target antigen.

Since balance between proliferation and deletion of auto-reactive $CD8^+$ T cells can be shifted towards an immunogenic response if $CD4^+$ T cell help is available, attempts to generate a strong anti tumour response will need to focus on the induction of tumour specific $CD4^+$ T cell responses in the induction of MUC1 specific responses.

Tumour immune escape and tumour induced tolerance at the level of the effector phase

Successful immunisation approaches also have to take into account that even if CTL have been primed in the draining lymph nodes (Figure 7.1;3), and have been able to home to the peripheral tissues (Figure 7.1;5) to attack tumour cells (Figure 7.1;6), tumour cells may still escape from the effector phase of a CTL response:

Newly primed CTL that can migrate to the peripheral tissues may be unable to penetrate the tumour milieu including surrounding connective tissues. This may be due to an alteration of the homing receptors at the tumour site, cytokine production, or even tumour vasculature being made up of tumour rather than endothelial cells lacking lymphocyte homing receptors (Maniotis et al., 1999). Once the CTL get to the tumour site, new tumour variants may arise which have down-regulated expression of the tumour antigen (Uyttenhove et al., 1983). Also, down regulation

et al., 1997; Seliger et al., 1997) loss of TAP expression (Cromme et al., 1994), TAP function (Chen et al., 1996) or proteasome subunits (Restifo et al., 1993) would have similar effects.

Even if appropriate recognition and activation of tumour-specific CTL occurs, they may still be ineffective due to immunosuppressive factors such as TGF-β (Ranges et al., 1987; Torre-Amoine et al., 1990), IL-10 (Becker et al., 1994; Matsuda et al., 1994), anergy induction or induction of apoptosis by FAS-L expression (O'Connell et al., 1996; Walker et al., 1997). Both TGF-β and IL-10 (Wojtowiczpraga et al., 1997) secretion but no FAS-L (CD95L)(Xerri et al., 1997) expression have been observed in breast cancers.

A final consideration is that each CTL will only go though a limited number of lytic cycles (Chen et al., 1992; Leong et al., 1996; Townsend and Allison, 1993). This implies that the immune response needs to be maintained to have a significant impact on any tumour. This will require continued presentation of the tumour antigen in a draining lymph node in a CTL priming context. Further more the tumour burden should be as small as possible.

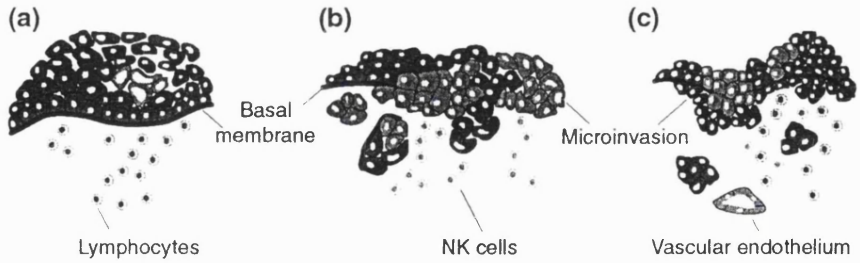


Figure 7.2: Model for stages of tumour escape form immune surveillance.

a) a HLA class I^+ tumour (red) comes under selective pressure from CTL mediated cytotoxicity and generates a HLA-deficient variant that is T-cell resistant but NK-cell susceptible by down regulation of HLA (orange). At this stage the tumour generates a new variant that has lost the HLA restriction element for the CTL response but expresses the other HLA alleles than confer resistance to NK lysis (green). This variant escapes both T-cell and NK attack and takes over the tumour cell population (C). (Adapted from (Garrido et al., 1997))

Down regulation of MHC class I expression should make tumour cells susceptible to attack by natural killer (NK) cells, which can lyse HLA class I-deficient targets. It has been shown that recognition, activation and destruction of target cells by NK cells in the circulation take only a few hours, which may be an important mechanism clearing HLA-negative tumour cells that reach the circulation during micro-metastasis formation. However, despite such NK surveillance, tumour cells clearly spread and metastasise This has been attributed to selection for tumour variants that are resistance to NK cell attack. (See Figure 7.2).

7.3 Conclusion

This thesis has shown that MUC1-specific CTL responses directed against the non-VNTR region of MUC1 can, in principle, differentiate between normal and MUC1-overexpressing tissues, with out the induction of significant auto-immunity. Several potential HLA-A*0201-restricted CTL epitopes from the non-VNTR region have been identified which may be useful as immunogens for the induction of MUC1-specific CTL in humans or for detection of MUC1-specific CTL responses in patients enrolled in MUC1 immunisation trials. For successful CTL mediated immunotherapy, several hurdles still need to be taken. Patients may be partially tolerant to MUC1 and more successful immunisation protocols need to be developed to induce strong and sustainable tumour-specific immune responses. The most important hurdle to cross in tumour immunotherapy in general is likely to be tumour heterogeneity in relation to escape from T-cell and NK cell-mediated immuno-surveillance. Tumours may have to be extensively characterised for TAP, HLA and tumour antigen expression. Treatment based on immunotherapy should start early in the disease and utilise an immunisation protocol that produces a specific CTL response, restricted by several different HLA alleles, in a way that promotes strong memory formation as well as a strong sustainable response. The current clinical trials using MUC1 as an immunogen, will hopefully be able to address some of these aspects.

Appendix

Name	Backbone	Insert	Methods	Notes
pMUC1	*β-actin promotor*	Full length MUC1 with 42 TRs	(Batra et al., 1991)	Used for cDNA vaccination experiments. Empty vector is pVO
pVO	β-actin promotor	none		Used for cDNA vaccination experiments. Empty vector is pVO
pMUC-TR	β-actin promotor	Full length MUC1 with 0TR	TR were removed by digest with Sma I.	Made by O. Rugatti
pMUC1-256	β-actin promotor	MUC1 without transmembrane and cytoplasmic tail	Removal of TM and cytoplasmic tail using AccI	Made by O. Rugatti
pMUC90-475	β-actin promotor			
pMUC1-90	pcDNA3.1 Invitrogen	MUC1 amino acids 1-90	HindIII- PVUII frgament into HINDIII-EcoRV	
pMUC1-39	pCR3.1 Invitrogen		PCR product	
pMUC1-30	pCR3.1 Invitrogen			
pMUC1-25	pCR3.1 Invitrogen			
pMUC1-21	pCR3.1 Invitrogen			

pMUC90-475

This construct was made by replacing the Hind III- Pvu II fragment encoding the first 90 amino acids of MUC1 with a double stranded oligonucleotide comprising the native MUC1 Kosak sequence, start coding and several linking aminoacids. For details see below:

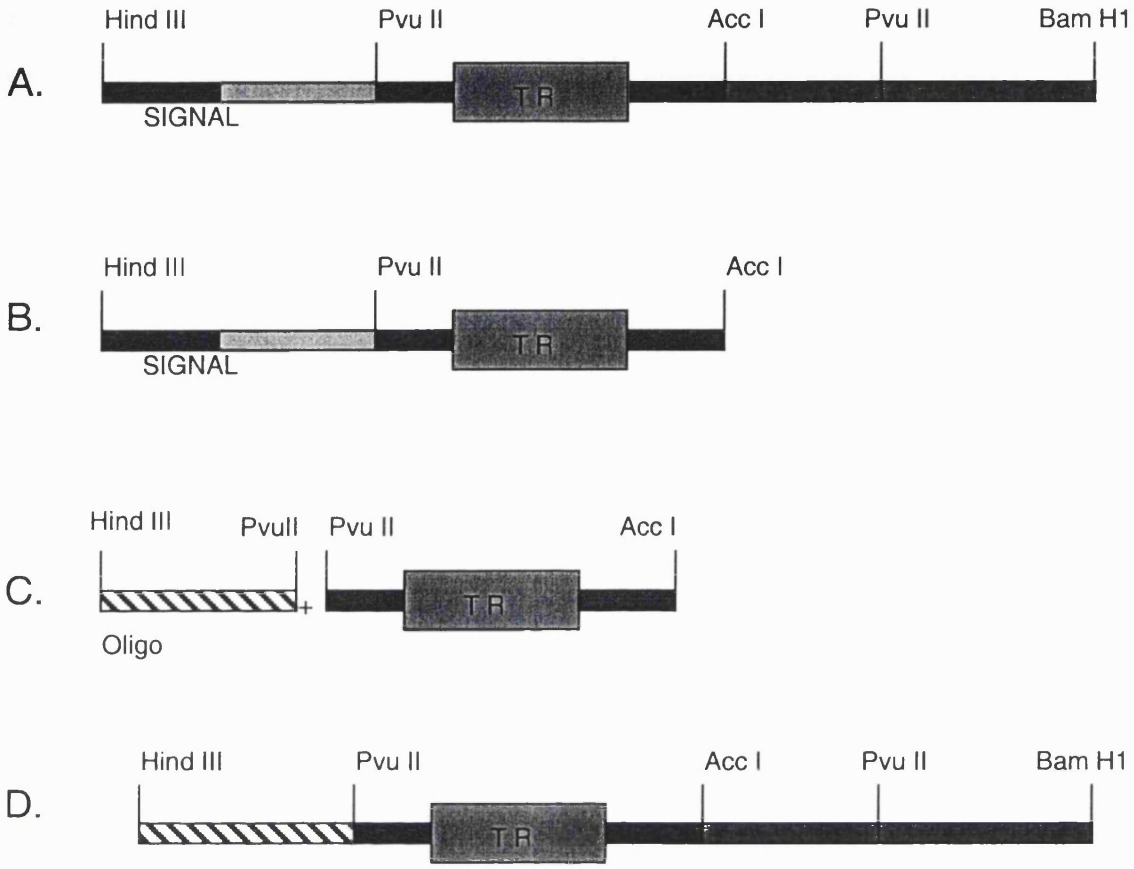


Figure 0.1: Cloning strategy for pMUC90-475

A.) shows the full length MUC1 cDNA insertt with 7 TRs. The signal sequence is part of the Hind III and Pvu II fragement.

B.) Since there is an additional Pvu II sites down stream of the TR, a fragment containing the signal peptide and the TRs was released using Hind III and Acc I restriction endonucleases and purified

C.) The Hind III- Acc I fragment was then cut with Pvu II to remove the signal sequence and and additional extracellular domain sequence.

D.) The Hind III - Pvu II fragment was then replaced by a synthetic double stranded oligonucleotide with the relevant annealing ends. The final insert (Hind III -> Bam H1) was moved into the an expression vector driven by the ß- Actin promoter for expression in mamalian cells .

pMUC1-90

This construct encodes the first 90 amino acids of MUC1 and was made by digestion of pMUC1 with Hind III and Pvu II. The Hind III lies within the multiple cloning site polylinker of pMUC1 and the Pvu II maps to nucleotide position 343 of the MUC1 cDNA. The resulting fragment of 388bp was purified and cloned into

pcDNA3.1 that had previously been digested with Hind III and EcoRV, purified and de phosphorylated. The original PvuII site was lost by ligation with the blunt ended EcoRV site (see Figure 0.2.)

The final construct encodes the following MUC1 derived amino acids shown in bold as well as 14 additional unrelated aminoacids derived from the pcDNA3.1 polylinker:

MTPGTQSPFFLLLLLTVLTVVTGSGHASSTPGGEKETSATQRSSVPSSTEKNAVS
MTSSVLSSHSPGSGSSTTQGQDVTLAPATEPASGS DPAQWRPLESRGPV

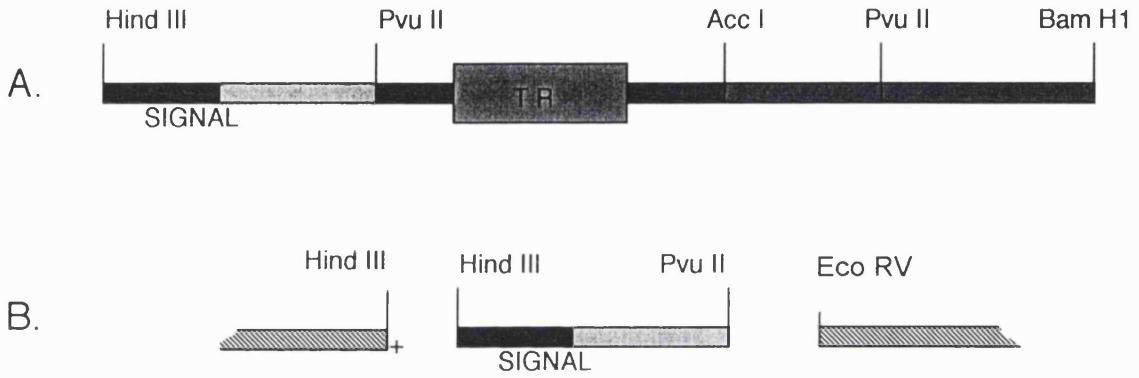


Figure 0.2: Cloning strategy for pMUC1-90

Length variant constructs

Constructs pMUC1-39, pMUC1-30, pMUC1-25 and pMUC1-21 were made by TA cloning of PCR products from pMUC1 encoding the indicated number of N-terminal amino acids of MUC1. The forward primer was designed in a way that the native MUC1 Kosak sequence was preserved. The reverse primers were chosen in a way that an in frame stop codon was present to ensure a protein product of defined length.

The PCR products were set up in a way that only a product of the desired length was detectable by agarose gel electrophoresis and directly cloned into pCR3.1-uni (Invitrogen) according manufacturers instructions.

Forward primer:

5' AAGCTTACCACCATGACACCGGGCACCCAGTCTCCTTTCTT 3'

Reverse primers:

pMUC1-39: 5' TCAAGCCGAAGTCTCCTTTTC 3'

pMUC1-30: 5' TCAGGTAGAGCTTGCATGACC 3'

pMUC1-25: 5' TCAACCAGAACCTGTAACAACTGTAAG 3'

pMUC1-21: 5' TCAACCGGATCCTGTAACAACTGTAAGCACTGTGA GGAGCAGCAG3'

References

Abdel-Motal, U. M., Berg, L., Rosen, A., Bengtsson, M., Thorpe, C. J., Kihlberg, J., Dahmen, J., Magnusson, G., Karlsson, K. A., and Jondal, M. (1996). Immunization with glycosylated Kb-binding peptides generates carbohydrate-specific, unrestricted cytotoxic T cells. Eur J Immunol *26*, 544-51.

Acres, R. B., Hareuveni, M., Balloul, J. M., and Kieny, M. P. (1993). Vaccinia virus MUC1 immunization of mice: immune response and protection against the growth of murine tumors bearing the MUC1 antigen. J Immunother *14*, 136-43.

Agrawal, B., Krantz, M. J., Parker, J., and Longenecker, B. M. (1998). Expression of MUC1 mucin on activated human T cells: implications for a role of MUC1 in normal immune regulation. Cancer Res *58*, 4079-81.

Agrawal, B., Krantz, M. J., Reddish, M. A., and Longenecker, B. M. (1998). Rapid induction of primary human CD4+ and CD8+ T cell responses against cancer-associated MUC1 peptide epitopes. Int Immunol *10*, 1907-16.

Agrawal, B., Reddish, M. A., Christian, B., VanHeele, A., Tang, L., Koganty, R. R., and Longenecker, B. M. (1998). The anti-MUC1 monoclonal antibody BCP8 can be used to isolate and identify putative major histocompatibility complex class I associated amino acid sequences [In Process Citation]. Cancer Res *58*, 5151-6.

Allison, J. P. (1994). CD28-B7 interactions in T cell activation. Curr. Opinion in Immunol. *6*, 414-419.

Allison, J. P., and Krummel, M. F. (1995). The Yin and Yang of T cell costimulation [see comments]. Science *270*, 932-3.

Apostolopoulos, V., Haurum, J. S., and MCKenzie, I. F. C. (1997). MUC1 peptide epitopes associated with five different H-2 class I molecules. Eur. J. Immunol. *27*, 2579-2587.

Apostolopoulos, V., Karanikas, V., Haurum, J. S., and McKenzie, I. F. (1997). Induction of HLA-A2-restricted CTLs to the mucin 1 human breast cancer antigen. J Immunol *159*, 5211-8.

Apostolopoulos, V., Loveland, B. E., Pietersz, G. A., and McKenzie, I. F. (1995). CTL in mice immunized with human mucin 1 are MHC-restricted. J Immunol *155*, 5089-94.

Arklie, J., Taylor Papadimitrious, J., Bodmer, W., Egan, M., and Millis, R. (1981). Differentiation antigens expressed by epithelial cells in the lactating breast are also detectable in breast cancers. Int J Cancer *28*, 23-9.

Bakker, A. B. H., Schreurs, M. W. J., De Boer, A. J., Kawakami, Y., Rosenberg, S. A., Adema, G. J., and Figdor, C. G. (1994). Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. J. Exp. Med. *179*, 1005-1009.

Bakker, A. B. H., Schreurs, M. W. J., Tafazzul, G., de Boer, A. J., Kawakami, Y., Adema, G. J., and Figdor, C. G. (1995). Identification of a novel peptide derived from the melanocyte GP100 antigen as the dominant epitope recognized by an HLA-A2.1-restricted anti-melanoma CTL line. Int. J. Cancer *62*, 97-102.

Balloul, J. M., Acres, R. B., Geist, M., Dott, K., Stefani, L., Schmitt, D., Drillien, R., Spehner, D., McKenzie, I., Xing, P. X., and et al. (1994). Recombinant MUC 1 vaccinia virus: a potential vector for immunotherapy of breast cancer. Cell Mol Biol Noisy le grand *1*, 49-59.

Bartek, J., Bartkova, J., Kyprianou, N., Lalani, E. N., Staskova, Z., Shearer, M., Chang, S., and Taylor Papadimitriou, J. (1991). Efficient immortalization of luminal epithelial cells from human mammary gland by introduction of simian virus 40 large tumor antigen with a recombinant retrovirus. Proc Natl Acad Sci U S A *88*, 3520-4.

Batra, S. K., Kern, H. F., Worlock, A. J., Metzgar, R. S., and Hollingsworth, M. A. (1991). Transfection of the human Muc 1 mucin gene into a poorly differentiated human pancreatic tumor cell line, Panc1: integration, expression and ultrastructural changes. J Cell Sci *100*, 841-9.

Becker, J. C., Czerny, C., and Brocker, E. B. (1994). Maintenance of clonal anergy by endogenously produced IL-10. Int Immunol *6*, 1605-12.

Bennett, E. P., Hassan, H., Mandel, U., Mirgorodskaya, E., Roepstorff, P., Burchell, J., Taylor-Papadimitriou, J., Hollingsworth, M. A., Merkx, G., van Kessel, A. G., Eiberg, H., Steffensen, R., and Clausen, H. (1998). Cloning of a Human UDP-N-Acetyl-alpha-D-Galactosamine:Polypeptide N- Acetylgalactosaminyltransferase That Complements Other GalNAc- Transferases in Complete O-Glycosylation of the MUC1 Tandem Repeat. J Biol Chem *273*, 30472-30481.

Bennett, S. R., Carbone, F. R., Karamalis, F., Miller, J. F., and Heath, W. R. (1997). Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J Exp Med *186*, 65-70.

Bennett, S. R. M., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F. A. P., and Heath, W. R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signaling. Nature *393*, 478-480.

Berry, N., Jones, D. B., Smallwood, J., Taylor, I., Kirkham, N., and Taylor Papadimitriou, J. (1985). The prognostic value of the monoclonal antibodies HMFG1 and HMFG2 in breast cancer. Br J Cancer *51*, 179-86.

Bevan, M. J. (1976). Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J. Exp. Med. *143*, 1283-1288.

Birkeland, S. A. (1997). De novo cancers complicating renal transplantation: experience in the Nordic countries. Ann Transplant *2*, 22-6.

Birkeland, S. A., Storm, H. H., Lamm, L. U., Barlow, L., Blohme, I., Forsberg, B., Eklund, B., Fjeldborg, O., Friedberg, M., Frodin, L., and et al. (1995). Cancer risk after renal transplantation in the Nordic countries, 1964- 1986. Int J Cancer *60*, 183-9.

Bizouarne, N., Balloul, J. M., Schatz, C., Acres, B., and Kieny, M. P. (1996). Immunotherapy of breast cancer using a recombinant vaccinia virus expressing the human MUC1 and IL2 genes. In Breast Cancer Advances in Biology and Therapeutics, F. Calvo, M. Crepin and H. Magdelenat, eds.: John Libbey Publishers.), pp. 309-313.

Bjorkman, P. J., and Parham, P. (1990). Structure, function, and diversity of class I major histocompatibility complex molecules. Annu. Rev. Biochem. *59*, 253-288.

Bogyo, M., Shin, S., McMaster, J. S., and Ploegh, H. L. (1998). Substrate binding and sequence preference of the proteasome revealed by active-site-directed affinity probes. Chem Biol *5*, 307-20.

Braga, V. M., Pemberton, L. F., Duhig, T., and Gendler, S. J. (1992). Spatial and temporal expression of an epithelial mucin, Muc-1, during mouse development. Development *115*, 427-37.

Brichard, V., Van Pel, A., Wolfel, T., Wolfel, C., De Plaen, E., Lethe, B., Coulie, P., and Boon, T. (1993). The tyrosinase gene codes for an antigen recognized by

autologous cytotoxic T lymphocytes on HLA-A2 melanomas. J. Exp. Med. *178*, 489-495.

Brockhausen, I., Yang, J. M., Burchell, J., Whitehouse, C., and Taylor Papadimitriou, J. (1995). Mechanisms underlying aberrant glycosylation of MUC1 mucin in breast cancer cells. Eur J Biochem *233*, 607-17.

Brodie, S. J., Lewinsohn, D. A., Patterson, B. K., Jiyamapa, D., Krieger, J., Corey, L., Greenberg, P. D., and Riddell, S. R. (1999). In vivo migration and function of transferred HIV-1-specific cytotoxic T cells [see comments]. Nat Med *5*, 34-41.

Brossart, P., Heinrich, K. S., Stuhler, G., Behnke, L., Reichardt, V. L., Stevanovic, S., Muhm, A., Rammensee, H. G., Kanz, L., and Brugger, W. (1999). Identification of HLA-A2-restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies. Blood *93*, 4309-17.

Burchell, J., Gendler, S., Taylor Papadimitriou, J., Girling, A., Lewis, A., Millis, R., and Lamport, D. (1987). Development and characterization of breast cancer reactive monoclonal antibodies directed to the core protein of the human milk mucin. Cancer Res *47*, 5476-82.

Burchell, J., and Taylor Papadimitriou, J. (1993). Effect of modification of carbohydrate side chains on the reactivity of antibodies with core-protein epitopes of the MUC1 gene product. Epithelial Cell Biol *2*, 155-62.

Burnet, F. M. (1970). The concept of immunological surveillance. Prog Exp Tumor Res *13*, 1-27.

Butcher, E. C., and Picker, L. J. (1996). Lymphocyte homing and homeostasis. Science *272*, 60-6.

Butterfield, L. H., Koh, A., Meng, W., Vollmer, C. M., Ribas, A., Dissette, V., Lee, E., Glaspy, J. A., McBride, W. H., and Economou, J. S. (1999). Generation of human T-cell responses to an HLA-A2.1-restricted peptide epitope derived from alpha-fetoprotein. Cancer Res *59*, 3134-42.

Carbone, F. R., and Gleeson, P. A. (1997). Carbohydrates and antigen recognition by T cells. Glycobiology *7*, 725-30.

Carbone, F. R., Kurts, C., Bennett, S. R., Miller, J. F., and Heath, W. R. (1998). Cross-presentation: a general mechanism for CTL immunity and tolerance. Immunol Today *19*, 368-73.

Carraway, K. L., and Hull, S. R. (1989). O-glycosylation pathway for mucin-type glycoproteins. Bioessays *10*, 117-21.

Cassel, D., and Forman, J. (1988). Linked recognition of helper and cytotoxic antigenic determinants for the generation of cytotoxic T lymphocytes. Ann. NY Acad. Sci. *532*, 51-60.

Cauley, L. S. (1995). Evidence for an early heavy chain intermediate in the assembly of H-2Db class I MHC molecules. Mol Immunol *32*, 137-46.

Cella, M., Sallusto, F., and Lanzavecchia, A. (1997). Origin, maturation and antigen presenting function of dendritic cells. Curr Opin Immunol *9*, 10-6.

Chapman, H. A. (1998). Endosomal proteolysis and MHC class II function. Curr Opin Immunol *10*, 93-102.

Chen, H. L., Gabrilovich, D., Tampe, R., Girgis, K. R., Nadaf, S., and Carbone, D. P. (1996). A functionally defective allele of TAP1 results in loss of MHC class I antigen presentation in a human lung cancer [see comments]. Nat Genet *13*, 210-3.

Chen, L., Ashe, S., Brady, W. A., Hellström, I., Hellström, K. E., Ledbetter, J., McGowan, P., and Linsley, P. S. (1992). Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. Cell *71*, 1093-1102.

Chen, L., Chen, D., Manome, Y., Dong, Y., Fine, H. A., and Kufe, D. W. (1995). Breast cancer selective gene expression and therapy mediated by recombinant adenoviruses containing the DF3/MUC1 promoter. J Clin Invest *96*, 2775-82.

Chen, P. W., Wang, M., Bronte, V., Zhai, Y., Rosenberg, S. A., and Restifo, N. P. (1996). Therapeutic antitumor response after immunization with a recombinant adenovirus encoding a model tumor-associated antigen. J. Immunol. *156*, 224-231.

Chicz, R. M., Urban, R. G., Gorga, J. C., Vignalli, D. A. A., Lane, W. S., and Strominger, J. L. (1993). Specificity and promiscuity among naturally processed peptides bound to HLA-DR molecules. J. Exp. Med. *178*, 27-47.

Chu, R. S., Askew, D., Noss, E. H., Tobian, A., Krieg, A. M., and Harding, C. V. (1999). CpG oligodeoxynucleotides down-regulate macrophage class II MHC antigen processing. J Immunol *163*, 1188-94.

Clausen, H., and Bennett, E. P. (1996). A family of UDP-GalNAc: polypeptide N-acetylgalactosaminyl-transferases control the initiation of mucin-type O-linked glycosylation. Glycobiology *6*, 635-646.

Cohen, E. P., and Kim, T. S. (1994). Neoplastic cells that express low levels of MHC class I determinants escape host immunity. Semin Cancer Biol *5*, 419-28.

Connor, M. E., and Stern, P. L. (1990). Loss of MHC class I expression in cervical carcinomas. Int. J. Cancer *46*, 1029-1037.

Correale, P., Walmsley, K., Zaremba, S., Zhu, M., Schlom, J., and Tsang, K. Y. (1998). Generation of human cytolytic T lymphocyte lines directed against prostate-specific antigen (PSA) employing a PSA oligoepitope peptide. J Immunol *161*, 3186-94.

Corthay, A., Backlund, J., Broddefalk, J., Michaelsson, E., Goldschmidt, T. J., Kihlberg, J., and Holmdahl, R. (1998). Epitope glycosylation plays a critical role for T cell recognition of type II collagen in collagen-induced arthritis. Eur J Immunol *28*, 2580-90.

Cromme, F. V., Airey, J., Heemels, M. T., Ploegh, H. L., Keating, P. J., Stern, P. L., Meijer, C. J. L. M., and Walboomers, J. M. M. (1994). Loss of transporter protein, encoded by the TAP-1 gene, is highly correlated with loss of HLA expression in cervical carcinomas. J. Exp. Med. *179*, 335-340.

Cromme, F. V., Meijer, C. J. L. M., Snijders, P. J. F., Uyterlinde, A., Kenemans, P., Helmerhorst, T., Stern, P. L., Van Den Brule, A. J. C., and Walboomers, J. M. M. (1993). Analysis of MHC class I and II expression in relation to HPV genotypes in premalignant and malignant cervical lesions. Br. J. Cancer *67*, 1372-1380.

D'Amaro, J., Houbiers, J. G., Drijfhout, J. W., Brandt, R. M., Schipper, R., Bavinck, J. N., Melief, C. J., and Kast, W. M. (1995). A computer program for predicting possible cytotoxic T lymphocyte epitopes based on HLA class I peptide-binding motifs. Hum Immunol *43*, 13-8.

Davis, H. L., Michel, M. L., and Whalen, R. G. (1993). DNA-based immunization induces continuous secretion of hepatitis B surface antigen and high levels of circulating antibody. Hum Mol Genet *2*, 1847-51.

Deck, B., Elofsson, M., Kihlberg, J., and Unanue, E. R. (1995). Specificity of glycopeptide-specific T cells. J Immunol *155*, 1074-8.

Deck, M. B., Sj#lin, P., Unanue, E. R., and Kihlberg, J. (1999). MHC-Restricted, Glycopeptide-Specific T Cells Show Specificity for Both Carbohydrate and Peptide Residues. J Immunol *162*, 4740-4744.

Diehl, L., den Boer, A. T., Schoenberger, S. P., van der Voort, E. I., Schumacher, T. N., Melief, C. J., Offringa, R., and Toes, R. E. (1999). CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy [In Process Citation]. Nat Med *5*, 774-9.

Dokurno, P., Bates, P. A., Band, H. A., Stewart, L. M. D., Lally, J. M., Burchell, J. M., Taylor-Papadimitriou, J., Snary, D., Sternberg, M. J. E., and Freemont, P. S. (1998). Crystal structure at 1.95 A resolution of the breast tumour-specific antibody SM3 complexed with its peptide epitope reveals novel hypervariable loop recognition. J Mol Biol *284*, 713-28.

Domenech, N., Henderson, R. A., and Finn, O. J. (1995). Identification of an HLA-A11-restricted epitope from the tandem repeat domain of the epithelial tumor antigen mucin. J Immunol *155*, 4766-74.

Drijfhout, J. W., Brandt, R. M., D'Amaro, J., Kast, W. M., and Melief, C. J. (1995). Detailed motifs for peptide binding to HLA-A*0201 derived from large random sets of peptides using a cellular binding assay. Hum Immunol *43*, 1-12.

Dudler, T., Altmann, F., Carballido, J. M., and Blaser, K. (1995). Carbohydrate-dependent, HLA class II-restricted, human T cell response to the bee venom allergen phospholipase A2 in allergic patients. Eur J Immunol *25*, 538-42.

Elliott, T., Cerundolo, V., Elvin, J., and Townsend, A. (1991). Peptide-induced conformational change of the class I heavy chain. Nature *351*, 402-406.

Engelhard, V. H. (1994). Structure of Peptides associated with class I and class II MHC molecules. Curr. Opin. Immunology *6*.

Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H. G. (1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature *351*, 290-6.

Feltkamp, M. C., Smits, H. L., Vierboom, M. P., Minnaar, R. P., de Jongh, B. M., Drijfhout, J. W., ter Schegget, J., Melief, C. J., and Kast, W. M. (1993). Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. Eur J Immunol *23*, 2242-9.

Fenteany, G., and Schreiber, S. L. (1996). Specific inhibition of the chymotrypsin-like activity of the proteasome induces a bipolar morphology in neuroblastoma cells. Chem Biol *3*, 905-12.

Fenteany, G., Standaert, R. F., Reichard, G. A., Corey, E. J., and Schreiber, S. L. (1994). A beta-lactone related to lactacystin induces neurite outgrowth in a neuroblastoma cell line and inhibits cell cycle progression in an osteosarcoma cell line. Proc Natl Acad Sci U S A *91*, 3358-62.

Fenton, R. G., and Longo, D. L. (1997). Danger versus tolerance: paradigms for future studies of tumor-specific cytotoxic T lymphocytes [editorial; comment]. J Natl Cancer Inst *89*, 272-5.

Finke, J., Ferrone, S., Frey, A., Mufson, A., and Ochoa, A. (1999). Where have all the T cells gone? Mechanisms of immune evasion by tumors. Immunol Today *20*, 158-160.

Finn, O. J., Jerome, K. R., Henderson, R. H., Pecher, G., Domenech, N., Magarian-Blander, J., and Barrat-Boyes, S. M. (1995). MUC-1 epithelial tumor mucin-based immunity and cancer vaccines. Immunol. Rev. *145*, 61-89.

Galli-Stampino, L., Meinjohanns, E., Frische, K., Meldal, M., Jensen, T., Werdelin, O., and Mouritsen, S. (1997). T-cell recognition of tumor-associated carbohydrates: the nature of the glycan moiety plays a decisive role in determining glycopeptide immunogenicity. Cancer Res *57*, 3214-22.

Garrido, F., Ruiz-Cabello, F., Cabrera, T., Perez-Villar, J. J., Lopez-Botet, M., Duggan-Keen, M., and Stern, P. L. (1997). Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol Today *18*, 89-95.

Gendler, S. J., Lancaster, C. A., Taylor Papadimitriou, J., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Lalani, E. N., and Wilson, D. (1990). Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. J Biol Chem *265*, 15286-93.

Gendler, S. J., Spicer, A. P., Lalani, E. N., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Boshell, M., and Taylor Papadimitriou, J. (1991). Structure and biology of a carcinoma-associated mucin, MUC1. Am Rev Respir Dis *144*, S42-7.

Gimmi, C. D., Freeman, G. J., Gribben, J. G., Gray, G., and Nadler, L. M. (1993). Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. Proc Natl Acad Sci U S A *90*, 6586-90.

Girling, A., Bartkova, J., Burchell, J., Gendler, S., Gillett, C., and Taylor Paradimitriou, J. (1989). A core protein epitope of the polymorphic epithelial mucin detected by the monoclonal antibody SM-3 is selectively exposed in a range of primary carcinomas. Int J Cancer *43*, 1072-1076.

Glithero, A., Tormo, J., Haurum, J. S., Arsequell, G., Valencia, G., Edwards, J., Springer, S., Townsend, A., Pao, Y. L., Wormald, M., Dwek, R. A., Jones, E. Y., and Elliott, T. (1999). Crystal structures of two H-2Db/glycopeptide complexes suggest a molecular basis for CTL cross-reactivity. Immunity *10*, 63-74.

Gong, J. L., Chen, D. S., Kashiwaba, M., Li, Y. Q., Chen, L., Takeuchi, H., Qu, H., Rowse, G. J., Gendler, S. J., and Kufe, D. (1998). Reversal of tolerance to human muc1 antigen in muc1 transgenic mice immunized with fusions of dendritic and carcinoma-cells. Proc Natl Acad Sci U S A *95*, 6279-6283.

Gordon, R. D., Mathieson, B. J., Samelson, L. E., Boyse, E. A., and Simpson, E. (1976). The effect of allogeneic presensitization on H-Y graft survival and in vitro cell-mediated responses to H-y antigen. J Exp Med 144, 810-20.

Graham, R. A., Burchell, J. M., Beverley, P., and Taylor-Papadimitriou, J. (1996). Intramuscular immunisation with MUC1 cDNA can protect C57 mice challenged with MUC1-expressing syngeneic mouse tumour cells. Int J Cancer *65*, 664-70.

Graham, R. A., Stewart, L. S., Peat, N. P., Beverley, P., and Taylor-Papadimitriou, J. (1995). MUC1-based immunogens for tumour therapy: development of murine model systems. Tumour Targeting *1*, 211-221.

Greenberg, P. D., and Cheever, M. A. (1984). Treatment of disseminated leukemia with cyclophosphamide and immune cells: tumor immunity reflects long-term persistence of tumor-specific donor T cells. J Immunol *133*, 3401-7.

Greenberg, P. D., Cheever, M. A., and Fefer, A. (1981). Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1+2- lymphocytes. J Exp Med *154*, 952-63.

Guerder, S., and Matzinger, P. (1992). A fail-safe mechanism for maintaining self-tolerance. J Exp Med *176*, 553-64.

Haltiwanger, R. S., Kelly, W. G., Roquemore, E. P., Blomberg, M. A., Dong, L. Y., Kreppel, L., Chou, T. Y., and Hart, G. W. (1992). Glycosylation of nuclear and cytoplasmic proteins is ubiquitous and dynamic. Biochem Soc Trans *20*, 264-9.

Hanke, T., Blanchard, T. J., Schneider, J., Hannan, C. M., Becker, M., Gilbert, S. C., Hill, A. V., Smith, G. L., and McMichael, A. (1998). Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. Vaccine *16*, 439-45.

Hanke, T., Neumann, V. C., Blanchard, T. J., Sweeney, P., Hill, A. V., Smith, G. L., and McMichael, A. (1999). Effective induction of HIV-specific CTL by multi-epitope using gene gun in a combined vaccination regime. Vaccine *17*, 589-96.

Hart, G. W., Kreppel, L. K., Comer, F. I., Arnold, C. S., Snow, D. M., Ye, Z., Cheng, X., DellaManna, D., Caine, D. S., Earles, B. J., Akimoto, Y., Cole, R. N., and Hayes, B. K. (1996). O-GlcNAcylation of key nuclear and cytoskeletal proteins: reciprocity with O-phosphorylation and putative roles in protein multimerization. Glycobiology *6*, 711-6.

Haurum, J. S. (1996). Recognition of peptides carrying post-translational modifications by class I MHC restricted cytotoxic T cells (Oxford, United Kingdom: University of Oxford,).

Haurum, J. S., Arsequell, G., Lellouch, A. C., Wong, S. Y., Dwek, R. A., McMichael, A. J., and Elliott, T. (1994). Recognition of carbohydrate by major histocompatibility complex class I-restricted, glycopeptide-specific cytotoxic T lymphocytes. J Exp Med *180*, 739-44.

Haurum, J. S., Tan, L., Arsequell, G., Frodsham, P., Lellouch, A. C., Moss, P. A., Dwek, R. A., McMichael, A. J., and Elliott, T. (1995). Peptide anchor residue glycosylation: effect on class I major histocompatibility complex binding and cytotoxic T lymphocyte recognition. Eur J Immunol *25*, 3270-6.

Heath, W. R., Kurts, C., Miller, J. F., and Carbone, F. R. (1998). Cross-tolerance: a pathway for inducing tolerance to peripheral tissue antigens. J Exp Med *187*, 1549-53.

Henderson, R. A., Michel, H., Sakaguchi, K., Shabanowitz, J., Apella, E., Hunt, D. F., and Engelhard, V. H. (1992). HLA-A2.1 associated peptides from a mutant cell line: a second pathway of antigen presentation. Science *255*, 1264-1266.

Ho, S. B., Shekels, L. L., Toribara, N. W., Kim, Y. S., Lyftogt, C., Cherwitz, D. L., and Niehans, G. A. (1995). Mucin gene expression in normal, preneoplastic, and neoplastic human gastric epithelium. Cancer Res *55*, 2681-90.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991). p53 Mutation in human cancers. Science *253*, 49-53.

Holt, G. D., Snow, C. M., Senior, A., Haltiwanger, R. S., Gerace, L., and Hart, G. W. (1987). Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked N-acetylglucosamine. J Cell Biol *104*, 1157-64.

Hombach, J., Pircher, H., Tonegawa, S., and Zinkernagel, R. M. (1995). Strictly transporter of antigen presentation (TAP)-dependent presentation of an immunodominant cytotoxic T lymphocyte epitope in the signal sequence of a virus protein. J Exp Med *182*, 1615-9.

Huang, A. Y. C., Bruce, A. T., Pardoll, D. M., and Levitsky, H. (1996). In vivo cross-priming of MHC class I-restricted antigens requires the TAP transporter. Immunity *4*, 349-355.

Huang, A. Y. C., Golumbek, P., Ahmadzadeh, M., Jaffee, E., Pardoll, D., and Levitsky, H. (1994). Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science *264*, 961-965.

Hughes, E. A., Ortmann, B., Surman, M., and Cresswell, P. (1996). The protease inhibitor, N-acetyl-L-leucyl-L-leucyl-leucyl-L- norleucinal, decreases the pool of major histocompatibility complex class I-binding peptides and inhibits peptide trimming in the endoplasmic reticulum. J Exp Med *183*, 1569-78.

Husmann, L. A., and Bevan, M. J. (1988). Cooperation between helper T cells and cytotoxic T lymphocyte precursors. Ann. NY Acad. Sci. *532*, 158-169.

Irvine, K. R., Parkhurst, M. R., Shulman, E. P., Tupesis, J. P., Custer, M., Touloukian, C. E., Robbins, P. F., Yafal, A. G., Greenhalgh, P., Sutmuller, R. P., Offringa, R., Rosenberg, S. A., and Restifo, N. P. (1999). Recombinant virus vaccination against "self" antigens using anchor- fixed immunogens. Cancer Res *59*, 2536-40.

Jenkins, M. K., and Johnson, J. G. (1993). Molecules involved in T cell costimulation. Curr. Opinion in Immunol.

Jensen, T., Galli-Stampino, L., Mouritsen, S., Frische, K., Peters, S., Meldal, M., and Werdelin, O. (1996). T cell recognition of Tn-glycosylated peptide antigens. Eur J Immunol *26*, 1342-9.

Jensen, T., Hansen, P., Galli-Stampino, L., Mouritsen, S., Frische, K., Meinjohanns, E., Meldal, M., and Werdelin, O. (1997). Carbohydrate and peptide specificity of MHC class II-restricted T cell hybridomas raised against an O-glycosylated self peptide. J Immunol *158*, 3769-78.

Jerome, K. R., Barnd, D. L., Bendt, K. M., Boyer, C. M., Taylor Papadimitriou, J., McKenzie, I. F., Bast, R. C., Jr., and Finn, O. J. (1991). Cytotoxic T-lymphocytes derived from patients with breast adenocarcinoma recognize an epitope present on the protein core of a mucin molecule preferentially expressed by malignant cells. Cancer Res *51*, 2908-16.

Kaklamanis, L., Leek, R., Koukourakis, M., Gatter, K. C., and Harris, A. L. (1995). Loss of transporter in antigen processing 1 transport protein and major histocompatibility complex class I molecules in metastatic versus primary breast cancer. Cancer Res *55*, 5191-4.

Kardon, R., Price, R. E., Julian, J., Lagow, E., Tseng, S. C., Gendler, S. J., and Carson, D. D. (1999). Bacterial conjunctivitis in Muc1 null mice. Invest Ophthalmol Vis Sci *40*, 1328-35.

Kawakami, Y., Eliyahu, S., Sakaguchi, K., Robbins, P. F., Rivoltini, L., Yannelli, J. R., Appella, E., and Rosenberg, S. A. (1994). Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J. Exp. Med. *180*, 347-352.

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

Keydar, I., Chen, L., Karby, S., Weiss, F. R., Delarea, J., Radu, M., Chaitcik, S., and Brenner, H. J. (1979). Establishment and characterization of a cell line of human breast carcinoma origin. Eur J Cancer *15*, 659-70.

Kihlberg, J., Elofsson, M., and Salvador, L. A. (1997). Direct synthesis of glycosylated amino acids from carbohydrate peracetates and Fmoc amino acids: solid-phase synthesis of biomedicinally interesting glycopeptides. Methods Enzymol *289*, 221-45.

Kirberg, J., Bruno, L., and von Boehmer, H. (1993). CD4+8- help prevents rapid deletion of CD8+ cells after a transient response to antigen. Eur J Immunol *23*, 1963-7.

Klein, G. (1991). Immunovirology of transforming viruses. Curr. Opin. Immunol. *3*, 665-673.

Kozak, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acid Research *12*, 857-872.

Kuby, J. (1994). Immunology, 2nd. ed. Edition (New York: W. H. Freeman and Company).

Kurts, C., Carbone, F. R., Barnden, M., Blanas, E., Allison, J., Heath, W. R., and Miller, J. F. (1997). CD4+ T cell help impairs CD8+ T cell deletion induced by cross- presentation of self-antigens and favors autoimmunity. J Exp Med *186*, 2057-62.

Kurts, C., Heath, W. R., Carbone, F. R., Allison, J., Miller, J. F. A. P., and Kosaka, H. (1996). Constitutive class I-restricted exogenous presentation of self-antigens in vivo. Journal of Experimental Medicine *184*, 923-930.

Kurts, C., Miller, J. F., Subramaniam, R. M., Carbone, F. R., and Heath, W. R. (1998). Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. J Exp Med *188*, 409-14.

Lehner, P. J., and Cresswell, P. (1996). Processing and delivery of peptides presented by MHC class I molecules. Curr Opin Immunol *8*, 59-67.

Leong, C., Marley, J., Loh, S., Robinson, B., and Garlepp, M. (1996). Induction and maintenance of T-cell response to a nonimmunogenic murine mesothelioma cell line requires expression of B7-1 and the capacity to upregulate class II major histocompatibility complex expression. Cancer Gene Ther *3*, 321-30.

Lewis, J. W., and Elliott, T. (1998). Evidence for successive peptide binding and quality control stages during MHC class I assembly. Curr Biol *8*, 717-20.

Ligtenberg, M. J., Kruijshaar, L., Buijs, F., van Meijer, M., Litvinov, S. V., and Hilkens, J. (1992). Cell-associated episialin is a complex containing two proteins derived from a common precursor. J Biol Chem *267*, 6171-7.

Ligtenberg, M. J., Vos, H. L., Gennissen, A. M., and Hilkens, J. (1990). Episialin, a carcinoma-associated mucin, is generated by a polymorphic gene encoding splice variants with alternative amino termini. J Biol Chem *265*, 5573-8.

Lloyd, K. O., Burchell, J., Kudryashov, V., Yin, B. W. T., and Taylor Papadimitriou, J. (1996). Comparison of O-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines. Demonstration of simpler and fewer glycan chains in tumor cells. J Biol Chem *271*, 33325-34.

Lu, P. J., Sundquist, K., Baeckstrom, D., Poulsom, R., Hanby, A., Meier-Ewert, S., Jones, T., Mitchell, M., Pitha-Rowe, P., Freemont, P., and Taylor-Papadimitriou, J. (1999). A novel gene (PLU-1) containing highly conserved putative DNA/chromatin binding motifs is specifically up-regulated in breast cancer. J Biol Chem *274*, 15633-45.

Lutcke, H. (1995). Signal recognition particle (SRP), a ubiquitous initiator of protein translocation. Eur J Biochem *228*, 531-50.

Lyko, F., Martoglio, B., Jungnickel, B., Rapoport, T. A., and Dobberstein, B. (1995). Signal sequence processing in rough microsomes. J Biol Chem *270*, 19873-8.

Mackay, C. R. (1991). T-cell memory: the connection between function, phenotype and migration pathways. Immunol Today *12*, 189-92.

Magarian-Blander, J., Ciborowski, P., Hsia, S., Watkins, S. C., and Finn, O. J. (1998). Intercellular and intracellular events following the MHC-unrestricted TCR recognition of a tumor-specific peptide epitope on the epithelial antigen MUC1. J Immunol *160*, 3111-20.

Maniotis, A. J., Folberg, R., Hess, A., Seftor, E. A., Gardner, L. M., Pe'er, J., Trent, J. M., Meltzer, P. S., and Hendrix, M. J. (1999). Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry [see comments]. Am J Pathol *155*, 739-52.

Martoglio, B., and Dobberstein, B. (1998). Signal sequences: more than just greasy peptides [In Process Citation]. Trends Cell Biol *8*, 410-5.

Martoglio, B., Graf, R., and Dobberstein, B. (1997). Signal peptide fragments of preprolactin and HIV-1 p-gp160 interact with calmodulin. Embo J *16*, 6636-45.

Marzo, A. L., Lake, R. A., Lo, D., Sherman, L., McWilliam, A., Nelson, D., Robinson, B. W., and Scott, B. (1999). Tumor antigens are constitutively presented in the draining lymph nodes. J Immunol *162*, 5838-45.

Marzo, A. L., Lake, R. A., Robinson, B. W., and Scott, B. (1999). T-cell receptor transgenic analysis of tumor-specific CD8 and CD4 responses in the eradication of solid tumors. Cancer Res *59*, 1071-9.

Matsuda, M., Salazar, F., Petersson, M., Masucci, G., Hansson, J., Pisa, P., Zhang, Q. J., Masucci, M. G., and Kiessling, R. (1994). Interleukin 10 pretreatment protects target cells from tumor- and allo- specific cytotoxic T cells and downregulates HLA class I expression. J Exp Med *180*, 2371-6.

Matzinger, P. (1994). Tolerance, danger, and the extended family. Annu. Rev. Immunol. *12*, 991-1045.

Michaelsson, E., Broddefalk, J., Engstrom, A., Kihlberg, J., and Holmdahl, R. (1996). Antigen processing and presentation of a naturally glycosylated protein elicits major histocompatibility complex class II-restricted, carbohydrate-specific T cells. Eur J Immunol *26*, 1906-10.

Michaelsson, E., Malmstrom, V., Reis, S., Engstrom, A., Burkhardt, H., and Holmdahl, R. (1994). T cell recognition of carbohydrates on type II collagen. J Exp Med *180*, 745-9.

Mitchison, N. A., and O'Malley, C. (1987). Three-cell-type clusters of T cells with antigen-presenting cells best explain the epitope linkage and non-cognate requirements of the *in-vivo* cytolytic response. European Journal of Immunology *17*, 1579-1583.

Morgan, D. J., Kreuwel, H. T., Fleck, S., Levitsky, H. I., Pardoll, D. M., and Sherman, L. A. (1998). Activation of low avidity CTL specific for a self epitope results in tumor rejection but not autoimmunity. J Immunol *160*, 643-51.

Mosse, C. A., Kittlesen, D. J., Skipper, J. C. A., Hendrickson, R., Hunt, D. F., Slingluff, C. L., and Engelhard, V. H. (1996). A tyrosinase epitope is processed after translation into the endoplasmic-reticulum. FASEB J *10*, 2523-2523.

Nakamori, S., Ota, D. M., Cleary, K. R., Shirotani, K., and Irimura, T. (1994). MUC1 mucin expression as a marker of progression and metastasis of human colorectal carcinoma. Gastroenterology *106*, 353-61.

Nieland, J. D., Da Silva, D. M., Velders, M. P., de Visser, K. E., Schiller, J. T., Muller, M., and Kast, W. M. (1999). Chimeric papillomavirus virus-like particles induce a murine self- antigen-specific protective and therapeutic antitumor immune response. J Cell Biochem *73*, 145-52.

Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng *10*, 1-6.

Nilsson, T., Pypaert, M., Hoe, M. H., Slusarewicz, P., Berger, E. G., and Warren, G. (1993). Overlapping distribution of two glycosyltransferases in the Golgi apparatus of HeLa cells. J Cell Biol *120*, 5-13.

Noto, H., Takahashi, T., Makiguchi, Y., Hayashi, T., Hinoda, Y., and Imai, K. (1997). Cytotoxic T-lymphocytes derived from bone-marrow mononuclear-cells of multiple-myeloma patients recognize an underglycosylated form of muc1 mucin. Int Immunol *9*, 791-798.

O'Connell, J., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. (1996). The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med *184*, 1075-82.

Ohashi, P. S., Oehen, S., Buerki, K., Pircher, H., Ohashi, C. T., Odermatt, B., Malissen, B., Zinkernagel, R. M., and Hengartner, H. (1991). Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. Cell *65*, 305-17.

Oldstone, M. B., Nerenberg, M., Southern, P., Price, J., and Lewicki, H. (1991). Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell *65*, 319-31.

Ormerod, M. G., Steele, K., Edwards, P. A., and Taylor Papadimitriou, J. (1984). Monoclonal antibodies that react with epithelial membrane antigen. J Exp Pathol *1*, 263-71.

Pamer, E., and Cresswell, P. (1998). Mechanisms of MHC class I--restricted antigen processing. Annu Rev Immunol *16*, 323-58.

Parker, K. C., Bednarek, M. A., and Coligan, J. E. (1994). Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol *152*, 163-75.

Peat, N., Gendler, S. J., Lalani, N., Duhig, T., and Taylor Papadimitriou, J. (1992). Tissue-specific expression of a human polymorphic epithelial mucin (MUC1) in transgenic mice. Cancer Res *52*, 1954-60.

Pekarek, L. A., Starr, B. A., Toledano, A. Y., and Schreiber, H. (1995). Inhibition of tumor growth by elimination of granulocytes. J Exp Med *181*, 435-40.

Pemberton, L. F., Rughetti, A., Taylor Papadimitriou, J., and Gendler, S. J. (1996). The epithelial mucin MUC1 contains at least two discrete signals specifying membrane localization in cells. J Biol Chem *271*, 2332-40.

Petersson, M., Charo, J., Salazar-Onfray, F., Noffz, G., Mohaupt, M., Qin, Z., Klein, G., Blankenstein, T., and Kiessling, R. (1998). Constitutive IL-10 production accounts for the high NK sensitivity, low MHC class I expression, and poor transporter associated with antigen processing (TAP)-1/2 function in the prototype NK target YAC-1. J Immunol *161*, 2099-105.

Petry, K. U., Scheffel, D., Bode, U., Gabrysiak, T., Kochel, H., Kupsch, E., Glaubitz, M., Niesert, S., Kuhnle, H., and Schedel, I. (1994). Cellular immunodeficiency enhances the progression of human papillomavirus-associated cervical lesions. Int J Cancer *57*, 836-40.

Pieters, J. (1997). MHC class II restricted antigen presentation. Curr Opin Immunol *9*, 89-96.

Plebanski, M., Gilbert, S. C., Schneider, J., Hannan, C. M., Layton, G., Blanchard, T., Becker, M., Smith, G., Butcher, G., Sinden, R. E., and Hill, A. V. (1998).

Protection from Plasmodium berghei infection by priming and boosting T cells to a single class I-restricted epitope with recombinant carriers suitable for human use. Eur J Immunol *28*, 4345-55.

Price, M. R., Rye, P. D., Petrakou, E., Murray, A., Brady, K., Imai, S., Haga, S., Kiyozuka, Y., Schol, D., Meulenbroek, M. F., Snijdewint, F. G., von Mensdorff-Pouilly, S., Verstraeten, R. A., Kenemans, P., Blockzjil, A., Nilsson, K., Nilsson, O., Reddish, M., Suresh, M. R., Koganty, R. R., Fortier, S., Baronic, L., Berg, A., Longenecker, M. B., Hilgers, J., and et al. (1998). Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, Calif., November 17-23, 1996. Tumour Biol *19*, 1-20.

Radvanyi, L. G., Shi, Y., Vaziri, H., Sharma, A., Dhala, R., Mills, G. B., and Miller, R. G. (1996). CD28 costimulation inhibits TCR-induced apoptosis during a primary T cell response. J. Immunol. *156*, 1788-1798.

Rammensee, H.-G., Falk, K., and Rötschke, O. (1993). Peptides naturally presented by MHC class I molecules. Annu. Rev. Immunol. *11*, 213-244.

Rammensee, H. G., Friede, T., and Stevanovic, S. (1995). MHC ligands and peptide motifs: first listing. Immunogenetics *41*, 178-228.

Ranges, G. E., Figari, I. S., Espevik, T., and Palladino, M. A., Jr. (1987). Inhibition of cytotoxic T cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha. J Exp Med *166*, 991-8.

Reis, C. A., David, L., Seixas, M., Burchell, J., and Sobrinho-Simoes, M. (1998). Expression of fully and under-glycosylated forms of MUC1 mucin in gastric carcinoma. Int J Cancer *79*, 402-10.

Ressing, M. E., Sette, A., Brandt, R. M., Ruppert, J., Wentworth, P. A., Hartman, M., Oseroff, C., Grey, H. M., Melief, C. J., and Kast, W. M. (1995). Human CTL

epitopes encoded by human papillomavirus type 16 E6 and E7 identified through in vivo and in vitro immunogenicity studies of HLA-A*0201-binding peptides. J Immunol *154*, 5934-43.

Restifo, N. P., Esquivel, F., Kawakami, Y., Yewdell, J. W., Mule, J. J., Rosenberg, S. A., and Bennink, J. R. (1993). Identification of human cancers deficient in antigen processing. J Exp Med *177*, 265-72.

Ridge, J. P., Dirosa, F., and Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4(+) t-helper and a t-killer cell. Nature *393*, 474-478.

Rivoltini, L., Kawakami, Y., Sakaguchi, K., Southwood, S., Sette, A., Robbins, P. F., Marincola, F. M., Salgaller, M. L., Yannelli, J. R., Appella, E., and et al. (1995). Induction of tumor-reactive CTL from peripheral blood and tumor-infiltrating lymphocytes of melanoma patients by in vitro stimulation with an immunodominant peptide of the human melanoma antigen MART-1. J Immunol *154*, 2257-65.

Rötschke, O., Falk, K., Stevanovic, S., Jung, G., Walden, P., and Rammensee, H.-G. (1991). Exact prediction of a natural T cell epitope. Eur. J. Immunol. *21*, 2891-2894.

Rottger, S., Bennett, E. P., White, T., Clausen, H., and Nilsson, T. (1996). Expression and immunolocalization of three human N-acetylgalactosaminyltransferases in HeLa cells. In 4th International Workshop on Carcinoma-Associated Mucins (Cambridge.

Rowse, G. J., Tempero, R. M., VanLith, M. L., Hollingsworth, M. A., and Gendler, S. J. (1998). Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. Cancer Res *58*, 315-21.

Rudensky, A. Y., Preston-Huriburt, P., Hong, S. C., Barkow, A., and Janeway, C. A. (1991). Sequence analysis of peptides bound to MHC class II molecules. Nature *353*, 622-626.

Salcedo, M., Momburg, F., Hammerling, G. J., and Ljunggren, H. G. (1994). Resistance to natural killer cell lysis conferred by TAP1/2 genes in human antigen-processing mutant cells. J Immunol *152*, 1702-8.

Salgaller, M. L., Afshar, A., Marincola, F. M., Rivoltini, L., Kawakami, Y., and Rosenberg, S. A. (1995). Recognition of multiple epitopes in the human melanoma antigen gp100 by peripheral blood lymphocytes stimulated in vitro with synthetic peptides. Cancer Res *55*, 4972-9.

Sallusto, F., Lanzavecchia, A., and Mackay, C. R. (1998). Chemokines and chemokine receptors in T-cell priming and Th1/Th2- mediated responses. Immunol Today *19*, 568-74.

Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M. D., Silverman, G. J., Lotz, M., Carson, D. A., and Raz, E. (1996). Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science *273*, 352-4.

Schoenberger, S. P., Jonges, L. E., Mooijaart, R. J., Hartgers, F., Toes, R. E., Kast, W. M., Melief, C. J., and Offringa, R. (1998). Efficient direct priming of tumor-specific cytotoxic T lymphocyte in vivo by an engineered APC. Cancer Res *58*, 3094-100.

Schoenberger, S. P., Toes, R. E. M., Mooijaart, R. J. D., Medema, J. P., Melief, C. J. M., and Offringa, R. Combined CTL and neutralizing antibody therapy efficiently eradicates TGF-ß producing tuors in vivo. Submitted.

Schoenberger, S. P., Toes, R. E. M., Vandervoort, E. I. H., Offringa, R., and Melief, C. J. M. (1998). T-cell help for cytotoxic T-lymphocytes is mediated by CD40-CD40l interactions. Nature *393*, 480-483.

Schoenberger, S. P., van der Voort, E. I., Krietemeijer, G. M., Offringa, R., Melief, C. J., and Toes, R. E. (1998). Cross-priming of CTL responses in vivo does not require antigenic peptides in the endoplasmic reticulum of immunizing cells. J Immunol *161*, 3808-12.

Schoenhals, G. J., Krishna, R. M., Grandea, A. G., 3rd, Spies, T., Peterson, P. A., Yang, Y., and Fruh, K. (1999). Retention of empty MHC class I molecules by tapasin is essential to reconstitute antigen presentation in invertebrate cells. Embo J *18*, 743-53.

Schumacher, T. N., De Bruijn, M. L., Vernie, L. N., Kast, W. M., Melief, C. J., Neefjes, J. J., and Ploegh, H. L. (1991). Peptide selection by MHC class I molecules. Nature *350*, 703-6.

Schwartz, R. H. (1992). Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. Cell *71*, 1065-8.

Schwientek, T., Almeida, R., Levery, S. B., Holmes, E. H., Bennett, E., and Clausen, H. (1998). Cloning of a Novel Member of the UDP-Galactose:beta-N-Acetylglucosamine beta1,4-Galactosyltransferase Family, beta4Gal-T4, Involved in Glycosphingolipid Biosynthesis. J Biol Chem *273*, 29331-29340.

Seliger, B., Maeurer, M. J., and Ferrone, S. (1997). Tap off - tumors on. Immunol Today *18*, 292-299.

Sensi, M., Traversari, C., Radrizzani, M., Salvi, S., Maccalli, C., Mortarini, R., Rivoltini, L., Farina, C., Nicolini, G., Wolfel, T., and et al. (1995). Cytotoxic T-lymphocyte clones from different patients display limited T-cell-receptor variable-

region gene usage in HLA-A2-restricted recognition of the melanoma antigen Melan-A/MART-1. Proc Natl Acad Sci U S A *92*, 5674-8.

Shamanin, V., Glover, M., Rausch, C., Proby, C., Leigh, I. M., zur Hausen, H., and de Villiers, E. M. (1994). Specific types of human papillomavirus found in benign proliferations and carcinomas of the skin in immunosuppressed patients. Cancer Res *54*, 4610-3.

Shen, Z., Reznikoff, G., Dranoff, G., and Rock, K. L. (1997). Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. J Immunol *158*, 2723-30.

Skipper, J. C., Hendrickson, R. C., Gulden, P. H., Brichard, V., Van Pel, A., Chen, Y., Shabanowitz, J., Wolfel, T., Slingluff, C. L., Jr., Boon, T., Hunt, D. F., and Engelhard, V. H. (1996). An HLA-A2-restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins. J Exp Med *183*, 527-34.

Smith, M., Burchell, J. M., Graham, R., Cohen, E. P., and Taylor-Papadimitriou, J. (1999). Expression of B7.1 in a MUC1-expressing mouse mammary epithelial tumour cell line inhibits tumorigenicity but does not induce autoimmunity in MUC1 transgenic mice [In Process Citation]. Immunology *97*, 648-55.

Solheim, J. C., Carreno, B. M., and Hansen, T. H. (1997). Are transporter associated with antigen-processing (tap) and tapasin class-i MHC chaperones. J Immunol *158*, 541-543.

Sogn, J. A. (1998). Tumor immunology: the glass is half full. Immunity 9, 757-63.

Sorensen, T., White, T., Wandall, H. H., Kristensen, A. K., Roepstorff, P., and Clausen, H. (1995). UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-

acetylgalactosaminyltransferase. Identification and separation of two distinct transferase activities. J Biol Chem *270*, 24166-73.

Soule, H. D., Vazguez, J., Long, A., Albert, S., and Brennan, M. (1973). A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst *51*, 1409-16.

Speir, J. A., Abdel-Motal, U. M., Jondal, M., and Wilson, I. A. (1999). Crystal structure of an MHC class I presented glycopeptide that generates carbohydrate-specific CTL. Immunity *10*, 51-61.

Spicer, A. P., Parry, G., Patton, S., and Gendler, S. J. (1991). Molecular cloning and analysis of the mouse homologue of the tumor- associated mucin, MUC1, reveals conservation of potential O- glycosylation sites, transmembrane, and cytoplasmic domains and a loss of minisatellite-like polymorphism. J Biol Chem *266*, 15099-109.

Spicer, A. P., Rowse, G. J., Lidner, T. K., and Gendler, S. J. (1995). Delayed mammary tumor progression in Muc-1 null mice. J Biol Chem *270*, 30093-101.

Stauss, H. J., Dahl, M., and Sadovnikova, E. (1997). Tumour-reactive cytotoxic T-lymphocytes specific for normal cellular proteins. Biochem Soc Trans *25*, 540-3.

Steinman, R. M. (1991). The dendritic cell system and its role in immunogenicity. Annu Rev Immunol *9*, 271-96.

Storrie, B., White, J., Rottger, S., Stelzer, E. H., Suganuma, T., and Nilsson, T. (1998). Recycling of golgi-resident glycosyltransferases through the ER reveals a novel pathway and provides an explanation for nocodazole-induced Golgi scattering. J Cell Biol *143*, 1505-21.

Suto, R., and Srivastava, P. K. (1995). A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. Science *269*, 1585-8.

Swallow, D. M., Gendler, S., Griffiths, B., Corney, G., Taylor Papadimitriou, J., and Bramwell, M. E. (1987). The human tumour-associated epithelial mucins are coded by an expressed hypervariable gene locus PUM. Nature *328*, 82-4.

Swallow, D. M., Gendler, S., Griffiths, B., Kearney, A., Povey, S., Sheer, D., Palmer, R. W., and Taylor Papadimitriou, J. (1987). The hypervariable gene locus PUM, which codes for the tumour associated epithelial mucins, is located on chromosome 1, within the region 1q21-24. Ann Hum Genet *51*, 289-94.

Taylor Papadimitriou, J., Peterson, J. A., Arklie, J., Burchell, J., Ceriani, R. L., and Bodmer, W. F. (1981). Monoclonal antibodies to epithelium-specific components of the human milk fat globule membrane: production and reaction with cells in culture. Int J Cancer *28*, 17-21.

Toes, R. E. M., Blom, R. J. J., Offringa, R., Kast, W. M., and Melief, C. J. M. (1996). Functional deletion of tumor-specific cytotoxic T lymphocytes induced by peptide immunization can lead to the inability to reject tumors. J. Immunol. *156*, 3911-3918.

Toes, R. E. M., Blom, R. J. J., van der Voort, E., Offringa, R., Melief, C. J. M., and Kast, W. M. (1996). Protective anti-tumor immunity induced by immunization with completely allogeneic tumor cells. Cancer Res. *56*, 3782-3787.

Toes, R. E. M., van der Voort, E. I. H., Schoenberger, S. P., Drijfhout, J. W., van Blois, L., Storm, G., Kast, W. M., Offringa, R., and Melief, C. J. M. (1997). Dendritic cells convert immuno-suppressive peptides into an effective and reliable antitumor vaccine. Submitted for publication.

Torre-Amoine, G., Beauchamp, R. D., Koeppen, H., Park, B. H., Schreiber, H., Moses, H. L., and Rowley, R. D. (1990). A highly immunogenic tumor transfected with a murine transforming growth factor B2 cDNA escapes immune surveillance. Proc. Natl. Acad. Sci. USA *87*, 1486-1490.

Townsend, S. E., and Alison, J. P. (1993). Tumor rejection after direct costimulation of $CD8^+$ T cells by B7-transfected melanoma cells. Science *259*, 368-370.

Townsend, S. E., and Allison, J. P. (1993). Tumor rejection after direct costimulation of CD8+ T cells by B7- transfected melanoma cells [see comments]. Science *259*, 368-70.

Traversari, C., Van der bruggen, P., Van den Eynde, B., Hainaut, P., Lemoine, C., Ohta, N., Old, L., and Boon, T. (1992). Transfection and expression of a gene coding for a human melanoma antigen recognized by autologuous CTL. Immunogenetics *35*, 145-152.

Uger, R. A., and Barber, B. H. (1997). Presentation of an influenza nucleoprotein epitope incorporated into the H-2Db signal sequence requires the transporter-associated with antigen presentation. J Immunol *158*, 685-92.

Unanue, E. R. (1992). Cellular studies on antigen presentation by class II MHC molecules. Curr. Opin. Immunol. *4*, 63-69.

Uyttenhove, C., Maryanski, J., and Boon, T. (1983). Escape of mouse mastocytoma P815 after nearly complete rejection is due to antigen-loss variants rather than immunosuppression. J Exp Med *157*, 1040-52.

van de Wiel-van Kemenade, E., Ligtenberg, M. J., de Boer, A. J., Buijs, F., Vos, H. L., Melief, C. J., Hilkens, J., and Figdor, C. G. (1993). Episialin (MUC1) inhibits cytotoxic lymphocyte-target cell interaction. J Immunol *151*, 767-76.

van der Burg, S. H., Ras, E., Drijfhout, J. W., Benckhuijsen, W. E., Bremers, A. J., Melief, C. J., and Kast, W. M. (1995). An HLA class I peptide-binding assay based on competition for binding to class I molecules on intact human B cells. Identification of conserved HIV-1 polymerase peptides binding to HLA-A*0301. Hum Immunol *44*, 189-98.

van der Burg, S. H., Visseren, M. J., Brandt, R. M., Kast, W. M., and Melief, C. J. (1996). Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. J Immunol *156*, 3308-14.

Van der Burg, S. H., Visseren, M. J. W., Offringa, R., and Melief, C. J. M. (1997). Do epitopes derived from autoantigens display low affinity for MHC class I? Immunol. Today *18*, 97-98.

van Elsas, A., van der Burg, S. H., van der Minne, C. E., Borghi, M., Mourer, J. S., Melief, C. J., and Schrier, P. I. (1996). Peptide-pulsed dendritic cells induce tumoricidal cytotoxic T lymphocytes from healthy donors against stably HLA-A*0201-binding peptides from the Melan-A/MART-1 self antigen. Eur J Immunol *26*, 1683-9.

van Hall, T., van de Rhee, N. E., Schoenberger, S. P., Vierboom, M. P., Verreck, F. A., Melief, C. J., and Offringa, R. (1998). Cryptic open reading frames in plasmid vector backbone sequences can provide highly immunogenic cytotoxic T-lymphocyte epitopes. Cancer Res *58*, 3087-93.

Varki, A. (1993). Biological roles of oligosaccharides: all of the theories are correct. Glycobiology *3*, 97-130.

Vierboom, M. P., Nijman, H. W., Offringa, R., van der Voort, E. I., van Hall, T., van den Broek, L., Fleuren, G. J., Kenemans, P., Kast, W. M., and Melief, C. J. (1997).

Tumor eradication by wild-type p53-specific cytotoxic T lymphocytes. J Exp Med *186*, 695-704.

Villunger, A., Egle, A., Marschitz, I., Kos, M., Bock, G., Ludwig, H., Geley, S., Kofler, R., and Greil, R. (1997). Constitutive expression of Fas (Apo-1/CD95) ligand on multiple myeloma cells: a potential mechanism of tumor-induced suppression of immune surveillance. Blood *90*, 12-20.

Visseren, M. J., van der Burg, S. H., van der Voort, E. I., Brandt, R. M., Schrier, P. I., van der Bruggen, P., Boon, T., Melief, C. J., and Kast, W. M. (1997). Identification of HLA-A*0201-restricted CTL epitopes encoded by the tumor-specific MAGE-2 gene product. Int J Cancer *73*, 125-30.

Vitiello, A., Marchesini, D., Furze, J., Sherman, L. A., and Chesnut, R. W. (1991). Analysis of the HLA-restricted influenza-specific cytotoxic T lymphocyte response in transgenic mice carrying a chimeric human-mouse class I major histocompatibility complex. J Exp Med *173*, 1007-15.

von Heijne, G. (1990). The signal peptide. J Membr Biol *115*, 195-201.

Walker, P. R., Saas, P., and Dietrich, P. Y. (1997). Role of Fas ligand (CD95L) in immune escape: the tumor cell strikes back. J Immunol *158*, 4521-4.

Wandall, H. H., Hassan, H., Mirgorodskaya, E., Kristensen, A. K., Roepstorff, P., Bennett, E. P., Nielsen, P. A., Hollingsworth, M. A., Burchell, J., Taylor-Papadimitriou, J., and Clausen, H. (1997). Substrate specificities of three members of the human UDP-N-acetyl- alpha-D-galactosamine:Polypeptide N-acetylgalactosaminyltransferase family, GalNAc-T1, -T2, and -T3. J Biol Chem *272*, 23503-14.

Wei, M. L., and Cresswell, P. (1992). HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides [see comments]. Nature *356*, 443-6.

Wentworth, P. A., Vitiello, A., Sidney, J., Keogh, E., Chesnut, R. W., Grey, H., and Sette, A. (1996). Differences and similarities in the A2.1-restricted cytotoxic T cell repertoire in humans and human leukocyte antigen-transgenic mice. Eur J Immunol *26*, 97-101.

Whitehouse, C., Burchell, J., Gschmeissner, S., Brockhausen, I., Lloyd, K. O., and Taylor-Papadimitriou, J. (1997). A transfected sialyltransferase that is elevated in breast cancer and localizes to the medial/trans-Golgi apparatus inhibits the development of core-2-based O-glycans. J Cell Biol *137*, 1229-41.

Wiedenfeld, E. A., Fernandez-Vina, M., Berzofsky, J. A., and Carbone, D. P. (1994). Evidence for selection against human lung cancers bearing p53 missense mutations which occur within the HLA A*0201 peptide consensus motif. Cancer Res *54*, 1175-7.

Wojtowiczpraga, S., Ziegler, H., Thale, R., Lucin, P., Muranyi, W., Flohr, T., Hengel, H., Farrell, H., Rawlinson, W., and Koszinowski, U. H. (1997). Reversal of tumor-induced immunosuppression - a new approach to cancer-therapy
A mouse cytomegalovirus glycoprotein retains MHC class-i complexes in the ergic/cis-golgi compartments. J Immunother *20*, 165-177.

Wolfel, T., Van Pel, A., Brichard, V., Schneider, J., Seliger, B., Meyer zum Buschenfelde, K. H., and Boon, T. (1994). Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. Eur J Immunol *24*, 759-64.

Xerri, L., Devilard, E., Hassoun, J., Mawas, C., and Birg, F. (1997). Fas ligand is not only expressed in immune privileged human organs but is also coexpressed with Fas in various epithelial tissues. Mol Pathol *50*, 87-91.

Xing, P. X., Prenzoska, J., and McKenzie, I. F. (1992). Epitope mapping of anti-breast and anti-ovarian mucin monoclonal antibodies. Mol Immunol *29*, 641-50.

Yee, C., Savage, P. A., Lee, P. P., Davis, M. M., and Greenberg, P. D. (1999). Isolation of High Avidity Melanoma-Reactive CTL from Heterogeneous Populations Using Peptide-MHC Tetramers. J Immunol *162*, 2227-2234.

Yewdell, J. W., Snyder, H. L., Bacik, I., Anton, L. C., Deng, Y., Behrens, T. W., Bachi, T., and Bennink, J. R. (1998). TAP-independent delivery of antigenic peptides to the endoplasmic reticulum: therapeutic potential and insights into TAP-dependent antigen processing. J Immunother *21*, 127-31.

Young, M. R., Wright, M. A., Lozano, Y., Matthews, J. P., Benefield, J., and Prechel, M. M. (1996). Mechanisms of immune suppression in patients with head and neck cancer: influence on the immune infiltrate of the cancer. Int J Cancer *67*, 333-8.

Zhai, Y., Yang, J. C., Kawakami, Y., Spiess, P., Wadsworth, S. C., Cardoza, L. M., Couture, L. A., Smith, A. E., and Rosenberg, S. A. (1996). Antigen-specific tumor vaccines. Development and characterization of recombinant adenoviruses encoding MART1 and gp100 for cancer therapy. J. Immunol. *156*, 700-710.