Tumor antigen processing and presentation on MHC Class II molecules for CD4 T cell recognition in health and disease

Thesis

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TUMOR ANTIGEN PROCESSING AND PRESENTATION ON MHC CLASS II MOLECULES FOR $CD4^+$ T CELL RECOGNITION IN HEALTH AND DISEASE

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STUDENT DECLARATION

None of the material I am now submitting has previously been submitted for a degree or any other qualification at this University or another institution.

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The results on the HLA-DRβ4 epitope and the dependence of presentation on the amount of MAGE-3 available for processing has been published in Cancer Immunology Immunotherapy. 2007 Jul 13, *(e-pub ahead of print)*.

The results from the patients and processing of MAGE-$3_{111\text{-}125}$ and MAGE-$3_{161\text{-}175}$ have been submitted for publication.

ABSTRACT

Animal models have shown that $CD4^+$ T cells are required to achieve effective, long-lasting immunity to cancer. Less is known on the role and functional state in human disease of tumor antigen specific $CD4^+$ T cells. These cells recognise epitopes from tumor antigens presented by MHC-II molecules on professional antigen presenting cells and also directly on tumor cells. MAGE-3 is a tumor specific antigen expressed in tumors of different histology but not healthy tissues and so is an ideal candidate for immunotherapeutic purposes. MAGE-3 CD4 naturally processed epitopes have been described.

The aims of the project were to analyse how processing influences the repertoire of MAGE-3 CD4 epitopes formed in different antigen expressing cell types and the role of MAGE-3 epitope specific $CD4^+$ T cells in the natural response of advanced melanoma patients.

Formation of epitopes through the exogenous pathway was differently influenced by endosomal proteases. Indeed, depending on the epitope studied cysteine and aspartic proteases lead to epitope formation or destruction affecting the repertoire presented *in vivo*. No clear data were obtained to understand the processing pathways in tumor cells, as formation of the studied epitope was unaffected by cytosolic proteases and did not require autophagy.

Most patients had circulating MAGE-3 specific $CD4^+$ T cells, mainly unpolarized or producing anti-inflammatory cytokines suggesting an impairment of the anti-MAGE-3 $CD4^+$ T cell response in advanced stages of disease. The repertoire of epitopes recognised confirmed the immunodominance of previously described epitopes and correlated well with the results of *in vitro* processing studies.

Collectively, these experiments show that anti-MAGE-3 $CD4^+$ T cell responses develop *in vivo* and that the repertoire of epitopes formed and recognised is influenced by endosomal proteases. Further studies are needed to investigate factors determining impairment of $CD4^+$ T cell function in advanced stages and ways to overcome this dysfunction.

LIST OF ABBREVIATIONS

MHC: major histocompatibility complex
APC: antigen presenting cell
TCR: cell receptor
CTL: Cytotoxic T lymphocyte
DC: Dendritic cell
IL: Interleukin
IFN: Interferon
TNF: Tumor necrosis factor
GM-CSF: Granulocyte-monocyte colony stimulating factor: GM-CSF
NKT: Natural killer T cells
T regs: Regulatory T cells
GITR: glucocorticoid-induced TNF receptor
SSX: synovial sarcoma X breakpoint
TAA: tumor associated antigen
CEA: carcinoembryonic antigen
EBNA: Epstein-Barr nuclear antigen
LMP: latent membrane protein
ER: endoplasmic reticulum
Ii: invariant chain
AEP: asparagine endopeptidase
MIIC: MHC-II class compartments
VIIC: multivesicular class II compartments
HLA: human leukocyte antigen
TAP: transported associated with antigen processing
CIITA: class II transactivator
DRiPs: defective ribosomal products
TPPII: tripeptidyl peptidase II
LAP: leucin aminopeptidase
PSA: puromycin sensitive aminopeptidase
BH: bleomycin hydrolase
TOP: thimet oligopeptidase
ERAAP: endoplasmic reticulum aminopeptidase associated with antigen processing
TTCF: tetanus toxoid C-terminal fragment
GILT: γ-IFN-inducible thiol protease
Cat: cathepsin
TCE: thymic cortical epithelial (cells)
MBP: myelin basic protein
AIF: apoptosis inducing factor
PI3K: phosphatidil-inositol-3-kinase
AP: Adaptor protein
mTOR: mammalian target of rapamycin
MAP-LC3: Microtubule-associated protein 1- light chain
Atg: autophagy related protein
MUC-1: mucin 1
NeoR: Neomycin phosphotransferase II
PTEN: Phosphate and tensin homologue
LCL: lymphoblastoid cell line
PBMC: peripheral blood mononuclear cell
NHS: normal human serum
FCS: fetal calf serum
TAP-L: transported associated with antigen processing-like

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1. INTRODUCTION

1.1. Anti-tumor immunity

T lymphocytes are major effectors cells in systemic antitumor immunity and recognise antigens displayed on the surface of body cells. Peptides are delivered to the cell surface through specialised host glycoproteins, the major histocompatibility complex (MHC), on tumor and antigen presenting cells (APCs). The peptides are produced after cleavage of antigens within the cells and are loaded onto MHC molecules; the events of this process will be described in greater detail in chapter 1.3. Two types of MHC, named MHC class I (MHC-I) and (MHC-II), exist. Each type has many alleles and will also be described in chapter 1.3. Recognition by T cells is triggered by interaction between the MHC complex and the T cell receptor (TCR). The TCR is associated with one of the two co-receptors that confers the ability to bind to MHC molecules. Through co-receptor CD8 the TCR interacts with MHC-I molecules while CD4 is required for interaction with MHC-II. The presence of the co-receptor divides T cells into two subsets that differ in role and cells they interact with.

As $CD8^+$ T cells can directly recognise tumoral cells expressing MHC-I molecules it was assumed that the predominant tumoricidal effector mechanism would be killing by $CD8^+$ cytotoxic lymphocytes (CTL). Experiments with animal models have indeed shown that adoptive transfer of tumor specific $CD8^+$ T cells, stimulated *in vitro*, is able to mediate anti-tumor immunity in tumor-bearing hosts (reviewed in (1)).

Many tumors are MHC-II negative and, as $CD4^+$ T cells require this complex to recognise cells, less attention was at first given to this class of lymphocytes. However,

better understanding of their function in the immune response led to an interest in $CD4^+$ T cells as key elements in the fight against cancer.

1.1.1. Role of $CD4^+$ T cells in antitumor immunity.

$CD4^+$ T cells are critical elements for priming $CD8^+$ T cells (2, 3), for immunity during chronic infection (4), for generation of memory T cells (5, 6) and for antibody production (7). $CD4^+$ T cells have been shown to play both a direct and an indirect role in antitumor immunity in animal models (Fig. 1).

$CD4^+$ T cells as helper cells. One of the most important roles of $CD4^+$ T cells is that of activating dendritic cells (DCs), for priming of cytotoxic $CD8^+$ T cells. For full activation of CTL interaction between $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes and DCs is necessary but contemporary interaction between three types of migratory cells is unlikely. Immature DCs endocytose antigens - including those from dead or dying tumoral cells - and migrate to the the lymphnode. In the lymphnode they will be recognised by activated $CD4^+$ T cells through interaction of MHC-II and CD80. This interaction stimulates up-regulation of CD40L on the T cell. Ligation of CD40L with CD40 on the DCs (8-11) confers enhanced antigen presentation and costimulatory activity to the DCs. The DCs are able to fully activate any specific $CD8^+$ T cell without requiring contemporary presence of $CD4^+$ T cells; these may detach and interact with another cell. 'Licensing' of DCs means also that a few $CD4^+$ T cells can license many APCs and these, in turn, can activate a multitude of CTLs.

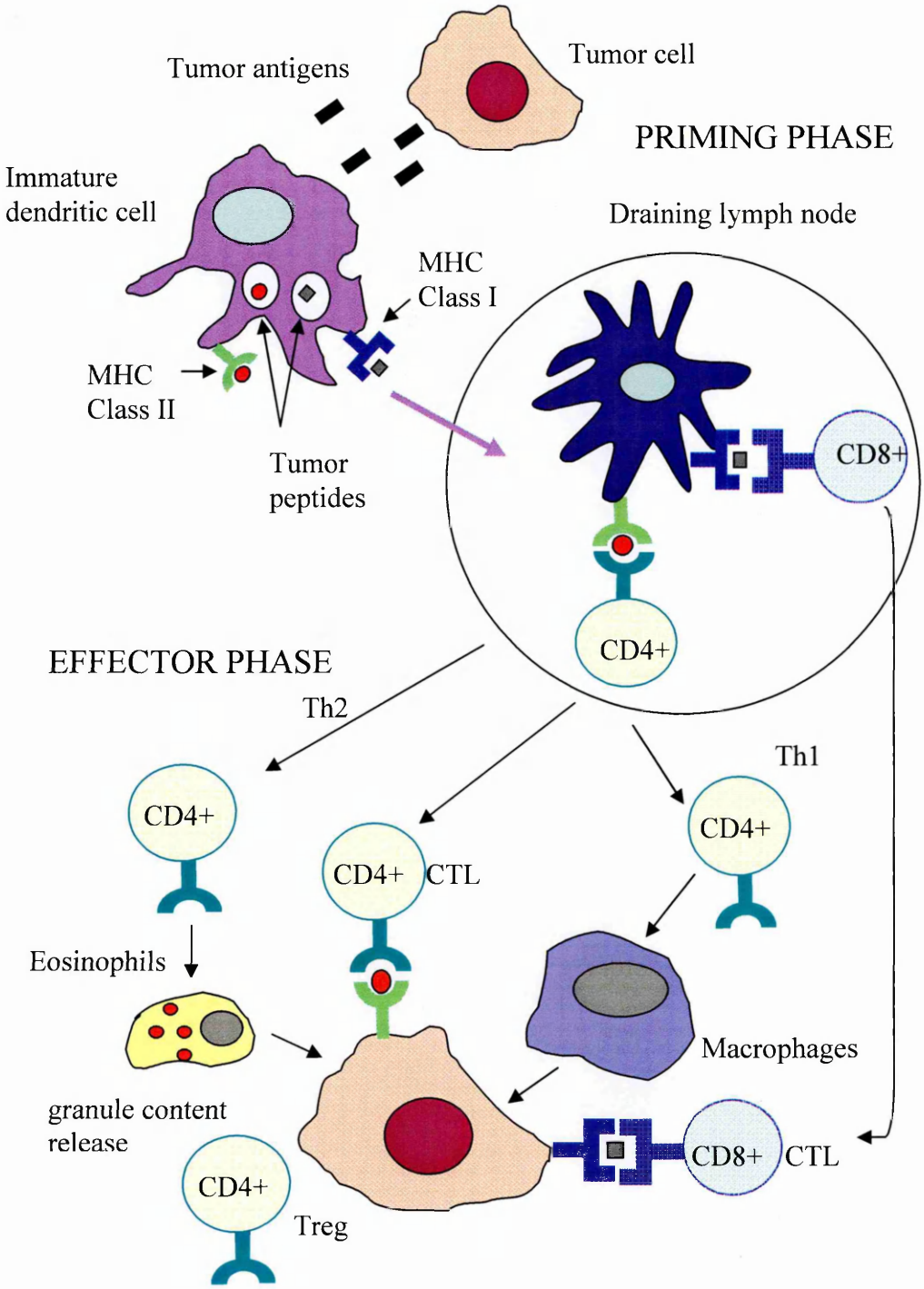


Figure 1. Immune response to tumors. Immature DCs that have endocytosed tumor antigens move to lymphnodes to mature and prime T cells. After the priming phase activated cells leave the lymphnode to begin the effector phase. Primed CD4+ T cells traffic to sites of tumor metastasis where they will stimulate eosinophils or macrophages, according to their Th phenotype; they could also interact directly with the cancerous cells. Shown are also suppressor CD4+ T reg cells.

$CD4^+$ T cells as mediators of tumor rejection. Based on the type of cytokine secreted $CD4^+$ T cells are either considered type 1 (Th1) or type 2 (Th2) helper cells. The first type secrets cytokines such as interleukin (IL) 2 (IL-2), interferon-γ (IFN-γ), tumor necrosis factor (TNF) whereas the second produces IL-4, IL-5, IL-10 and IL-13. The two stimulate different types of cells (CTLs or B cells with antibody production) (12).

Th1 $CD4^+$ T cells, with their ability to induce CTL responses, are clearly important in tumor rejection. In animal models, adoptive transfer of tumor specific $CD4^+$ T cells led to elimination of cancer. In a study by Nishimura et al., tumors showed cell necrosis and infiltration by neutrophils and eosinophils after transfer of tumor specific CD4+ T cells; necrosis was mediated by $CD8^+$ T cells because in recipient mice depleted of $CD8^+$ T cells no rejection was seen (13). In other models, tumor specific $CD4^+$ T cells (induced by a modified tumor secreting granulocyte/monocyte colony stimulating factor, GM-CSF) showed both Th1 and Th2 phenotype and both were required for tumor rejection. Cytokines produced by these cells activated eosinophils and macrophages, leading to production of superoxide and nitric oxide followed by tumoral cell death (14).

$CD4^+$ T cells can promote tumor cell destruction in a CD8-independent manner. Th2 $CD4^+$ T cells regulated cytotoxic activity in experimental lung metastases through infiltrating eosinophils (15). Neutrophil action against tumors has also been shown to be mediated by $CD4^+$ T cell activity (16). $CD4^+$ T cells, but not $CD8^+$ T cells, are required for natural killer T (NKT)-dependent tumor rejection (17).

Direct cytotoxic activity has been shown for $CD4^+$ T cells, mainly *in vitro* (18-20) but also *in vivo* in mice (21). The role of this activity may be relevant for haematological malignancies such as leukaemia *in vivo.* It is less clear in the case of solid tumors, as most are MHC-II negative (although expression may be induced in an inflammatory environment and direct recognition and destruction take place).

$CD4^+$ cells as suppressors of tumor destruction. Increasing attention has been given to a subset of T cells, called T regulatory (Tregs), whose primary function is to regulate the peripheral immune system activity against foreign and self antigens (reviewed in (22, 23)). These cells were first identified as suppressor cells because their removal caused the onset of a variety of autoimmune diseases. Tregs have been divided into subtypes and the best studied subtype expresses CD25 (the IL-2 receptor alpha chain), CTLA-4, Toll-like receptors, a glucocorticoid-induced TNF receptor (GITR) and Foxp3. They are known as $CD4^+$ $CD25^+$ $Foxp3^+$ and represent approximately 5-10% of peripheral $CD4^+$ T cells. Antibody-mediated removal of Tregs in mouse tumors resulted in improved specific anti-tumor immunity (24, 25). In a melanoma adoptive immunotherapy model transfer of $CD4^+CD25^+$ effectively prevented $CD8^+$ T cell-mediated tumor destruction; transfer of $CD4^+CD25^-$ cells did not have this effect (26). Taken together these data strongly support the idea that these cells interfere with tumor rejection. Recent studies have shown high levels of Tregs in the blood and/or the tumor environment of patients with melanoma, Hodgkin's lymphoma, breast cancer and carcinoma of the lung, gastrointestinal tract, pancreas and ovary. Indeed, a large-scale study of ovarian carcinoma showed the accumulation in tumors of $CD4^+CD25^+$ T cells that suppressed tumor-specific T cell immunity. Notably there was a strong inverse correlation between the number of Tregs in the tumor biopsy and patient survival (27).

Studies on the Treg subtype Tr1 have only just begun but they too have been reported in association with cancer in animal models and in humans (28, 29). In melanoma patients Tr1–like $CD4^+$ T cells have been isolated and found to secrete IL-10 in response to recognition of MHC-II restricted tumor antigens (30, 31). IL-10 acts by stopping up-regulation of MHC molecules and co-stimulatory molecules on APCs; it also blocks T cells by inhibiting their proliferation and cytokine production. Less characterised are Th3 Tregs (22). They are known to secrete tumor growth factor β

(TGF-β) that inhibits T cell proliferation, cytokine production and cytotoxicity acting at all stages of T cell differentiation. TGF-β can also act on APCs by suppressing maturation, IFN–γ production and MHC-II expression.

Activation of Tregs requires antigen specific stimulation through the TCR in the presence of IL-2 (32); once active the cells do not require antigen and are suppressive in a non specific manner, targeting both Th1 and Th2 $CD4^+$ T cells and also NKT cells (33-35).

1.1.2 Th profile of responding $CD4^+$ T cells in spontaneous response to tumors.

An important issue in anti-tumor immunity is the Th profile of $CD4^+$ T cells. A high level of Tregs, as discussed in 1.1.1, seems correlated to poor clinical outcome. Th1 and Th2 responses also have different effects on tumor rejection. Although some Th2 $CD4^+$ T cells are able to mediate tumor rejection, experiments in mice unable to mount a Th2 response showed the animals to be far more resistant to cancer compared to their wild type counterparts (36). Immune responses skewed to a Th1 phenotype and away from a Th2 are thought optimal for tumor rejection, a conclusion based on the efficient tumoricidal activity of CTLs and on many studies demonstrating that induction of Th1 responses leads to tumor rejection, while Th2 responses are associated to tumor progression (37).

In the spontaneous immune response of humans to cancer expression of certain antigens appears associated to a Th1 and/or Th2 response.

$CD4^+$ T cells isolated from patients whose cancers expressed SSX-2 (product of one of the synovial sarcoma X breakpoint genes, a family of cancer – testis antigens) were found to release IFN-γ upon stimulation with the protein (38). In patients whose tumors

expressed a related protein, SSX-4, $CD4^+$ T cells also appeared to have a Th1 phenotype (39).

NY-ESO-1 is a cancer-testis antigen expressed in different types of tumor but not healthy tissues. Spontaneous responses are found in 50% of patients with cancers expressing this antigen that elicits both humoral and cellular immunity. A survey on patients showed that the antibody response was correlated to the $CD4^+$ T cell response and that the T cells had a strictly Th1 phenotype (40) also confirmed by other studies (41). This antigen is however also capable of inducing mixed Th1/Th2 responses (42); indeed, in a study enrolling patients with ovarian epithelial carcinoma, Th2 responses were found only associated with Th1 and never alone. The prognosis of patients with mixed response was no worse than that of patients with Th1; the Th2 $CD4^+$ T cells could be considered productive collaborators of Th1 $CD4^+$ T cells in anti-tumor immunity (43).

On the contrary, a different pattern was observed in renal cell carcinoma and melanoma expressing the tumor antigen MAGE-6. This protein is also a cancer-testis antigen and belongs to the family of MAGE proteins (described in 1.4). Patients with progressive disease were found to have a Th2 response against MAGE-6, while patients with no evidence of disease had a Th1 phenotype. Patients that had undergone surgery changed from a Th2 to a Th1 phenotype, showing that, in the presence of the tumor, responses were clearly skewed towards a Th2 phenotype resulting in poorer prognosis (44). Little information is available about the response to the other members of this family; in the case of MAGE-3 a Th1 response was seen but the studies performed were concentrated on checking vaccinated melanoma patients.

Viewed together, these data suggest that both types of response could be important and that Th2 response may not always be linked to progression of disease.

1.2 Tumor associated antigens

Tumor associated antigens (TAA) have been identified in a number of tumors; expression of some is restricted almost entirely to tumors while others can be found in healthy tissue as well. These antigens have been divided into groups on the basis of their pattern of expression (reviewed in (45)).

Cancer/testis antigens. These antigens are encoded by genes activated in many histologically different tumors but not in normal tissues; prototypes of this group are the MAGE genes, expressed in placental trophoblasts and testicular germ cells; as these cells do not express MHC molecules (46, 47) the antigens can be safely defined as tumor-specific. MAGE proteins, and MAGE-3 in particular, will be described in detail in chapter 1.4. This group includes GAGE, BAGE, the SSX family and NY-ESO-1 (mentioned in 1.1.2). Their pattern of expression and presence in many different types of tumor makes them ideal for immunotherapy.

Differentiation antigens. Antigens belonging to this group are tyrosinase, Melan-A/MART-1, gp100/Pmel17, gp57/TRP-1 and TRP-2; these antigens are also expressed in melanocytes. Patients experiencing tumor regression often have vitiligo, a local skin depigmentation caused by death of melanocytes. This type of cell is found in the eye so using these antigens for immunotherapy is not without risks.

Antigens from mutated ubiquitous proteins. These mutated proteins are probably involved in oncogenesis, as they have been found in independent tumors and influence activity of the proteins themselves. The mutated form of CDK4, for example, is unable to bind to its inhibitor and so alters the cell cycle (48). The mutated form of β-catenin is

more stable than the non mutated one and forms complexes with transcription factors; the complexes probably act on some other target enhancing proliferation or stopping apoptosis (49). CASP-8 is needed for FAS and TNF-1 mediated apoptosis; the mutated version is not as efficient as the wild type protein, allowing cells to grow indefinitely (50). Other genes belonging to this group include MUM-1 and the chimeric protein bcr-abl, found in some leukaemias.

Antigens from over-expressed genes. Some CTLs have been found to recognise antigens encoded by non-mutated genes expressed at different levels in normal and tumoral tissue. One of these genes is HER-2/neu, expressed at high levels in approximately 30% of breast and ovarian cancer (51); lymphocytes infiltrating some ovarian carcinomas were specific for an HER-2/neu peptide (52, 53). Another member of this group is p53, whose concentration is increased in many tumor cells because half-life of mutant forms is extended compared to wild type (51). A CTL clone, raised after *in vitro* priming, was able to lyse tumor cells over-expressing p53 (54). The carcinoembryonic antigen (CEA) is an oncofetal protein expressed in normal colon epithelium and in most gut carcinomas; it contains at least one epitope recognised by T cells from immunised patients (55).

Viral antigens. Certain families of viruses (such as herperviruses and papillomaviruses) can cause persistent infection and cancer. Cancer such as nasopharyngeal carcinoma and Hodgkin's disease express the antigens Epstein-Barr nuclear antigen 1 (EBNA1) and latent membrane proteins (LMP) derived from the Epstein-Barr herpesvirus (56, 57). Cervical cancer is associated to infection by papillomaviruses with expression of immunogenic early proteins (58).

1.3. Antigen processing

Processing of an antigen comprises all the events that, starting from a whole antigenic molecule, lead to the formation of peptides that can be loaded onto an MHC molecule. As mentioned in chapter 1, there are two types of MHC molecules, MHC-I and MHC-II, closely related in three-dimensional structure despite differences in subunit structure. The two differ from each other also in distribution, allowing them distinct functions in antigen presentation, binding of peptides from different intracellular sites and activation of different subsets of T cells.

MHC-I molecules, found on almost all nucleated cells, contain peptides derived from endogenously synthesized proteins. In MHC-II-peptide complexes, commonly found on professional APCs, the peptide usually derives from an exogenous antigen. However, as MHC-II molecules can be expressed by non professional APCs as well (see section 1.3.1.3), peptides can also derive from an endogenously expressed protein.

1.3.1. MHC-II molecules.

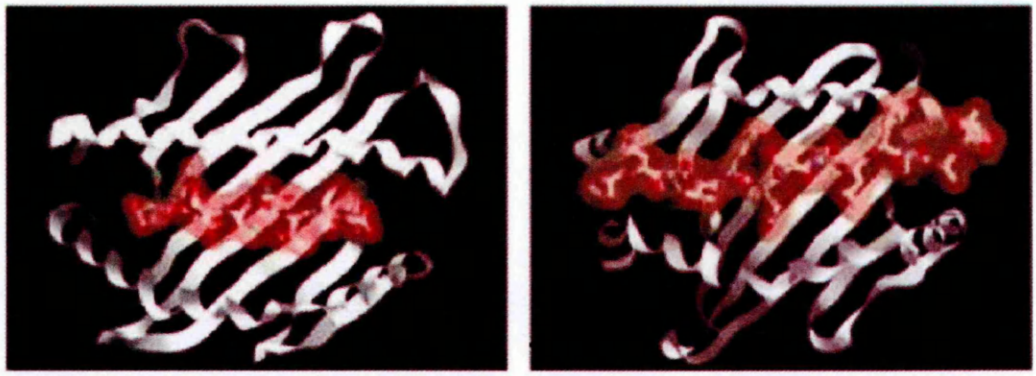
MHC-II molecules are dimers made of an α and a β subunit of slightly different molecular weight (34 kD and 29 kD respectively) that are not covalently bound (reviewed in (59)). Each chain has two extracellular domains, α1 and α2 in the α chain and β1 and β2 in the β chain. Each chain also has a transmembrane domain with a short intracellular tail, showing a different structure compared to MHC-I molecules, where the second subunit, β2-microglobulin, does not have a transmembrane domain. α1 and β1, the distal domains, form the peptide binding groove; α2 and β2, the proximal domains, resemble immunoglobulin constant domains both in 3-dimensional structure and aminoacid sequence. The β2 domain contains the site where the CD4 molecule binds through its D1 domain.

The peptide binding groove of MHC-II molecules (formed by the α1 and β1 domains) is made of two segmented α-helices (one from each domain) over antiparallelel β-strands. The groove is open at both ends and its depth is linked to the alleles expressed.

Peptides presented by MHC-II molecules are usually 13 to 17 aminoacids in length and lie in an extended conformation along the groove. They are kept in place in two ways: by peptide side chains that protrude into the MHC's pocket and by binding of the peptide backbone with side chains of the conserved residues of MHC-II molecules that line the groove. Crystallisation studies suggest that a minimal class-II bound peptide is held in place by side chains at residues 1, 4, 6 and 9, called anchor residues (usually denoted as P1, P4, P6 or P9). As the groove is open the peptide lies in an extended conformation and binding restrictions are fewer compared to MHC-I molecules, where the closed structure restricts peptide length. In MHC-I molecules peptides are eight to ten aminoacids long and the small variations in length observed are due to peptide kinking and protruding above the binding groove. Binding is stabilised by interactions between the amino- and carboxy-terminals of the peptide and the helices (60-62).

Despite the fewer binding restrictions in MHC-II molecules compared to MHC-I molecules a pattern can generally be detected for MHC-II peptides. For example, anchor residue P4 binding the HLA-DR*03 molecule is a negatively charged aminoacid (such as aspartic and glutamic acid) whereas P9 holds a hydrophobic residue (such as phenylalanine, tyrosine, proline or leucine).

A representation of the two classes of MHC molecule is shown in Figure 2.



A B

Figure 2. MHC-peptide complexes. Three-dimensional model of the peptide-binding region in MHC-I (A) and MHC-II (B) molecules; the peptide is red (*taken from Immunobiology, Janeway et al., 5th edn, Elsevier Science Ltd/Garland Publishing, 2001*).

1.3.1.1. Assembly of MHC-II molecules. Nascent α and β chains are directed into the endoplasmic reticulum (ER) by signal peptides. As they are synthesised they associate rapidly (within three minutes) with calnexin that stabilises them (63). Calnexin temporarily binds to the invariant chain molecule (referred to as 'Ii' from here on) as well, that is also produced inside the ER. The Ii forms trimers and each monomer binds to an immature α:β complex in the peptide binding region. This further stabilises the immature forms of the MHC-II and avoids their binding to peptides and or to any other protein present in the ER. Calnexin will dissociate from the nonameric complex as the last α:β dimer is added (63). Dissociation coincides with egress of the complex from the ER. The first eighty aminoacids of the Ii contain the signal for transport of this large nonameric complex to the Golgi apparatus and then on to the endosomal/lysosomal compartment, where peptides are formed. Cleavage of the Ii-nonameric complex is typically started in endosomes, where the action of asparagine endopeptidase (AEP) produces a 22 kD fragment (Iip22) in human cells (64). Proteases other than AEP (but like AEP not sensitive to the cysteine protease inhibitor leupeptin) may also cleave the Ii. The Iip22 fragment is then cleaved by cysteine cathepsins (generally cathepsins S or

V, depending on cell type) to form a minimal class II associated Ii derived peptide (CLIP, residues 81-104) that is bound to the α:β dimer in the peptide binding groove.

Removal of CLIP is catalysed by HLA-DM. This molecular chaperone is encoded in MHC locus and its overall structure resembles that of MHC-II molecules. It is a heterodimer of an α and β chain that have the classic domains of MHC-II molecules (65). They do not form an open peptide-binding groove; indeed, crystallisation studies have shown that the groove is closed at the ends, with a central pocket. The pocket contains an aminoacid triad closely reminiscent of the active site of serine proteases. There is apparently no room for a peptide to bind however so whether this is a vestige of a catalytic site or a site able to bind some ligand is not known. The overall structure of HLA-DM is similar to that of HLA-DR molecules but it has a lateral surface with partially exposed tryptophan residues. This is consistent with the possible formation of a DM-DR dimer; the tryptophan residues, interacting with residues of the HLA-DR molecule will then trigger a conformational change leading to release of CLIP and binding of a peptide (65). HLA-DM works as a 'peptide editor' favouring the formation of complexes of highest stability and therefore playing a role in shaping the repertoire of MHC-II bound peptides (66, 67).

HLA-DM catalyses release of CLIP and loading of a suitable antigenic peptide in the late endosome/lysosome-like compartment, defined MHC class II compartments or MIICs (68). These vesicles, 200 to 400 nm in diameter, contain multiple internal membranes, remniscient of classical multivesicular lysosomes. They were first identified in B cells and contain some markers of lysosomes, such as the lysosome-associated membrane proteins (LAMPs) but were devoid of Ii-chain, MHC-I and markers of the receptor recycling pathway (such as transferrin). They are distinct from multivesicular class II compartments or CIIVs, that more closely resemble endosomes (68, 69); they are devoid of lysosomal markers and contain some markers of the

recycling pathway. Loading of antigenic peptides can occur in either of these compartments (70).

There is evidence that loading of antigenic peptides can occur in an HLA-DM independent manner on recycling MHC-II molecules (71). On the whole there seems to be more than way to the formation of the MHC-II-peptide complex in its later stages.

The MHC-II-peptide complex can be produced in two ways (reviewed in (72)) that are equally possible and not mutually exclusive.

Peptide production and loading. In this model proteases work on the antigen cutting it into progressively smaller fragments. All these peptides can be loaded onto the MHC-II molecule: the ones with the highest affinity will bind first. The stable complex will then be sent to the cell surface. This was the first model proposed and was based mainly on analogies with MHC-I loading and is likely to occur in lysosomes, where most of the antigen has been well processed.

Protein fragment binding and trimming. As proteins unfold sites for attachment will be exposed to MHC-II molecules in search of a peptide. Presumably the competition between the MHC molecules will be won by the one with the highest affinity for an epitope in the exposed stretch. Binding could then expose or favour processing by other proteases; the regions flanking the sequence bound to the MHC would be trimmed by enzymes. If the first binding is stable enough the combination may be dominant in responsiveness for the whole antigen. This competition is known as 'determinant capture' because locally MHC molecules compete to capture a single pro-determinant. At the same time the large pro-determinant may contain more than one region ready to bind with MHC molecules. In this case the most available region with the highest affinity for the MHC molecule will predominate. In this type of competition, known as 'competitive capture', where different determinants of the antigen compete for MHC molecules, both affinity and availability play a crucial role.

On the whole the peptides that are actually loaded onto MHC-II molecules depend largely on a number of chance events, such as the shape of the antigen, the presence of a distinct set of proteases in the compartment they are in and competition between proteolytic enzymes and MHC molecules for binding to the processing site of the antigen.

The encounter with pro-determinants in more than one site (early and late endosomes, lysosomes) allows interaction with the antigen in all its forms (as long, partly processed fragment or as short, highly processed peptides). This maximises the chances of presentation of the highest and most varied yield of determinants to $CD4^+$ T cells.

1.3.1.2. Genetic organisation of the MHC locus. In humans the genes encoding for MHC molecules are located on chromosome 6 and span $4x10^6$ bp; more than a hundred genes are included in this region. The gene for β2 microglobulin is in chromosome 15 (reviewed in (59)).

MHC genes (also called HLA in humans, from 'human leukocyte antigen') are polygenic, as they encode proteins with different ranges of peptide binding specificity. They are also polymorphic, as there are multiple alleles for each gene; they are probably among the most polymorphic genes known.

The class I regions contains the genes for HLA-A, -B and -C. There are over a hundred known alleles and their distribution is often linked to race: the HLA-A1 allele, for example, is common among Caucasians and HLA-A24 in Asians (73). The class III region has genes encoding for the complement proteins C4 (A and B), C2 and Factor B other than the genes TNF α and β and for 21-hydroxylase, plus a number of other genes showing little polymorphism and whose function is not clear yet.

The class II region contains genes that encode for subunits of the proteasome and for the peptide transporters TAP1 and TAP2. There are also the genes for the subunits of

HLA-DM. The remaining genes encode for the proteins that make up MHC-II molecules, HLA-DP, HLA-DQ and HLA-DR. As each of these has an α and a β subunit there are two genes per locus.

The locus for HLA-DR can contain an extra β gene that binds to the α chain, increasing the variability of MHC-II molecules and therefore the peptides they can present. The two β genes differ, as one, HLA-DRβ1, is highly polymorphic (over one hundred alleles are known and probably more exist) whereas there is little allelic diversity for the second HLA-DRβ: only three alleles exist. From here on the HLA-DRβ1 chain will be referred to simply as 'HLA-DR' followed by the number; the extra β gene will be described as 'HLA-DRβ' and the number. The extra β chain is associated only with certain HLA-DR types: HLA-DR*04, *07 and *09 express HLA-DRβ4, HLA-DR*15 and HLA-DR*16 express HLA-DRβ5; HLA-DR*03, *11, *13 express HLA-DRβ3 but HLA-DR*01, *08 and *10 are not associated with any extra β chain (73). The two β chains, bound to the α chain, function as distinct restriction elements and exhibit different $CD4^+$ T cell reactivity (74, 75). Their level of expression is different as HLA-DRβ1 molecules are expressed three to five times more than other HLA-DRβ molecules (76).

The high number of MHC-II that can present antigens to the immune systems makes it extremely unlikely that any pathogen could mutate its structure enough to avoid presentation to the immune system by at least one of them.

1.3.1.3. Expression and regulation of MHC molecules. The distinct distribution among cells of MHC-I and MHC-II molecules reflects the different effector functions of the T cells that recognise them. Class I molecules are normally found on all nucleated cells. The level of expression varies in different cell types (it is high on cells of the immune system but lower on hepatocytes and kidney cells). Expression can be

upregulated by cytokines, such IFN-α and IFN-β. Double-strand RNA (found in cells only during viral infection) is a strong stimulus to produce these interferons leading to upregulation of expression of MHC-I molecules, of TAP proteins and certain subunits of the proteasome (reviewed in (59)), increasing the chances of presenting a viral peptide to $CD8^+$ T cells for destruction of the infected cell.

As the main function of $CD4^+$ T cells is to activate other effector cells of the immune system MHC-II molecules are found only on cells of the immune system like DCs, B cells and macrophages (reviewed in (77)), or cells related to them (such as cells of brain microglia, derived from macrophages). Cells from other tissues can, however, be induced to express class II molecules after cytokine production and some cancerous cells have constitutive expression.

Upregulation of HLA-DR, -DP and –DQ is coordinated with upregulation of the Ii molecule and the HLA-DM chaperone. A common activation region is shared by all the genes for these proteins in the DNA upstream of the coding sequence. The activation motif is made of four regions, named W, X and Y boxes. These boxes are bound by transcription factors. The RFX factor, a heterodimer made of a 75 and a 36 kD chain, binds to the first half of the X box (X1). The second half of the box, X2, is bound by the X2BP factor. The two factors bind cooperatively, the interaction of the complex being much stronger than interaction of the proteins separately. The complex also interacts with the heterotrimeric factor NF-Y, known to bind to the minor groove of the DNA and distort it. As the NF-Y complex-Y box interaction is weaker compared to the RFX-X2BP-X box interaction it is likely that the latter is more important in the first interactions at class II promoters. RFX is also known to weakly bind the W box but this binding is greatly enhanced by NF-Y, suggesting that the whole class II regulatory region interacts with multiple RFX factors.

The promoter regions among the MHC-II genes are very similar but not identical: for example, there are isotype-specific differences concerning the X and Y boxes in the promoters of HLA-DRβ1 genes and HLA-DRβ3, β4 and β5. This results in the X1, X2 and NF-Y factors having different affinity for the promoters of these genes, leading to different levels of expression of the β chains (78).

All these factors are ubiquitously expressed but their presence on the DNA does not trigger transcription. The last factor required for this is the Class II Transactivator (CIITA), a large protein of 1130 aminoacids with an amino-terminal acidic domain, a proline-threonine-serine rich domain and a GTP binding domain. The protein itself does not bind the DNA and uses its acidic domain to interact with the RFX factors and activate transcription (79, 80). CIITA is present only in class II expressing cells and appearance of its mRNA was found to precede appearance of MHC-II molecules on the cell surface (81), so it may be the limiting factor in class II regulation.

Expression of MHC-II molecules may be increased after exposure to cells to IFN-γ (82) and, in certain cell types, also by IL-4 and IL-13 (82, 83). IFN-γ induces transcription of CIITA (81).

Expression may also be decreased by cytokines like IL-10 (83), TGF-β (84) and IFN-β (85), that works in a CIITA independent manner.

1.3.2. Processing of antigens through the endogenous pathway

Proteins – antigens – that reside in the cytosol or nucleus are considered 'endogenous' as they are produced and kept inside the cell, where they will be degraded at the end of their life. The events that lead to the formation of an MHC-peptide complex starting from an endogenous protein will be referred to as 'processing through the endogenous pathway': as the focus of this PhD project is the study of CD4 epitopes that MHC-peptide complex will be an MHC-II-peptide complex. This pathway is opposed to 'processing through the exogenous pathway', a definition used to indicate all those events leading to the formation of MHC-II-peptide complexes starting from an exogenous antigen carried into the cell. As the final product – an MHC-II-peptide complex – is the same the two pathways connect and overlap at some stage. The possible points of interaction will be described later. The classical MHC-I peptide producing pathway is shown in Figure 3; some of the peptides (or precursor peptides) produced in this pathway could be delivered for loading onto MHC-II molecules.

All endogenous proteins are potential targets for the proteases present in the cytosol, regardless of the MHC complex their peptides will eventually be loaded onto. Short-lived proteins are typically targeted for destruction by the proteasome (86). Newly synthesized proteins that fail to reach their native state (due to mistranslations, truncations, improper folding, etc) are also targeted for destruction. This particular pool of proteins has been given the collective name of 'defective ribosomal products' (DRiPs) (reviewed in (87)). DRiPs from cytosolic proteins are cleaved into smaller peptides by the proteasome and other cytosolic proteases, then transported into the ER via TAP. Proteins directed into the ER may here fail to reach their proper conformation and will be dislocated from the ER to the cytosol for degradation by the proteasome. Transport of the proteins occurs through the Sec61 translocon (88). Improperly folded or defective glycoproteins are also dislocated to the cytosol. Removal of N-linked

glycans from defective glycoproteins, mediated by peptide:N-glycanase (89), can proceed at either site of the ER membrane and depends on the glycoprotein substrate (90); the proteasome is capable of degrading glycoproteins without prior removal of their glycans (91). Peptide fragments from both glycosilated and non glycosilated proteins can therefore be presented to the immune system.

1.3.2.1. The standard proteasome. The largest multicatalytic proteolytic complex in eukaryotes is the proteasome; a 26S structure made of a regulatory 19S particle and a 20S core (reviewed in (92)). The 19S regulatory particle caps the proteasome at one or both ends and modulates entry of substrates into the core; it is also needed for recognition of ubiquitin-tagged proteins and protein unfolding prior to entry. The 20S core is a hollow barrel-shaped structure made of four rings. The two outer rings are identical and made of seven α subunits. The two inner rings are made of different β subunits; of these seven, three have catalytic activity, each with a distinct substrate specificity: tryptic (cleaving after basic aminoacids) catalysed by subunit β2, chimotryptic (cleaving after hydrophobic aminoacids), catalysed by subunit β5, and caspase-like (after acidic aminoacids), catalysed by subunit β1. The genes for all these subunits are in the MHC gene locus and are constitutively active.

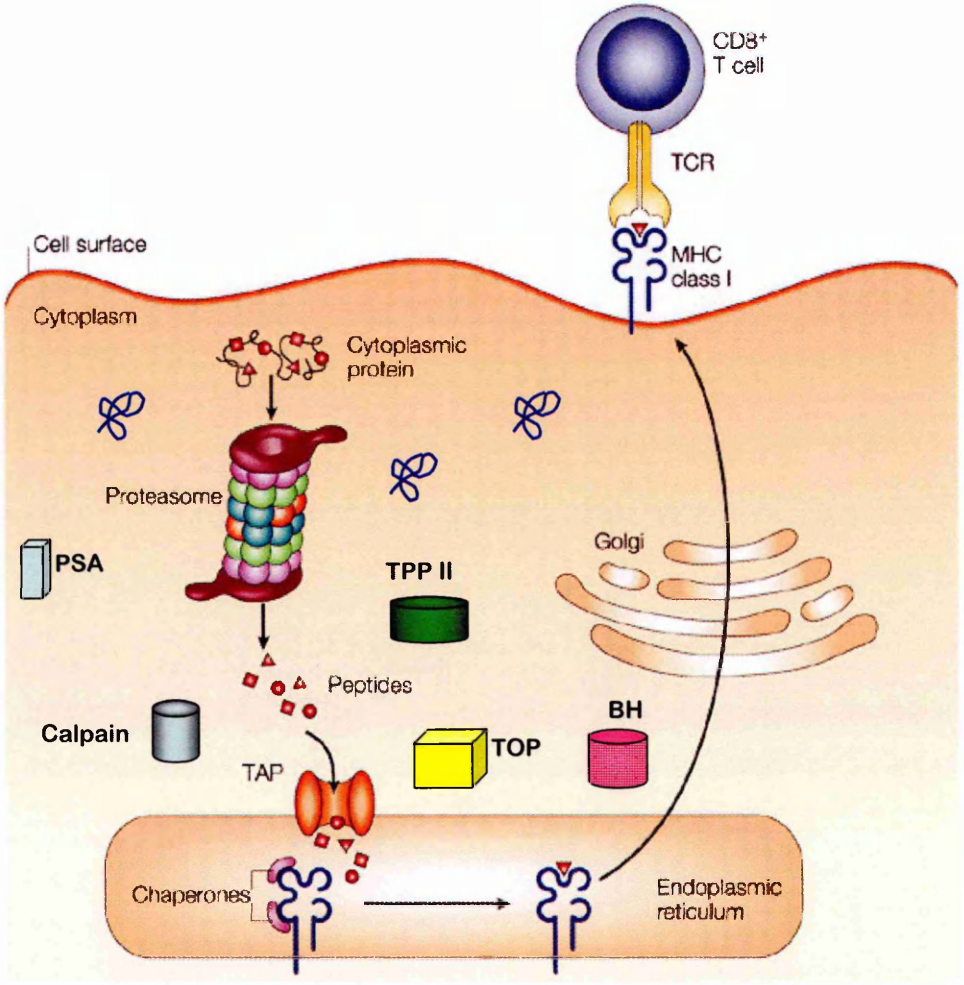


Figure 3. Processing through the endogenous pathway. Proteins are subjected to the proteolytic activity of cytosolic proteases and carried into the ER for loading onto MHC-I class molecules for presentation to $CD8^+$ T cells. Some peptides from endogenous proteins will be loaded onto MHC-II molecules following a pathway not identified yet. PSA: puromycin sensitive aminopeptidase, TPP II: tripeptidyl peptidase II, BH: bleomycin hydrolase, TOP: thimet oligopeptidase, TAP: transporter associated with antigen processing *(picture adapted from Nature Immunology, 2004, 5: 661).*

Proteins are targeted to the proteasome by ubiquitination (93). This process involves attacking one or more ubiquitin molecules to the ε-amino group of lysines in the protein; this is carried out by the E1, E2 and E3 system. Substrates for the proteasome are typically short lived proteins (86).

Ubiquitination makes delivery to the cytosol quicker but in general any protein residing in the cytosol could be degraded by the proteasome. Proteins from viruses that replicate and assemble in the cytosol are also substrates for proteasomal activity. DCs

retrotranslocate endocytosed proteins into the cytosol for processing by the proteasome (94); this allows them to prime $CD8^+$ T cells for antigens they could find on the surface of cells infected by pathogens (reviewed in (95)).

Proteins will be cleaved by the proteasome into progressively smaller fragments. Some of these peptides will bind to TAP and be delivered inside the ER for loading onto MHC-I molecules. The complex will be then transported to the cell surface. The presence of a complex with a peptide of viral, bacterial or simply unexplained origin will lead to destruction of the presenting cell.

1.3.2.2. The immunoproteasome. Exposure of cells to certain cytokines, such as IFN-γ, causes changes in the composition of proteasomal subunits: β1, β2 and β5 are replaced by their inducible counterparts β1i, β2i, β5i (coded by genes LMP2, MECL1 and LMP7 respectively) to form a new structure called 'immunoproteasome' (96). Compared to its standard counterpart the immunoproteasome has higher tryptic and chymotryptic activity but a much lower caspase-like activity; this is partly replaced by new specificity for branched-chain aminoacids, like leucine and isoleucine. Hydrophobic aminoacids are more favoured anchor residues, so production of more peptides with the right anchor residue should improve presentation. Immunoproteasomes are indeed expressed constitutively in APCs like DCs and B cells (96).

1.3.2.3. Differences in processing between the two proteasomes. As in mature DCs the main type of proteasome is the immunoproteasome $CD8^+$ T cells can be primed preferentially for the peptides produced by this sort of complex, influencing the CD8 epitope hierarchy (97). Experiments in immunoproteasome deficient mice confirm this (98). Lack of inducible subunits is not, however, incompatible with life, as cells

negative for the immunoproteasome do not show any metabolic disorder or decrease in production of MHC-I-peptide complexes (99, 100). Indeed, some antigenic peptides are produced regardless of the type of proteasome in the cell (99); for example, presentation of the epitope NY-ESO-$1_{157-165}$ (from tumor antigen NY-ESO-1) is not affected by the presence or absence of β1i and β5i (101). For others there is difference, deriving from the different substrate specificity of the two complexes.

Some epitopes are produced preferentially in cells with a standard proteasome, such as epitope 26-35 of Melan-A, a protein from human melanocytes (97). The human MAGE-C2 protein has a class I epitope in the 336-344 region that is destroyed by the standard proteasome, perhaps because the aminoacid sequence is rich in acidic residues and so a good substrate for the caspase-like activity (102). The tumor antigen MAGE-3 also contains an epitope that is sensitive to proteasomal activity. MAGE-$3_{271-279}$ is produced efficiently in melanoma cells only when the proteasome is partly inhibited by the proteasomal inhibitor lactacystin (103).

The immunoproteasome was found to be essential for production of the HLA-B40 restricted 114-122 epitope from the tumor antigen MAGE-3. Even more specifically, subunit β5i was required for formation of this epitope, as transfection of 293-EBNA lines with β5i alone was enough for production (104). The immunoproteasome has been shown to destroy epitopes from other TAAs, such as $gp100_{209-217}$ and $tyrosinase_{369-377}$, as their hydrophobic-rich sequence is probably recognised and cleaved. These epitopes are therefore more likely to be found on the surface of cells expressing the standard proteasome (102).

1.3.2.4. Tripeptidyl-peptidase II (TPP II). A large, active cytosolic protease is TPP II (105). This rod-shaped complex, with a channel in the centre, is a serine-peptidase and removes tripeptides from the free amino-terminal of peptide fragments, with tryptic and

chimotryptic specificity. It has been shown to be more active in murine cells with impaired proteasome function, although it cannot compensate for severe or total loss of proteasomal activity (106). It could play a role in production of class I epitopes. Peptides loaded onto HLA-A3 and HLA-A11, for example, generally have a lysine in the carboxyl terminal position and production of these peptides is not impaired even when the tryptic activity of the proteasome is inhibited (107). It was also essential for processing of the influenza virus nucleoprotein $NP_{147-155}$ epitope (108). Other experiments, however, show that TTP II is not required for correct processing of other epitopes, such as $OVA_{258-265}$, in murine cells (109).

Taken together, the data gathered so far suggest that TPP II plays a role in antigen processing but not one that is essential for generation of most of the presented epitopes.

1.3.2.5. Effects of other proteases. Proteasomes (both standard and not) will produce peptides of different length whose aminoacid in the carboxy-terminal is usually a suitable anchor residue for loading. Most 'post proteasomal' activity will be trimming of peptides in their amino-terminal region. Leucine aminopeptidase (LAP) is an IFN-γ inducible cytosolic aminopeptidase (110). It was thought to be important in trimming of antigenic peptides but experiments with LAP null mice have shown that it is not essential (111) so its role is probably redundant. Other cytosolic proteases are known. Puromycin sensitive aminopeptidase (PSA) is a metalloprotease conserved in all species (112) with broad tissue specificity (113). Bleomycin hydrolase (BH), first identified for its capacity to inactivate the anticancer drug bleomycin, is also a well conserved cysteine protease found in many tissues (114). Both have been shown to be involved in correct processing of a viral epitope (VSV NP_{52-59}) so their aminopeptidase activity could be important in antigen processing (115). Calpains are cytosolic (but also transmembrane) calcium-dependant cysteine proteases involved in modulation of signal

transduction and transcription pathways (116) and have been implicated in autoimmune disorders such as arthritis and multiple sclerosis (117).

Not all proteolytic activity will favour production of antigenic peptides. The metallo-endoprotease thimet oligopeptidase (TOP), for example, has been shown to rapidly and efficiently degrade antigenic peptides both *in vitro* (118) and, most importantly, also *in vivo* (119). It could therefore be a significant factor limiting peptide presentation. On the whole, the extent of antigen presentation is closely related to the balance between the proteolytic activities that generate and destroy epitopes.

After transport inside the ER peptides can still be trimmed in their aminoterminal region. In human cells this is probably carried out by the metallo-proteinase endoplasmic reticulum aminopeptidase associated with antigen processing, ERAAP (reviewed in (120)). In humans there are two ERAAP-like proteins, ERAP1 and ERAP2. ERAP1 is a 930 aminoacid glycoprotein whose expression is ubiquitous but higher in spleen, thymus and liver, paralleling MHC-I expression. Its substrate specificity is for peptides nine to ten or more aminoacids in length. ERAP2 is a glycosilated protein whose pattern of expression is slightly more restricted than ERAP1, being found mainly in the thymus and in leukocytes; occasionally ERAP1 and ERAP2 form heterodimeric complex for the digestion of particularly large peptides. Both proteins are upregulated upon IFN-γ stimulation but their roles are not overlapping. The data obtained so far on the role of ERAPs in proteolysis of antigenic peptides is contradictory.

1.3.2.6. Proteasomal activity in the nucleus. Short lived transcription factors (such as the ones induced in response to a specific signal) must be inactivated and eliminated rapidly once the signal ceases. Coherently with this, proteasomes have been described inside the nucleus and seem to be located near the sites of most active transcription

(121) and not in the nucleolus or the nuclear envelope; nuclear proteins are known to have MHC-I epitopes (48). The digested peptides are then transported into the cytoplasm; from here on the peptides will follow the fate of their cytosol-produced counterparts.

1.3.2.7. Effects of cytosolic proteases on MHC-II epitope processing. In DCs endocytosed material is translocated into the cytosol for processing and cross-presentation of antigens on MHC-I molecules, so 'exogenous' antigens will feel the effects of the immunoproteasome and other proteases. The role in MHC-II epitope processing of cytosolic proteases is less characterised but well known. Two influenza epitopes, $HA_{302-313}$ and NA_{79-93}, were produced in a proteasome and TAP dependent manner in DCs but not in cells with less permeable endosomes, such as fibroblasts (122). In human cells, proteasomal activity has been reported necessary for proper production of an immunodominant epitope of glutamate decarboxylase ($GAD_{273-285}$) when this antigen is delivered endogenously in lymphoblastoid cell lines (LCLs). As these are B cells, the activity is probably that of the immunoproteasome (123). Calpains also appear required for this epitope (123) in the same cells.

1.3.3. Processing through the exogenous pathway

The events that lead to the formation of an MHC-II-peptide complex from an antigen endocytosed from the external milieu are here referred to as 'processing through the exogenous pathway. As antigens travel towards the lysosomal compartment where MHC-II molecules mature they encounter different enzymes and environmental conditions, starting from the near-neutral, mildly acidic early endosomes to the highly acidic lysosomes (see Figure 4). These organelles are rich in proteases that have different optimal activity pH: proteases cathepsin B and cathepsin D, for example, could start attacking an antigen in early endosomes and in acidic lysosomes respectively. Some compact, globular antigens could be particularly difficult to unfold and could require a cleavage by a specific protease (such as the tetanus toxoid C-terminal fragment, TTCF (124)). Even if an antigen proved particularly resistant to protease action and it would however begin unfolding in an acidic pH. GILT (γ-IFN-inducible lysosomal thiol protease) is active at low pH and can catalyse reduction of disulfide bonds required for complete denaturation, making sites accessible to proteolysis (125). Once the peptides are produced and the MHC-II molecules loaded they will be delivered to the cell surface for recognition by $CD4^+$ T cells (see Figure 4). Most mammalian proteases are cysteine proteases of the cathepsin family but some cathepsins are aspartic proteases; some are cysteine proteases but not cathepsins. Their expression and activity vary according to cell type and stimulus.

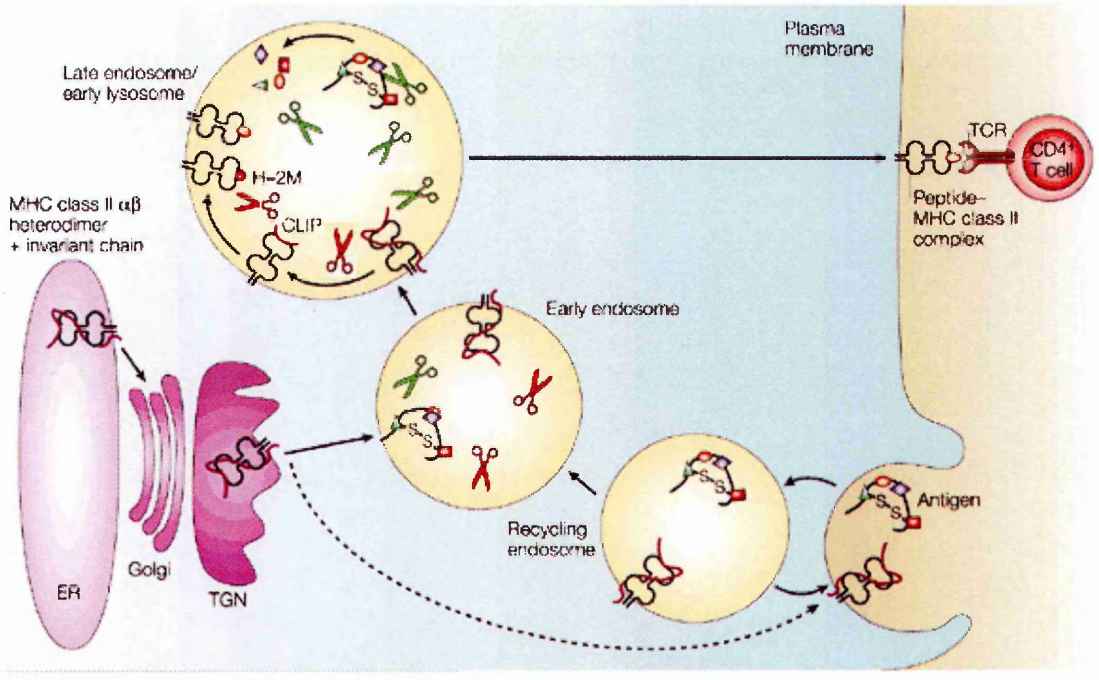


Figure 4. Processing through the exogenous pathway. Endocytosed antigens are processed in endosomes and lysosomes and peptide fragments loaded onto MHC-II molecules for recognition by $CD4^+$ T cells. Scissors represent proteases *(picture adapted from Nature Immunology Reviews, 2003, 3:472).*

1.3.3.1. Cathepsin S (catS). This protease plays a major role in late processing of the Ii-MHC-II molecule in all cells of the immune system (126) except for thymic cortical epithelial (TCE) cells, where its activity is not detectable. CatS null mice show a considerable impairment of antigen presentation and accumulation of incompletely processed MHC-II molecules in cells (127). It is unique among cathepsins as its expression is up-regulated by IFN-γ (128).Other than a role in maturation of MHC-II molecule catS is also able to process antigens, as in murine systems it is required for specific subsets of antigens (129), including production of the HEL_{30-44} epitope (130). CatS is currently regarded as a very promising target for the treatment of autoimmune diseases such as rheumatoid arthritis and bronchial asthma (131). CatS is able to degrade elements of the extra-cellular membrane such as elastin and collagen (132) and, unlike most other cathepsins, it is fully active even at neutral pH (133). It is therefore

thought important in tissue remodelling other than playing an important role in cells of the immune system.

1.3.3.2. Cathepsin L (catL). In murine TCE cells transformation of Ii to CLIP is catalysed by catL (134); in human cells its role is taken by cathepsin V, which shares a high homology with catL (135, 136). This protease is not active in other cells of the immune system (such as B cells and DCs) although it is expressed (137, 138). It is active in cells not belonging to the immune system, where it is able to process antigens and influence presentation of subsets of some (129). Variation in catL activity is linked to some cancers including prostate (139) and colorectal cancers (140) and melanoma (141), where it can promote degradation of extracellular matrix and basement membrane that precedes tumor metastasis.

1.3.3.3. Cathepsin B (catB). This enzyme is abundant and active in lysosomes suggesting it functions primarily as a component of the protein degradation system. Some studies have shown that it is dispensable in antigen processing (142); indeed, catB negative mice show no defect in antigen processing, strongly suggesting that it plays no specific role in MHC-II maturation. Rather than not involved it is likely to simply be redundant. Extensive studies have shown changes of catB levels in human tumors suggesting a role in invasion and metastasis (140, 141, 143). Additionally, there is evidence of its involvement in rheumatoid arthritis (144).

1.3.3.4. Asparagine endopeptidase (AEP). This protease is a cysteine protease unrelated to cathepsin family but grouped with caspases, separases and some bacterial proteases (145). It is unique among cysteine proteases because it is not sensitive to inhibitors such as leupeptin or E64 (146) and among lysosomal proteases because its

cleavage site (the carboxyl side of an asparagines residue) is very specific when compared to relatively non-specific sequences cleaved by cathepsins. It is required in the initial steps of the Ii-MHC-II complex maturation in B cells and DCs, where its activity is particularly high; its role in other cell types may be less important (64). It is involved in antigen processing, as it is required for the correct processing of TTCF (124, 147) and its activity determines the amount of myelin basic protein (MBP) epitope MBP_{85-99} presented to $CD4^+$ T cells in TCE cells (148). It therefore contributes to the formation of the MHC-II-peptide complex by affecting MHC-II molecule maturation and processing of antigen.

1.3.3.5. Cathepsin D (catD). This ubiquitous lysosomal enzyme contains an aspartic acid residue in its active site and plays an important role in protein degradation. Its role in antigen processing is redundant (as catD negative cells do not show impaired antigen presentation) (142) but it is specifically required to produce some epitopes (149-151). There is also evidence that aspartyl protease activity can destroy epitopes rather than produce them (152). Expression of catD is constitutive in almost all cells but can be regulated by molecules such as estrogen (153). CatD is recognised as a worthwhile therapeutic target for cancer research as this protease is involved in many tumor progression steps, including cancer cell proliferation, angiogenesis and apoptosis (154). Recently a novel apoptotic pathway has been demonstrated whereupon catD triggers Bax activation and consequently induces the selective release of mitochondrial apoptosis inducing factor (AIF) responsible for the early apoptotic pathway (155).

1.3.3.6. Cathepsin E (catE). An aspartic protease found in the perinuclear compartment in DCs (and therefore in the ER) (156) and, to a lesser extent, in some endosomal compartments (157), catE was found to be upregulated late in human B cell activation

(158). It has been shown to be involved in processing of ovalbumin in murine B cells (159) and in DCs (specifically, it was involved in production of the $OVA_{323-339}$ peptide (157)) and could have a negative, destructive effect on processing of some epitopes (152), as was seen for catD. It was specifically required for generation of the $OVA_{266-281}$ in murine microglia (but not for Ii processing) (160) but its exact role in processing in cells not of the immune system or not derived from those remains to be elucidated.

1.3.3.7. Other enzymes. Enzymes other than the ones mentioned above populate the endosomal lysosomal compartment (reviewed in (161)). Cathepsin H is known to be present and active in almost all cells; it is not involved in MHC-II maturation but probably in antigen processing. Its optimal pH is around 7, suggesting that this enzyme is most likely to be active in mildly acidic early endosomes. Its levels of activity have also been linked to prognosis of some types of cancer (141).

A number of other cathepsins have been described but their role in antigen processing is not fully understood yet. CatX, for example, has a structure similar to catB but its role in the immune response requires experimental confirmation. CatC is involved in maturation of progranzymes to their catalytically active form in natural killer cells and active CTLs rather than processing antigen in APCs (162). Cathepsin W is expressed mainly in NK cells and $CD8^+$ lymphocytes, where it is probably localised in the ER. As its expression is low in the thymus and in $CD4^+$ T cells it is thought to be involved in thymic selection of $CD8^+$ T cells (163).

1.3.4. Delivery of antigens to the MHC-II compartment

Antigens can be delivered to the MHC-II compartment in at least two ways, depending on their origin: if they are outside the cell they will enter through endocytosis and be directed into the lysosomal compartment; if they are endogenous antigens they will be most probably delivered to the MHC-II compartment via autophagy.

1.3.4.1. Endocytosis. Molecules from the outer milieu enter the cell in ways dependent on cell type and molecule size: for example, small molecules such as sugars, aminoacids and ions will cross the plasma membrane using specific channels. Larger molecules and particles are carried into the cell in membrane bound vesicles deriving from plasma membrane invagination. This process is called 'endocytosis' and can be divided into two broad categories: phagocytosis (the uptake of large particles) and pinocytosis (uptake of fluids and solutes).

1.3.4.1.1. Phagocytosis. This process is conducted mainly by specialised cells such as macrophages, monocytes and neutrophils and its main function is to eliminate large pathogens (such as bacteria or yeast) and large debris (dead cells, etc, reviewed in (164)). Specific cell surface receptors and signalling cascades (mediated by Rho-family GTPases) are involved in this highly regulated process (reviewed in (165)): in phagocytosis of bacteria, the Fc receptors of macrophages are activated by the antibodies bound to bacterial surface antigens. The resulting signal cascade will lead to Cdc 42 activating the protein Rac triggering actin assembly and formation of membrane extensions; these will engulf the antibody-coated pathogen, forming a phagosome, by 'zipping' and membrane fusion. The signal cascade will also have activated the cell's inflammatory response, so that once inside the phagosome the pathogens will be destroyed by bactericidal molecules (such as hydrolases, acids and free radicals).

Phagocytosis is also used to eliminate apoptotic cells, especially by macrophages (reviewed in (166)). At least seven surface molecules are involved in recognition of apoptotic cells, including lectins, Sr-A, integrins, the receptor for phophatidyl serine (PSR) and the tyrosine kinase receptor MER. Inside the cell phagocytosis is mediated by the p130-cas/CrkII/Dock180 complex with Rac-I, that controlles downstream activation of tyrosine kinases and phosphatidil-inositol-3 kinase (PI3K).

Triggering a signal cascade through these receptors does not activate the cell's inflammatory response (166). Phagocytosis is carried out mainly by macrophages; other cells, such as DCs and fibroblasts, are less efficient (167).

1.3.4.1.2. Pinocytosis. This process allows entry of molecules of different sizes and occurs in at least four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and caveolae- and clathrin-independent endocytosis.

Macropinocytosis. Molecules over 1 μm can enter this way. It is more efficient way to absorb solutes compared to clathrin-mediated and caveolae-mediated endoytosis (168). Activation of DCs starts an intense and prolonged macropinocytotic activity, allowing these cells to sample large amounts of extracellular milieu and fulfil their role in immune surveillance (95). It is a process characterised by intense membrane ruffling, dependent on actin and activation of Rac (reviewed in (169)). Large circular ruffles form and close off in a PI3K-dependent manner to form macropinosomes. These vesicles will shrink in size due to loss of water through aquaporins. The content of the micropinosome may then be delivered to the MHC-II loading compartment for degradation or released into the cytosol for processing by the proteasome.

Clathrin mediated endocytosis. This process occurs constitutively in all mammalian cells and it is one of the better characterised types of endocytosis (reviewed in (170)). Clathrin forms a triskelion shaped structure, made of three heavy clathrin molecules and three light ones. The triskelions require 'coat' proteins for assembly into vesicles: the assembly proteins (APs) are divided into two classes, monomeric AP180 and the heterotetrameric adaptor complexes. There are four structurally related adaptor protein complexes (AP1-4) but only AP2 is directly involved in clathrin coated vesicle formation. The coat proteins, clathrin, AP2 and AP180 are able to select cargo and form a vesicle. The closing of the vesicle is probably catalysed by dynamin, a large GTPase with several domains, including a GTPase effector domain and activation domain. At later stages of coated vesicle formation dynamin is thought to assemble into a ring round the neck of the deeply invaginated coated pits. GTP-hydrolysis causes conformational changes that allow the protein to act.

Clathrin-mediated endocytosis is involved in the continuous uptake of essential nutrients and its role in delivering antigens is not clear.

Caveolae-mediated endocytosis. Caveolae are flask-shaped invaginations of the membrane than can contain particles up to 120 nm in size (reviewed in (170)). The main protein is caveolin, a dimeric protein that binds cholesterol; it inserts a loop into the inner layer of the plasma membrane to form a striated caveolin coat on the surface of the membrane invaginations. Caveolin null mice have a relatively mild phenotype, with some tissues showing a hyperproliferative response but normal development (171). This and other evidence led to supposing that caveolin might negatively regulate caveolae uptake rather than promote it (172). Caveolin is tyrosine phosphorilated by Src *in vivo* but it is not known if this triggers internalisation (173). As caveolae are slowly

internalised in most cells and carry a small volume it is unlikely that this process plays a significant part in fluid-phase uptake and antigen entry.

Clathrin and caveolae independent endocytosis. This type of endocytosis has been observed in lymphocytes, neurons and neuroendocrine cells (reviewed in (170)). More studies are required to elucidate the functions and mechanisms of this process.

1.3.4.2. Autophagy. Metabolically active cells have two ways of eliminating proteins: through ubiquitination and destruction by the proteasome or via destruction in lysosomes. Proteasome degradation is generally used for short-lived proteins (such as activated forms of proteins involved in signal cascades). Degradation in lysosomes is usually for long-lived proteins; delivery to the lysosomal compartment occurs through autophagy, a normal metabolic event in all eukaryotic cells. Damaged organelles (such as mytochondria) and protein aggregates containing long-lived proteins are also targeted for autophagic destruction but the exact targeting signals have not been defined yet.

1.3.4.2.1. Regulation. In normal conditions autophagy is kept at a basic level and contributes to the general turnover of cellular components (reviewed in (174)). In starvation this process may be increased to produce aminoacids needed for building essential proteins. Hormone stimulation also affects autophagy: insulin inhibits the process while glucagons increase it. Knowledge on the molecular regulation of this complex multi-step process is fragmented and incomplete and more than one signalling pathway has been implicated in its regulation. A relatively well known pathway is the PI3K-AKT-mTOR pathway (reviewed in (175)). PI3K type I, in the absence of growth factors, promotes the formation of pre-autophagosomal factors. Growth factors lead to

activation of PI3K that activates the serine kinase AKT3 that in turn activates the mammalian target of rapamycin (mTOR) kinase resulting in inhibition of autophagy; the cellular factors working downstream of mTOR have not been identified with certainty yet.

1.3.4.2.2. Mechanisms of autophagy. Three types of autophagy have been described: chaperone-mediated autophagy, microautophagy and macroautophagy.

In chaperone-mediated autophagy cytosolic proteins are delivered to lysosomes by LAMP-2A (176, 177) aided by cytosolic and lysosomal members of the Hsc70 family (178, 179). Signal peptides are required for sorting proteins to the lysosomes in this type of autophagy (180).

Microautophagy consists in uptake of cytoplasm on the lysosomal surface via budding into the lysosomal lumen; little else has been characterised.

In macroautophagy 'isolation membranes' (a single layer membrane) elongate and envelop a portion of cytoplasm with all its contents (see Figure 5).

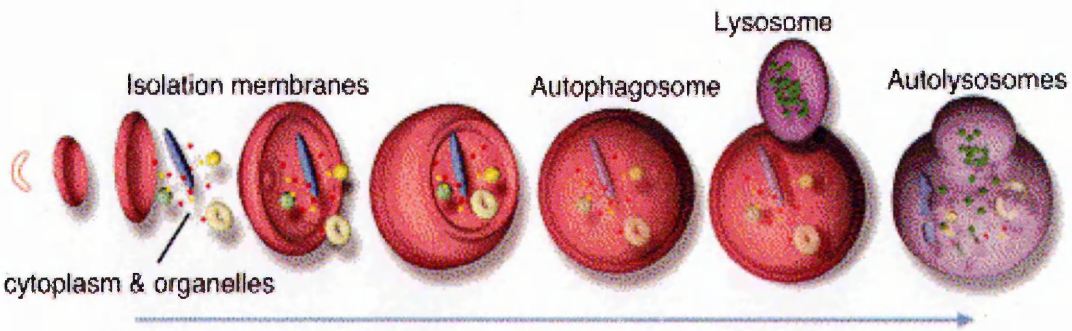


Figure 5. Macroautophagy. Isolation membranes close and merge with lysosomes for digestion of enveloped contents *(taken from Biochemical Biophysical Research Communications, 2004, 313: 453).*

Fusion on the membrane tips will lead to the formation of a double membrane structure called autophagosome (approximately 1 μm in diameter). The origin of the membrane is unknown; it is possible that it has different origin depending on cell type

and on autophagy-inducing stimulus. Two ubiquitin-like systems are used in this type of autophagy. The first system is made of autophagy-related protein Atg5 that couples to Atg12 (181). The complex then localises on the concave side of the elongation membrane and is probably involved in curving of the membrane itself. Upon closure this complex must be released, as it is not found in mature autophagosomes. The second system is the maturation of newly-synthesised Atg8 (better known as microtubule-associated protein 1-light-chain 3, MAP1-LC3) (182). Atg4 cleaves the C-terminal 22 aminoacids to form an 18 kD protein (LC3-I) that is then modified by Atg3 and Atg7 to a 16 kD form, LC3-II. LC3-II is linked to phosphatidylethanolamine in the autophagosomal membrane (183). LC3-II remains in the autophagic vesicle and is degraded in lysosomes; it is, to date, the best autophagosome marker.

This short-lived organelle (half-life of eight minutes) will then merge with a lysosome and deliver its contents to the proteases within for digestion.

1.3.4.2.3. Role of autophagy in class II antigen processing. Independently of how it occurs autophagy is a direct path for delivery of endogenous proteins to the MHC-II compartment. Cytoplasmic and nuclear antigens, such as complement C5 (184), EBNA-1 (185), tumor antigen mucin 1 (MUC-1) (186) and selection marker neomycin phosphotranferase II (neoR) (187) have been shown to localise to autophagic vesicles; production of an epitope from this last antigen is also sensitive to wortmannin, an inhibitor of autophagy. In LCLs two epitopes from LC3-II (188) and five from glyceraldehyde-3-phosphate-dehydrogenase (189) and many other endogenous proteins (such as actin, syntaxin 6, α-enolase and ubiquitin), have been eluted from HLA-DR molecules (188) clearly showing that not only does delivery through autophagy occur but also processing and loading onto MHC-II molecules.

Most experiments have been carried out on professional APCs (DCs for MUC-1 and LCLs for the rest). As autophagy is a ubiquitous cellular process it is possible that non-professional APCs expressing MHC-II molecules (during inflammation or after exposure to IFN-γ) could process and present endogenous protein epitopes to $CD4^+$ T cells.

The role of autophagy in antigen presentation on MHC-II molecules on tumoral cells (especially solid tumors) is more difficult to define. In these cells basal autophagy appears decreased compared to normal cells; indeed, at least two gene products, the phosphate and tensin homologue, PTEN (190, 191), and BECN1 (192), that directly affect autophagy regulation have been found mutated in a number of solid tumors, causing a decrease in autophagy. This could be explained by the tumoral cell's need, in the first stages, to undergo a higher level of protein synthesis rather than protein destruction. Tumoral and healthy cells show the same autophagy response under starvation conditions; this would help cells survive in the middle of large cancerous masses, where vascularisation and nutrient availability is limited. It is therefore possible that presentation of endogenous proteins (including TAAs) is low in the outer parts of a tumoral mass and high in the centre but where $CD4^+$ T cells are less likely to be. However, tumoral cells from solid tumors can and do present endogenous proteins on MHC-II molecules *in vitro* at least (193, 194), so this path of delivery could play a role in formation and presentation of epitopes on the surface of cancer cells *in vivo* as well.

1.3.4.3. Other routes of delivery. Some experiments in mice with modified tumoral cells suggest that endogenous antigen peptides transported into the ER may be loaded onto MHC-II molecules and that this process is much more efficient if the Ii chain is not expressed; the antigen peptides they carried were from cytosolic but also nuclear antigens, probably loaded onto empty MHC-II dimers (195). These MHC-II-peptide

molecules, lacking the Ii signal peptide, are presumably delivered to the cell surface after glycosilation. The Ii chain has a very high affinity for the MHC-II molecules but it is constant in its sequence. In each cell there is more than one MHC-II allele and the various molecules may have a higher or lower affinity for the Ii chain itself depending on the allele expressed. A peptide with a particularly high affinity for the groove may bind before the Ii chain; as happened in the murine system described above these complexes may find their way to the cell surface.

It is theoretically possible that some antigenic peptide may 'drift' towards the Golgi network and the MHC-II compartment having escaped loading onto MHC-I molecules or retrotranslocation from the ER and avoiding protease activity. There is no data so far to confirm (or disprove) the theory above.

1.4. MAGE PROTEINS

MAGE ('melanoma antigen') genes are cancer-germline tumor specific antigens, silent in normal adult tissues but expressed in male germline cells. The antigens are strictly tumor specific, because the only normal cells that express them, germline cells and placental trophoblasts, do not express MHC class I molecules and are therefore incapable of presenting antigens to T cells (46, 47). The first member of the MAGE family of TAA, MAGE-1, was described over fifteen years ago (196). Since then, the number of MAGE genes has increased considerably. There are three families of MAGE genes, MAGE-A, MAGE-B and MAGE-C.

MAGE-A proteins (referred to as MAGE proteins from here onwards) form a multigenic family of proteins whose genes are clustered on the q28 locus of the X chromosome (197). The average gene structure, spanning on average 4.5 kb, is made of three exons, two shorter ones and the third containing the open reading frame in its 5' region. MAGE-2 and MAGE-10 differ in gene structure compared to other MAGE-A gene because they contain an extra exon (198, 199). The activation of all these genes in cancer is usually the result of demethylation of their promoters, correlated to a general demethylation of the genome (200, 201).

MAGE proteins have a general weight of 40 to 50 kD. They do not have a localisation signal and are generally cytosolic proteins (202). MAGE-10 (199) and MAGE-11 (203) are nuclear proteins: MAGE-10 has a SV40 large T antigen type nuclear localisation signal but MAGE-11 does not and, to date, the exact nuclear localisation signal of these proteins has not been identified.

1.4.1. MAGE-3.

The existence of a gene for this protein was first described in a melanoma cell line by van der Bruggen et al. (196) but the exact sequences of gene and protein were described only three years later (204). The MAGE-3 gene has the standard structure of MAGE-A genes, with two short exons and a longer one containing the open reading frame. The protein itself is 314 aminoacids in length with a molecular weight of 48 kD. It is devoid of any known localisation signal and has been shown to be in the cytoplasm (202, 205). Its function is currently unknown but as it is expressed during embryogenesis it is probably involved in development. It is not expressed in adult healthy tissues, with the exception of placenta and testis, both tissues that do not express MHC molecules (46, 47). This protein is expressed in a number of solid tumors such as melanomas, carcinoma of the lung, bladder, oesophagus, head and neck (reviewed in (45)), thyroid (206), hepatocellular carcinoma (207) and gastro-intestinal cancer (208). Its pattern of expression makes it an ideal candidate for tumor immunotherapy.

1.4.1.1. MHC-I restricted epitopes of MAGE-3. Interest in MAGE-3 also stemmed from the identification of epitopes from this protein found in MHC-I molecules on the surface of cancer cells recognised by CTLs; indeed, the first MAGE was actually identified starting from a CTL from a patient with the capacity to lyse cells transfected with cosmid libraries derived from the DNA of the patient's own tumor (196). The first epitope characterised was MAGE-$3_{168\text{-}176}$, recognised in association with HLA-A1 (204) and but also with HLA-B35 (209); the almost identical epitope MAGE-$3_{167\text{-}176}$ is presented in association with HLA-B18 and HLA-B44 (210). Other MHC-I epitopes have been identified since then (reviewed in (211)), some distinct, others, like MAGE-$3_{112\text{-}120}$ (212), MAGE-$3_{113\text{-}121}$ (208) and MAGE-$3_{114\text{-}122}$ (104), partially overlapping and

with different restrictions (HLA-A2, HLA-A24 and HLA-B40 respectively). A complete list of the MHC-I epitopes of MAGE-3 is in Table 1.

The fact that a same (or almost identical) epitope can be presented by more than one allele increases the number of patients eligible for immunotherapy, specifically for peptide-based immunotherapy.

Given the importance of $CD4^+$ T cells in induction and maintenance of the immune response in anti-tumor immunity, addition of peptides containing MHC-II restricted epitopes could improve the efficacy of anti-tumor vaccination. This prompted a new search, the search for MHC-II restricted epitopes.

1.4.1.2. MHC-II restricted epitopes of MAGE-3. The first MHC-II epitopes of this protein to be described were identified following two different approaches. One was a 'classical' approach, where $CD4^+$ T cells were induced from a healthy donor by stimulation with autologous DCs and the whole recombinant protein (213). Responding clones were then checked for specificity by measuring reactivity to a panel of overlapping MAGE-3 peptides scanning the whole protein. This lead to the identification of two overlapping HLA-DR*13 restricted epitopes, MAGE-$3_{114\text{-}127}$ and MAGE-$3_{121\text{-}134}$, both naturally processed through the exogenous pathway, as the $CD4^+$ T cells responded to DCs pulsed with the whole protein but did not recognise HLA-DR-matched, MAGE-3 expressing tumors.

The second approach used a novel technique. Like MHC-I peptides, that, for a given allele, have preferred residues at the anchoring position of the peptide-binding groove, MHC-II peptides also have consensus anchor residues (214). This knowledge allowed the production of an algorithm and the design of a software, TEPITOPE, able to predict which parts of a protein are most likely to contain an MHC-II epitope, among the 25 alleles contained in its algorithm (215). This prediction was carried out for MAGE-3.

HLA	HLA frequency (%)	peptide	position
A1	26	EVDPIGHLY	168-176
A2	44	FLWGPRALV	271-279
A2	44	KVAELVHFL	112-120
A24	20	TFPDLESEF	97-105
A24	20	VAELVHFLL	113-121
B18	6	MEVDPIGHLY	167-176
B35	20	EVDPIGHLY	168-176
B37	3	REPTVKAEML	127-136
B40	6	AELVHFLLL	114-122
B44	21	MEVDPIGHLY	167-176
B52	5	WQYFFPVIF	143-151
Cw7	41	EGDCAPEEK	212-220
DP4	75	KKLLTQHFVQENYLEY	243-258
DQ6	63	KKLLTQHFVQENYLEY	243-258
DR*01	18	ACYEFLWGPRALVETS	267-282
DR*04	24	FFPVIFSKASSSLQL	146-160
DR*07	25	FFPVIFSKASSSLQL	146-160
DR*11	25	GDNQIMPKAGLLIIV	191-205
DR*11	25	TSYVKVLHHMVKISG	281-295
DR*13	19	AELVHFLLLKYRAR	114-127
DR*13	16	LLKYRAREPVTKAE	121-134

Table 1. Naturally processed epitopes of MAGE-3. The percentages of HLA frequencies are referred to Caucasians.

and peptides corresponding to the predicted sequences were synthesized and used to stimulate $CD4^+$ T cells from whole PBMCs. The $CD4^+$ T cell line specific for MAGE-$3_{281-295}$ obtained this way recognised autologous APCs pulsed with the whole recombinant protein, showing that the predicted epitope was naturally processed and presented. Furthermore the cell line exerted cytolytic activity against HLA-DR-matched, MAGE-3 expressing melanomas, meaning that it is also processed through the endogenous pathway (193).

The number of MAGE-3 MHC-II epitopes and the alleles they bind to has increased since these first experiments. The whole protein, 'classical' approach led to the identification of the HLA-DP4-restricted MAGE-$3_{243-258}$ epitope (194) and to the HLA-DR1-restricted MAGE-$3_{267-282}$ epitope (216).

Other MAGE-3 epitopes were identified using prediction software (TEPITOPE and others). MAGE-$3_{146-160}$ was found to be contain naturally processed epitopes in association with HLA-DR*04 and HLA-DR*07 (19, 217). MAGE-$3_{191-205}$ contains a naturally processed epitope in association with HLA-DR*11 (19). TEPITOPE had also predicted that MAGE-$3_{111-125}$ would bind to HLA-DR*01, HLA-DR*04 and HLA-DR*11 (19) and the MAGE-$3_{111-125}$ peptide was able to activate HLA-DR*11 restricted $CD4^+$ T cells (19). This epitope closely overlaps the naturally processed MAGE-$3_{114-127}$ described by Chaux and co-workers (213), so it is possible that the epitopes recognised by the $CD4^+$ T cells specific for the two peptides are the same. A list of MAGE-3 with all its naturally processed MHC-II epitopes and their restriction alleles is in Table 1.

It is worthy of notice that all the naturally processed epitopes found so far are produced through the exogenous pathway and most are also presented on the surface of melanoma cells. MAGE-$3_{114-127}$ is the exception here, as it does not appear processed through the exogenous pathway. The MAGE-$1_{282-292}$ epitope also displays similar behaviour (218) and so do some other tumor antigen epitopes unrelated to MAGE

proteins (39, 219). The function of these epitopes *in vivo* is unclear, although it is reasonable to envisage a role both in induction of anti-MAGE-3 responses as well as helper and indirect effector functions.

TEPITOPE had also predicted that MAGE-$3_{161-175}$ and MAGE-$3_{171-185}$ would be promiscuous; two $CD4^+$ T cell lines specific for those regions were obtained from a melanoma patient (19). The epitopes within however did not appear to be naturally processed through either processing pathway and were described as 'cryptic'. Other epitopes of this type have been described (39); their function, if any, has not been explained yet.

1.4.1.3. Natural and vaccine induced response to MAGE-3. Spontaneous response to melanomas may occur, although not very frequently (220). To determine the impact of antitumor immunity in spontaneous or vaccine induced tumor regression T cell precursor frequencies is evaluated. Few studies have addressed the spontaneous anti-MAGE-3 T cells response in healthy donors and patients. The number of $CD8^+$ T cell precursors in healthy individuals was around $4x10^{-7}$ (221) and $CD4^+$ T cell frequency was around 10^{-6} or lower (222), lower than the frequency for some other antigens such as NY-ESO-1 (223).

Different types of MAGE-3 vaccines have been used. In a peptide-based vaccine without adjuvants a CTL response was seen in only two of the seven responding patients, with CTL frequency reaching $5x10^{-6}$ and $4x10^{-5}$ (224, 225).

Another vaccination strategy used a recombinant canarypox virus, ALVAC, containing the sequence for the HLA-A1 restricted MAGE-3 epitope. Among the four patients with regression, CTL responses were seen in three (226, 227).

Both types of vaccination produced monoclonal responses, as seen by TCR Vβ usage; all responses were stable, in some cases for months.

Another strategy was to use monocyte-derived peptide-pulsed DCs (228); in this case a polyclonal response was seen in three patients showing regression, with frequencies ranging from $3x10^{-6}$ to 10^{-3}. No response was seen in patients without regression (229). A CTL response to MAGE-$3_{271-279}$ was induced also when peptide-pulsed $CD34^+$ DCs were used (230). MHC-II peptides have been included in DC-peptide vaccines and have proven able to induce $CD4^+$ T cell responses: MAGE-$3_{243-258}$ specific cells reached a frequency of $7x10^{-4}$ after vaccination (222, 231).

Adjuvants can improve the response to, as seen after vaccination with IL-12 for the response to MAGE-$3_{271-279}$ (232). Vaccination using incomplete Freund's adjuvant and GM-CSF gave a good response towards MAGE-$1_{243-258}$ and MAGE-$10_{254-262}$ (233).

Use of adjuvants seems particularly important when trying to elicit a $CD4^+$ T cell response. Using the adjuvant SBAS-2 with the whole recombinant protein in twenty-four patients all produced antibodies, whereas in the control group without adjuvant production was seen only in one individual (234). A $CD4^+$ T cell response was observed after administration of whole recombinant protein alone and frequencies of specific cells in the blood reached $1,5x10^{-5}$ (235).

Summing all the trials described above the overall clinical response was low, between 5 and 10%, even taking into account the low number of patients involved. However, considering the number of patients with regression and those with T cell responses there appears a clear correlation, showing that the vaccines are able in a restricted number of patients to induce or increase the number of TAA specific T cells. Reasons responsible for the limited clinical success (i.e. immunsuppressive factors) are now being studied.

2. AIMS OF THE RESEARCH PROJECT

$CD4^+$ T cells are critical elements for priming $CD8^+$ T cells, for immunity during chronic infection, for generation of memory T cells and for antibody production. Animal models have shown $CD4^+$ T cells to play both a direct and an indirect role in antitumor immunity. Presentation of tumor antigens to $CD4^+$ T cells *in vivo* occurs through cross-presentation on MHC-II molcules by DCs that have endocytosed necrotic or apoptotic tumoral cells. Some tumors express MHC-II molecules and are also able to present peptides from tumor antigens directly to $CD4^+$ T cells.

The goal of my project was to study processing of tumor antigens within professional and non professional APCs and to evaluate how processing impact on the repertoire of epitope formed *in vivo* for presentation to $CD4^+$ T lymphocytes in cancer patients.

The first of the project's specific aims was to investigate the mechanisms leading to production of CD4 epitopes from MAGE-3, to study the factors that, from a whole intact protein, will allow the formation of stable MHC-II-peptide complexes carrying MAGE-3 peptides for presentation to $CD4^+$ T cells. MAGE-3 has been chosen for its expression pattern and because has been already used for immunotherapeutic purposes. Presentation on professional (such as DCs and macrophages) and non professional (tumoral cells) APCs with different content of proteolytic enzymes has been evaluated.

The second of the project's specific aims was to characterise the qualitative and quantitative response in the natural anti-MAGE-3 response of melanoma patients. Quantitative response analysis reveals how good priming for a given epitope is *in vivo*. Qualitative response analysis yields information on the effectors' function, whether it is skewed towards productive or non productive antitumor immunity. Th1, Th2, Th3/Tr or nonpolarised responses have been associated with a different outcome of the disease as the cytokines released promote or inhibit tumoricidal activity.

The whole project aimed at gathering as much information as possible on how epitopes from MAGE-3 are produced and their role in the anti-tumor response in patients. This information will possibly allow medical doctors to manipulate the immune response and shift the balance to a more favourable outcome of the disease.

3. MATERIALS AND METHODS

3.1. Subjects and cells. HLA-DR types of LCLs, cancer cell lines, donors and patients were identified by molecular or serologic typing. The LCLs used were: BM21 (DR*11), HOM (DR*01), KT14 (DR*09), Mundula (DR*13) kindly provided by K. Fleischhauer (Hospital San Raffaele, Milan, Italy); Pitout (DR*07), purchased from the European Collection of Cell Culture (Salisbury, UK); DAS (DR*04), kindly provided by J. Anholts (LUMC, Leiden, Netherlands). Melanoma cell line HT144 (DR*04, *07) was purchased from the ATCC (Rockville, MD, USA) while cell line MD TC (DR*04, *11) was obtained in our laboratory from a melanoma patient. The $CD4^+$ T cell lines were induced from two healthy subjects (donor 1: DR*01, *07, donor 2: DR*11) and from a melanoma patient (011: DR*10, *11). The HLA-DR types of all melanoma patients enrolled in this study were identified and are reported in table 2 (see Results).

The mediums used were IMDM for LCLs and RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA) for melanoma cell line HT144, in both cases supplemented with heat-inactivated serum (10%), L-glutamine (2mM), penicillin (100 U/mL) and streptomycin (50 μg/mL; Biowhittaker, Walkersville, MD). Medium was supplemented with fetal calf serum (FCS) for LCLs and melanoma, with normal human serum (NHS) for $CD4^+$ T cell lines. Both sera were from Biowhittaker. Unless specified, glutamine, penicillin and streptomycin at the above concentrations were used in mediums for all cells.

3.2. *In vitro* propagation of anti-MAGE-3 $CD4^+$ T lines and clones from healthy donors and melanoma patients. $20x10^6$ peripheral blood mononuclear cells (PBMCs) were cultured for 7 days in RPMI 1640, supplemented with heat-inactivated human serum (10%), containing MAGE-3 peptide pools (1μg/mL of each peptide). The reactive blasts were isolated on a Percoll gradient, expanded in IL-2-containing medium

(25 U/mL; Lymphocult, Biotest Diagnostic, Dreieich, Germany), and restimulated at weekly intervals with the same amount of peptides plus irradiated (3000 rad) autologous PBMCs as APCs. The peptides used contained the following sequences of MAGE-3: 111-125, 141-155, 146-160, 156-170, 161-175, 171-185, 191-205, 281-295, 286-300. All these peptides had been used in the previous studies conducted in our laboratory. Peptides 21-35 and 251-265 were not included, as according to the TEPITOPE algorithm (215) they are predicted to have a lower binding affinity and had previously failed to elicit any response.

Once established the cell lines were cloned by limiting dilution: 100 μl/well containing 50,000 irradiated PBMCs from 2 allogenic donors and an average of 5, 1 or 0.5 T cells were plated in 96/well plates in 10% NHS RPMI with 250 U/ml IL-2 and 1 μg/ml phyto-hemoagglutinin A (PHA). Fresh medium containing only IL-2 was added after 1 week. Plates were screened daily and growing clones transferred to new wells. Three weeks from the first stimulation all wells were restimulated with 0.5 μg/ml PHA, PBMCs and IL-2. When resting, T cells were assayed for specificity.

The 12-residue peptides used to determine the minimum recognised sequence in the 156-175 region of MAGE-3 contained the following sequences: 153-164, 154-165, 155-166, 156-167, 157-168, 158-169, 159-170, 160-171, 161-172, 162-173, 163-174, 164-175, 165-176, 166-177, 167-178, 168-179.

The $CD4^+$ T cell line specific for MAGE-$3_{281-300}$ was a gift from Dr Monica Moro (Experimental Unit, Cancer Immunotherapy and Gene Therapy Program, DIBIT directed by Drs. Giulia Casorati and Paolo Dellabona). Between $2x10^5$ and 10^6 cells were restimulated at two-three week intervals in 12-well plates with $3x10^6$ irradiated allogenic PBMCs (a mixture of three donors) and 10^6 irradiated LG2 cells. The cells were grown in the same medium described above supplemented with the anti-CD3 antibody OKT3 (Orthobiotech, Raritan, NJ) (30 ng/ml) and IL-2 (200 U/ml).

3.3. TCR Vβ usage. Cells were coloured with the IoMark beta test (Becton Dickinson, Sunnyvale, CA) solutions. Each solution contains a mixture of three antibodies against three vβs; each antibody is either FITC-conjugated, PE-conjugated or FITC- and PE-conjugated. The eight mixtures recognise most of the vβs; the missing ones were checked via PCR, according to published protocols (236). 100,000 cells were lysed in Trizol (Invitrogen, Carlsbad, CA) to extract the mRNA; cDNA was obtained by reverse transcription using Moloney's murine leukaemia virus retranscriptase (Promega, Madison, WI) by incubating for one hour at 37° C in appropriate buffer followed by inactivation of the enzyme by heating to 65°C for ten minutes. Ten μg/reaction were amplified using forward primers specific for the vβ genes and a Cβ downstream-specific oligonucleotide. Amplification was carried out using TaqGold polymerase (Promega), in the appropriate buffer, in 35 cycles, with the following phases: melting (94°C, 30 minutes), annealing (61°C, 30 minutes) and extension (72°C, 30 minutes). PCR products (10 μl) were visualized on a 1.5% agarose gel.

3.4. Recombinant viruses and infection of LCLs and melanoma cells. The PG13 packaging cells producing retroviral vectors LXΔSN-M3 or LXΔSN-IiM3 were a kind gift from Dr Catia Traversari. The retroviruses contain either the full length MAGE-3 sequence under the control of the 5' LTR promoter or a fusion between the first eighty aminoacids of the human Ii and MAGE-3 followed by the truncated form of the human low-affinity nerve growth factor receptor (ΔNGFr) under the control of the SV40 promoter. For transduction, LCLs were co-cultivated with irradiated packaging cells producing vectors for 72 h in the presence of polybrene (0,8 mg/ml). Melanoma cells were incubated with supernatant from cells producing the Ii construct in the presence of polybrene (0,8 mg/ml). A pure population of transduced cells was obtained by immunoselection. Cells were incubated with supernatant from the anti-ΔLNGFr Mab

20.4 (ATCC) for 20 minutes at 4°C, washed in PBS then incubated with sheep anti-mouse antibody conjugated to magnetic beads (Dynal Biotech, Oslo, Norway). Cell-containing tubes were passed on a magnet dividing cells as bound and unbound. Bound cells were expanded and ΔLNGFr expression checked by FACS.

3.5. Proliferation assays. $CD4^+$ T cells and autologous irradiated PBMCs or HLA-DR-matched homozygous LCLs as APCs were diluted at a 1:10 or 1:5 ratio, respectively. The single peptides were added at a final concentration of 10 μg/ml, the recombinant MAGE-3 protein (kindly provided by Dr Pierre van der Bruggen, Ludwig Institute for Cancer Research, Brussels, Belgium) or the recombinant E6 protein from the human papilloma virus 18 (HPV 18), were added at a final concentration of 20 μg/mL. DCs were obtained from PBMC after monocyte separation via adherence and grown for a week in RPMI supplemented with 1% heat inactivated human serum, IL-4 (500 U/ml) and GM-CSF (800 U/ml). On day six, $2.5x10^4$ cells were fed with $5x10^4$ cells, either LCL or tumors that had been lysed by three cycles of freeze-thawing, and incubated overnight in the same medium with the addition of TNF-α (1 μg/ml). After removing old medium $5x10^3$ lymphocytes were added in RPMI supplemented with 10% heat inactivated human serum. Triplicate wells with $CD4^+$ T cells alone and APCs alone were used as controls. Three wells with $CD4^+$ T cells plus APCs did not receive any stimulus to determine the basal growth rate. After 48 h, the cultures were pulsed for 16 h with [3H]TdR (1 mCi/well, 6.7 Ci/mol; Amersham Corp., Milan, Italy). The cells were collected with a FilterMate Universal Harvester (Packard, USA) in specific plates (Unifilter GF/C; Packard), and the thymidine incorporated was measured in a liquid scintillation counter (TopCount NXT; Packard).

All assays with cells from patients were carried out in X-VIVO 15 medium supplemented with 3% heat-inactivated NHS.

To study the role of lysosomal and cytosolic enzymes in the presentation of MAGE-3 epitopes APCs were treated with specific inhibitors. For tumoral line HT144 (wild type or transduced) 1 confluent well (previously seeded with 100,000 cells) from a 48-well plate was used per condition; in the case of LCLs 500,000 cells were used per condition. The inhibitors used were: CA074, leupeptin, Z-FY(tBu)-DMK (hereon referred to as catL inhIII), pepstatin A, leupeptin, lactacystin, MG132, calpeptin, AAF-chloromethylketone (AA-CFK), wortmannin, 3-methyl-adenine (3MA) and bortezomib. Leupeptin, wortmannin and 3MA were purchased from Sigma (Highland, IL); all the rest were purchased from Calbiochem (Darmstadt, Germany). Inhibitors were resuspended in DMSO or water according to the manufacturer's instructions. Velcade (bortezomib) was a kind gift from Dr Marco Bregni (Hospital San Raffaele, Milan, Italy); it had been resuspended in water.

All cells were incubated at 37°C for 18-20 hours before use in the proliferation assay. Vitality in treated cells was checked by Trypan Blue (Sigma) staining compared to untreated controls and the presence of HLA-DR molecules was tested by FACS analysis.

3.6. ELISA assays. For IFN-γ and GM-CSF detection the Biosource kits were used (CHC1234 and CHC0904 respectively, Biosource Europe, SA, Nivelles, Belgium). ELISA plates (Maxisorp, Nunc, Wiesbaden, Germany) were coated overnight with 100 µl of 4 µg/ml in PBS of anti-IFN-γ and 1 µg/ml for anti-GM-CSF at 4°C; all other steps were carried out at room temperature. Plates were washed three times with washing solution (0.9% NaCl-0,1% Tween) and blocked with PBS/BSA 0,5% solution for 2 hours. Plates were always washed before addition of new reagents. Samples were diluted in PBS/BSA 0.5%/Tween 0.1%; 100 µl/well of sample was added, together with 50 µl/well and 0,4 µg/ml of biotinylated anti-cytokine antibody diluted in the same

buffer, for 2 hours. Plates were incubated with 100 µl/well streptavidin-HRP (horseradish peroxidase), diluted as suggested for 30 minutes before addition of 100 µl/well TMB. Plates were read in a 680 Model reader (Biorad, Hercules, CA) at a wavelength of 622 nm. A standard curve was created by reading the absorbance of known quantities of cytokine (ranging from 2000 pg/ml to 32 pg/ml).

IL-5 was detected using the MabTech (Miltenyi Biotec, USA) kit. ELISA plates were coated with 2 µg/ml anti-IL-5 antibody in 100 µl/well PBS by overnight incubation at 4°C. All successive steps were carried out at room temperature and with 100 µl/well (with the exception of plate blocking, where 200 µl were used). Washes were carried out between every incubation with PBS/Tween 0.05%. Plates were blocked for an hour in PBS/BSA 0.1%; this buffer was also used for all successive dilutions. Samples and standards were added and incubated for 2 hours; standards ranged from 1000 pg/ml to 15 pg/ml. Plates were washed and biotinylated anti-IL-5 antibody at 1 µg/ml added for an hour, followed by more washes and an hour incubation with streptavidin-HRP. TMB was used as substrate and absorbance read at a wavelength of 622 nm in a 680 Model reader (Biorad).

3.7. Western blot. $2x10^5$ cells (either wild type LCLs, transduced LCLs or tumors), were boiled in loading buffer and loaded onto 10% polyacrilamide gel before tranferring to a nitrocellulose membrane. The membrane was blocked by overnight incubation at 4°C in a 5% milk, 0.5% Tween PBS solution. Three 10 minute washes were carried out at room temperature in a PBS/0.1% Tween solution between every incubation. The membrane was stained with the anti-MAGE antibody 57B (kindly provided by Dr G. Spagnoli, Basel, Switzerland and described in (202)) for two hours, followed by one hour's incubation with a goat anti-mouse antibody conjugated to horseradish peroxidase

(Southern Biotechnology Associates, USA) and developed using ECL (Amersham Biosciences, UK).

3.8. Flow cytometry. Cells were stained with specific antibodies and analysed within 12 hours of staining. In the case of intracellular staining, cells were fixed in 4% paraformaldehyde for 20 minutes at 4° C, washed with PBS/FCS 2% and permeabilised for 20 minutes on ice with a PBS/BSA 0.1%/saponin 0.5% solution, washed in the same solution and stained with appropriate antibodies. They were then washed twice with the same solution and twice in PBS-BSA 0.1%. Cytofluorimetric analyses were performed on a FACStarPlus (Becton Dickinson). ΔLNGFr was stained with supernatant from the ATCC Mab 20.4 then with FITC-goat antimouse immunoglobulin (SouthernBiotech, USA). All the following antibodies were purchased from Becton Dickinson: anti-CD4-FITC, anti-CD4-Cy5, anti-CD8-PE, anti-CD45RA-FITC, anti-CD45RO, anti-CD3-FITC, anti-IL5-PE, anti-IFNγ-APC. Anti-CD4-QR was purchased from Sigma.

To separate cells expressing Vβ14 from those expressing Vβ6 the $CD4^+$ T cells were stained for 30' at 4°C with the solution containing the Ab specific for Vβ14. In the case of LCL-IiM3, LCLs were stained with the anti-ΔLNGFr Mab 20.4, followed by the FITC-conjugated anti-mouse Ab. The two fractions (positive and negative or high and low expression) were separated in a FACSVantage SE (Becton Dickinson) using the DIVA software.

3.9. Enzyme activity assays. Inhibitor treated LCLs or HT144 cells were washed once in PBS, pelleted and lysed in Cytobuster (Calbiochem) for one hour on ice. Protein concentration was determined in a standard BCA protein assay (Pierce. Rockford, IL). The activities of cathepsins B, D and L were determined using the Calbiochem assay kits (CBA 001, 002, 023 respectively), according to the manufacturer's instructions. In each test an internally quenched fluorogenic substrate (Z-Phe-Arg-AMC, Z-Arg-Arg-

AMC and Mca-GKPILFFRLI-Lys(DNP)-D-Arg-NH2) was used. Upon cleavage by the enzyme the fluorogenic moiety containing a molecule of 7-amido-4-methyl-coumarin or the is released and its concentration measured as relative fluorescence units (RFU). Using the kit's buffers samples were plated in a 96-well plate and incubated at room temperature for 30 minutes with gentle shaking. The fluorogenic substrate was then added and plates were incubated for an hour at 37°C before reading in a Victor 3 reader (Perkin Elmer, Waltham, MA) at an excitation wavelength of 360-380 nm and emission wavelength of 440-460 nm.

To determine activity of cathepsin B and L samples (diluted in the provided buffers) were plated in a 96-well plate and incubated at room temperature for 30' with gentle shaking. The fluorogenic substrate was then added and plates were incubated for an hour at 37°C before reading.

In the case of cathepsin D fluorimetric plates coated with an anti-cathepsin D antibody were used. These plates were incubated for 30 minutes at room temperature with samples or with known concentration of active cathepsin D as standard. Plates were washed and the fluorogenic substrate added and incubated at 37°C for an hour before reading.

In all cases a calibration curve was generated by measuring the RFU produced by known concentrations of active enzyme. Activity in the samples was expressed as ng/ml active protein per mg/ml total protein. All samples were assayed in duplicate.

3.10. Confocal microscopy. Wild type and transduced HT144 cells were grown on coverslips to 70% confluence then fixed by incubating for 20 minutes in PBS/PFA 4% at 4°C. After washes with PBS they were permeabilised for an hour in a PBS/BSA 1%/saponin 0.2% solution at room temperature (as all successive steps). They were then washed in a PBS/BSA 1%/saponin 0.1% solution; this solution was used for all

successive washes and as diluent for all antibodies. Coverslips were incubated for 2 hours with the following primary antibodies: mouse anti-MAGE-3 B57 antibody; rabbit anti-LAMP1 (abCAM, Cambridge, UK), rabbit anti-calnexin (Sigma) or rabbit anti-calreticulin (Stressgen, Victoria, BC, Canada). After washes the following secondary antibodies were added for 1 h: anti-mouse Alexa 648 (Invitrogen), anti rabbit-FITC (abCAM). After three other washes coverslips were fixed on slides using Mobiol and read in a Leica microscope.

3.11. *In vitro* restimulation assays of $CD4^+$ T cells from melanoma patients and cord bloods. PBMC from cord bloods were isolated from whole blood after centrifugation on a Ficoll gradient (Lympholite, Cedarlane, Hornby, Canada) and tested for CD45RA/RO expression. Samples from melanoma patients were obtained as apheresis. $CD4^+$ T cells were separated from other cells by magnetic separation using Miltenyi reagents. Total PBMC were incubated with magnetically conjugated anti-CD4 antibodies (80 µl of PBS/BSA 0.5%/EDTA 2mM and 20 µl antibody solution per every 10^7 total cells) for 20 minutes at 4°C, washed with the PBS/BSA 0.5%/EDTA 2mM solution and resuspended in 0.5 ml of the same, before loading onto a previously wet MiniMACS column set in the magnet to elute $CD4^-$ cells. $CD4^+$ T were eluted after magnet removal. Both fractions were washed and the $CD4^-$ fraction irradiated. Cells were resuspended in X-VIVO 15 medium supplemented with 3% inactivated NHS then plated in 96-well plates at a ratio of 1:3 $CD4^+/CD4^-$, with $5x10^4$ $CD4^+$ T cells/well. MAGE-3 peptides 111-125, 146-160, 161-175, 171-185, 191-205, 243-258, 281-300 were added at a final concentration of 10 µg/ml. Controls contained only medium or PHA 10 µg/ml. Six wells were plated per condition. Half the medium was replaced after seven days with new medium containing IL-2 (final concentration of 25 U/ml). After other seven days medium was removed and tested for cytokine production. If positive,

new medium was added and cells grown for another week, then tested for specificity in a standard cytokine release/proliferation assay.

4. RESULTS

4.1. ANTIGEN PROCESSING: TOOLS FOR THE STUDY

To study the events leading to the formation of MAGE-3 CD4 epitopes I first produced the necessary tools (*i.e.* MAGE-3 expressing APCs and MAGE-3 specific $CD4^+$ T cells). Previous studies (19, 193, 194, 213, 217) have shown that MAGE-3 specific $CD4^+$ T cells recognise MAGE-3 epitopes produced through both the endogenous and/or the exogenous pathways, depending on the epitope. Indeed, $CD4^+$ T cells may be activated through MAGE-3 cross-presentation by DCs (*i.e.* professional APC) or directly by MAGE-3 and MHC-II expressing tumor cells (*i.e.* non-professional APC). As I was interested in studying production of MAGE-3 CD4 epitopes through both pathways for my studies I used APCs expressing MAGE-3 either in the cytoplasm or in the endosomal/lysosomal compartment and $CD4^+$ T cells specific for MAGE-3 epitopes produced through either one or both pathways.

4.1.1. Production and characterization of the MAGE-3 expressing cells.

To study processing of MAGE-3 as an endogenous protein cells expressing MAGE-3 in the cytoplasm were used. I used either melanoma cells constitutively expressing MAGE-3 (HT144 and MD TC) or homozygous LCLs engineered to express MAGE-3 in the cytoplasm. Homozygous LCLs allow the study of antigen processing in cells mainly expressing the proper MHC-II restricting allele, therefore reducing the interference of multiple alleles competing for binding of MAGE-3 peptides within the APC.

I engineered LCLs to express MAGE-3 by transduction with the retroviral vector containing the MAGE-3 gene. The retroviral vector, as explained in chapter 3, contains

the gene for MAGE-3 and for a truncated form of the nerve growth factor receptor (ΔNGFr). The sequence of the vector is shown in Figure 6A. After infection transduced cells express both foreign proteins. The ΔNGFr is a surface molecule and its expression can be monitored through FACS analysis. Selection of ΔNGFr expressing cells (through magnetic sorting via mouse anti-ΔNGFr antibody and magnetically labelled anti-mouse beads) allowed me to obtain a pure line of transduced cells starting from a mixed population (Fig. 6 C-D).

LCLs line DAS (DRβ1*04, DRβ4*01) and BM21 (DRβ1*11, DRβ3*02) were engineered. MAGE-3 protein expression in the engineered LCLs was verified by Western blot analysis. Figure 7 shows that transduction of MAGE-3 negative wild type LCLs was successful, as a band of the appropriate length was present (Fig. 7A-B, middle lanes). Cells transduced with this construct will be referred to as LCL-M3 hereon (DAS-M3 and BM21-M3). Two types of APCs (LCLs and melanoma cells) were therefore available to study processing through the endogenous pathway.

To study processing of MAGE-3 as an exogenous protein the best cells are DCs after processing and presentation of soluble MAGE-3 protein or necrotic or apoptotic MAGE-3 expressing cells. Indeed, I performed some experiments with these cells (see 4.1.2.1.3, 4.1.2.2, 4.2.1.4 and 4.2.2). In order to obtain unlimited number of cells needed for extensive testing I decided to mimic the physiological exogenous pathway by expressing MAGE-3 in the endosomal/lysosomal compartment of suitable cells.

To this aim I engineered the wild type LCLs and melanoma cells described above by using the retroviral vector containing the Ii construct (the structure of the construct is shown in Figure 6B). As described for LCL-M3, LCLs and melanoma cells expressing the IiM3 protein and melanoma were obtained by sorting ΔNGFr expressing cells. The presence of a band corresponding to the expected molecular weight for the MAGE-3 invariant chain protein was verified by Western blot analysis (Fig. 7A-B-C, right lanes).

These transduced cells will hereon be referred to as LCL-IiM3 (DAS-IiM3, BM21-IiM3 and HT144-IiM3). Western blot analysis also showed the amount of protein produced by the two constructs (M3 and IiM3) to be comparable.

To verify that MAGE-3 was correctly transported to the endosomal/lysosomal compartment I performed confocal microscopy analysis on transduced HT144 cells. Figure 8 shows a diffuse cytoplasmic distribution of MAGE-3, as expected for the constitutive expression, and in the lysosomal compartment, as shown by co-localisation with LAMP and as expected from the construct.

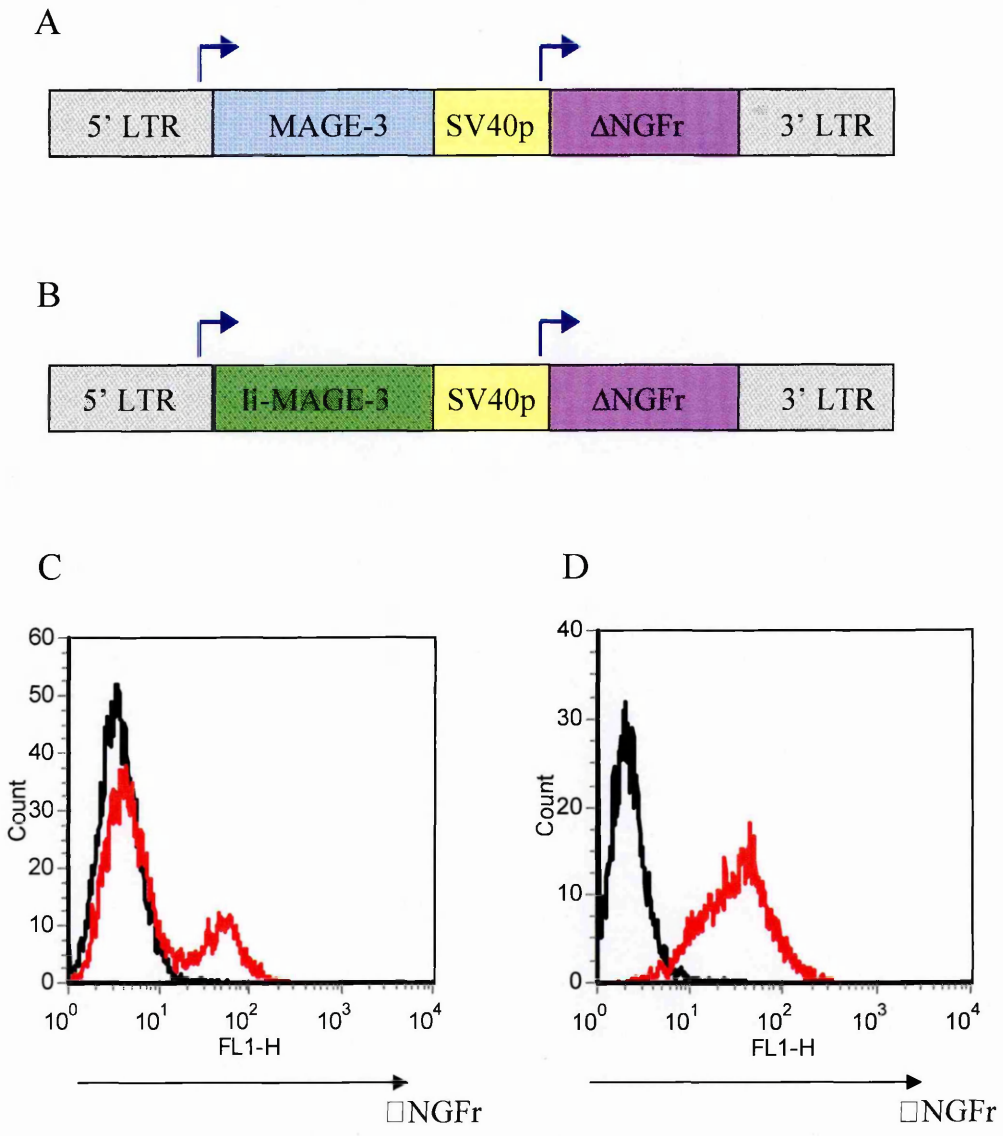


Figure 6. Generation of MAGE-3 and Ii-MAGE-3 expressing cells. LCLs BM21 and DAS and melanoma HT144 were transduced with retroviral constructs containing wild type MAGE-3 or the fusion protein Ii-MAGE-3. Transduced cells were separated on the basis of ΔNGFr expression through magnetic bead separation. A: Vector containing full length MAGE-3. B: Vector containing fusion protein Ii-MAGE-3. C and D: ΔNGFr expression in cells before separation (C) and after (D). FACS curves are representative of all transduced cells. LTR: long terminal repeat; SV40p: simian virus 40 promoter; ΔNGFr, truncated nerve growth factor receptor.

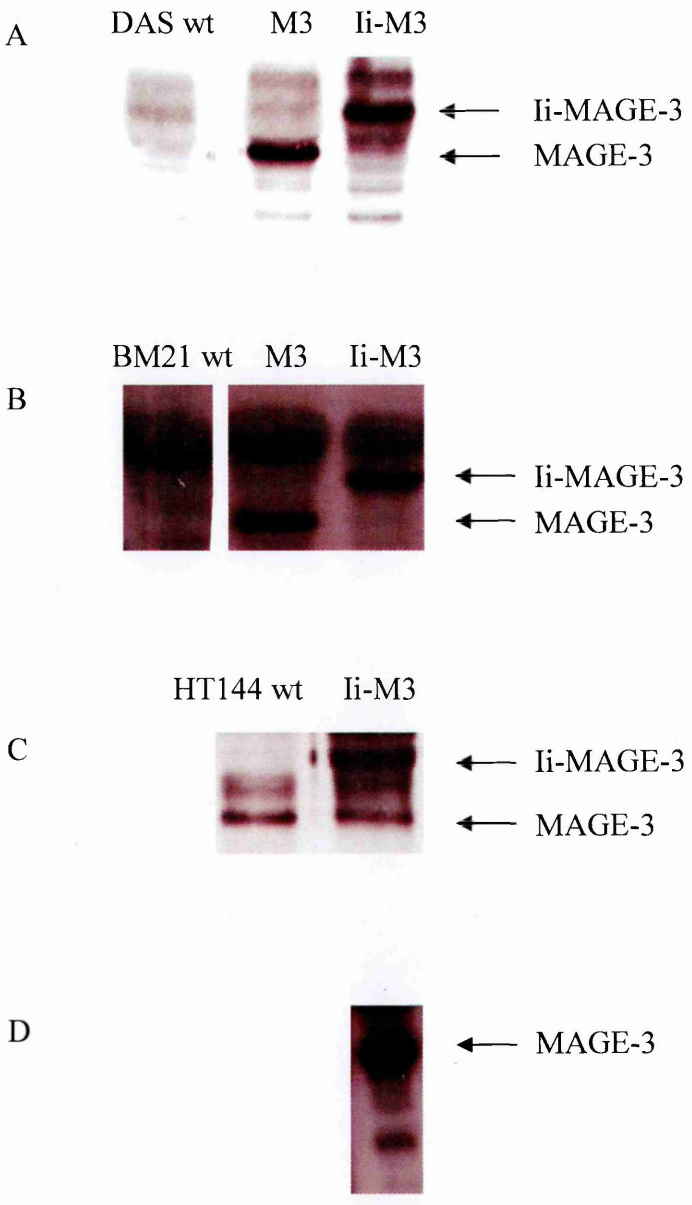


Figure 7. Western blots of transduced cells. Lysates from LCLs and melanoma cells (wild type and transduced) were run on a standard acrylamide gel then transferred to a nitrocellulose membrane for staining with the MAGE specific antibody B57. A: wild type and engineered DAS LCLs (left lane, wt; middle lane, DAS-M3; right lane, DAS-IiM3). B: wild type and engineered BM21 LCLs (left lane, wt; middle lane, BM21-M3; right lane, BM21-IiM3). C: wild type and engineered HT144 melanoma cells (left lane, wt; right lane HT144-IiM3). The two proteins of the corresponding molecular weight for M3 and IiM3 are indicated. D: MAGE-3 expression in MD TC melanoma cells.

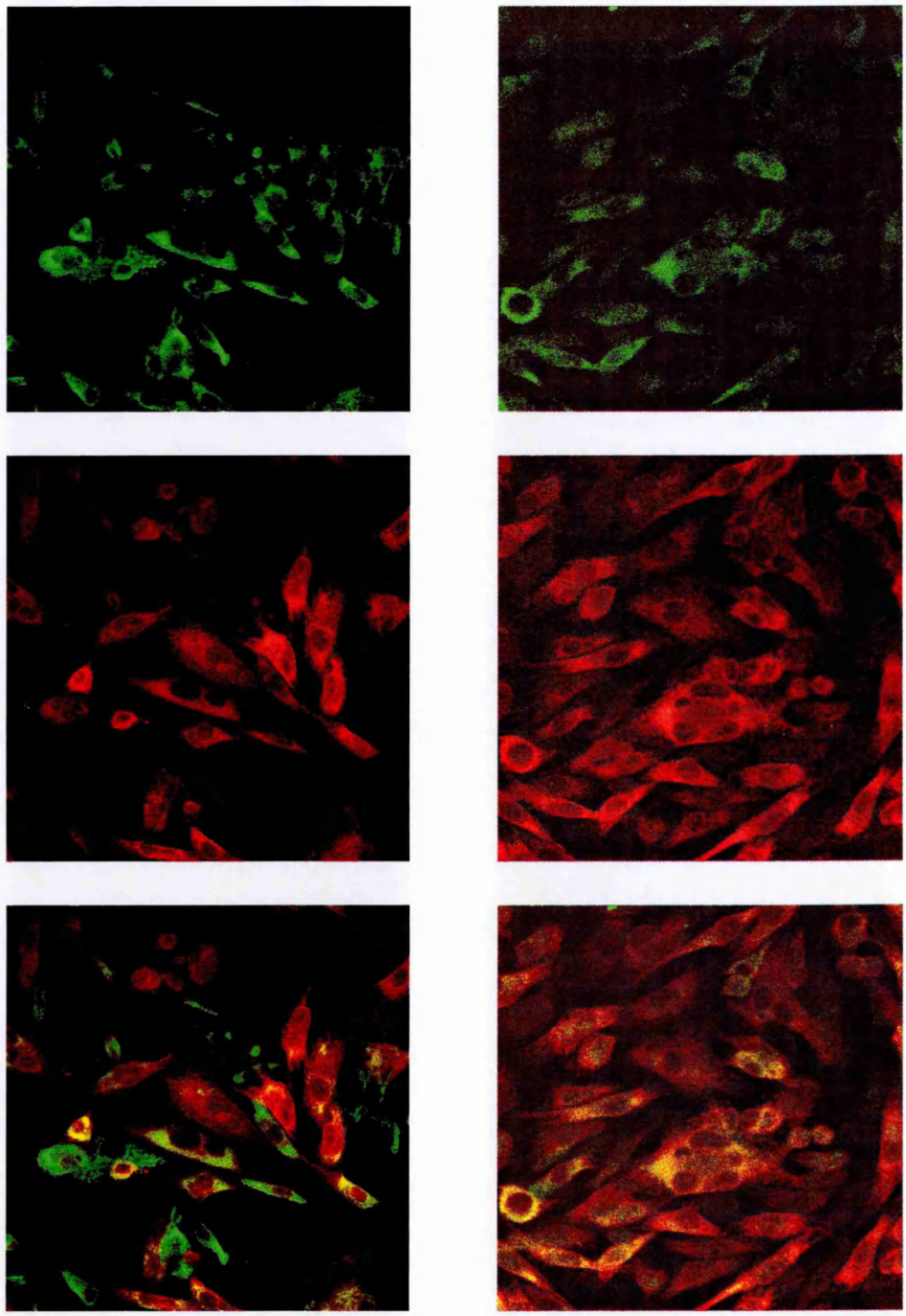


Figure 8. Staining of HT144-IiM3. Semi-confluent HT144-IiM3 slides were stained with mouse antibody 57B and rabbit anti-calreticulin or rabbit anti-LAMP followed by staining with PE-conjugated anti-mouse and FITC-conjugated anti-rabbit antibody. Co-localising antibodies appear yellow. Left column panels: MAGE-3 (red) and calreticulin (green). Right column panels: MAGE-3 (red) and LAMP (green).

4.1.2. Establishment and characterization of $CD4^+$ T cell lines and clones specific for MAGE-3 epitopes.

$CD4^+$ T cells specific for MAGE-$3_{161-175}$ and MAGE-$3_{289-300}$ were obtained from two healthy donors (Donor 1 and Donor 2); $CD4^+$ T cells specific for MAGE-$3_{111-125}$ were obtained from a metastatic melanoma patient (011, Table 2).

4.1.2.1. MAGE-$3_{156-175}$ specific $CD4^+$ T cells. PBMCs from Donor 1 (DRβ1*01, *07, DRβ4*01) were stimulated *in vitro* with a pool of nine MAGE-3 peptides (MAGE-3 pool) corresponding to sequences 111-125, 141-155, 146-160, 156-170, 161-175, 171-185, 191-205, 281-295 and 286-300, which were previously shown to be immunogenic (19, 193). PBMC were cultured for 7 days, activated cells were then enriched by a density gradient, expanded in the presence of IL-2 and weekly re-stimulated with irradiated MAGE-3 pool-pulsed autologous PBMCs. After 2 cycles of stimulation, I obtained a polyclonal line that comprised only $CD4^+$ T cells (Fig. 9A).

The repertoire of epitopes recognised by polyclonal $CD4^+$ T cells was determined by testing their reactivity to each peptide forming the pool in the presence of autologous LCLs as APCs. MAGE-$3_{141-155}$ and MAGE-$3_{191-205}$ were weakly (although significantly) recognised; a strong, specific response was found to overlapping sequences MAGE-$3_{156-170}$ and MAGE-$3_{161-175}$ (Fig. 9B).

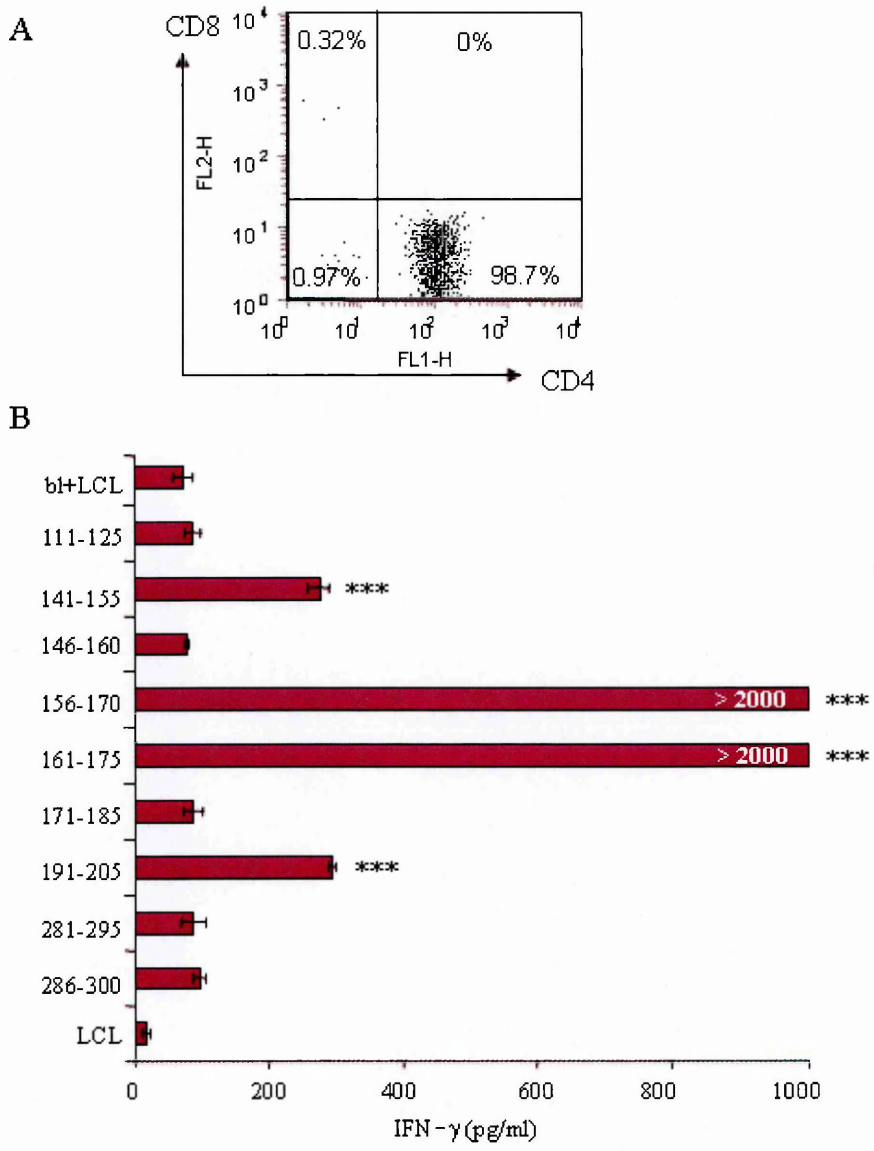


Figure 9. Characterisation of the MAGE-3 peptide pool primed polyclonal CD4$^+$ T cell line. A: FACS analysis of CD4$^+$ T cells after two weeks of stimulation *in vitro* with the MAGE-3 peptides. B: epitope repertoire. CD4$^+$ T cells were challenged with LCLs pulsed singularly with each peptide forming the pool and tested for IFN-γ release. The blanks (*i.e.* the level of IFN-γ release of CD4$^+$ T cells in the presence of unpulsed LCLs) are expressed as bl+LCL. The data are means of triplicate determinations ± SD and are representative of several experiments. Responses significantly higher than the blanks are indicated as: ***p>0,001 (determined by unpaired, one-tailed Student's *t* test).

4.1.2.1.1. Selection of $CD4^+$ T cell clones specific MAGE-$3_{156-175}$. To obtain $CD4^+$ T cells with single specificity the polyclonal cell line was cloned by limiting dilution. I obtained seventeen oligoclonal cell lines; all recognized only the two overlapping MAGE-$3_{156-170}$ and MAGE-$3_{161-175}$ sequences (Fig. 10). No line recognised only one of the two peptides, suggesting that the epitope recognised lies within the overlapping region.

To determine the degree of oligoclonality of the cells, the lines were tested for the Vβ expressed in their TCR by FACS and PCR analysis (Fig. 11 and Fig. 12A). All cell lines expressed predominantly the Vβ6 chain; among others the most represented was the Vβ14 with percentages ranging from 0.81% to 17.54% in six. Vβ6 positive $CD4^+$ T cells isolated via cell sorting exhibited the same recognition behaviour (Fig. 12B), while Vβ14 positive cells were unspecific.

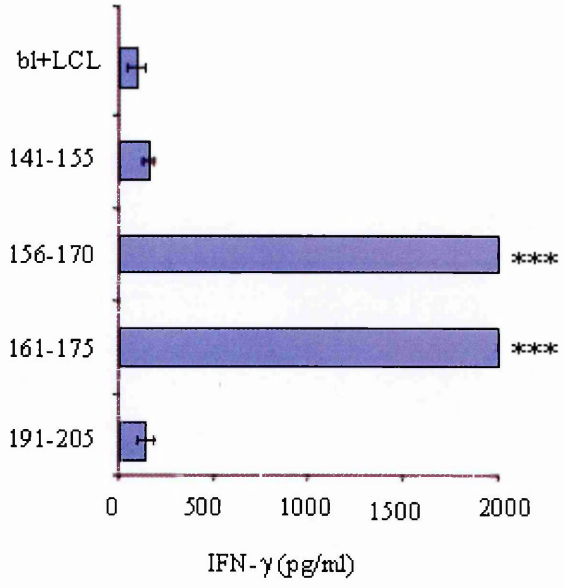


Figure 10. Screening of oligoclonal cell lines obtained from cloning of the polyclonal CD4$^+$ T cell line. CD4$^+$ T cells were challenged with LCLs pulsed singularly with each peptide recognised by the polyclonal cell line and tested for IFN-γ release. The blanks (*i.e.* the level of IFN-γ release of CD4$^+$ T cells in the presence of unpulsed LCLs) are expressed as bl+LCL. Responses significantly higher than the blanks are indicated as: ***p>0,001 (determined by unpaired, one-tailed Student's *t* test). The reactivity shown for oligoclonal cell line 5 is representative of all lines tested.

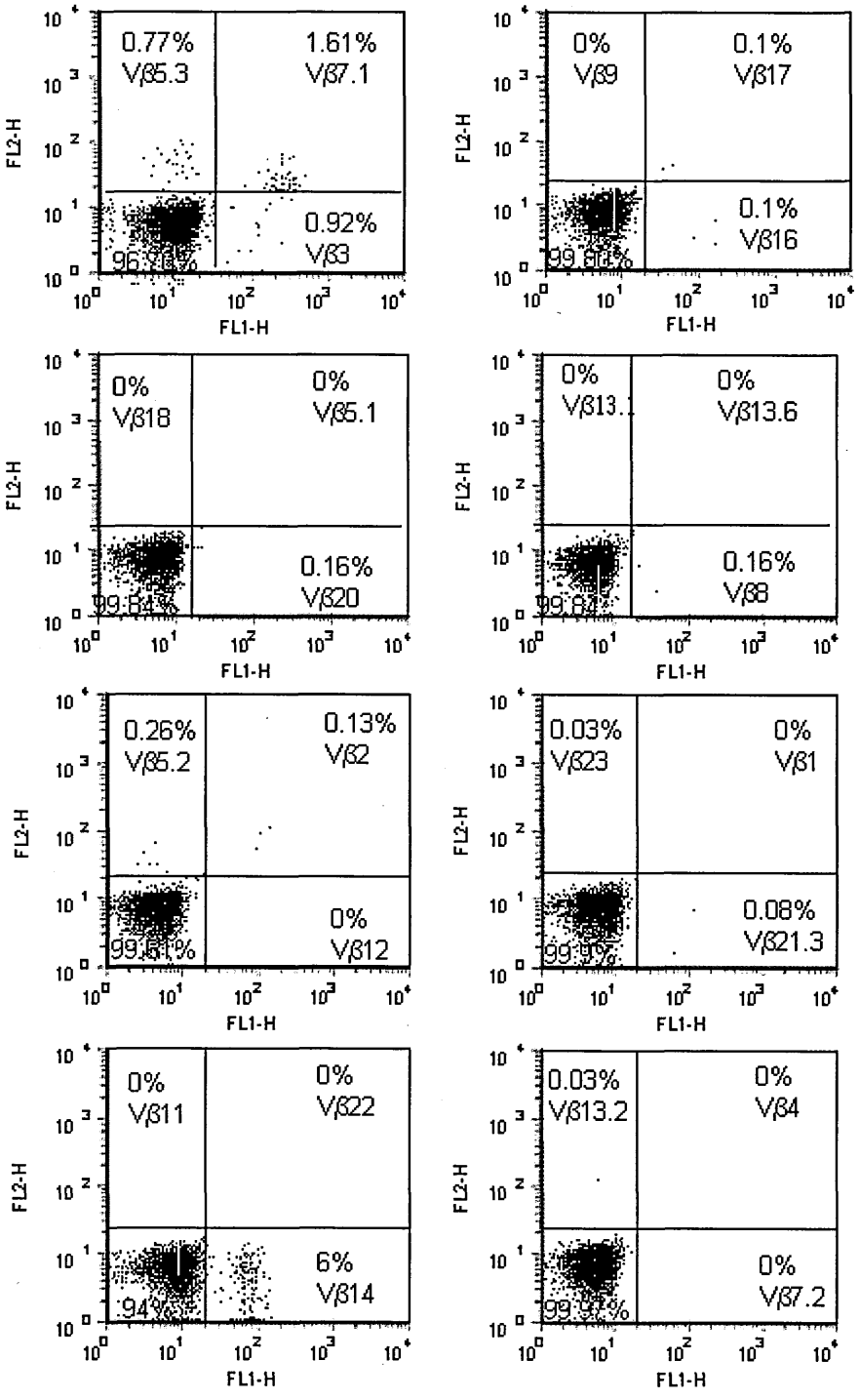


Figure 11. Vβ expression in oligoclonal cell lines. Vβ usage was determined by flow cytometric analysis using the Iotest Beta Mark kit, following manufacturer's instructions. Representative results of oligoclonal cell 5 are shown.

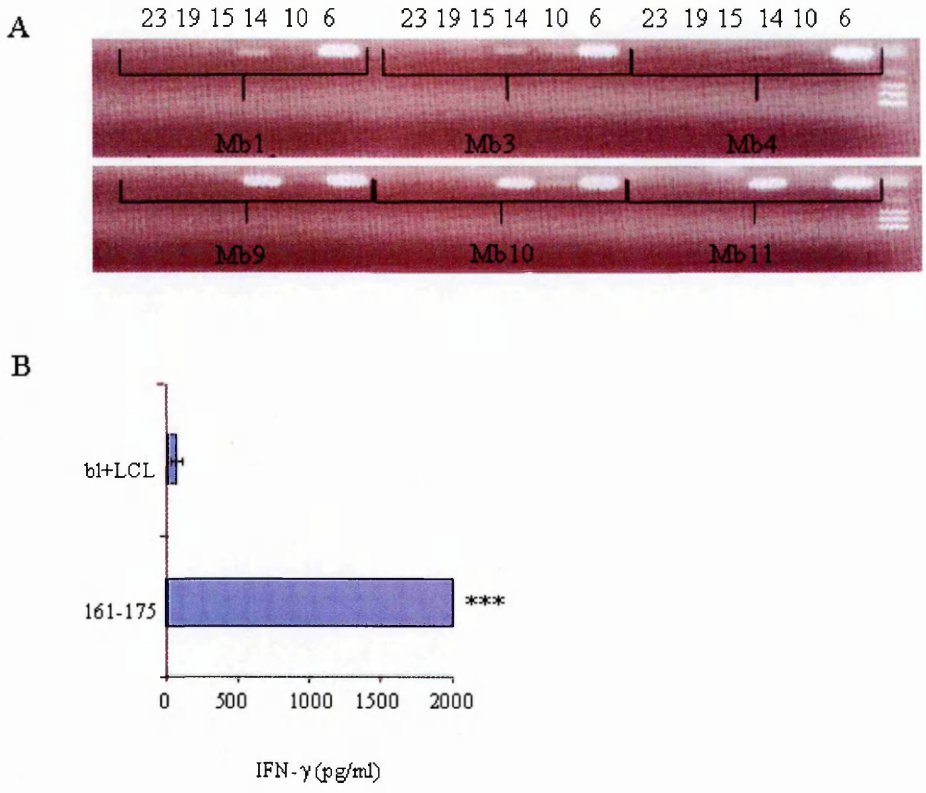


Figure 12. MAGE-$3_{161-175}$ specific $CD4^+$ T cells are Vβ6 positive. A: PCR analysis for Vβ usage using primers for specific the Vβ 6, 10, 14, 15, 19 and 23 chains. B: Vβ6 expressing T cells were separated from Vβ14 expressing T cells via cell sorting and challenged with unpulsed LCLs or LCLs pulsed with MAGE-$3_{161-175}$. Reactivity was measured as IFN-γ release. Responses significantly higher than the blanks are indicated as: ***p>0,001 (determined by unpaired, one-tailed Student's *t* test).

4.1.2.1.2. Characterisation of oligoclonal MAGE-$3_{161-175}$ specific $CD4^+$ T cells. To identify the HLA-DR restricting allele for overlapping sequences MAGE-$3_{156-170}$ and MAGE-$3_{161-175}$, $CD4^+$ T cells were challenged in proliferation and IFN-γ release assays with LCLs, homozygous for each of the two HLA-DRβ1 (DR*01, DR*07) alleles expressed by the donor, pulsed with individual peptides. Presentation of the two sequences occurred in association with HLA-DR*07 (Fig. 13A). Since HLA-DR*07 is associated with HLA-DRβ4, to discriminate between the two presenting molecules, I also tested reactivity of $CD4^+$ T cells in the presence of LCLs HLA-DRβ4 positive but bearing different HLA-DRβ1 molecules (Fig. 13B). $CD4^+$ T cells recognised all peptide-pulsed HLA-DRβ4 expressing cells but not HLA-DRβ4 negative cells, thus demonstrating that HLA-DRβ4 is the restricting allele.

To identify the epitope shared between MAGE-$3_{156-170}$ and MAGE-$3_{161-175}$, I first tested the functional avidity of $CD4^+$ T cells for the two peptides by challenging the oligoclonal cell lines in the presence of LCLs with increasing concentrations of peptides. The concentration of peptide requested to reach the half maximal stimulation (EC_{50}) was as follows: MAGE-$3_{156-170}$ (0,55 μg/ml), $MAGE_{161-175}$ (0,06 μg/ml) (Fig. 14). This result strongly suggests that the antigenic epitope is better comprised within sequence MAGE-$3_{161-175}$. Sixteen peptides were then synthesised, each 12-mer long overlapping of 11 residues and spanning region MAGE-$3_{153-179}$ (Fig. 15), and recognition of them was tested as previously described. MAGE-3 peptides 161-172, 162-173 and 164-175 were strongly recognized; MAGE-3 peptides 158-169, 159-170, 160-171 and 163-174 were reproducibly and significantly recognized, although to a much lower extent (Fig. 16). Dose-response curves for truncated peptides confirmed MAGE-3 peptides 161-172, 162-173 and 164-175 as the best binders with EC_{50} ranging from 0,38 to 0,52 μg/ml; while $CD4^+$ T cells showed very low functional avidity for the other recognized MAGE-3 peptides (EC_{50} ranging from 4 to 6 μg/ml)(Fig. 17). Several

epitope frames within MAGE-$3_{156-170}$ and MAGE-$3_{165-176}$ were predicted by TEPITOPE (215) as promiscuous MHC class II binders. Predicted P1 anchors for MAGE-$3_{156-170}$ were L_{158}, L_{160}, V_{161} and F_{162}; predicted P1 anchors for MAGE-$3_{161-175}$ were V_{161}, F_{162}, I_{164}, L_{166} and M_{167}. Truncated peptide recognition experiments showed loss of recognition at peptide MAGE-$3_{165-176}$, which does not contain I_{164}. This result, along with the curve-response experiments, strongly support the possibility that I_{164} is the P1 anchor for the epitope recognized. Indeed, $CD4^+$ T cells showed the highest functional avidity for the original peptide MAGE-$3_{161-175}$, followed by truncated peptides 161-172, 162-173 and 164-175; in all of these the epitope frame starting with P1 anchor I_{164} is better accommodated in the HLA-DR groove with extended C- or both C- and N-termini sequences. The only exception was recognition of peptide 163-174, which consistently induced a lower level of IFN-γ production by $CD4^+$ T cells; data confirmed by the very low functional avidity of the cells for this peptide.

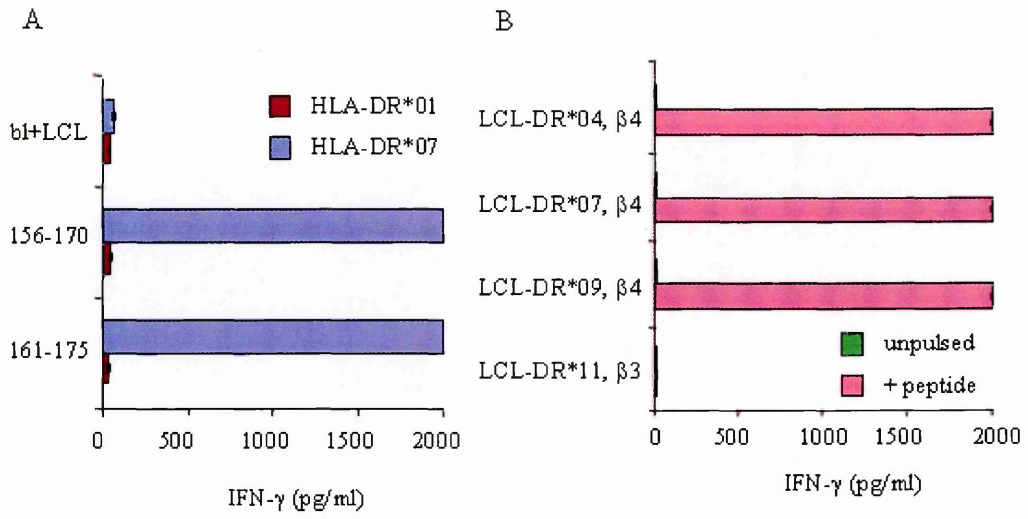


Figure 13. HLA-DR restriction of MAGE-3$_{156\text{-}170/161\text{-}175}$ specific oligoclonal CD4$^+$ T cells. A: CD4$^+$ T cells were challenged with peptides in the presence of LCLs homozygous for each of the HLA-DRβ1 alleles expressed by the donor. B: CD4$^+$ T cells were challenged with the reactive peptide in the presence of HLA-DRβ4*01 positive or negative LCLs. The data are means of duplicate determinations ± SD and are representative of three experiments.

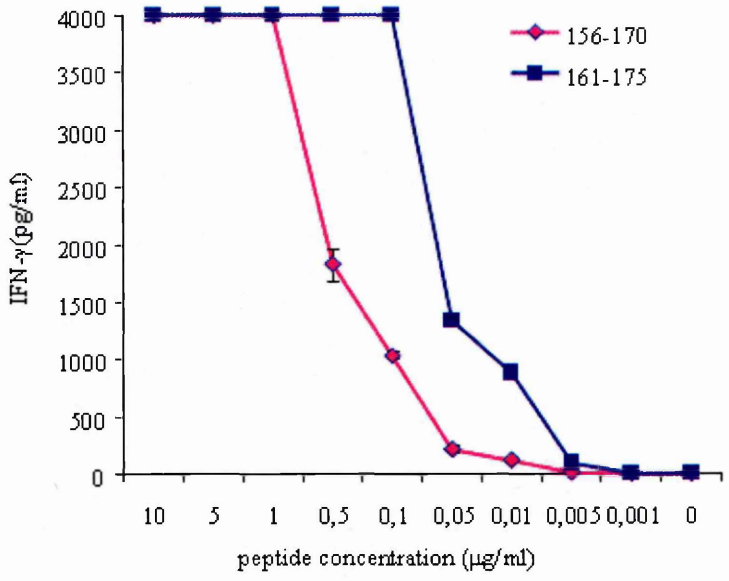


Figure 14. Peptide titration curve. $CD4^+$ T cells were challenged with titrated doses of the indicated peptides and IFN-γ release measured. The blank (the basal level of IFN-γ release of $CD4^+$ T cells in the presence of the unpulsed LCLs) is expressed as '0'. The data are means of duplicate determination ± SD.

```
156-170     SSLQLVFGIELMEVD
161-175             VFGIELMEVDPIGHL

153-164     KASSSLQLVFGI
154-165      ASSSLQLVFGIE
155-166       SSSLQLVFGIEL
156-167        SSLQLVFGIELM
157-168         SLQLVFGIELME
158-169          LQLVFGIELMEV
159-170           QLVFGIELMEVD
160-171            LVFGIELMEVDP
161-172             VFGIELMEVDPI
162-173              FGIELMEVDPIG
163-174               GIELMEVDPIGH
164-175                IELMEVDPIGHL
165-176                 ELMEVDPIGHLY
166-177                  LMEVDPIGHLYI
167-178                   MEVDPIGHLYIF
168-179                    EVDPIGHLYIFA
```

Figure 15. Sequences of truncated MAGE-3 peptides. Original overlapping MAGE-$3_{156\text{-}170}$ and MAGE-$3_{161\text{-}175}$ and truncated sequences spanning region 153-179 are reported. The aminoacids predicted by TEPITOPE as P1 anchor residues are highlighted in red.

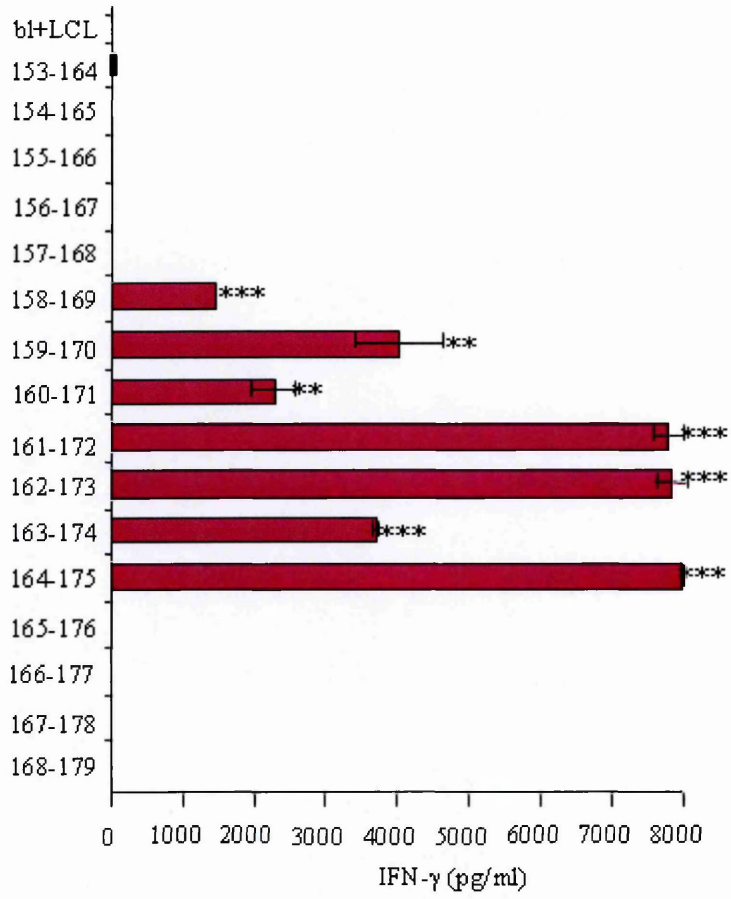


Figure 16. Evaluation of the epitope frame recognized by $CD4^+$ T cells specific for overlapping peptides MAGE-$3_{156-170}$ and MAGE-$3_{161-175}$. $CD4^+$ T cells were cultured in the presence of HLA-DRβ4*01 positive LCLs and each single MAGE-3 peptide shown in Fig. 15 and tested for IFN-γ release. The blanks (*i.e.* the basal level of IFN-γ release of $CD4^+$ T cells in the presence of unpulsed LCLs) are expressed as bl+LCL. The data are means of triplicate determinations and are representative of three experiments. Responses significantly higher than the blanks are indicated as: **0,001<p<0,05, ***p<0,001 (determined by unpaired, one-tailed Student's *t* test).

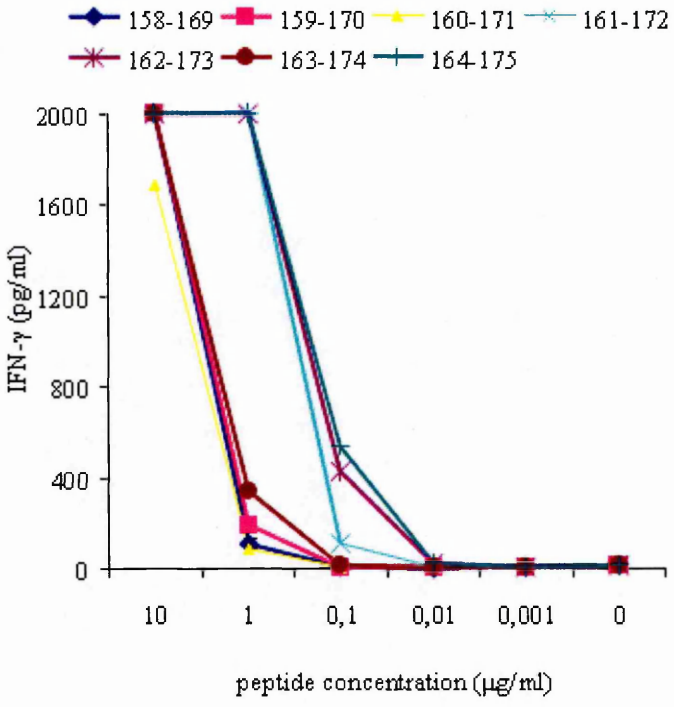


Figure 17. Peptide titration curves for truncated peptides recognized by CD4$^+$ T cells specific for overlapping MAGE-3$_{156-170}$ and MAGE-3$_{161-175}$ sequences. CD4$^+$ T cells were challenged with titrated doses of the indicated peptides and IFN-γ release measured. The basal level of IFN-γ release of CD4$^+$ T cells in the presence of the unpulsed LCLs) is expressed as '0'. The data are means of duplicate determination ± SD and are representative of three experiments.

4.1.2.1.3. MAGE-$3_{161-175}$ contains a naturally processed epitope. To determine if overlapping sequences MAGE-$3_{156-170}$ and MAGE-$3_{161-175}$ contain a naturally processed epitope(s) I tested the reactivity, measured as IFN-γ release or ^{3}H-thymidine incorporation, of $CD4^{+}$ T cells to different MAGE-3 expressing cells (*i.e.*; DRβ4 positive LCL-M3 or LCL-IiM3; wild type HT144 melanoma cells or HT144-IiM3); PBMC pulsed with the whole recombinant protein and autologous DCs loaded with lysates from cells expressing MAGE-3 (LCL-M3) (Fig. 18).

$CD4^{+}$ T cells showed a strong recognition of LCL-IiM3 and HT144-IiM3 whereas they failed to release IFN-γ in the presence of LCL-M3 or wild type HT144 cells (Fig. 18A-B). $CD4^{+}$ T cells also specifically proliferated in the presence of autologous PBMCs pulsed with the whole recombinant MAGE-3 protein (Fig. 18C) and, although to a much lower extent, responded with IFN-γ production to DCs pulsed with lysates from MAGE-3 expressing cells but not from wild type LCLs (Fig. 18D).

Collectively these experiments demonstrate that MAGE-$3_{161-175}$ contains a naturally processed epitope formed through the exogenous pathway. Lack of recognition of LCL-M3 or wild type HT144 was not due to lack of antigen expression; indeed, western blot analysis showed that the amount of protein expressed in LCLs expressing MAGE-3 in the cytoplasm or in the endosomal/lysosomal compartment is comparable (Fig. 7A). HT144 melanoma cells appear to express a lower quantity of antigen but the $CD4^{+}$ T cells failed to recognise any HLA-DRβ4, MAGE-3 expressing tumor (data not shown), including MD TC, where there is strong expression of antigen (Fig. 7D). HT144 melanoma cells constitutively express MHC class II molecules, as verified by FACS analysis (data not shown).

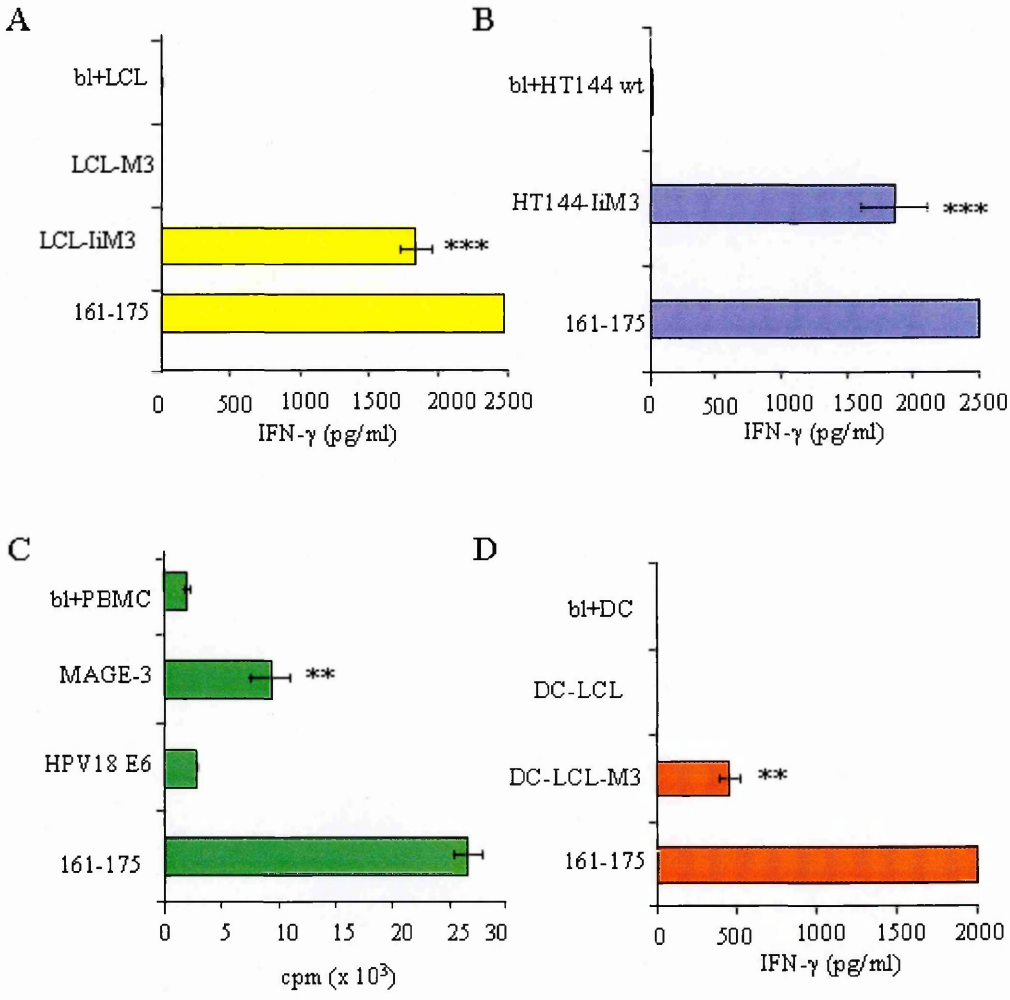


Figure 18. Overlapping sequences MAGE-$3_{156-170}$ and MAGE-$3_{161-175}$ contain a naturally processed epitope. $CD4^+$ T cells were challenged with cells expressing MAGE-3 either in the cytoplasm or in the endosomal-lysosomal compartment and tested for IFN-γ release or radioactive thymidine incorporation. The data are means of triplicate determinations ± SD and are representative of three experiments. Responses significantly higher than the blanks are indicated as: **0,001<p<0,05, ***p<0,001 (determined by unpaired, one-tailed Student's *t* test). A: Response to LCL-M3 and LCL-IiM3. Wild type LCLs were used as negative control. B: Response to wild type (HT144) and engineered HT144 melanoma cells (HT144-IiM3). Peptide-pulsed HT144 cells were used as positive control for presentation capability of HT144 cells. C: Response to PBMCs pulsed with recombinant protein MAGE-3. PBMC pulsed with HPV18 E6 or the peptide were used as negative and positive controls. Unpulsed PBMC as blanks. D: Response to DCs loaded with lysates from MAGE-3 expressing cells. DCs loaded with lysate from wild type LCLs or the MAGE-$3_{161-175}$ peptide were added as negative and positive controls, respectively.

4.1.2.1.4. Correlation between expression of MAGE-3 by APCs and intensity of recognition by MAGE-$3_{161-175}$ specific $CD4^+$ T cells. A possible explanation for the different intensity of recognition of autologous DCs loaded with lysate from MAGE-3 expressing cells compared to cells engineered to express the antigen in the endosomal-lysosomal compartment is that a different amount of protein is available for processing and presentation with MHC class II molecules. To test this hypothesis LCL-IiM3 were sorted on the basis of their surface expression of ΔNGFr (Fig. 19A). I next analysed recognition of these sorted cells by MAGE-$3_{161-175}$ specific $CD4^+$ T cells. $CD4^+$ T cells recognized cells with both low and high expression of ΔNGFr, but the intensity of recognition was considerably lower for low ΔNGFr expressing cells (Fig. 19B). MAGE-3 expression in high and low ΔNGFr expressing cells was verified by Western blot (Fig. 19D). As expected, cells expressing high levels of ΔNGFr had a higher amount of MAGE-3, whereas low expression of ΔNGFr was associated with a lower expression of protein. These results strongly support that the amount of protein available for processing determines the intensity of recognition.

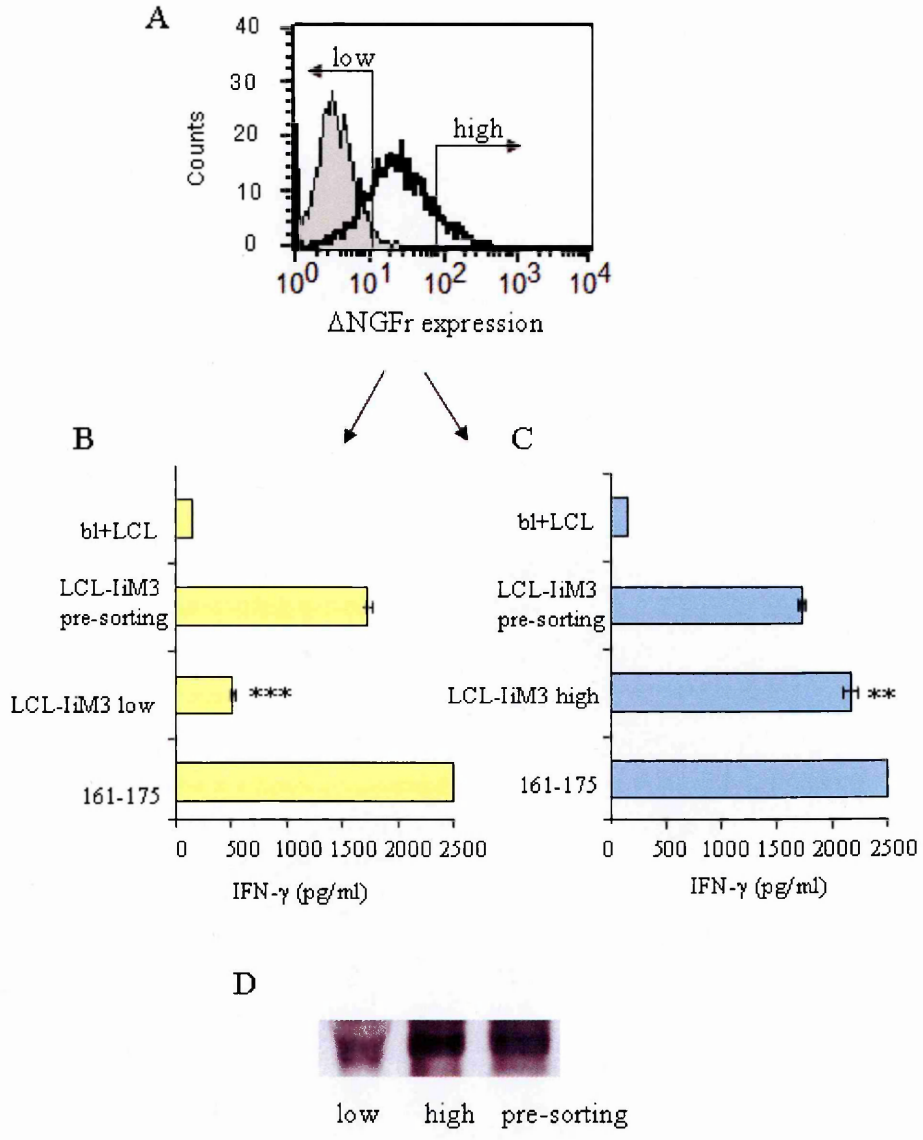


Figure 19. The amount of MAGE-3 expressed by engineered LCLs determines the intensity of recognition by CD4$^+$ T cells. A. LCL-IiM3 were sorted on the basis of high or low ΔLNGFr expression. B. CD4$^+$ T cells were challenged with LCL-IiM3 expressing low (B) or high (C) level of ΔLNGFr and MAGE-3 (D), and tested for IFN-γ release. The basal level of IFN-γ release of CD4$^+$ T cells in the presence of the wild type LCLs) is expressed as bl+LCL. Positive controls were peptide-pulsed LCLs and LCL-IiM3 before sorting. The data are means of duplicate determinations ± SD and are representative of two experiments. Responses significantly higher than the blanks are indicated as: **0,001<p<0,05, ***p<0,001 (determined by unpaired, one-tailed Student's *t* test). D. Western blot analysis for MAGE-3 of sorted cells.

4.1.2.2. MAGE-$3_{111-125}$ specific $CD4^+$ T cells. MAGE-$3_{111-125}$ specific $CD4^+$ T cells were obtained from melanoma patient 011 (Table 2; DRβ1*10, *11, DRβ3*02) by stimulation of purified $CD4^+$ T cells in the presence of autologous $CD4^+$-depleted PBMCs as APCs plus 10 μg/ml peptide. Peptide specific recognition and HLA-DR restriction were tested after 3 cycles of stimulation. $CD4^+$ T cells specifically produced IL-5 but not IFN-γ in the presence of autologous LCLs pulsed with MAGE-$3_{111-125}$ (data not shown). To identify the HLA-DR restricting allele $CD4^+$ T cells were challenged in IL-5 release assays with LCLs, homozygous for each of the HLA-DRβ1 (DR*10, DR*11) and HLA-DRβ3*02 alleles expressed by the donor, pulsed with peptide. As shown in Figure 20A presentation of MAGE-$3_{111-125}$ occurred in association with HLA-DR*11.

The 111-125 sequence overlaps the 114-127 sequence shown by Chaux et al. (213) to contain a naturally processed epitope produced through the exogenous pathway and presented in association with HLA-DR*13. I therefore tested cross-recognition of MAGE-$3_{114-127}$ by MAGE-$3_{111-125}$ specific $CD4^+$ T cells. Figure 20B shows that the two peptides share an epitope, as the $CD4^+$ T cell line was able to recognise both peptides with similar intensity. MAGE-$3_{111-125}$ specific $CD4^+$ T cells were then tested for recognition of the native epitope. I confirmed that the region contains an epitope naturally processed through the exogenous pathway, as the $CD4^+$ T cells responded specifically to autologous DCs loaded with lysates from MAGE-3 expressing melanoma MD TC but not to the HLA-DR*11-matched tumor MD TC (Fig. 21).

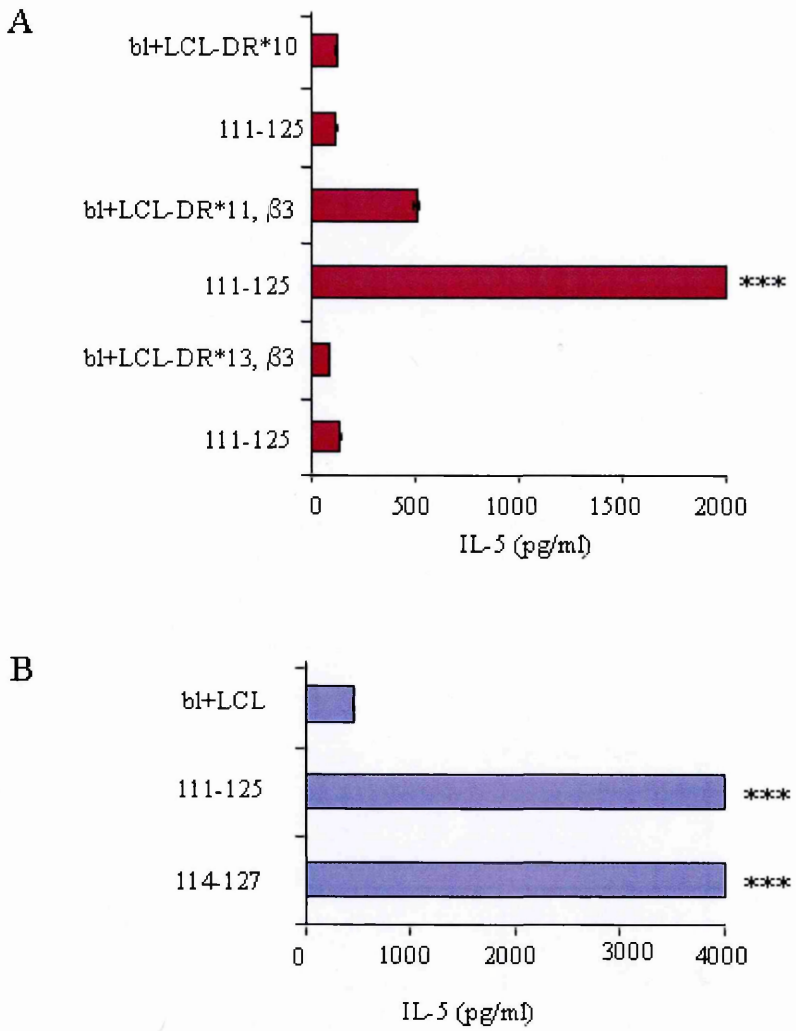


Figure 20. HLA-DR retriction of MAGE-$3_{111-125}$ specific $CD4^+$ T cells. $CD4^+$ T cells were challenged with peptide pulsed LCLs expressing each of the HLA-DRβ1 expressed by the donor or HLA-DRβ3 matched and tested for IL-5 release (A). The blanks (*i.e.* the level of IL-5 produced in the presence of unpulsed LCLs) is expressed as bl+LCLs. B: cross-recognition by MAGE-$3_{111-125}$ specific CD4+ T cells of overlapping peptide MAGE-$3_{114-127}$. Responses significantly higher than the blanks are indicated as: ***p<0,001 (determined by unpaired, one-tailed Student's *t* test)

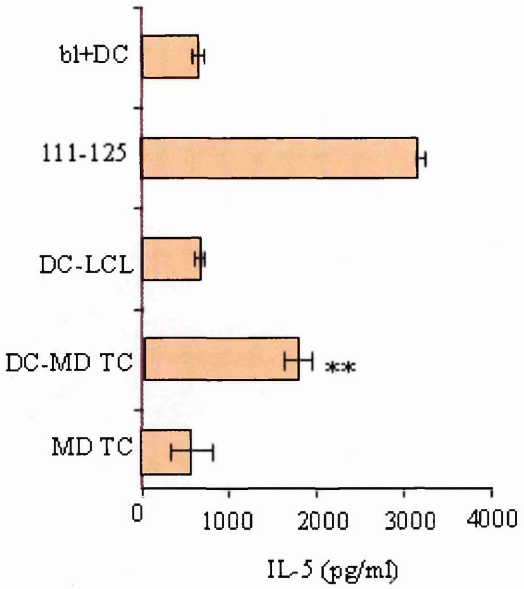


Figure 21. MAGE-$3_{111-125}$ contains a naturally processed epitope through the exogenous pathway. $CD4^+$ T cells were challenged with DCs loaded with lysates from MAGE-3 expressing melanoma MD TC and with MAGE-3 expressing tumor MD TC. DCs loaded with lysate from wild type LCLs or the peptide were added as negative and positive controls, respectively. IL-5 release was measured. The data are means of duplicate determinations ± SD and are representative of three experiments. Responses significantly higher than the blanks are indicated as: **0,001<p<0,05 (determined by unpaired, one-tailed Student's *t* test).

4.1.2.3. MAGE-$3_{289-300}$ specific $CD4^+$ T cells. The HLA-DR*11 restricted MAGE-$3_{289-300}$ specific oligoclonal 6.22 cell line was a kind gift from Dr Monica Moro (Experimental Unit, Cancer Immunotherapy and Gene Therapy Program, DIBIT directed by Drs. Giulia Casorati and Paolo Dellabona).

The presence of CD4 naturally processed epitope(s) in the 281-300 region of MAGE-3 has been described previously (19, 193). TEPITOPE (215) analysis had predicted several potential epitope frames for HLA-DR*11 within this sequence (Fig. 22). To verify if this region contains more than one epitope Dr. Moro stimulated the PBMC from Donor 2 (DRβ1*11, DRβ3*02) with peptides corresponding to overlapping sequences MAGE-$3_{281-295}$ and MAGE-$3_{286-300}$ and obtained by limiting dilution of a polyclonal cell line several clones recognising either one of the peptides (MAGE-$3_{281-295}$ and MAGE-$3_{286-300}$) or both.

Three clones (6.93, 2E5.53 and 6.22), representative for each type of behaviour, were further characterised. Overlapping truncated peptides spanning the 281-300 region were synthesized to identify the minimal recognized sequence(s) by the different clones (Fig. 22). $CD4^+$ T cells were tested for IFN-γ release in the presence of autologous LCLs and each truncated peptide. In agreement with TEPITOPE prediction, experiments shown in Figure 23 clearly demonstrate the presence of three different epitopes within MAGE-$3_{281-300}$ (*i.e.* 282-294, 284-298 and 289-300). Each clone was then tested for recognition of the native epitope. Clone 6.93 did not recognise MAGE-3 when processed either through the endogenous (LCL-M3 and HLA-DR*11 expressing melanoma MD TC) or the exogenous (LCL-IiM3) pathways (Fig. 24, right panels). Clone 2E5.53 recognised only MAGE-3 processed through the exogenous pathway (LCL-IiM3, Fig. 24, middle panels). Clone 6.22 recognised MAGE-3 when processed through both pathways (LCL-M3, LCL-IiM3 and HLA-DR*11 expressing MD TC) (Fig. 24, left panels).

I decided to use clone 6.22 recognising MAGE-$3_{289-300}$ for processing studies because it is a prototype for epitopes formed through both the endogenous and exogenous pathways. Before using it for further experiments I completed its characterisation. Peptide titration experiments with increasing concentration of the MAGE-$3_{281-300}$ peptide showed that the concentration of peptide requested to reach the half maximal stimulation (EC_{50}) was 0,2 μg/ml (Fig. 25), demonstrating an overall intermediate avidity. TCR Vβ receptor usage analysis also verified that the clone was in fact an oligoclonal line with 85.43% expression of Vβ22 chain (Fig. 26).

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281-300          TSYVKVLHHMVKISGGPHIS

277-288      ALVETSYVKVLH
278-289       LVETSYVKVLHH
279-290        VETSYVKVLHHM
280-291         ETSYVKVLHHMV
281-292          TSYVKVLHHMVK
282-293           SYVKVLHHMVKI
283-294            YVKVLHHMVKIS
284-295             VKVLHHMVKISG
285-296              KVLHHMVKISGG
286-297               VLHHMVKISGGP
287-298                LHHMVKISGGPH
288-299                 HHMVKISGGPHI
289-300                  HMVKISGGPHIS
290-301                   MVKISGGPHISY
291-302                    VKISGGPHISYP
292-303                     KISGGPHISYPP
293-304                      ISGGPHISYPPL
294-305                       SGGPHISYPPLH
295-306                        GGPHISYPPLHE
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Figure 22. Sequences of peptides spanning the 277-306 region of MAGE-3. The aminoacids predicted by TEPITOPE as P1 anchor residues for HLA-DR*11 are highlighted in red.

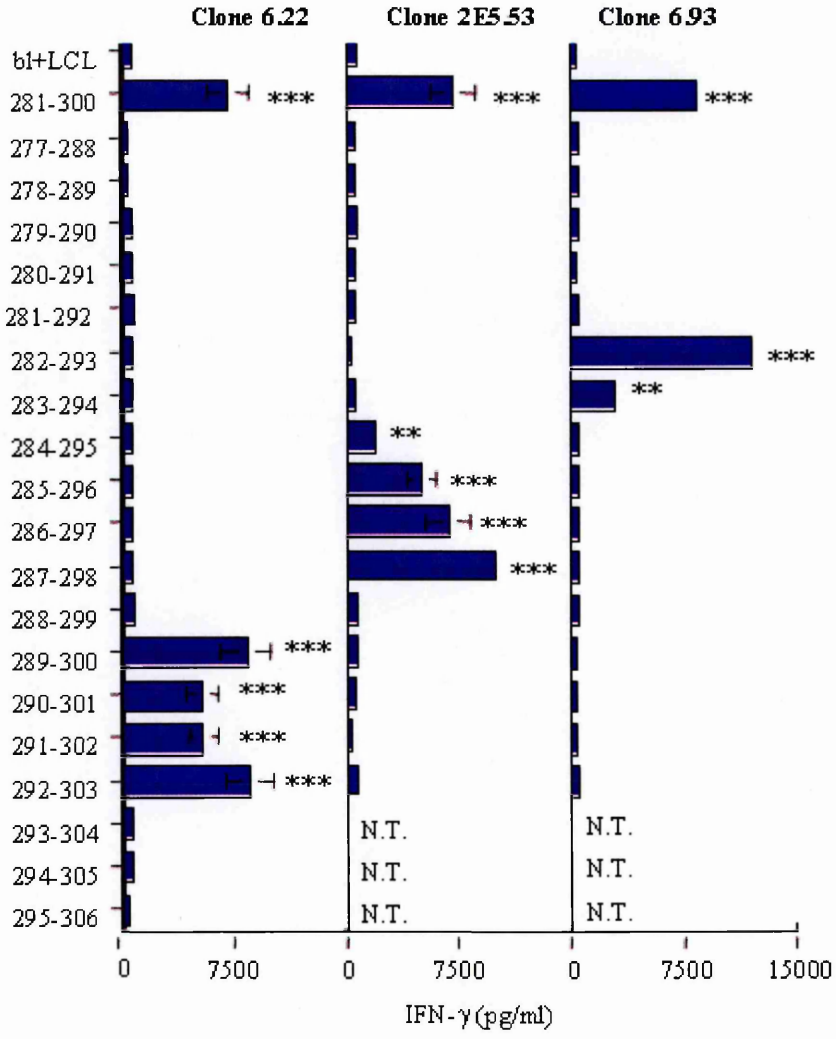

Figure 23. Identification of the epitopes in the 281-300 region of MAGE-3 recognized by CD4$^+$ T cells clones specific for MAGE-3$_{281\text{-}300}$. CD4$^+$ T cells were cultured in the presence of HLA-DR*11 positive LCLs and each single MAGE-3 peptide shown in Fig. 22 and tested for IFN-γ release. The blanks (*i.e.* the basal level of IFN-γ release of CD4$^+$ T cells in the presence of unpulsed LCLs) are expressed as bl+LCL. The data are means of triplicate determinations and are representative of three experiments. N.T.: not tested.

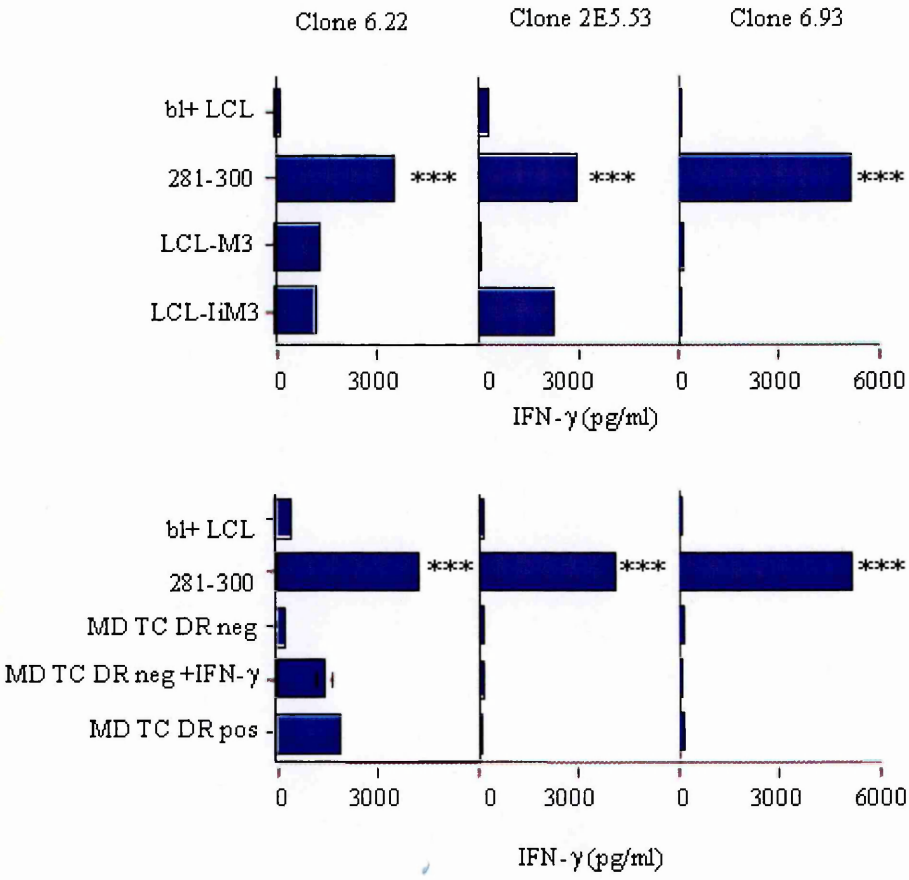

Figure 24. MAGE-$3_{281\text{-}300}$ contains naturally processed and not naturally processed epitopes. MAGE-$3_{281\text{-}300}$ specific clones were challenged with cells LCLs expressing MAGE-3 in the cytoplasm (LC-M3) or the endosomal/lysosomal compartment (LCL-IiM3) (upper panels) and MAGE-3 and HLA-DR expressing melanoma (MDTC) (lower panels) and tested for IFN-γ release. The blanks (*i.e.* the basal level of IFN-γ release of CD4+ T cells in the presence of unpulsed LCLs) are expressed as bl+LCL. Peptide-pulsed LCLCs or MDTC were used as positive controls. The data are means of triplicate determinations ± SD and are representative of at least three experiments.

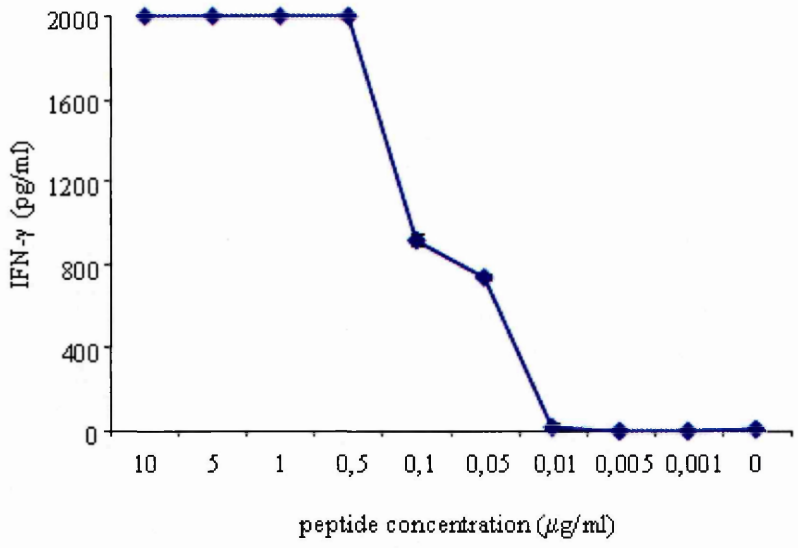

Figure 25. Peptide titration curve for MAGE-$3_{281-300}$ specific $CD4^+$ T cell line 6.22. $CD4^+$ T cells were challenged with titrated doses of the indicated peptides and IFN-γ release measured. The blank (*i.e.* the basal level of IFN-γ release of $CD4^+$ T cells in the presence of the unpulsed LCLs) is expressed as 0. The data are means of duplicate determination ± SD and are representative of at least three experiments.

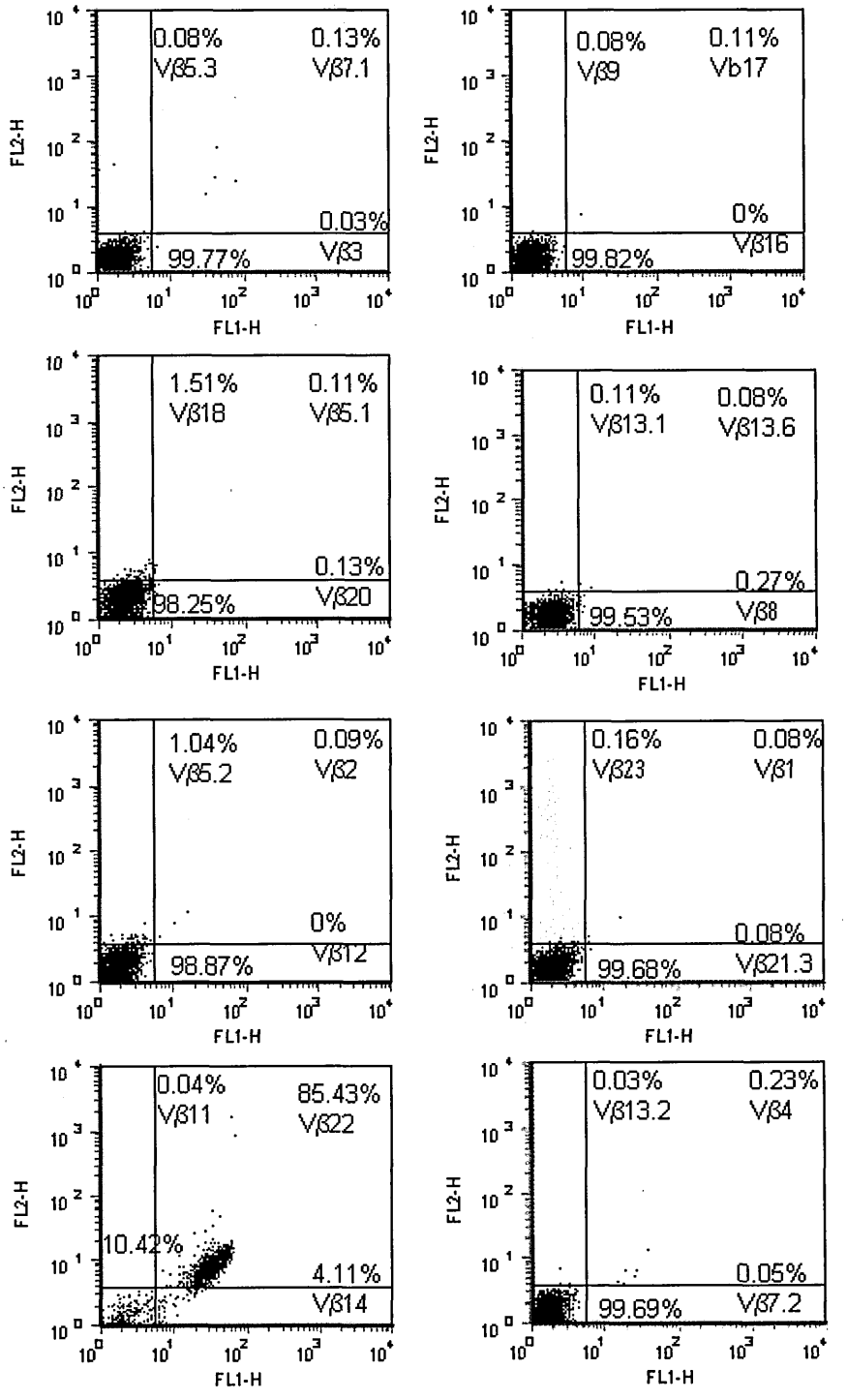

Figure 26. Vβ usage in MAGE-$3_{281-300}$ specific $CD4^+$ T cell line 6.22. Vβ usage was determined by flow cytometric analysis using the Iotest Beta Mark kit, following manufacturer's instructions.

4.2. STUDY OF PROCESSING THROUGH THE EXOGENOUS PATHWAY: EPITOPES MAGE-$3_{161-175}$ and MAGE-$3_{111-125}$

To study processing through the exogenous pathway the enzymes involved in processing were inhibited in MAGE-3 expressing APCs and the reactivity of the MAGE-3 specific $CD4^+$ T cells to these treated APCs was measured. The enzymes were inhibited with inhibitors specific for one protease if possible or with generic inhibitors if not. Specific inhibitors were available for cathepsins B and L; the commercially available inhibitor for cathepsin S was very toxic and poorly soluble so it was not used. Generic inhibitors included leupeptin, that acts on cysteine cathepsins, and pepstatin A, specific for the family of aspartic proteases.

The two epitopes MAGE-$3_{161-175}$ and MAGE-$3_{111-125}$ described above (Sections 4.1.2.1 and 4.1.2.2) were studied.

4.2.1. MAGE-$3_{161-175}$

4.2.1.1. Effect of cysteine proteases on MAGE-$3_{161-175}$ processing. LCLs and HT144 cells expressing MAGE-3 in the endosomal/lysosomal compartment were incubated with non-toxic concentrations of inhibitors for 18-20 hours before being used as APCs for $CD4^+$ T cells recognition in standard proliferation and IFN-γ release assays. Treated and untreated wild type LCL and LCL-M3, wild type HT144 cells and peptide-pulsed cells were always included as controls. Upon treatment with leupeptin, a generic inhibitor of cysteine proteases, a considerable decrease of recognition was observed for both LCL-IiM3 (Fig. 27A) and HT144-IiM3 (Fig. 27B), suggesting that at least one cysteine protease plays an important role in the correct formation of this epitope.

Recognition of peptide-pulsed leupeptin-treated or untreated APCs was always comparable (not shown), suggesting that the treatment with the inhibitor did not affect the antigen presenting capability of inhibitor treated APCs.

When specific inhibitor CA074 of cathepsin B was used no significant decrease was seen in either LCLs (Fig. 28A) or HT144 melanoma cells (Fig. 28B), although the inhibitor was correctly working, especially at higher concentrations (Fig. 28C), suggesting that cathepsin B is not essential for correct formation of the epitope in either cell type.

A similar pattern of recognition was seen when inhibiting cathepsin L with catL inh III, a specific inhibitor of cathepsin L, in melanoma cells. This enzyme is probably also not required in processing of this epitope (Fig. 29A). Enzyme activity was measured in both melanoma cells and LCLs. Cathepsin L has been shown to be present but not active in human primary B cells and LCLs (137, 237). No activity was detected in LCLs using the fluorimetrc assay described in chapter 3, even using high concentrations of protein lysate, thus confirming what described by others using different methods (Fig. 29B). As there was no active enzyme to influence processing no experiments were performed using LCLs as APCs.

As control for presentation capability I also evaluated if inhibitors could affect HLA-DR expression. FACS analysis, shown in Fig. 30, confirmed that this was not the case. Cathepsin B has not, in fact, been shown to be involved in processing of MHC-II molecules (142). In murine cells cathepsin L has been found necessary only in thymic epithelial cells; its role in other cells has not been identified exactly (134). Instead, the generic inhibitor leupeptin works on cathepsin S, that is involved in maturation of MHC-II molecules (126). However, with the concentrations used, the amount of MHC-II molecules on the cell surface appeared normal (Fig. 30A and C). Small variations were observed but were not reproducible or related to inhibitor dose.

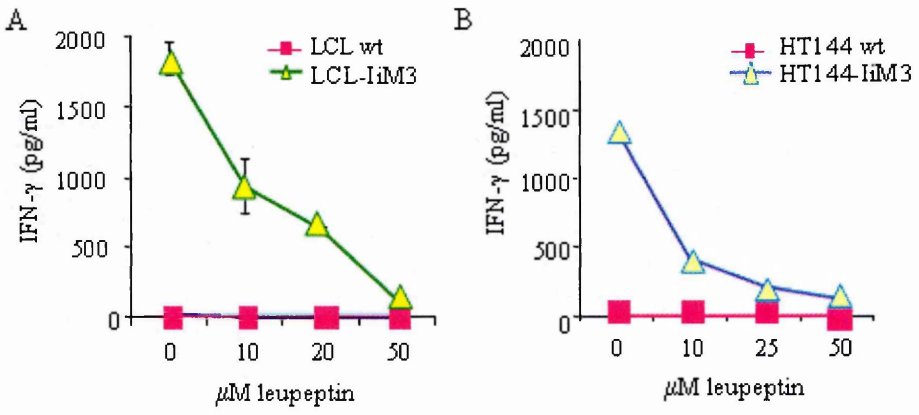

Figure 27. Effect of cysteine cathepsin inhibition on presentation of MAGE-$3_{161-175}$. APCs were treated with increasing concentrations of the cysteine cathepsin inhibitor leupeptin for 18-20 hours. MAGE-$3_{161-175}$ specific $CD4^+$ T cells were then added and IFN-γ release measured. The APCs used were LCL-IiM3 (A) and HT144-IiM3 (B). Treated wild type cells were used as negative control: the response towards both was identical (data not shown). The data are means of duplicate determination ± SD and are representative of at least three experiments.

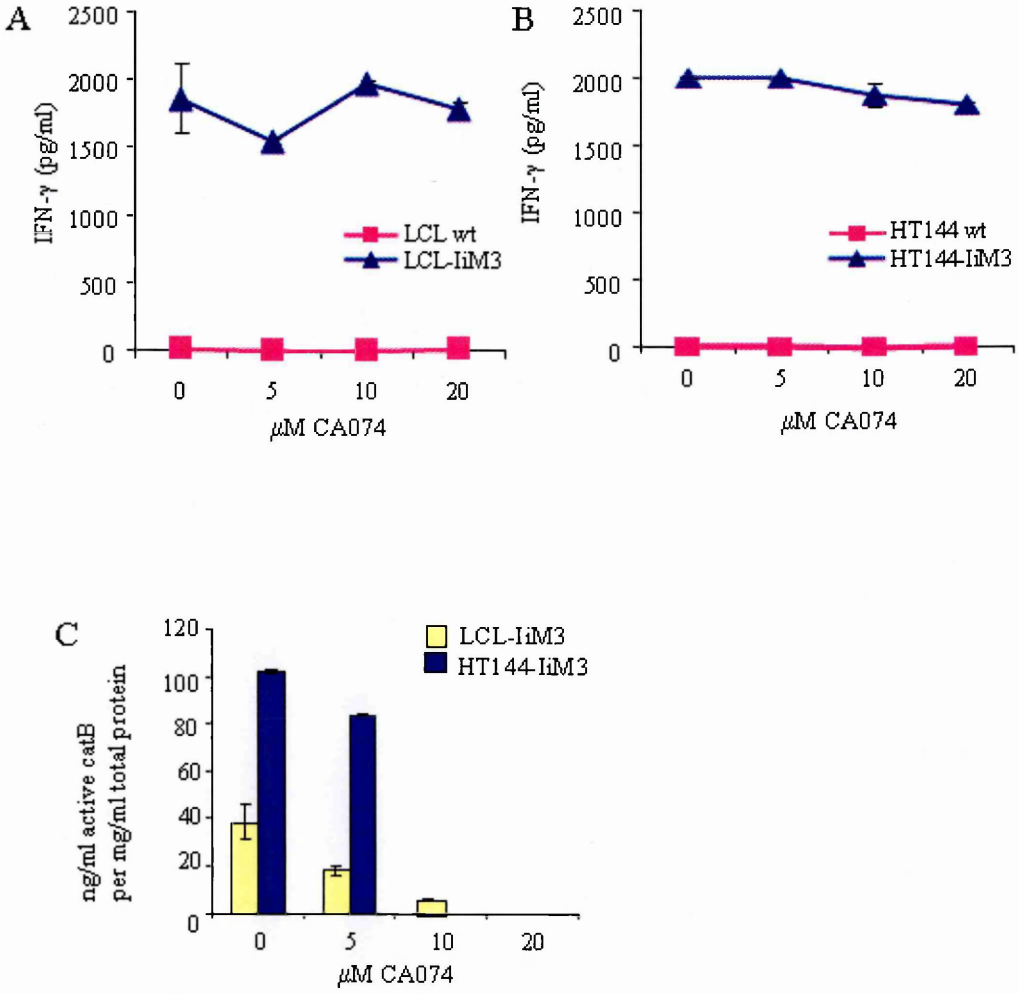

Figure 28. Effect of cathepsin B inhibition on presentation of MAGE-3$_{161-175}$. APCs were treated with increasing concentrations of the cathepsin B inhibitor CA074 for 18-20 hours. MAGE-3$_{161-175}$ specific CD4$^+$ T cellswere then added and IFN-γ release measured. The APCs used were LCL-IiM3 (A) and HT144-IiM3 (B). Treated wild type cells were used as negative controls. Peptide-pulsed treated or untreated cells were used as positive control: the response towards both was identical (data not shown). The data are means of duplicate determination ± SD and are representative of at least three experiments. C: After 18-20 hours incubation with CA074 cells were lysed and activity of cathepsin B measured as ng/ml active cathepsin B per mg/ml total protein.

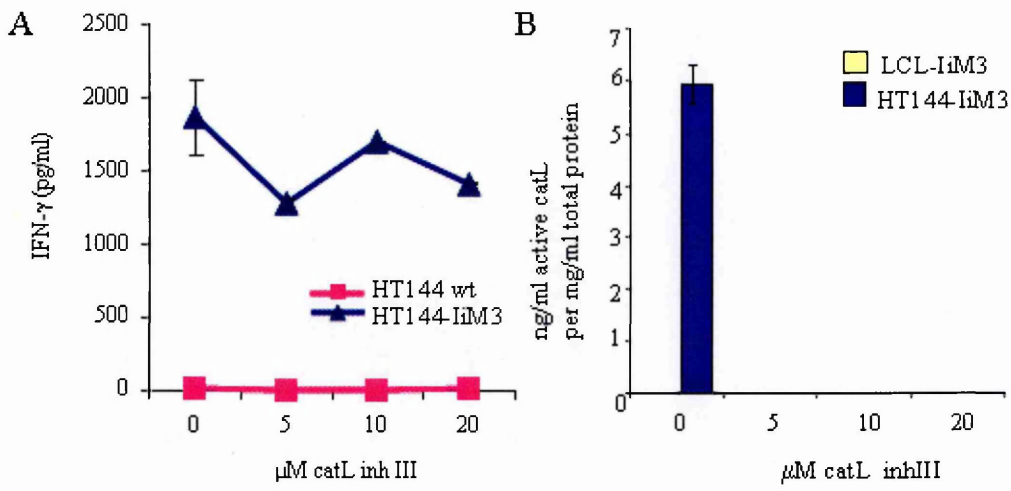

Figure 29. Effect of cathepsin L inhibition on presentation of MAGE-$3_{161-175}$. A: HT144 cells were treated with increasing concentrations of the cathepsin L inhibitor catL inhIII for 18-20 hours. MAGE-$3_{161-175}$ specific $CD4^+$ T cells were then added and IFN-γ release measured. Treated wild type cells were used as negative controls. Peptide-pulsed treated or untreated cells were used as positive control: the response towards both was identical (data not shown). The data are means of duplicate determination ± SD and are representative of at least three experiments. B: After 18-20 hours incubation with inhibitor cells were lysed and activity of cathepsin L measured as ng/ml active cathepsin L per mg/ml total protein.

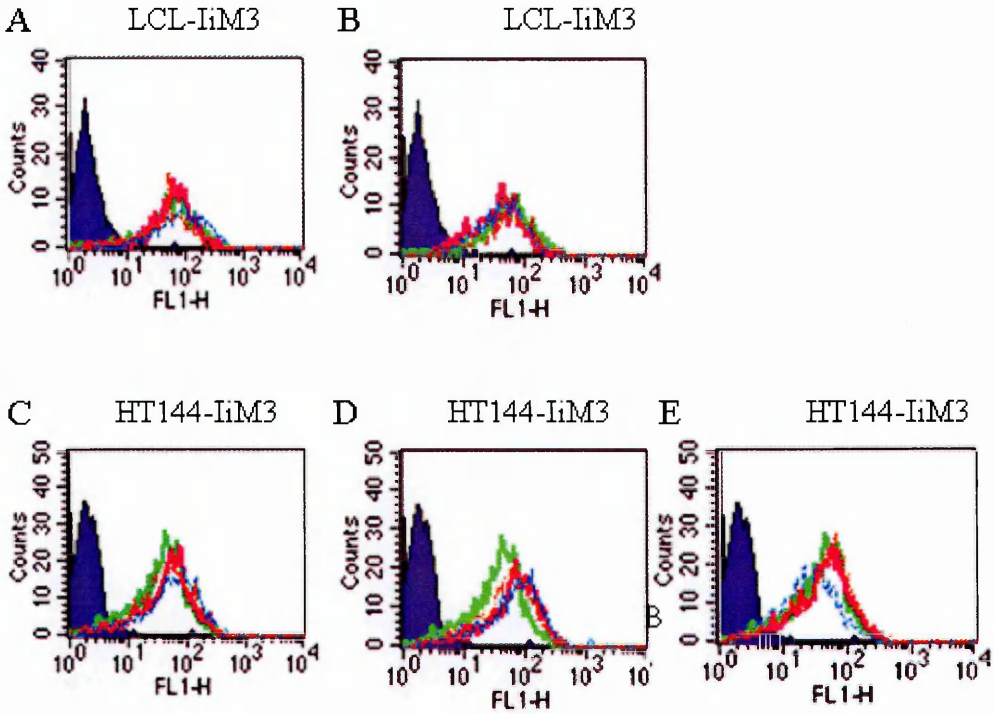

Figure 30. HLA-DR expression on inhibitor treated cells. FACS analysis was used to measure changes of HLA-DR molecules on the cell surface. Full purple line: negative control; untreated cells: in green; pink, orange and blue: cells treated with increasing concentrations of inhibitor (5, 10 and 20 μM for cathepsin B inhibitor CA074 and cathepsin L specific inhibitor catL inhIII; 10, 20 and 50 μM for leupeptin). A: leupeptin treated LCLs. B: CA074 treated LCLs. C: leupeptin treated melanoma cells. D: CA074 treated melanoma cells. E: catL inhIII treated melanoma cells.

4.2.1.2. Effect of aspartic proteases on MAGE-$3_{161-175}$ processing. LCLs and HT144 melanoma cells (wild type and transduced) were treated with increasing concentrations of the generic aspartic protease inhibitor pepstatin A then used as APCs for recognition by $CD4^+$ T cells. Variations in processing and presentation were measured as IFN-γ release. Treatment of APCs with the generic inhibitor of aspartic proteases pepstatin A led to a dramatically improved recognition of LCL-IiM3 (Fig. 31A) and HT144-IiM3 (Fig. 31B). This improved recognition was nonetheless associated to a decrease in cathepsin D activity, as shown by activity measurements (Fig. 32A) and it was not associated to variation in HLA-DR surface expression (Fig. 32B).

4.2.1.3. Combined inhibition of aspartic and cysteine proteases. Contemporary administration of leupeptin and pepstatin A to LCL-IiM3 (Fig. 33A) and HT144-IiM3 (Fig. 33B) was comparable to that of leupeptin alone, suggesting a predominant activity of leupeptin sensitive enzymes in MAGE-$3_{161-175}$ formation. The inhibitory effect of leupeptin was evident after an 8-hour treatment in both types of APC used (Fig. 34A-B) whereas the epitope-presentation promoting effect of pepstain A was proportional to the incubation time with the inhibitor (Fig. 34C-D).

4.2.1.4. Effect of proteases on processing of MAGE-$3_{161-175}$ epitope in DCs. DCs were loaded with lysates from MAGE-3 negative and MAGE-3 expressing cells. Leupeptin and pepstatin A were added and left for 18-20 hours before adding $CD4^+$ T cells as described above. In agreement with results showed for LCL-IiM3 and HT144-IiM3 (Fig. 27 and Fig. 31), presentation was abolished when cells loaded with MAGE-3 expressing lysates were treated with leupeptin but when cells were treated with pepstatin A presentation improved even more dramatically than what was seen using

other APCs (Fig. 35). This finding is of particular relevance as DCs are the ones most likely to present the epitope *in vivo*.

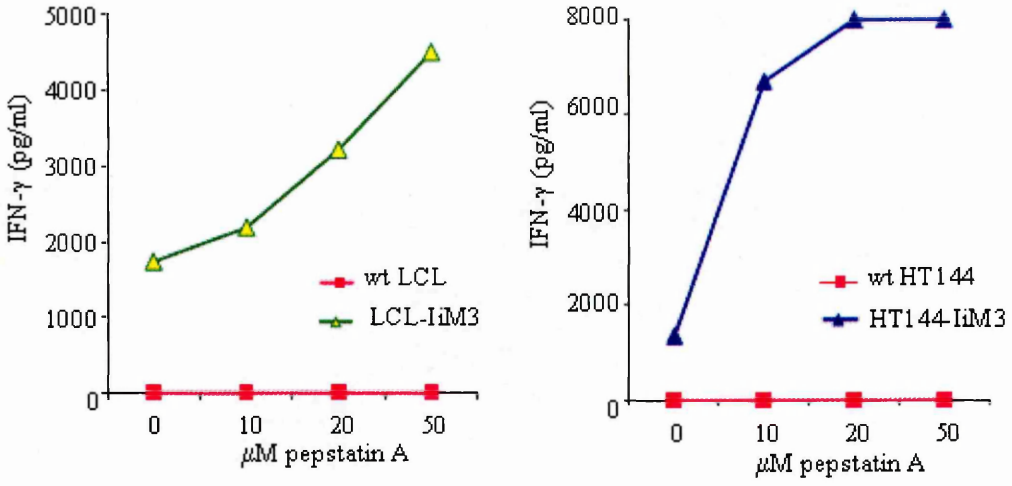

Figure 31. Effect of cathepsin D inhibition on presentation of MAGE-$3_{161-175}$. APCs were treated with increasing concentrations of the aspartic protease pepstatin A for 18-20 hours. MAGE-$3_{161-175}$ specific $CD4^+$ T cells were then added and IFN-γ release measured. The APCs used were LCL-IiM3 (A) and HT144-IiM3 (B). Treated wild type cells were used as negative controls. Peptide-pulsed treated or untreated cells were used as positive control: the response towards both was identical (data not shown). The data are means of duplicate determination ± SD and are representative of at least three experiments.

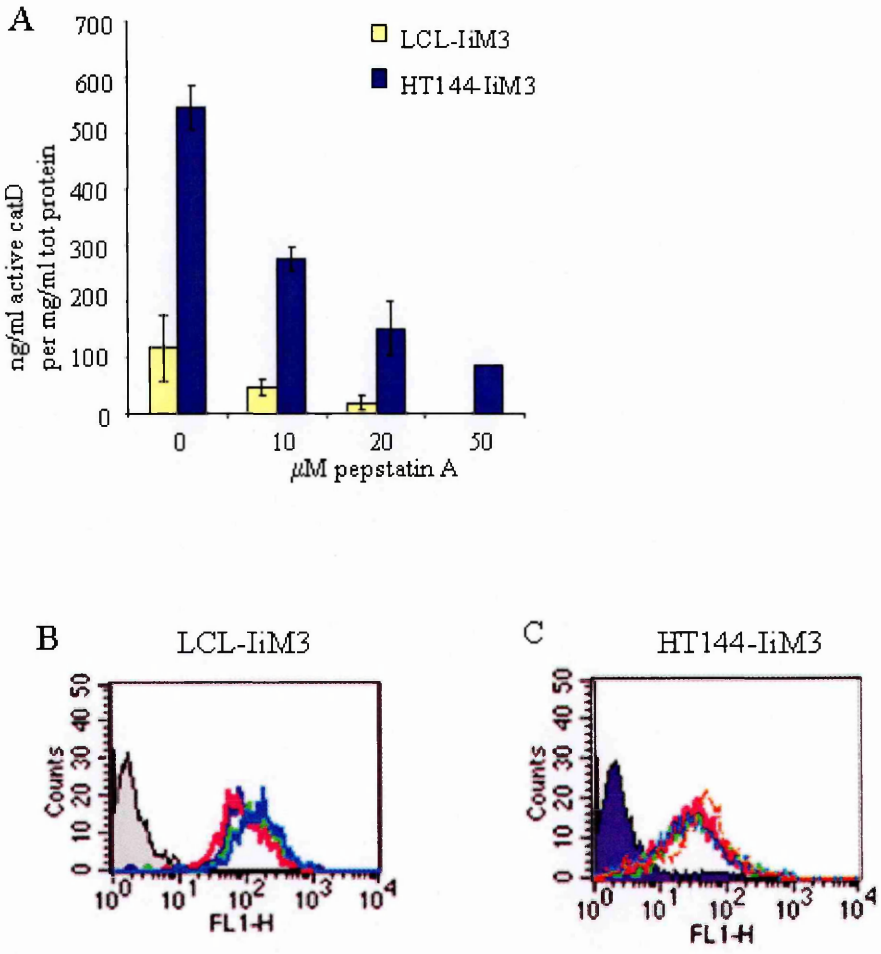

Figure 32. Activity and HLA-DR expession of pepstatin A treated cells. A: After 18-20 hours incubation with aspartic protease pepstatin A cells were lysed and activity of catD measured as ng/ml active catD per mg/ml total protein. B: HLA-DR expression on LCL-IiM3. Untreated cells: green; pink: 10 μM, purple: 20 μM; blue: 50 μM inhibitor. The full line is the negative control. (C): HLA-DR expression on HI144-IiM3. Untreated cells: green; pink: 10 μM; orange: 20 μM; blue: 50 μM inhibitor. The full line is the negative control.

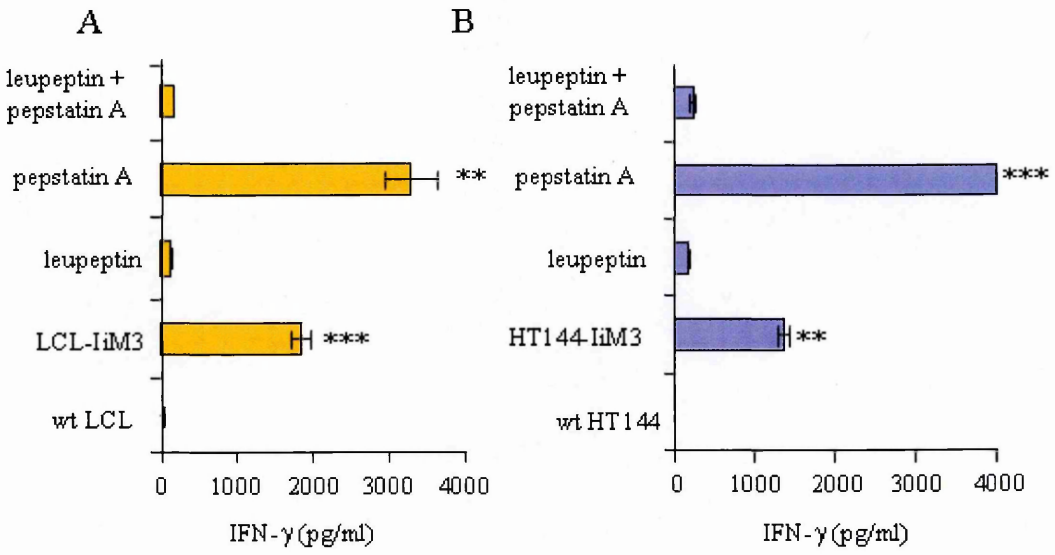

Figure 33. Combined effect of inhibitors on MAGE-3$_{161-175}$ processing. CD4$^+$ T cells were challenged with APCs treated with 50 μM leupeptin and 50 μM pepstatin A and IFN-γ release measured. The data are means of duplicate determination ± SD. Responses significantly higher than the blanks are indicated as: **0,001<p<0,05, ***p<0,001 (determined by unpaired, one-tailed Student's *t* test). A: LCLs as APCs. B: HT144 cells as APCs.

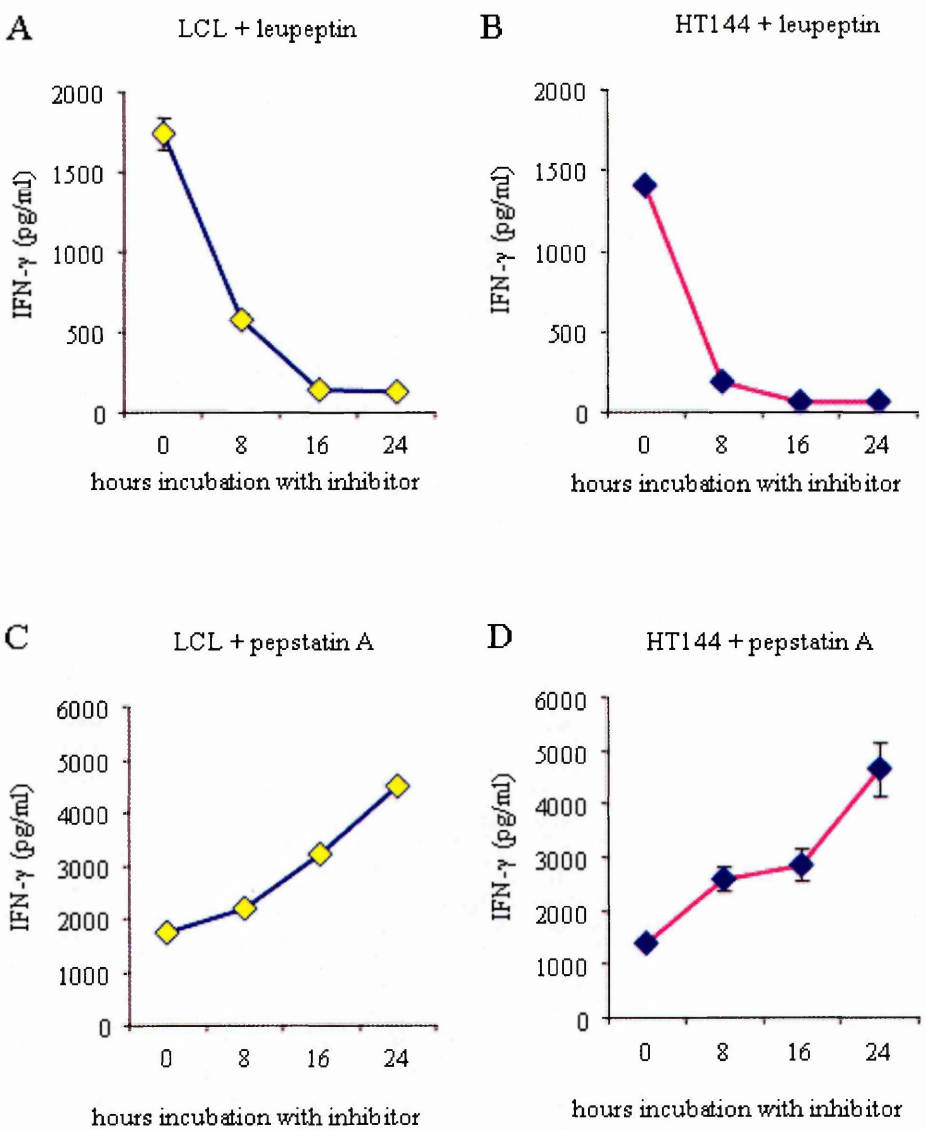

Figure 34. Time dependence of MAGE-3$_{161-175}$ processing. CD4$^+$ T cells were challenged with LCL-IiM3 and HT144-IiM3 treated with 50 μM leupeptin or pepstatin A for 8, 16 or 24 hours before the assay. A and B: IFN-γ release after treatment with leupeptin. C and D: IFN-γ release after treatment with pepstatin A. The data are means of duplicate determination ± SD and are representative of two experiments.

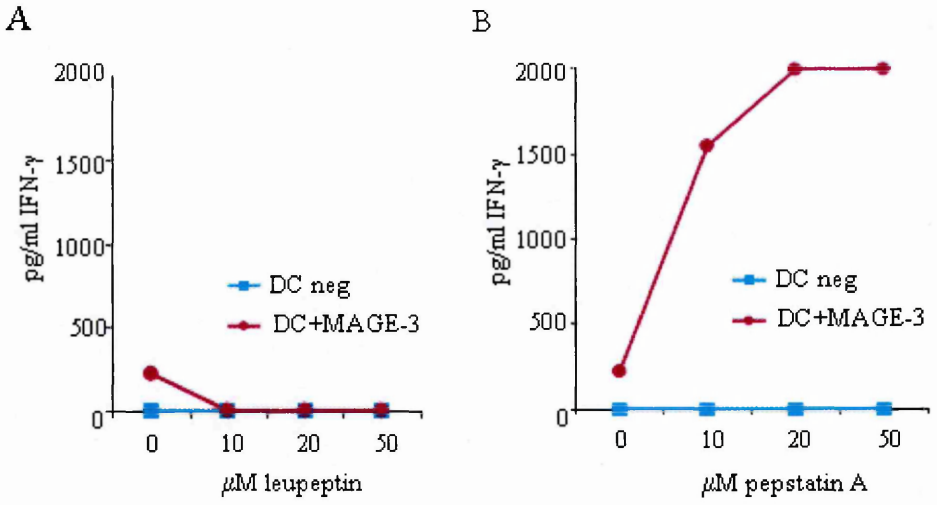

Figure 35. Protease inhibition effects on MAGE-3$_{161-175}$ processing in DCs. MAGE-3$_{161-175}$ specific CD4$^+$ T cells were challenged with DCs loaded with lysates from MAGE-3 negative or MAGE-3 expressing cells and treated with protease inhibitors. A: IFN-γ release after DC treatment with cysteine cathepsin inhibitor leupeptin. B: IFN-γ release after DC treatment with aspartic protease inhibitor pepstatin A. The data are means of duplicate determination ± SD and are representative of two experiments.

4.2.2. MAGE-$3_{111-125}$

4.2.2.1 Effect of cysteine and aspartic proteases on processing of MAGE-$3_{111-125}$.

Due to limited amount of MAGE-$3_{111-125}$ specific $CD4^+$ T cells from patient 011, I was not able to perform extensive experiments as I did for the MAGE-$3_{161-175}$ epitope. Therefore for processing of MAGE-$3_{111-125}$ I tested recognition of MAGE-3 loaded autologous DCs because the most relevant APCs *in vivo*. DCs from patient 011 were loaded with lysates from MAGE-3 negative and MAGE-3 expressing cells; inhibitors of cysteine cathepsins and aspartic proteases were added as described above (4.2.1.4). Treatment with 10 μM of leupeptin was enough to lead to abolishment of recognition (Fig. 36A). A cysteine protease is therefore required to correctly produce this epitope, as was seen for MAGE-$3_{161-175}$, but in this case the amount of inhibitor required is much less. Inhibition of aspartic proteases with pepstatin A used caused (as with leupeptin) abolishment of recognition (Fig 36B), a very different effect compared to that seen for formation of MAGE-$3_{161-175}$. These results suggest that formation of the MAGE-$3_{111-125}$ epitope depends on both leupeptin- and pepstatin A-sensitive enzymes.

Collectively the experiments on antigen processing through the exogenous pathway show how two epitopes from the same antigen (*i.e.* MAGE-3) can be processed differently: whereas MAGE-$3_{161-175}$ is produced by a cysteine protease and destroyed by an aspartic one MAGE-$3_{111-125}$ requires both types of enzyme to be produced.

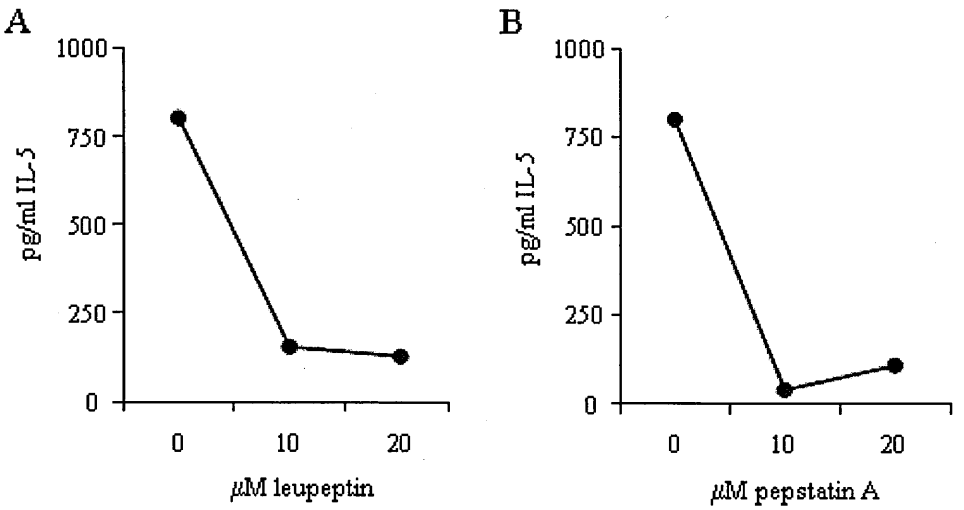

Figure 36. Protease inhibition effects on MAGE-$3_{111-125}$ processing in DCs. MAGE-$3_{111-125}$ specific $CD4^+$ T cells challenged with DCs loaded with lysates from MAGE-3 expressing cells and treated with protease inhibitors. DCs loaded with lysates of MAGE-3 negative cells and peptide-pulsed cells were used as negative and positive controls (data not shown). A: IL-5 release after DC treatment with cysteine cathepsin inhibitor leupeptin. B: IL-5 release after DC treatment with aspartic protease inhibitor pepstatin A. The data are means of duplicate determination ± SD and are representative of two experiments.

4.3. STUDY OF PROCESSING THROUGH THE ENDOGENOUS PATHWAY: EPITOPE MAGE-$3_{289-300}$

To study processing through the endogenous pathway I followed the same strategy used for the exogenous pathway. The enzymes involved in processing were inhibited in APCs and the reactivity of the $CD4^+$ T cells to these treated APCs was measured. As the MAGE-$3_{289-300}$ epitope (Fig. 24, left panels) is, in fact, produced through both processing pathways experiments were performed using both LCL-M3 and LCL-IiM3 as APCs. The melanoma MD TC was not consistently recognised therefore I decided not to use it.

4.3.1. MAGE-$3_{289-300}$

4.3.1.1. Effect of the proteasome on processing of MAGE-$3_{289-300}$. Proteasomal activity was inhibited using three types of inhibitor: lactacystin, MG132 and bortezomib. These inhibitors affect both the standard proteasome and the immunoproteasome in different ways (238, 239). In these experiments, peptide-pulsed inhibitor treated cells were included as controls and in all cases were able to elicit the same response as peptide-pulsed untreated cells (data not shown).

Lactacystin is a well-known inhibitor that inhibits tryptic and chymotryptic activities (238). Treatment of LCL-M3 with non-toxic concentrations of lactacystin caused only a modest change in presentation (Fig. 37A).

MG132 (Cbz-leu-leu-leucinal) binds to the β1 and β5 subunits of the standard proteasome and to their counterparts in the immunoproteasome (239). It also inhibits the tryptic activity of the immunoproteasome by completely blocking subunit β2i.

Treatment of LCL-M3 with MG132 did not decrease presentation of the epitope (Fig. 37B).

Bortezomib, the active principle in Velcade (used to treat refractory multiple myeloma), does not interfere with tryptic activity, but it binds and inhibits subunits β5, β5i and also β1, blocking chymotryptic activity and caspase-like activity (239). The epitope presenting capacity of bortezomib treated cells was significantly increased compared to their untreated counterparts, suggesting that chymotriptic activity destroys the epitope (Fig. 37C).

The effect on presentation of the epitope by treated LCL-IiM3 did not greatly differ from that seen for presentation by LCL-M3 (Fig. 37A-B-C).

4.3.1.2. Inhibition of TPP II and calpains. Tripeptidyl peptidase has been implied in antigen processing, as described in chapter 1.3.2.4, but reports on its exact function are contradictory (108, 109). LCL-M3 treated with non-toxic concentrations of the inhibitor AA-CMK did not show any significant change in antigen presenting capacity (Fig. 38A). The cells, however, were very sensitive to this compound so extremely low concentrations had to be used; they may not have been enough to inhibit the enzyme completely.

The effect on presentation of the epitope by treated LCL-IiM3 closely resembled that seen for presentation by LCL-M3 (Fig. 38A).

Calpains may also be involved in antigen processing, as shown by Lich et al (123); LCL-M3 treated with a specific inhibitor did not elicit any change (Fig. 38B). These enzymes may therefore not be involved in processing of this epitope.

The effect on presentation of the epitope by treated LCL-IiM3 was similar to that seen for presentation by LCL-M3 (Fig. 38B).

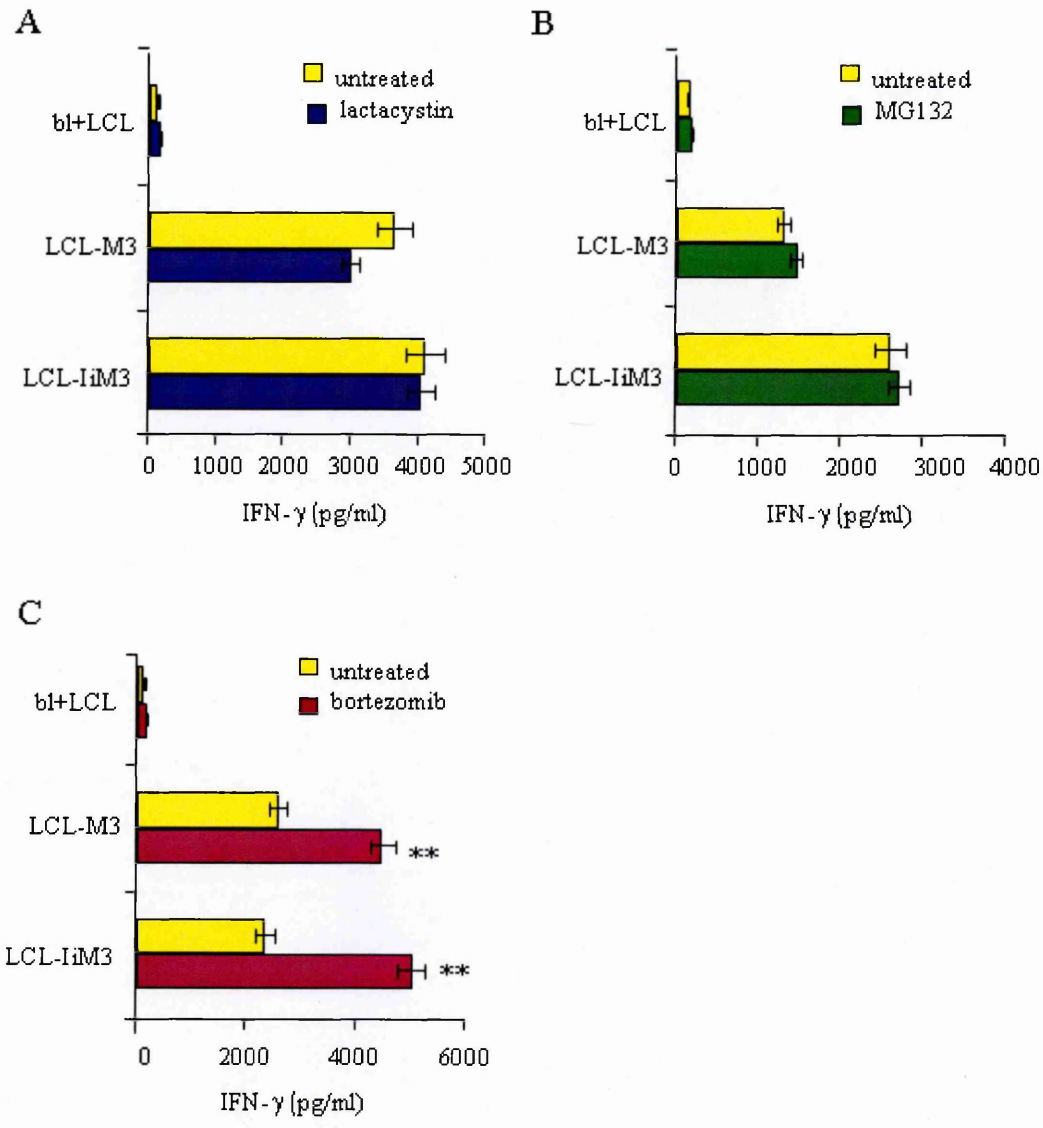

Figure 37. Effects of proteasome inhibition on MAGE-3$_{289\text{-}300}$ processing. MAGE-3$_{289\text{-}300}$ specific CD4$^+$ T cells were challenged with cells expressing MAGE-3 either in the cytoplasm (LCL-M3) or in the endosomal-lysosomal compartment (LCL-IiM3) after treatment with proteasomal inhibitors. Peptide-pulsed cells were always added as positive controls (data not shown) and blanks (bl+LCL) as negative control. The data are means of duplicate determinations ± SD and are representative of three experiments. Responses significantly higher than the blanks are indicated as: **0,001<p<0,05, (determined by unpaired, one-tailed Student's *t* test). A: IFN-γ release after treatment with lactacystin. B: IFN-γ release after treatment with MG132. C: IFN-γ release after treatment with bortezomib.

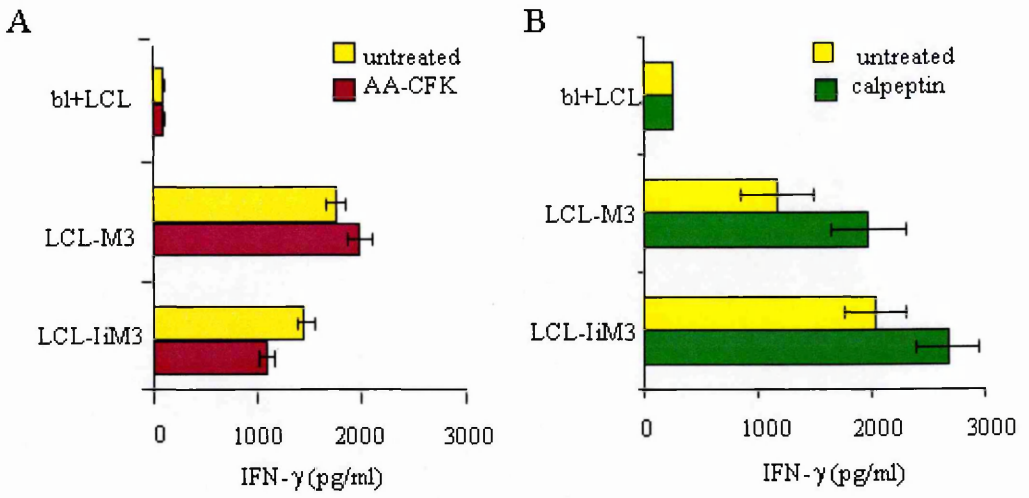

Figure 38. Effects of cytosolic protease inhibition on MAGE-$3_{289-300}$ processing. MAGE-$3_{289-300}$ specific $CD4^+$ T cells were challenged with cells expressing MAGE-3 in the cytoplasm (LCL-M3) or in the endosomal-lysosomal compartment (LCL-IiM3) after treatment with inhibitors of cytosolic proteases. Peptide-pulsed cells were always added as positive controls (data not shown) and blanks (bl+LCL) as negative control. The data are means of duplicate determinations ± SD and are representative of two experiments. A: IFN-γ release after treatment with AA-CFK, inhibitor of TPP II. B: IFN-γ release after treatment with calpetin, an inhibitor of calpains

4.3.1.3. Effect of autophagy inhibition on processing. Autophagy is a normal process occurring in all metabolically active cells. During the process single membrane structures (the 'isolation' membranes) envelop portions of cytoplasm and organelles (like mitochondria) and form autophagosomes. The contents of the autophagic vesicles are digested after fusion with lysosomes. Autophagy has been hypothesized to be involved in MHC class II processing of cytoplasmic tumor antigens (240). The two best known inhibitors of autophagy are wortmannin and 3-methyl-adenine. Experiments using LCL-M3 treated with these compounds showed a significant increase in recognition, suggesting that autophagy removes MAGE-3 from the pathway leading to the formation of the 289-300 epitope (Fig. 39A-B). No difference on presentation of the epitope by treated LCL-IiM3 was seen compared to presentation by LCL-M3 (Fig. 39A-B).

4.3.1.4. Inhibition of lysosomal enzymes. As mature MHC-II molecules are only found in the endosomal/lysosomal compartment endogenous MAGE-3 (or its partially processed products) will eventually encounter the enzymes present in this compartment. Therefore LCL-M3 cells were treated with inhibitors of cathepsin B, with leupeptin and with pepstatin A. Cathepsin B inhibition (data not shown) and inhibition of cysteine cathepsins with leupeptin caused an increase in recognition in both LCL-M3 and LCL-IiM3 (Fig. 40A).

Inhibition of aspartic proteases with pepstatin A had no effect in presentation in LCL-M3 but improved it in LCL-IiM3 (Fig. 40A-B). This suggests that aspartic proteases have different roles in processing of this epitope from the two pathways.

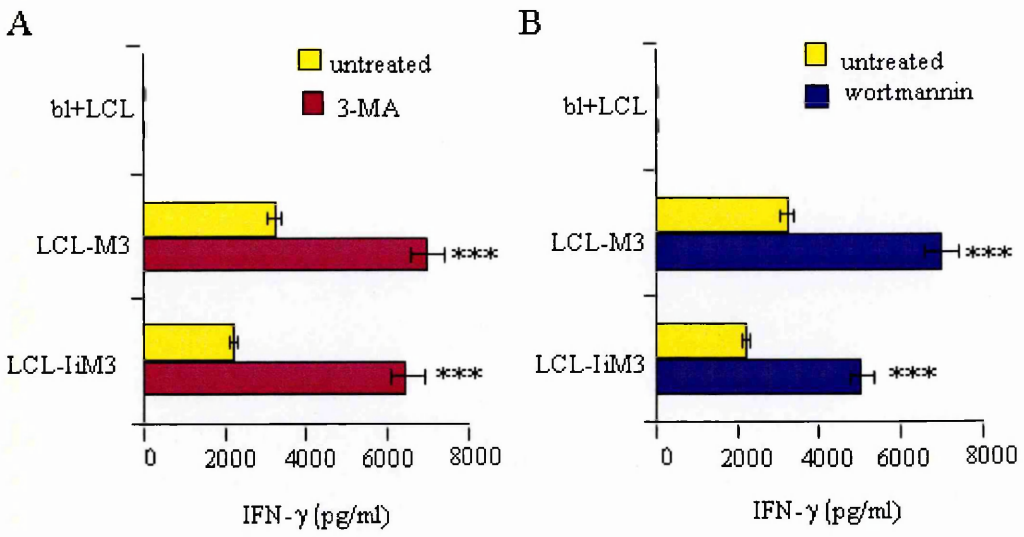

Figure 39. Effects of autophagy inhibition on MAGE-$3_{289\text{-}300}$ processing. MAGE-$3_{289\text{-}300}$ specific $CD4^+$ T cells were challenged with cells expressing MAGE-3 in the cytoplasm (LCL-M3) or in the endosomal-lysosomal compartment (LCL-IiM3) after treatment with proteasomal inhibitors. Peptide-pulsed cells were always added as positive controls (data not shown) and blanks (bl+LCL) as negative control. The data are means of duplicate determinations ± SD and are representative of three experiments. Responses significantly higher than the blanks are indicated as: ***p<0,001 (determined by unpaired, one-tailed Student's *t* test). A: IFN-γ release after treatment with 3-MA. B: IFN-γ release after treatment with wortmannin.

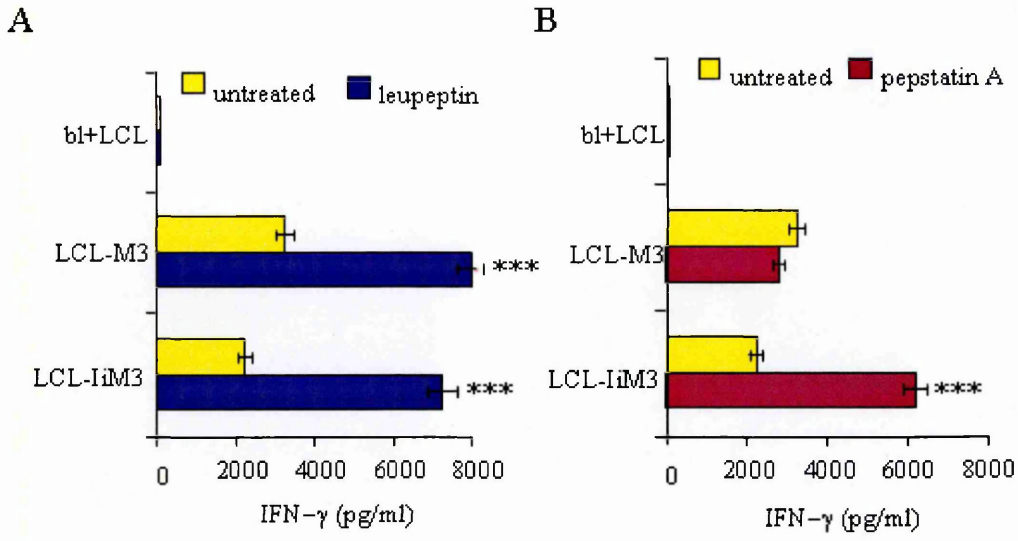

Figure 40. Effects of lysosomal protease inhibition on MAGE-$3_{289-300}$ processing. MAGE-$3_{289-300}$ specific $CD4^+$ T cells were challenged with cells expressing MAGE-3 in the cytoplasm (LCL-M3) or in the endosomal-lysosomal compartment (LCL-IiM3) after treatment with inhibitors of lysosomal proteases. Peptide-pulsed cells were always added as positive controls (data not shown) and blanks (bl+LCL) as negative control. The data are means of duplicate determinations ± SD and are representative of three experiments. Responses significantly higher than the blanks are indicated as: ***p<0,001 (determined by unpaired, one-tailed Student's *t* test). A: IFN-γ release after treatment with leupeptin. B: IFN-γ release after treatment with pepstatin A.

4.4. PATIENTS' RESPONSE TO THE MAGE-3 EPITOPES

To study the role of the examined epitopes in the spontaneous anti-cancer response I tested the responses to MAGE-3 peptides in a panel of eleven advanced melanoma patients. Patients' characteristics are described in Table 2. The MAGE-3 peptides used were 111-125, 146-160, 161-175, 191-205, 243-258, and 281-300 and corresponded to sequences previously identified by my laboratory (19, 193), by others (194, 213, 217) or in the current study as containing naturally processed epitopes, plus peptide MAGE-$3_{171\text{-}185}$ which apparently does not contain a natural epitope.

$CD4^+$ T cells were cultured in the presence of $CD4^+$-depleted PBMCs and each single peptide in several replicates. At day 7, low dose IL-2 was added in culture and the cells were cultured for another 7 days without further antigen stimulation. Naïve $CD4^+$ T cells from cord bloods were also tested to verify if the culture system induced *in vitro* priming. At day 14, the culture supernatant was removed for cytokine (GM-CSF, IL-5 and IFN-γ) release assays and ^{3}H-thymidine incorporation counted.

Naïve $CD4^+$ T cells from cord blood did not significantly proliferate (not shown) or produce cytokines in response to any peptide (Fig. 41).

$CD4^+$ T cells from most of the patients were producing one or another cytokine in the presence or one or more peptides (not shown). To verify if cytokine production was specific $CD4^+$ T cells were cultured as described above and then tested in the absence or presence of peptide and autologous PBMCs.

Of the eleven patients studied three (003, 013 and 015) showed no specific response while the remaining eight (002, 004, 008, 010, 011, 017, 022 and 026) responded in different ways, both as type and quantity of cytokine produced (Figs. 42 and 43). Most patients produced IL-5, suggesting a Th2 response or a mixed response, as in two cases (002 and 004) GM-CSF was also secreted. Three patients (017, 022 and 026) produced

only GM-CSF and therefore probably had not developed a polarised response. Only one (008) showed a clear Th1 response producing IFN-γ only.

Table 3 summarises the patients' responses. The peptide to elicit the highest number of specific responses was MAGE-$3_{111-125}$, followed by MAGE-$3_{191-205}$ and MAGE-$3_{281-300}$. One patient (008) responded to MAGE-$3_{146-160}$, also known to contain a naturally processed epitope (19, 217). One response to MAGE-$3_{171-185}$ was seen (patient 002). No response was seen to MAGE-$3_{243-258}$ or to MAGE-$3_{161-175}$.

For three patients (008, 017 and 022) it was possible to quantify by intracellular staining the enrichment of specific $CD4^+$ T cells after three weeks in culture. In the case of cells specific for MAGE-$3_{146-160}$ the percentage of peptide specific cells was 6.56% (Fig. 44A). Lower but sizeable percentages of MAGE-$3_{191-205}$ (Fig. 44B) and MAGE-$3_{281-300}$ (Fig. 44C) specific cells (*i.e.* 3,24% and 2,83%, respectively) were clearly detectable.

It is worthy of note that MAGE-$3_{111-125}$, the epitope that requires both classes of protease for correct processing, was the one to elicit the highest number of specific responses. Equally noteworthy is that MAGE-$3_{161-175}$ elicited no response at all, probably because aspartic proteases hinder correct processing of the epitope.

Patient	Stage	Neoplastic lesions	MHC-II typing
002	IV	bone, hepatic, pulmonary, splenic, lymph nodes	HLA-DR*04, *13
003	IV	bone	HLA-DR*07, *12
004	IV	head&neck, pulmonary, abdominal, arm, leg	HLA-DR*03, *07
008	IV	pulmonary	HLA-DR*01, *08
010	IV	pulmonary	HLA-DR*11, *16
011	IV	skin	HLA-DR*10, *11
013	IV	abdomen	HLA-DR*01, *13
015	IV	right lombar	HLA-DR*01, *11
017	IV	no evidence of disease	HLA-DR*08, *15
022	IV	lymph nodes	HLA-DR*07, *11
026	IV	pulmonary, lymph nodes	HLA-DR*07, *11

Table 3. Patients' characteristics.

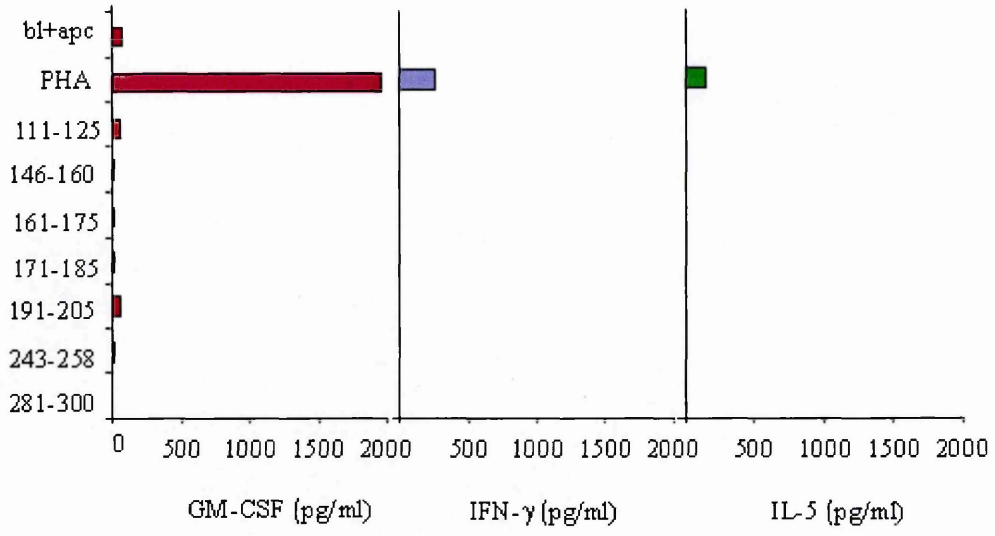

Figure 41. Response to MAGE-3 peptides of naïve CD4+ T cells from cord blood. CD4+ T cells were challenged with each single peptide in the presence of autologous APCs (CD4 depleted PBMCs). After 14 days of culture supernatants were tested for cytokine (GM-CSF, IFN-g and IL-5) release. The basal level is indicated as bl+APC.

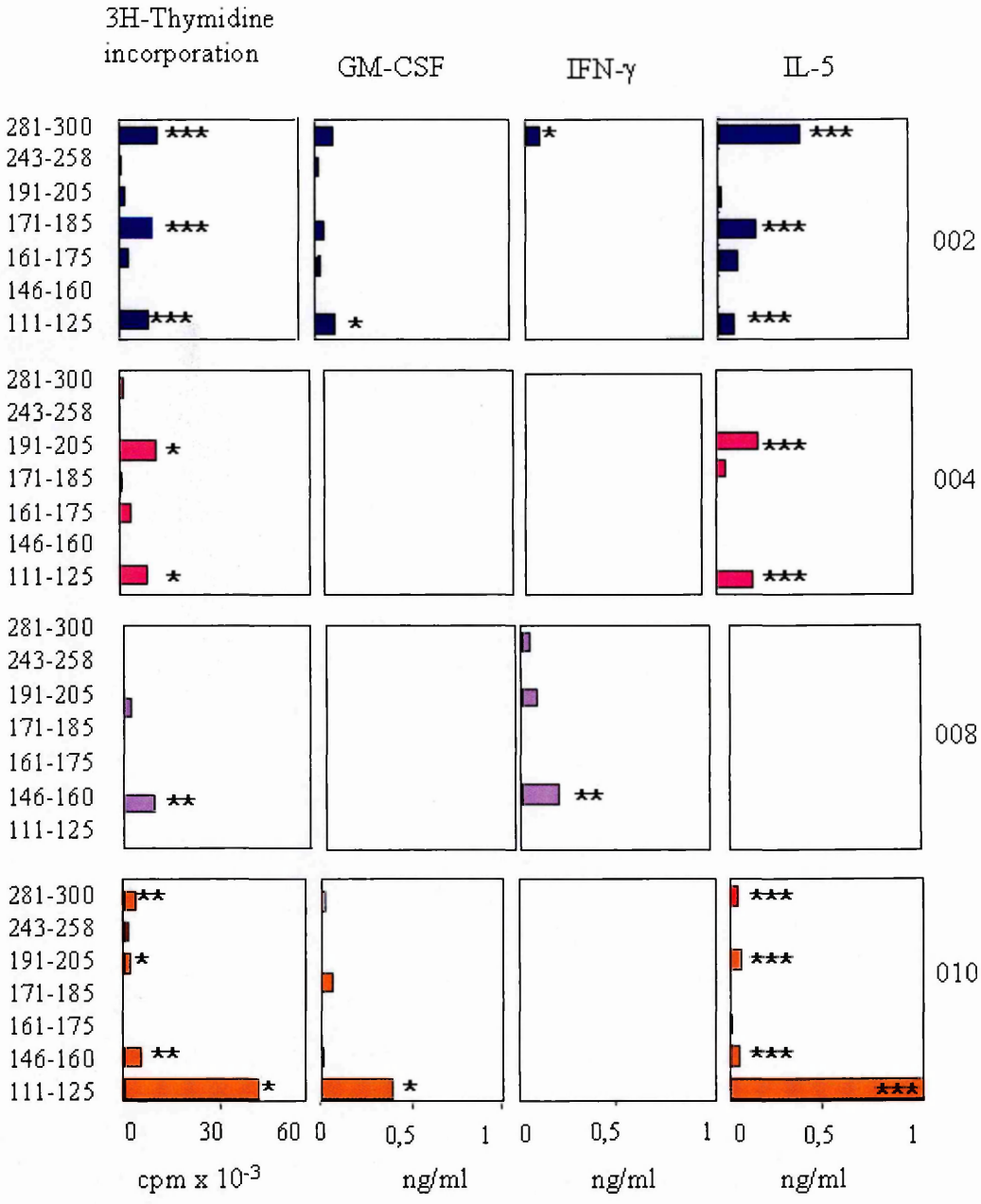

Figure 42. Patients' response to MAGE-3 peptides. $CD4^+$ T cells were purified from the patients' PBMCs and cultured with each single MAGE-3 peptides. After 3 weeks in culture $CD4^+$ T cells were washed and re-challenged with autologous PBMCs in the absence or presence of peptide. The data are means of duplicate determinations ± SD. The blanks (ie, the basal level of proliferation or cytokine release of $CD4^+$ T cells in the presence of unpulsed APCs) have been subtracted from each culture. Responses significantly higher than the blanks are indicated as: *0,05<p, **0,001<p<0,05, ***p<0,001 (determined by unpaired, one-tailed Student's *t* test). The number by each graph identifies the patient.

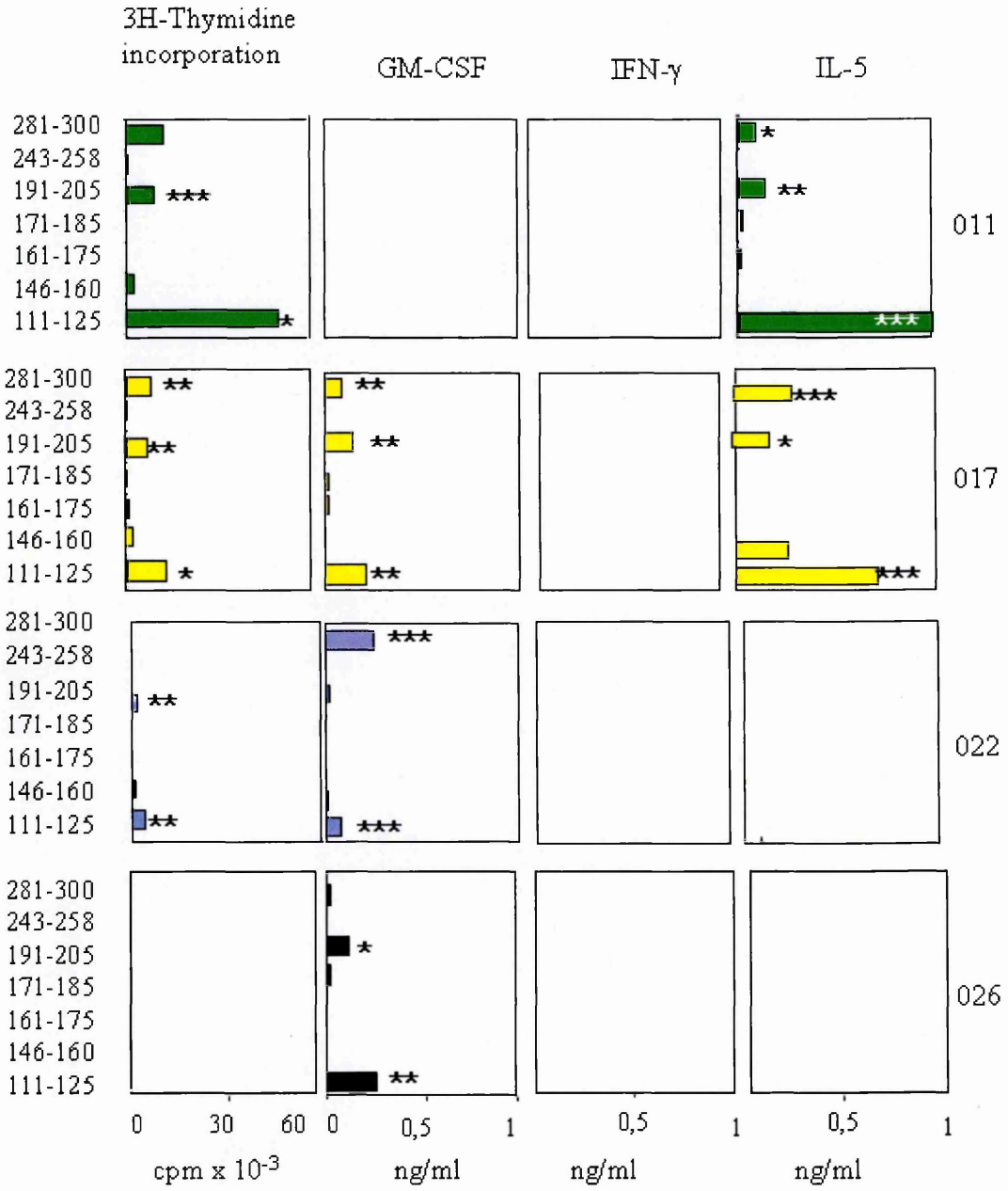

Figure 43. Patients' response to MAGE-3 peptides. $CD4^+$ T cells were purified from the patients' PBMCs and cultured with each single MAGE-3 peptides. After 3 weeks in culture $CD4^+$ T cells were washed and re-challenged with autologous PBMCs in the absence or presence of peptide. The data are means of duplicate determinations ± SD. The blanks (ie, the basal level of proliferation or cytokine release of $CD4^+$ T cells in the presence of unpulsed APCs) have been subtracted from each culture. Responses significantly higher than the blanks are indicated as: *0,05<p, **0,001<p<0,05, ***p<0,001 (determined by unpaired, one-tailed Student's *t* test). The number by each graph identifies the patient.

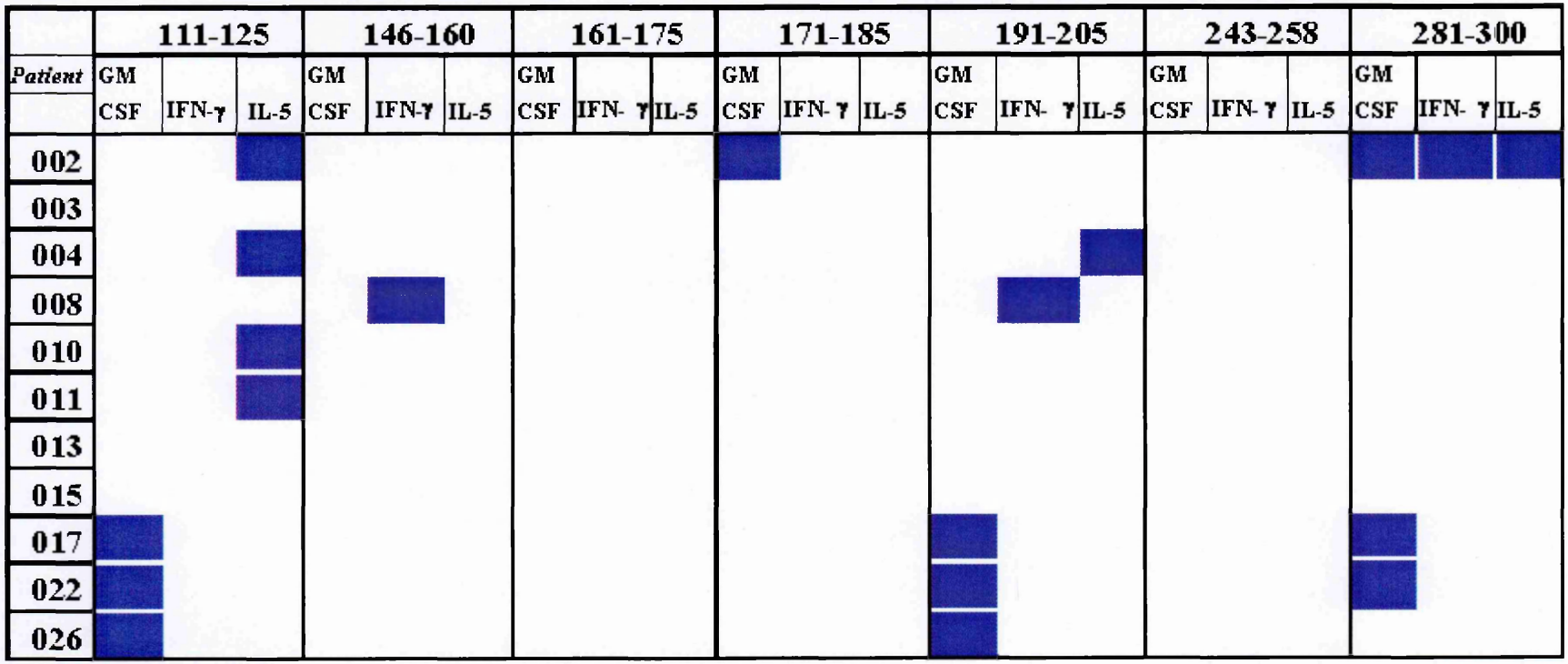

Table 3. Patients' response

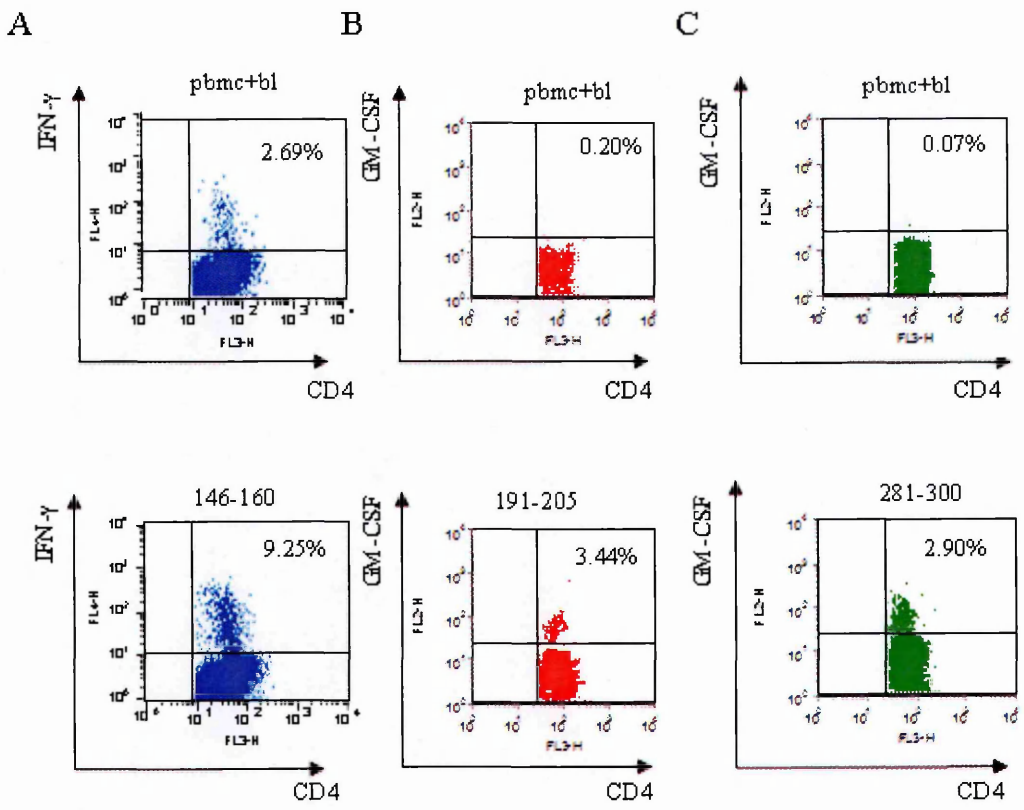

Figure 44. Quantification of the response to MAGE-3 peptides. Specific cytokine production of $CD4^+$ T cells after three weeks' culture. Cells were stimulated for 6 hours before fixing and staining. A: $CD4^+$ T cells from 008 specific for MAGE-$3_{146-160}$ (upper panel: unstimulated; lower panel: stimulated with peptide). B: $CD4^+$ T cells from 017 specific for MAGE-$3_{191-205}$ (upper panel: unstimulated; lower panel: stimulated with peptide). C: $CD4^+$ T cells from 022 specific for MAGE-$3_{281-300}$ (upper panel: unstimulated; lower panel: stimulated with peptide).

5. DISCUSSION

Processing for the formation of MHC-II epitopes has been studied for model antigens (such as ovalbumin) but very little is known about processing of tumor antigens, even the better characterised ones such as MAGE-3, for which many MHC-II epitopes have been described. MAGE-3 was a logical choice to study processing and formation of CD4 epitopes from a tumor antigen with the aim of using that knowledge to manipulate the patients' immune response.

To address this issue three previously identified MAGE-3 regions known to contain epitopes (111-127, 161-175 and 281-300 (19, 193, 213)) were selected to study processing of this antigen through the endogenous and the exogenous pathways.

Earlier work in my laboratory had identified an immunodominant epitope in the 111-125 region presented by HLA-DR*01, DR*04, and DR*11 (19). In this study I showed that MAGE-$3_{111\text{-}125}$ is naturally processed only through the exogenous pathway, as lysate-loaded DCs, but not an HLA-DR expressing matched tumor, elicited a response. Similar behaviour among MAGE-3 epitopes has been reported for MAGE-$3_{114\text{-}127}$, presented in association with HLA-DR*13 (213). The two overlapping regions (111-125 and 114-127) share a common epitope(s), as MAGE-$3_{111\text{-}125}$ specific $CD4^+$ T cells recognised (with similar intensity) APCs pulsed with either of the two peptides (Fig. 20).

Characterisation of the epitope in the 161-175 region in the present work showed that it presented by HLA-DRβ4. Previous work in my laboratory had identified an HLA-DR*04 restricted epitope in this same region that did not appear naturally processed. The HLA-DR β4 restricted epitope is produced only after loading of DCs with lysates of MAGE-3 containing cells or after processing of the whole recombinant

protein. Specific $CD4^+$ T cells also recognised cells engineered to express MAGE-3 in the endosomal/lysosomal compartment, meaning that the epitope is produced through the exogenous pathway. On the contrary HLA-DR β4 matched tumors and LCL-M3 were not recognised.

Characterisation of region 281-300 with truncated peptides showed the presence of three distinct epitopes: 282-294, 284-298 and 289-300. As the previously described for the MAGE-$3_{111\text{-}125}$ and MAGE-$3_{161\text{-}175}$ epitopes, MAGE-$3_{284\text{-}298}$ is also produced only through the exogenous pathway. Cells specific for MAGE-$3_{289\text{-}300}$, instead, were able to recognise cells engineered to express MAGE-3 in the endosomal/lysosomal compartment but also MHC-II expressing tumors, showing that this epitope is naturally processed through both the endogenous and the exogenous pathway. Epitope 282-294 was found not to recognize a naturally processed epitope as specific $CD4^+$ T cells were able to recognise peptide-pulsed cells but not cells that had processed the whole antigen protein. It is possible that this epitope is produced *in vivo* but operated by APC not tested in the reported *in vitro* studies, therefore it might be considered cryptic. Epitopes with these characteristics have been described for other tumor antigens (like SSX-4 (39)) as well and their role in tumor rejection remains to be elucidated.

The results reported for the MAGE-$3_{161\text{-}175}$ stress the importance of using reliable tools when testing recognition of native epitopes by peptide specific $CD4^+$ T cells. Indeed, MAGE-$3_{161\text{-}175}$ specific $CD4^+$ T cells poorly recognised lysate-loaded DCs but showed good response to B cells and tumoral cells expressing MAGE-3 in the endosomal/lysosomal compartment. The difference in recognition intensity could be due to competition among HLA-DR molecules for peptides inside the cell, to different amounts of protein available for processing and to different enzymes active in the intracellular compartments. The three causes are not mutually exclusive.

Within a cell MHC molecules compete for loading of the peptide/protein fragment and as a result the molecules with the highest affinity will bind to the peptide (72). Competition among MHC molecules could account for lower recognition of lysate loaded DCs. It is interesting to note that MAGE-$3_{161-175}$, besides several predicted MHC class II binding epitopes, contains the HLA-A1 (MAGE-$3_{168-176}$) and HLA-B44 (MAGE-$3_{167-175}$) binding epitopes (204, 211). HT144 melanoma cells and autologous DCs express HLA-A1 and HLA-B44, respectively. Furthermore the three different APCs used in this study have HLA-DRβ4 as common allele but express different HLA-DRβ1 alleles (DR*04 in LCLs, DR*04 and DR*07 in melanoma cells and DR*01 and DR*07 in autologous DCs). Therefore competition among several alleles with different binding affinity may have affected the repertoire of epitopes displayed by these cells.

The antigen available could also be relevant. DCs endocytose MAGE-3 from the extracellular environment so the amount of MAGE-3 actually available for uptake (and processing) by DCs from melanoma lysates *in vitro* (or after natural necrosis/apoptosis *in vivo*) could easily be less than that available in transduced cells. Sorting of transduced cells by expression of ΔNGFr allowed selection of two populations of cells with different expression of MAGE-3. The intensity of recognition was proportional to the amount of protein in the cell, strongly supporting the idea that lower recognition of DCs might be related to a lower amount of MAGE-3 to process.

The amount of protein available for processing, however, is not the only factor to determine the number of MHC-II-specific peptide complexes present on the cell surface. Processing is dependent on the environment of the intracellular compartments and this, in turn, is determined by cell type and external influences, such as stimulation by molecules that can affect activity and expression of certain proteases (128, 153, 241). Abundant and widely expressed lysosomal enzymes are cathepsins that can be divided

into two large groups on the basis of the aminoacid in their active site (cysteine or aspartic acid).

Inhibition of cysteine cathepsins abolished presentation by DCs of MAGE-$3_{111-125}$, revealing that at least one of these enzymes is essential for production of this epitope. Similarly, inhibition of cysteine cathepsins considerably reduced presentation of MAGE-$3_{161-175}$. Decrease in presentation was seen in the three different types of APCs (LCL-IiM3, HT144-IiM3 and DCs loaded with lysates from MAGE-3 expressing cells) used. Specific inhibition of cysteine cathepsins B and L led only to a mild decrease in recognition. It is therefore likely that these enzymes are redundant or not involved in processing of this epitope.

I was not able to determine which enzyme is specifically required for processing of the 161-175 epitope. Cathepsin S, given its abundance and ubiquitous presence in different cell types, is a good candidate as 'antigen processor'. Other cysteine cathepsins (such as cathepsin C and H), sensitive to leupeptin, could also be important. It is however likely, given the broad specificity of cleaved substrates, that any of these proteases leads to correct processing of the epitope; indeed, there are very few examples where epitopes from other antigens (such as lysozyme and TTCF (130, 147, 151)) have been shown to require specific proteases for proper processing.

While the effect of cysteine cathepsin inhibition for the two 'exogenous' epitopes was similar, causing decrease in production and presentation, inhibition of aspartic proteases had very different effects. Presentation of MAGE-$3_{111-125}$ was abolished, meaning that an aspartic protease is required for correct production of the epitope, whereas presentation of MAGE-$3_{161-175}$ was surprisingly improved, suggesting that one (or more) of these enzymes has a destructive effect. However, destructive activity of aspartic proteases was not responsible for the lack of recognition of wild type melanoma

cells expressing MAGE-3 in the cytosol. Indeed, melanoma cells were never recognised, even after treatment with inhibitor.

Within the cell production of epitopes from a given antigen is the result of protein cleavage and destruction of an epitope may be necessary for the production of others. Negative effects of enzymes on epitope presentation have been described before (148, 149, 152). The studied region of MAGE-3 is rich in hydrophobic residues and aspartic proteases such as cathepsins D and E exhibit a preference for cleaving such regions (149). Cathepsin D is a good candidate as the protease responsible for destruction of MAGE-$3_{161\text{-}175}$, as it is abundantly expressed in lysosomes whereas data about cathepsin E cellular distribution are contradictory (156, 157). Most importantly, the increase was highest in DCs, suggesting that the low intensity of recognition of these cells could be due to a higher aspartic protease activity (compared to the other APCs used) other than to a lower amount of protein available for processing. The relevance of a possible role *in vivo* of aspartic proteases in destruction of epitope MAGE-$3_{161\text{-}175}$ was confirmed by the study of the repertoire of MAGE-3 epitopes recognised by melanoma patients where no MAGE-$3_{161\text{-}175}$ specific $CD4^+$ T cells were found.

Production of most MAGE-3 MHC-II epitopes, such as MAGE-$3_{289\text{-}300}$, occurs also in cells expressing MAGE-3 as an endogenous protein. Being a cytosolic protein MAGE-3 is a target of the proteasome and of other cytosolic proteases (TPP II and calpains). However, processing does not seem required for production of MAGE-$3_{289\text{-}300}$ by the better characterised cytosolic proteases; indeed, blocking of proteasomal chymotryptic activity by the drug bortezomib improved presentation. Proteasomal activity has been shown to be important for correct production of some MHC-II epitopes (123) but not to hinder it.

Being processed also in the endosomal/lysosomal compartment (where loading of MHC-II molecules takes place), MAGE-3 is a substrate for cysteine and aspartic

enzymes. The effects of these enzymes differ and depend on the route of delivery of the protein. Aspartic proteases do not hinder epitope production if MAGE-3 is an endogenously produced protein. If MAGE-3 is an exogenously delivered protein then both classes of enzyme will destroy the epitope. Nonetheless none of the proteases studied is required for the correct formation of the epitope.

Production of an MHC-II epitope from an endogenous protein could occur after delivery to the endosomal/lysosomal compartment through autophagy, as was seen with neoR (187). Instead, autophagy seems to subtract MAGE-3 from the intracellular compartment where production of the epitope or its precursor occurs.

A comprehensive explanation for the production of MAGE-$3_{289\text{-}300}$ is to assume that, after processing by an unknown or not identified protease in the cytosol, the protein/fragment is carried into the lysosome not by autophagy but perhaps by direct import via TAP-L, a peptide importer present on the surface of lysosomes and directly linking the cytosol to the lysosomal compartment. Here it would be further processed by unidentified proteases and loaded onto MHC-II molecules and carried to the surface.

TAP-L (transporter associated with antigen processing like) is a large protein belonging to the ATP-binding cassette (ABC) transporter family and is related to TAP1 and TAP2, the subunits of TAP (242, 243). Its peptide specificity is very broad, being able to transport peptides ranging from six to fifty-nine aminoacids, with a slight preference for peptides about twenty-three residues long. The fragment containing the 289-300 epitope could be transported into a lysosome through this transporter. Proving this hypothesis is however difficult in the absence of TAP-L specific inhibitors.

The natural response to an antigen reveals how good presentation (and priming) of its epitopes is. The response to MAGE-3 peptides containing epitopes was analysed in a panel of eleven melanoma patients, all in stage IV of disease. Eight of the eleven patients tested showed a specific response to one or more peptides. Seven of the eight

responding patients showed a specific response to MAGE-$3_{111-125}$, five to MAGE-$3_{191-205}$, three to MAGE-$3_{281-300}$, and one to MAGE-$3_{146-160}$ and MAGE-$3_{171-185}$, respectively. No response was seen to MAGE-$3_{161-175}$ in any patient.

The predominant response to MAGE-$3_{111-125}$ and the lack of responses to MAGE-$3_{161-175}$ well reconcile the results that I obtained *in vitro* for processing and presentation of the two epitopes. Indeed MAGE-$3_{111-125}$ was produced by both aspartic and cysteine proteases. Conversely, the MAGE-$3_{161-175}$ epitope was destroyed by an aspartic protease and this effect was particularly pronounced when processing in MAGE-3 loaded DCs was studied, suggesting the importance of the destructive phenomenon *in vivo*.

Experiments of anti-MAGE-3 response *in vivo* in patients also confirmed data obtained so far on naturally processed epitopes. $CD4^+$ T cells specific for MAGE-$3_{191-205}$ could recognise a native epitope produced through both the exogenous and the endogeous pathway (19). Peptide MAGE-$3_{281-300}$ also elicited a response in three patients. It was not possible to verify which of the three epitopes (282-294, 284-298, 289-300) in the 281-300 region was the one to elicit a response, as the peptide used spanned the whole length of that region. Only one patient responded to MAGE-$3_{146-160}$, another region known to contain a naturally processed epitope (19, 217). No patient responded to MAGE-$3_{243-258}$, previously shown by Schultz et al. (194) to be naturally processed; one patient recognised MAGE-$3_{171-185}$ apparently not naturally processed (19).

Taken together, all the experimental data regarding processing of epitopes and response of patients fit very well with previously obtained results and with the suggestion that the destructive effect of aspartic proteases would limit presentation of the MAGE-$3_{161-175}$ epitope.

An interesting element of this study was characterisation of the natural response to MAGE-3 in terms of cytokine production, as previous studies have analysed the

response only in vaccinated patients. GM-CSF, either alone or with IL-5, was secreted by six of the eight responsive patients tested. One patient produced both Th1 and Th2 cytokines; only one patient had a Th1 response. Collectively, these data demonstrate that the spontaneous anti-MAGE-3 $CD4^+$ T cells response in advanced melanoma patients is skewed towards an unpolarised or anti-inflammatory response.

Skewing of cancer response to a Th2 phenotype has been reported in melanoma and renal cell carcinoma (44, 244, 245) and it has been related to an escape mechanism adopted by the tumor itself (for example, by secretion of cytokines like TGF-β).

All patients in this study were in stage IV and the response seen may represent the response in advanced stages of disease. In earlier stages the anti-MAGE-3 $CD4^+$ T cell response might differ both in the epitope recognised and in the cytokine produced. Further studies to investigate this issue are worthwhile.

A more comprehensive identification of the epitopes eliciting the highest number of responses will be obtained by increasing the total number of patients analysed; enrolling patients at different disease stages will enable determination of which epitopes are presented at each stage and of the quality of the response, possibly with correlation to the outcome of the disease. Discovering how every relevant epitope is processed might allow to devise strategies to favour or block its production.

All this information, jointly with the correct understanding of how to overcome tumor escape mechanisms and skewing towards an unfavourable Th type response, will allow manipulation of patients' immune response by more effective vaccination strategies and possibly change of the disease outcome.

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