Molecular mechanisms governing CD4+ T cell recognition of human papilloma virus (HPV) E6 in cervical carcinoma

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SUMMARY

Genital human papillomavirus (HPV) infection is common and the majority of infected individuals successfully clear with this virus. But in less than 5% of the cases the infection by high-risk HPV induces cervical cancer, which is preceded by a phase of persistent HPV infection during which the host immune system fails to eliminate the virus. Effective eradication of HPV-positive tumours may require both CD8+ and CD4+ T-cell-mediated immune responses. Specific CD4 T cell responses against the protein E6 of the HPV16 have been described in healthy subjects, but appear absent in patients with cervical cancer. This suggests the importance of specific CD4+ T-cell responses against E6 protein for the clearance of the virus.

The aim of this project was to investigate the molecular interactions involved in T cell recognition of the HPV16 E6127-141 (DKKQRFHNIRGRWTG) peptide, in particular, mapping the important amino acids for T cell receptor (TCR) and MHC contact.

Initially, this was to be done directly by studying the biophysical interactions between soluble TCR and MHC molecules. Several constructs were made with the pBACgus expression vector in an attempt to generate soluble HLA-DR1/HPV16 E6127-141 complexes. These vectors were used to generate recombinant baculovirus to infect insect cells, but unfortunately no stable MHC:peptide complexes were expressed. .

Since the biophysical approach was unsuccessful, the impact of changes in the peptide structure was assessed using functional T cell activation (IFN-γ secretion). Two separate T-cell clones against HPV16 E6127-141 were tested against truncated versions of the peptide. The results show that the amino acids from the Cterminal end are essential for functional activation of both T-cell lines. Conversely, the amino acids from the Nterminal end do not appear to be as important. These variations in the responses induce by truncated peptides could be due to effects on TCR contact or MHC binding. However, molecular modelling of the HPV16 E6127-141 associated with the MHC complex suggests that the Cterminal end of the peptide contributes to MHC binding.. These two clones were tested against variants of HPV16 E6127-141 from different High risk HPV types (HPV31, 52, 33, 58, 67). Three types of response could be demonstrated. Indeed two peptides (HPV31, 52) have a null effect on the two T-cell clones, two others (HPV33, 58) have a partial agonist effect and one peptide (HPV67), has an antagonist effect on the T-cell clone. Based on these limited results, a model mechanism for viral immune evasion is proposed, whereby co-infection or sequential infection by different high risk HPV types could induce antagonistic effects on T cell responses. This could potentially inhibit effective clearance of a single HPV type, leading to viral persistence and disease.

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ABBREVIATIONS

AcNPV	Autographa californica Nuclear Polyhedrosis Virus
APC	Antigen Presenting Cell
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
bp	base pairs
BLCL	B-cell line (EBV immortalised)
BSA	Bovine Serum Albumin
cADPR	cyclic ADP ribose
CaMK	Calmodulin-dependent kinase
CD	Cluster of Differentiation
CDR	Complementarity Determining Region
CIN	Cervical Intraepithelial Neoplasia
CLIP	Class II Associated Invariant-Chain Peptide
CMV	Cytomegalovirus
COPV	Canine Oral Papillomavirus
CRAC	Calcium-Release-Activated Calcium
CREB	Cyclic-AMP-Responsive-Element-Binding protein
CTL	Cytotoxic T-lymphocyte
DAG	diacylglycerol
DC	Dendritic Cell
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
dNTP	desoxyribonucleotides
ds	double stranded
DTH	Delayed-Type Hypersensitivity
EDTA	Ethylenediaminetetra acetic acid
EBV	Epstein-Barr Virus
ELISA	Enzyme-linked immunoabsorbent assay
ELISpot	Enzyme-linked immunospot assay
ER	Endoplasmic Reticulum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FOXP3+	
GADS	growth-factor-receptor-bound-protein-2-related adaptor protein
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HCV	Hepatitis C virus
HPV	Human Papillomavirus
HR	High risk (Human papillomavirus)
ICAM	Intercellular adhesion molecule
IKK	inhibitor of NF-κB kinase
IL	Interleukin
IFA	Incomplete Freunds adjuvant
IFN	Interferon
Ig	Immunoglobulin
InsP3	inositol-1,4,5-trisphosphate

IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITK	interleukin-2-inducible T-cell kinase
kb	Kilobase
kD	kilo Dalton
LAT	Linker for activation of T-cells
LCR	Long control region
LC	Langerhans cell
LEEP	Loop Electrosurgical Excision Procedure
LFA	Lymphocyte function associated antigen
LPS	lipopolysaccharide
LR	Low risk (Human Papillomavirus)
MCP-1	Monocyte Chemo-attractant Protein-1
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MVA	vaccinia virus Ankara
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
NK	Natural killer cell
OD	Optical density
OR	Origin of replication
ORF	Open reading frame
PAMPs	Pathogen-Associated Molecular Patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PD	hemophilus influenza protein D
PE	Phycoerythrin
pfu	Plaque forming unit
PHA	Phytohaemagglutinin
PI3K	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PtdIns(4,5)P2	phosphadylinositol-4,5-bisphosphate
PRR	Pattern Recognition Receptors
PV	Papillomaviruses
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RAG	Recombination activating protein
RYR3	ryanodine receptor 3
SPL76	SRC-homology-2-domain-containing leukocyte protein
of	76 kD
STIM1	stromal interaction molecule 1
RSS	Recombination Signal Sequences
TAP	Transporter associated with antigen processing
TCR	T-cell Receptor
TGF-β	Transforming growth factor-β
Th	T-helper
TIL	Tumour-Infiltrating Lymphocytes
TLR	Toll Like Receptor
TNF	Tumour necrosis factor

Treg	T-regulatory cell
VLP	Virus-like particles
VIN	Vulval intraepithelial neoplasia
VAIN	Vaginal intraepithelial neoplasia

TABLE OF CONTENTS

INTRODUCTION

In 1910 the first proof of a relationship between viruses and tumours was made. Then, Rous described a malignant chicken sarcoma, which could be propagated by transplanting its cells. These multiplied in their new hosts and formed tumours. The tumour cells yielded a causative virus (Rous 1967). This work was followed by the studies of Shope, who showed that the rabbit papillomavirus could induce giant warts on the skin and they might be true tumours (Shope 1966). Between 1960 and 1970 several studies demonstrated a viral aetiology for certain cancers (Southam 1963). In the 1970s, Harald zur Hausen postulated a role for, and then found HPV-DNA in cervical cancers (zur Hausen 2002, Haverkos, 2005). In the 1980s his group was the first to isolate HPV-16 DNA or HPV-18 DNA in more than 70% of cervical cancer biopsies from german patients (Durst, Gissmann et al. 1983; Boshart, Gissmann et al. 1984). It is now known that HPV-16 and HPV-18 play a major role in the aetiology of cervical cancer, the second most common cancer in women worldwide (Franco and Harper 2005), indeed Walboomers et al showed in a cervical cancer screening study that in 99.8% of cases HPV-16 or HPV-18 DNA could be detected (Meijer, Rozendaal et al. 2000).

1-PAPILLOMAVIRUSES:

Papillomaviruses (PV) were classified in a common family the papovaviruses, it was in the 1980s after sequencing and functional studies that the family "papillomaviruses" (*Papillomaviridae*) became officially recognized by the International Council on Taxonomy of Viruses (Bernard 2005). PVs have been found in more than 20 different mammalian species, as well as in birds and reptiles and their classification is based on nucleotide sequence homology (Doorbar 2005). Because of their medical

importance, HPV have been the most extensively studied, and more than 100 different types have now been identified. To qualify as a distinct HPV type, the nucleotide sequence of the HPV L1 gene must differ from all other HPV types by at least 10% (de Villiers, Fauquet et al. 2004). The viruses are absolutely species specific and also exquisitely tissue tropic with a predilection for infection of either cutaneous or internal squamous mucosal surfaces (Stanley 2001). Furthermore the HPV can be separated into high or low risk types depending upon their oncogenic potential.

Table 1: Example of the most frequently studied papillomavirus types and their biological and clinical properties (Bernard 2005).

Types	Properties
Low risk	
-HPV-2, -27, -57	-Common skin warts, frequently in genital warts of children
-HPV-7, -40, -43	-Low risk mucosal and cutaneous lesions
-HPV-6, -11, -13, -44, -74	-Benign mucosal lesions. Some of these can progress malignantly
-HPV-1, -63	-Cutaneous lesions, frequently in footwarts
High risk	
-HPV-16, -18 -31, -33, -35, -52, -58.	-High risk malignant cervical HPV-16 most prevalent HPV type in cervical malignancy
-HPV-5, -8	-Cutaneous benign and malignant lesions in EV and immune-suppressed patients

Infection of cutaneous epithelia with a low risk HPV leads to the formation of warts, which are hyperproliferative lesions. In this way common skin warts are frequently found in infection with HPV-2, -27, and –57 (de Villiers, Fauquet et al. 2004). Infection with a high risk HPV leads to the formation of carcinomas, for example HPV-5 and HPV-8 induce malignant lesions in epidermodysplasia verruciforms and immune-suppressed patients (Bernard 2005).

About 30 types regularly or sporadically infect the genital tract and they can be divided into those predominately associated with benign ano-genital warts or condylomata, HPV-6, -11 and those associated with ano-genital cancers and intra-epithelial lesions particularly of the cervix (Stanley 2001). The first HPV types isolated directly from cancer biopsies of the cervix were the HPV-16 and –18 (zur Hausen 2002), since then greater than 99% of all cervical cancers have been found to be HPV positive with particular high risk HPV (-16, -18, -31, -45, -52, -58, etc) (Barnard and McMillan 1999). The comparison of HPV type distribution in high-grade squamous intraepithelial lesion shows a prevalence of the HPV-16 and –18 types. This could be interpreted either these types have a greater potential to induce malignant transformation or they have a greater potential to evade the host immune system (Clifford, Smith et al. 2003).

1-1-Epidemiology of the "high risk" HPV infections

Cervical cancer is the second most common cancer worldwide (471 000 annual cases, 233 000 deaths) (Parkin 2001), and high-risk HPV can be found in a growing proportion of patients with cervical cancer, approaching 99%, but is not yet found in every patient with disease (Haverkos 2005). HPV is so common that more than half of all

sexually active adults will be infected in their lifetime, although young, sexually active women bear the brunt of both infection and clinical complications (Ault 2006). In UK, as in other countries, the seroprevalence for high-risk HPV declines with increasing age. Nevertheless, even if the seroprevalence of the high-risk HPV is important in the young population in 80% of the cases the individual clears the infection, but it is not completely understood why some HPV lesions persist and progress in cancer. A number of genetic and/or immunological and environmental co-factors have been involved in increasing the risk of disease progression. Reproductive history, hormonal contraceptive use and smoking (Cotton, Sharp et al. 2007) can play a role in the progression of disease. Furthermore it has been demonstrated that high risks of squamous cell cervical cancer were associated with DRB1*1001, DRB1*1101 and DQB1*0301 and decreased risks were associated with DRB1*0301 and DRB1*13 (Madeleine, Brumback et al. 2002).

1-2-Genome of HPV:

Papillomaviruses are a group of small non-enveloped DNA tumour viruses with a virion size of approximately 55nm in diameter. All the viral particles contain a double-stranded, circular DNA genome of approximately 8 kb that can be divided into 3 major regions: early, late, and a long control region (LCR or noncoding region) (figure 1). The early region of HPV genomes occupies over 50% of the virus genome from its 5' half and encodes six common open reading frames (Zheng and Baker 2006) that translate individual proteins named E1, E2, E4, E5, E6, and E7. The late region of the HPV genome, covering almost 40% of the virus genome, codes for the L1 and L2 proteins. L1 is the major capsid protein and L2 the minor capsid protein (Stanley 2001). The LCR

region, a segment of about 850 bp (10% of HPV genome), has no protein-coding function, but bears the origin of replication as well as multiple transcription factor binding sites that are important in regulation of RNA polymerase II-initiated transcription from viral early as well as late promoters (Zheng and Baker 2006) The individual functions of each protein are briefly summarized in table 2.

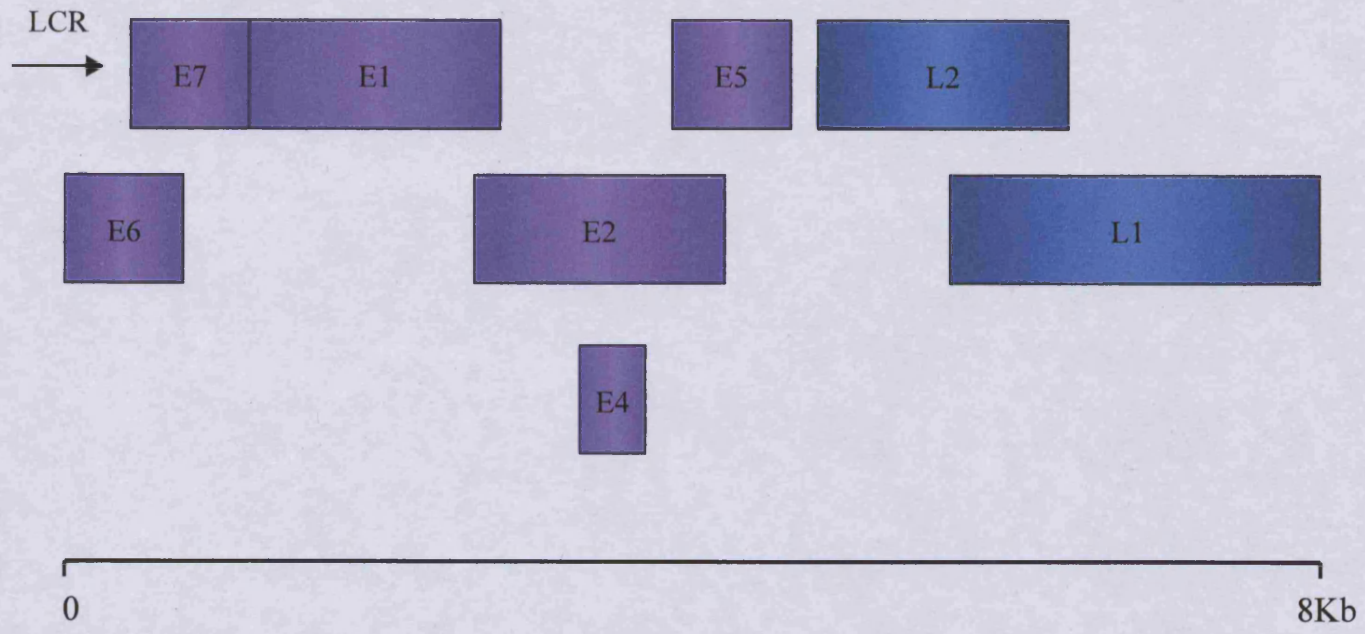


Figure 1:Genome organisation of high-risk HPV16. Early ORFs are indicated in purple, while capsid genes are shown in blue (Stanley 2001)

Table 2: High-risk HPV protein functions

Protein	Proposed functions	Mode of action
E1	Viral genome replication	-DNA helicase and ATPase activity, necessary to unwind DNA at the OR allowing DNA replication (Chiang, Ustav et al. 1992; Hughes and Romanos 1993).
E2	Viral genome replication	-DNA binding transcription factor interacting with motifs within LCR (McBride, Romanczuk et al. 1991). -Acts as either transcriptional repressor or activator (Bernard, Bailly et al. 1989; Demeret, Desaintes et al. 1997). -Interaction with E1 protein vital for OR recognition (Chiang, Ustav et al. 1992).
E4	Cytoskeletal changes	-Interacts with the keratin cytoskeleton causing collapse of the cytokeratin matrix, which facilitates virion release (Doorbar, Ely et al. 1991).
E5	Cellular growth Immune evasion	-Interacts with epidermal growth factor receptor (Crusius, Auvinen et al. 1998). -Inhibits endosome acidification (Straight, Herman et al. 1995). -Down regulates HLA class I and interferes with HLA class II expression (Zhang, Li et al. 2003; Ashrafi, Haghshenas et al. 2005).
E6	Inhibition of apoptosis	-Interacts with p53 leading to its degradation (Scheffner, Werness et al. 1990), with Bak, p300/CBP, MAGI-1... (Mantovani and Banks 2001)
	Keratnocyte immortalisation	-Induces telomerase activity (Klingelhutz, Foster et al. 1996).
E7	Activation of cell cycle Keratnocyte immortalisation	-Binds to Rb and induce its degradation resulting in the loss of cell cycle control at the G_1/S-phase transition (Boyer, Wazer et al. 1996).
L1	Viral capsid protein	-Major coat protein (360 copies per capsid) (Doorbar and Gallimore 1987).
L2	Viral capsid protein	-Minor coat protein (12 copies per capsid) (Doorbar and Gallimore 1987).

1-3-Viral cycle of HPV:

Normal squamous human epithelia grow as stratified layers and only the cells of the basal layers are able to actively divide. During these mitoses one of the daughter cells migrates upward and starts her differentiation while the other remains in the basal layers (Hebner and Laimins 2006). As differentiation progresses, the cell begins to synthesize and accumulate high-molecular-weight keratins. This accumulation induces the nuclear envelope break down and the cell ceases to function and exists only as keratin-filled sacs, and is in the highest strata of differentiated epithelium.

HPV virions initially infect the basal layers of epithelia via micro-abrasions and enter cells via an unidentified receptor; however it is known that heparin sulphate mediates the initial attachment of virions to cells (Longworth and Laimins 2004). HPV infection of the cells leads to the establishment, as episomes, of viral genome in the cellular nucleus (Doorbar 2005). All these events result in the activation of the early HPV promoter and of a cascade of viral gene expression, which ends up with the production of approximately 20 to 100 extrachromosomal copies of viral DNA per cell. The expression of specific "early" viral genes (such as E5, E6 and E7) results in enhanced proliferation of the infected cells and their lateral expansion (zur Hausen 2002). In parallel, one of the infected daughter cells migrates into the upper layers of the epithelium and begins to differentiate, though it remains active in the cell cycle (Hebner and Laimins 2006). As HPV positive cells differentiate, the late promoter is activated and induces the expression of the late genes, which leads to high-level amplification of the viral genome. The virus continues its migration in the epithelium with the daughter cell and in the uppermost

layers, viral DNA is packaged into newly formed capsid and progeny virions are released from the cell (Doorbar 2005).

In a model of mucosal PV infections (inoculation of dogs with COPV) these viral cycles result in the formation of epithelial lesions, between weeks 8 and weeks 12 of infection there is an infiltration of lymphocytes and lesion regression takes place, and at week 16 the infected area has the appearance of uninfected epithelium (Nicholls, Moore et al. 2001). A similar pattern may also occur in humans and the importance of the immune system in controlling the spread of HPV-associated disease is well established, this will be described in section 3.

In absence of regression these lesions of the epithelium can persist and progress to cancer. When we observe the prevalence of the HPV infection in general population the proportion of lesions that progress to cancer is very low, but when the patient does not successfully resolve this infection, such lesions can progress to cervical intraepithelial neoplasia grade 1 (CIN 1), and may progress further to CIN2, CIN3 and cancer (Doorbar 2005). These cellular transformations are principally due to the E6 and E7 genes and their respective proteins.

The HPV viral cycle is summarized in figure 2 and figure 3 shows the expression of HPV genes during the cell differentiation.

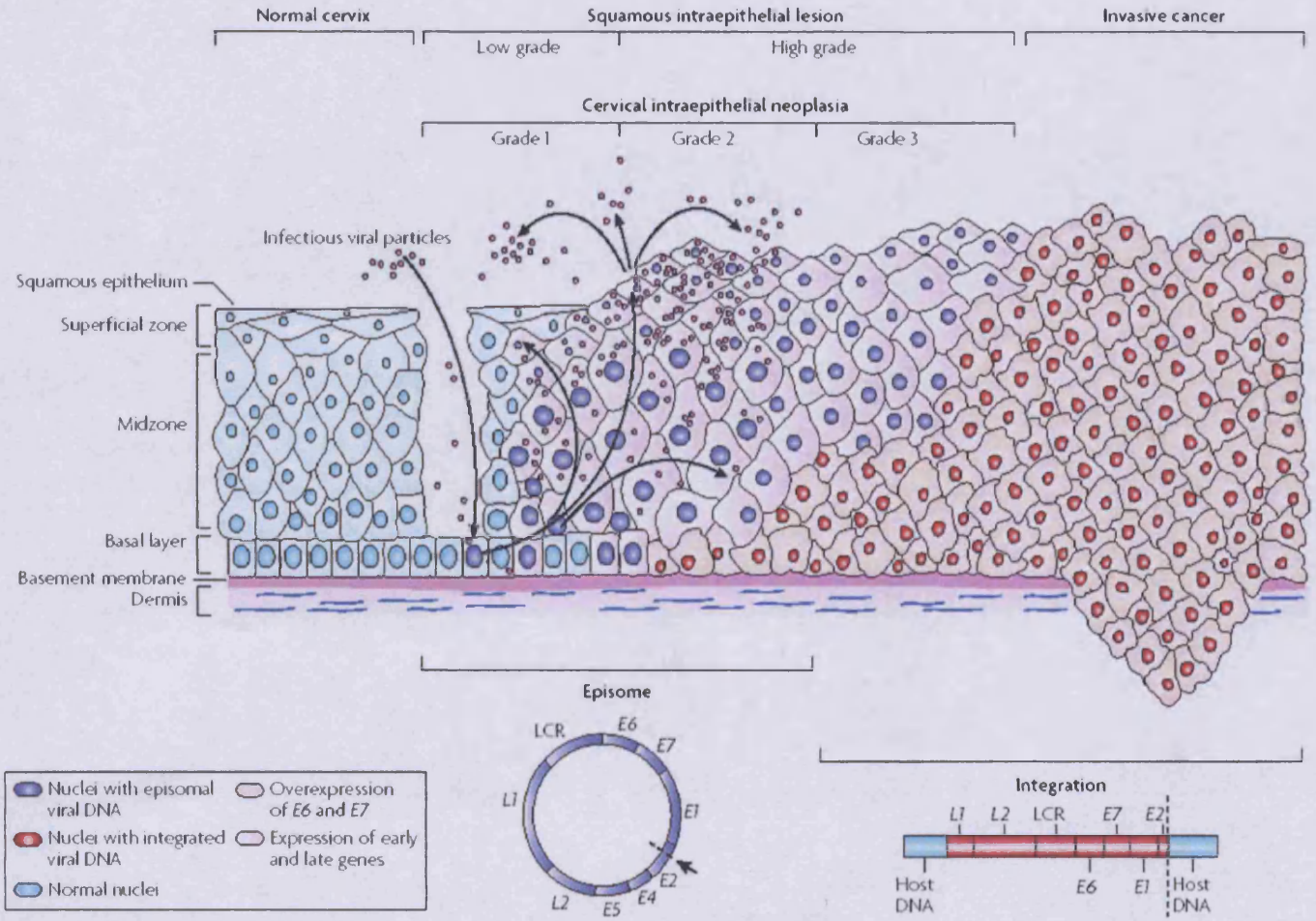


Figure 2: Viral cycle of HPV from Woodman, 2007.

HPV is thought to access the basal cell through micro-abrasions in the cervical epithelium. Following infection, the early HPV genes are expressed and the viral DNA replicates from episomal DNA (purple nuclei).In the upper layers of epithelium the viral genome is replicated further, and the late genes are expressed. L1 and L2 encapsidate the viral genomes to form progeny virions in the nucleus. The shed virus can then initiate a new infection. The progression of untreated lesions to microinvasive and invasive cancer is associated with the integration of the HPV genome into the host chromosomes (red nuclei), with associated loss or disruption of E2, and subsequent up-regulation of E6 and E7 oncogene expression.

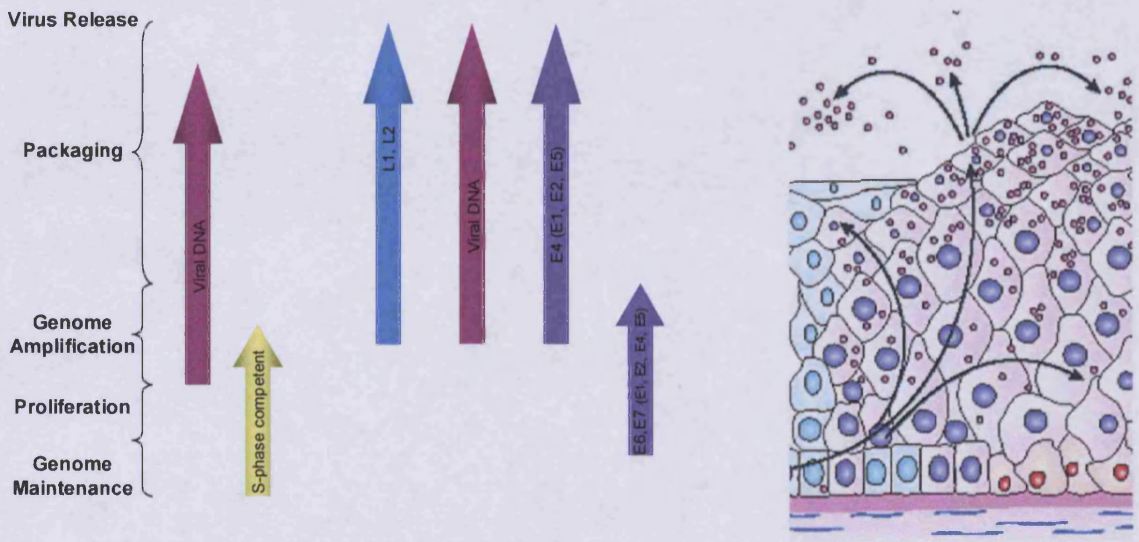


Figure 3: Expression of each HPV genes during infection (Woodman 2007, Doorbar et al. 2005)

1-4-The E6 and E7 genes and their proteins

The E6-E7 region is present in all PVs and contains genes that code for transforming proteins expressed in the initial stages of the infection, before the infected cells start to differentiate (Garcia-Vallve, Alonso et al. 2005).

The E6 proteins of high-risk HPVs contain approximately 150 amino acids and include 2 zinc finger-like motifs Cys-X-X-Cys (Longworth and Laimins 2004). The high-risk E6 proteins are localised to both the nucleus and the cytoplasm of HPV-infected keratinocytes (Hebner and Laimins 2006). The functions of the protein are principally to prevent the induction of apoptosis of the infected cells. In this goal the E6 protein is able to associate with the protein p53, which is a tumour suppressor factor involved in regulation of both the G_1/S and G_2/M cell cycle check point (Oren 2003). E6 binds to p53 in a ternary complex with the ubiquitin ligase, which induces the ubiquitination of p53 and subsequently its degradation by the proteasome. The E6 protein can also induce the decrease of pro-apoptotic proteins including Bak or Bax (Mantovani and Banks 2001). As a consequence, the presence of E6 is considered a predisposition factor in the development of HPV-associated cancers, allowing the accumulation of risk errors in host cells DNA (Doorbar 2005). Furthermore E6 may contribute to the development of metastatic tumours by disrupting normal cells adhesion. A summary of the functions is presented in the figure 4.

The E7 proteins of high-risk are found predominantly in the nucleus of infected cells and are approximately 100 amino acids in size. In transgenic mice it has been shown that expression of this protein by itself is enough to transform cells at a low frequency (Riley, Duensing et al. 2003). One of the main functions of the E7 protein in the HPV

cycle life is the binding and the degradation of the Rb family proteins (Hebner and Laimins 2006). The Rb protein is a negative regulator of the cell cycle that normally prevents the S-phase entry by associating with the E2F family of transcription factors. On progression from G_1 into the S-phase, some cyclin-kinase complexes phosphorylate Rb, resulting in release of Rb from E2F complex and transcription of genes involved in the synthesis of DNA (Longworth and Laimins 2004). The E7 protein induces the destruction of the Rb protein by the proteasome and leads the cells in the cell cycle, the proposed model is E7 binds to and recruits calpain to Rb. E7 activates calpain cleavage Rb, which results in the release of E2F and the degradation of Rb by the proteasome (Darnell, Schroder et al. 2007). E7 can also bind other proteins involved in the cell proliferation including the histone deacetylalases and the cyclin-dependent kinase inhibitors (p21 cip1 and p27kip1), which leads the cell in cellular cycle.

1-5-The natural history of cervical HPV infection

In reality the period between the infection by HPV and the appearance of lesions is highly variable and can vary from weeks to months (Stanley 2001), and fortunately 80% of HPV infections are transient (Schiffman and Kjaer 2003). The natural history of cervical carcinogenesis may be summarised in the following schema:

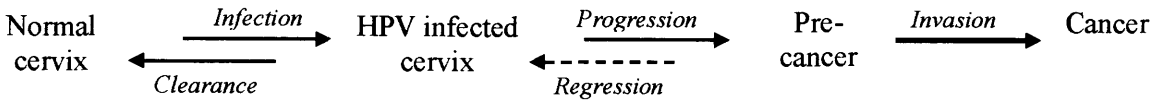


Figure 4: An epidemiologic model of cervical carcinogenesis. The persistence of oncogenic HPV types is necessary for progression and invasion (Schiffman and Kjaer 2003).

Sexual contact with an infected partner is necessary for the transmission of the HPV. HPV must be persistent in host epithelial cells during few months to few years before development of a cervical cancer (Ault 2006). During this phase no HPV DNA is detectable by conventional molecular tests, and in 80% of the cases there is a clearance of the pathogen. In uncommon cases, compared with clearance, the latency phase is followed by a phase of persistence, which is defined by the detection of the same HPV type or variant two or more times over a certain period (between few months and a year). During the persistence phase the lesions can progress to CIN with different grades (1 to 3) (Woodman, Collins et al. 2007). In CIN 1 only the lower third of the epithelium is affected, whilst in CIN 2 the lower two thirds of the epithelial structure is altered. In these low grade CIN the virus is still maintained as an episome in the keratinocytes and the early and late proteins are produced and some new virus is synthesised (figure 2). However, high grade lesions (CIN 3) are associated with an integration of the viral genome in the DNA of the host cell. In several cases this integration occurs in or close to known genes, most frequently in intronic regions (Wentzensen, Vinokurova et al. 2004). This integration is associated with a deregulated expression of HPV E6 and E7 genes in epithelial stem cells, which leads to major chromosomal instability and aneuploidy. The progression from CIN 3 to invasive cancer occurs when transformed cells breach the basement membrane of the epithelial tissue, allowing spread to other anatomical sites.

CIN 1 lesions can spontaneously regress without any treatment in 60% of cases, in contrast the CIN 3 lesions regression occurs in only 33% of the cases (Ostor 1993). For the patients with cervical cancer, the survival rates are closely related to the stage of

disease. Following treatment, the 5 years survival rate of women with invasive cancer is ~20%, whilst for women with very early stage the 5 years survival rate is 95%.

2-IMMUNE SYSTEM

Host defence is a partnership between innate immunity (phagocytes, soluble proteins as cytokines, complement, and epithelial barriers) and adaptive immunity (antibodies, cytotoxic effector cells, and auxiliary cells). Without coordination between both systems an efficient defence can not be observed. Indeed the innate immune system detects the pathogen and acts as the first line of defence.

2-1-Innate immunity

The innate immune response is not specific for pathogen; therefore it has evolved several strategies of self/nonself discrimination, based on the recognition of molecular patterns demarcating infectious nonself, as well as normal and abnormal self.

In vertebrate animals three strategies of immune recognition can be described in terms of recognition of "microbial nonself", recognition of "induced or altered self" and recognition of "missing self" (Medzhitov and Janeway 2002).

2-1-1-Recognition of "microbial nonself" and "induced or altered self"

The basis of microbial nonself recognition lies in the ability of the host to recognize conserved products of microbial metabolism that are unique to micro-organisms and are not produced by the host. Indeed pathogens express conserved molecules that are essential products for their physiology. These invariant structures are

referred to as pathogen-associated molecular patterns (PAMPs). Some known examples of these conserved molecules are lipopolysaccharide (LPS) of the gram-negative bacteria and the peptidoglycan of the gram-positive bacteria. These PAMPs are recognised by receptors of the innate immune system called pattern recognition receptors (PRRs). Nevertheless it is important to note that PAMPs are basically not unique to pathogens and are expressed by both pathogenic and non-pathogenic micro-organisms. This is due to the essential role of these molecules for microbial survival and they are incapable of sustaining mutations. The exact mechanisms that allow the host to "tolerate" non-pathogenic micro-organisms remain largely unknown. It has been hypothesized that compartmentalization (e.g., confinement of micro-flora to the lumina side of the intestinal epithelium), as well as anti-inflammatory cytokines, such as transforming growth factor-β (TGF- β) and interleukin IL-10, have an important role in this process (Medzhitov 2001).

The innate immune system uses various PPRs that are expressed on the cell surface, in intracellular compartments, or secreted into the blood stream and tissue fluids. Currently we can distinguish seven protein families: the C-type lectins, the leucine-rich proteins, the scavenger receptors, the pentraxins, the lipid transferases, the integrins and the toll like receptors (TLRs) (Medzhitov and Janeway 1997). The principal functions of these molecules are the activation of the leptin pathway of the complement, the opsonisation of the microorganism due to activation of the classical complement pathway, phagocytosis, activation of pro-inflammatory signalling pathways and induction of apoptosis. Lot of PPRs are expressed on the cell surface on specialized antigen-presenting cells (APCs), such as dendritic cells (DCs), or macrophages and plays a critical role in the initiation of the adaptive immune response (Medzhitov and Janeway

2002). Indeed these molecules are able to recognize their own specific ligand, which induces the upregulation of cell-surface expression of co-stimulatory molecules (CD80 and CD86) and of major histocompatibility complex class II (MHC II) molecules. TLRs also induce expression of cytokines (such as IL-12), chemokines, their receptors, and trigger many other events associated with DCs maturation. All these upregulations due to the TLRs contribute to the differentiation of activated T cells into T helper T_h1 effector cells. It is not yet known whether TLRs have any role in the induction of T_h2 responses.

2-1-2-Recognition of "missing self"

This concept of "missing self" was proposed to explain why natural killer (NK) cells preferentially kill target cells that express few or no MHC class I. These cells are a type of lymphocyte that mediates innate immunity against pathogens and tumours (Cerwenka and Lanier 2001). NK cells express on their membrane a vast number of receptors that control their activation. Indeed the activation of these cells will depend on a tuned balance between the activating and the inhibitory signals provided by a large number of cell surface receptors belonging to immunoglobulin (Ig)-like and C-type lectin receptor families (Andoniou, Andrews et al. 2006). During some viral infections a down regulation of class I MHC may be observed at the surface of certain cells, this kind of event can disturb the tuned balance between activating and inhibitory signals received by the NK cells and induce their activation, the same profile may be shown in cancer cells (Pena, Alonso et al. 1990). The activated NK cells will mediate their activities through the release of cytokines (IFNγ, TNFα, IL-5, IL-10...) or cytolysis of the targets via the exocytosis of granzymes A and B, and have been believed unable to mount a recall or

memory response when re-exposed to the same antigenic challenge (Andoniou, Andrews et al. 2006).

In conclusion all these recognitions of events also control innate or non-adaptive host defence mechanisms that keep infection in check during the several days required for adaptive immune response to become effective (Janeway 1992).

2-2-Adaptive immunity

Adaptive immunity is mediated by T and B-lymphocytes bearing clonally distributed antigen receptors and these responses are induced by the innate immunity (Medzhitov and Janeway 1997). The recognition of the PAMPs by the PRRs has two actions. In a first instance there is the induction of expression of co-stimulatory molecules on the APC surface, as CD80 and CD86, and the expression of other molecules such as adhesion molecules, which are evolved in the formation of the immunological synapse. In a second instance the production of pathogen-specific signals in the form of MHC-peptide complexes (Medzhitov and Janeway 1997). Furthermore the successful engagement of innate immunity induces an inflammation with the secretion of different cytokines such as IL-1, type I IFNs (α and β), IL-6, TNFα, and IFNγ, which are helpful for the activation of the T cells (Hoebe, Janssen et al. 2004). This cytokine environment associated with the interaction between the TCR and the MHC lead to the linear T lymphocytes differentiation into T_h1 or T_h2 cells. Then in primary immune response activated T cells proliferate and, depending on the duration of stimulation by APCs and cytokines environment, reach hierarchical levels of differentiation characterized by

distinct effector function, homing and survival capacity (Lanzavecchia and Sallusto 2000).

2-2-1-Processing and presentation of the antigen

In the absence of ongoing inflammatory and immune responses, DCs constitutively patrol through the blood, peripheral tissues, lymph and secondary lymphoid organs. In peripheral tissues the DCs take up self and nonself antigens, process them into proteolytic peptides, and load them onto class I or II MHC molecules (Guermonprez, Valladeau et al. 2002). Most of the peptides loaded onto class I MHC are generated by proteasome degradation of newly synthesized ubiquitinated proteins. The resulting peptides are transferred to the ER by specialized peptides transporters, TAP, and load on a new class I MHC under the control of complex composed of several ER resident chaperons (including tapasin, calnexin, calreticulin) (Cresswell, Bangia et al. 1999). Once associated to peptides, class I MHC molecules are rapidly transferred through the Golgi apparatus to the plasma membrane (figure 6).

In parallel to this presentation of endogenous antigens by the class I MHC, another mechanism has been discovered 30 years ago (Bevan 1976), called cross-priming. This process is the presentation of exogenous peptides by the class I MHC (Basta and Alatery 2007). This occurs in the APCs and several mechanisms are proposed to explain this kind of event. One of them is the release of the pathogen to the cytosol from the phagosome or phagolysosome (Kovacsovics-Bankowski and Rock 1995).

Peptide loading on class II MHC occurs through a different pathway. Soon after synthesis in the ER, three α/β MHC class II dimers associate with a trimer of invariant

(Ii) chains, which ensure that ER derived proteins do not bind to the ER membrane anchored MHC II molecule (Cresswell 1996, Romagnoli, 1993). This nonameric complex exits the ER and pass through the Golgi apparatus before being transported to the endocytic pathway, under the influence of transport signals present in the cytoplasmic region of the Ii chain (Guermonprez, Valladeau et al. 2002). Like MHC I, during the formation of these complexes, calnexin is present to add stability to the newly formed molecule (Anderson and Cresswell 1994). Ii has a second function, which starts with the dissociation of MHC II from calnexin and results in the transport of the MHC II molecule from the ER to endosomal compartments for peptide loading. In order for peptide loading to occur, Ii must be removed from the MHC II peptide-binding groove. This is mediated through the activities of cathepsin S (Villadangos, Bryant et al. 1999), which cleaves Ii in a number of steps. Ii is then released from the MHC II molecule through the actions of the class II associated invariant-chain peptide (CLIP), which performs the final Ii cleavage. This also releases the MHC II molecule from the membrane. In order for MHC II to bind to endosomally derived proteins, an MHC-like molecule called DM must first associate. This association leads to the dissociation of CLIP from the MHC II peptide-binding groove, allowing endosomally processed peptide fragments to bind. MHC DM (Kropshofer, Hammerling et al. 1999) also facilitates a process termed "peptide-editing", which ensures that only stable MHC II peptide complexes are expressed and transported to the cell surface for potential TCR interactions (Busch, Doebele et al. 2000) (figure 6). Like MHC I, many self-peptides are presented on the surface of APCs expressing MHC II. With this in mind, the control, or deletion of self-reactive T cells is vitally important for immunogenic tolerance.

The APCs and principally the DCs, which are in contact with the pathogens prime and present the antigen to the T cells via the class I or class II MHC. These mechanisms induce the maturation of the DC, which is characterized by the up-regulation of a number of adhesion molecules, such as ICAM-1, ICAM-3 or LFA-3 as well as co-stimulatory molecules (B7.1 and B7.2), that are respectively required for the migration of the DC and the priming of T cells (Timmerman and Levy 1999).

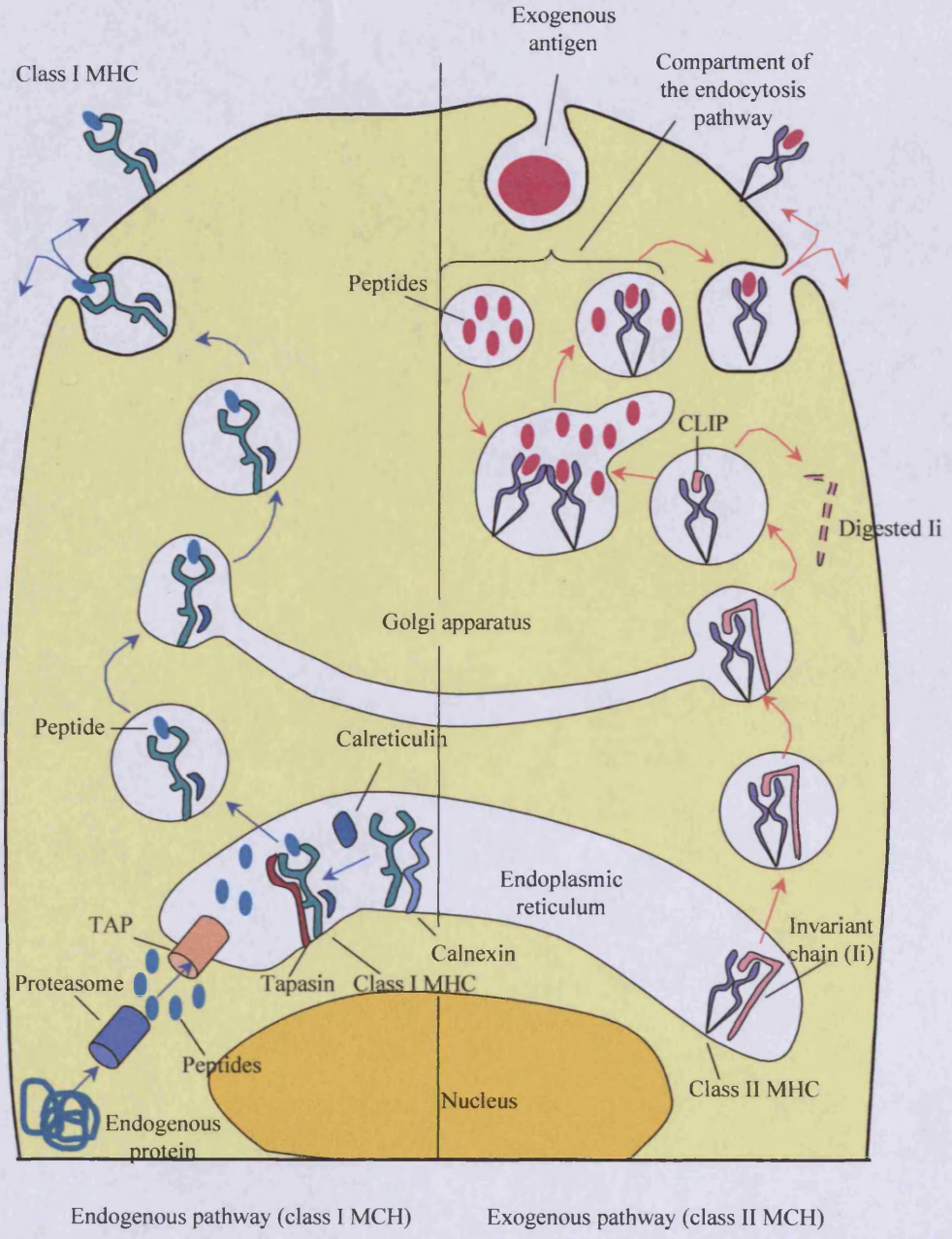


Figure 6: Model of the two pathways for the presentation of the antigen by the class I MHC and the class II MCH (adapted from Immunology, 4th edition, Janis Kuby, 2000).

2-2-2-Genetics and structure of MHC class I

The human HLA region is located on the chromosome 6 and extends over $4x10^6$ base pairs of DNA. It can be divided in 3 regions and the first contains the class I MHC with the HLA-A, -B, -C, -E, -F and –G. These molecules are able to present endogenous peptides derived from an intracellular pathogen to CD8+ T cells. MHC I comprise of a α-domain and β2-macroglobulin (β2m). β2m is comprised mainly of β-sheets, and it associates loosely with the MHC complex (Figure 7A). Peptides are presented in a groove between by the MHC α1 and α2-domains formed from two long α-helices and a β-sheet domain, which provides the floor of the binding groove. The α3-domain, which is comprised mainly of β–sheets, is linked to the cell surface *via* a transmembrane-domain.

The peptide binding groove has been described in terms of pockets (A to F) which form important contacts with the peptide (Figure 7B). Generally, the B and F pockets are the most important in anchoring the N and C terminals, respectively, of the peptide. This interaction is often very selective, with only certain peptide residues in certain positions being compatible with the MHC binding groove. This anchoring stabilises the peptide, allowing central residues to bulge out away from the MHC surface for TCR recognition (Figure 7C) (Saper, Bjorkman et al. 1991; Reid, McAdam et al. 1996; Cole, Rizkallah et al. 2006).

2-2-3- Genetics and structure of MHC class II

These molecules are encoded by the chromosome 6 and are divided in 3 families HLA-DR, HLA-DQ and HLA-DP. These molecules are able to present exogenous peptides derived from an extra-cellular pathogen to CD4+ T cells. MHC II is very similar

in overall structure to MHC class I, although they are formed from a α and β-chain, both with two distinct domains, rather than a three domains α-chain and β2m. (figure 8A). The α1 and β1-domains form the peptide binding groove, which is topologically similar to pMHC I. The main difference between the grooves is in the characterisation of the MHC II binding pockets. For MHC II molecules, the binding pockets are described as 1 to 9 (Jones, Fugger et al. 2006) (Figure 8B), although the main concepts are similar compared to the defined MHC I pockets A to F. The α2 and β2-domains, which are comprised mostly of β-sheets, link the molecule to cell surface, *via* a transmembrane domain, and add to its overall stability. The main structural difference between the two molecules is in the peptide conformation. MHC II peptides are generally more buried within the groove and have a less prominent central bulge, resulting in a theoretically less favourable surface for TCR docking (Figure 8C) (Silver, Guo et al. 1992; Rudolph and Wilson 2002; Rudolph, Stanfield et al. 2006).

The structures of MHC complexes have provided answers to key questions concerning their overall conformation and antigen presentation. From such studies it has become clear that certain MHC types are restricted in terms of the peptide sequences they can successfully present. This is mostly due to the identification of important peptide anchor residues that form specific interactions with the MHC binding groove. In the case of pMHC I, for example, the interactions of peptide position 2 with the MHC B-pocket, and the terminal peptide residue with MHC F-pocket are of particular importance. Therefore, only certain residues at these positions will support stable antigen presentation.

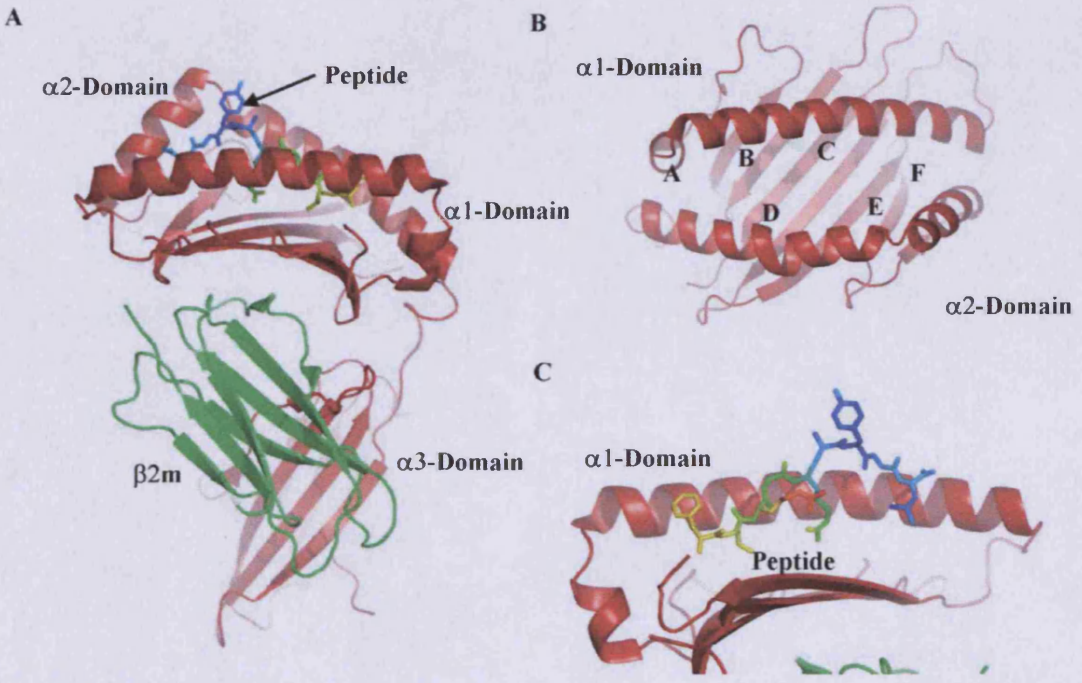


Figure 7: A–Crystal structure of HLA-B8 EBNA to represent the overall structure of pMHC (Cole, Rizkallah et al. 2006). pMHC Is are composed of an α–chain (brick red) and β2m (green), with the peptide (coloured using Wilson B factor which is a measure of residue flexibility (red to blue, high to low)) presented in a 'peptide groove' formed between two α-helices of the α1 and α2-chains. B–The MHC binding pockets (A-F) which form specific interactions with conserved peptide anchor residues. C–The peptide conformation in MHC I molecules is characterised by a prominent central bulge which forms the main platform for the antigen specific TCR interaction.

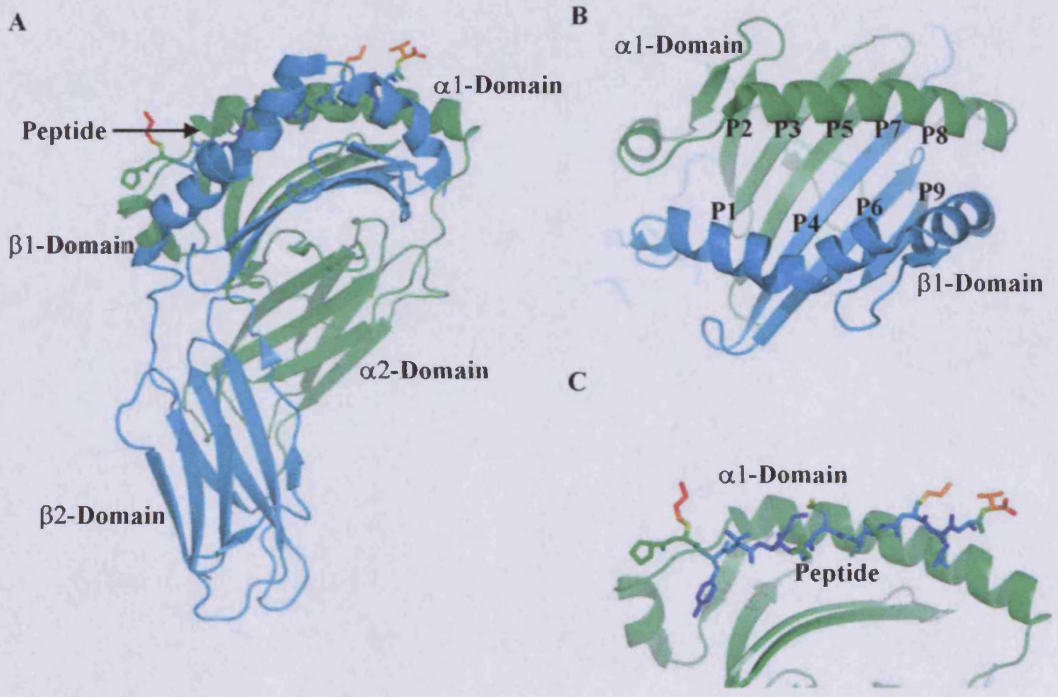


Figure 8: A–Crystal structure of HLA-DR1 HA to represent the overall structure of pMHC II (Hennecke, Carfi et al. 2000). pMHC IIs are composed of a α-chain (green) and a β-chain (cyan), with the peptide (Wilson B factor) presented in a 'peptide groove' formed between two α-helices of the α1 and β1-chains. B–The MHC binding pockets (1-9) which form specific interactions with conserved peptide anchor residues. C–The peptide conformation in MHC II molecules is flatter compared to MHC I, although the exposed surface available for antigen specific TCR interactions is similar.

2-2-4-Structure and functions of HLA class II

Polymorphism in HLA class II molecule, as well class I, is principally located in the amino acids of the peptide binding groove, which contribute to the formation of peptide binding pocket. This polymorphism induces spatial and chemical characteristics of the binding pocket and permits a preferential accommodation of the antigenic peptide in the groove of the HLA class II molecule (Nielsen, Lundegaard et al. 2004). The amino acids residues or the peptide epitopes that interact in the pockets of the binding groove are known as anchor residues and constitute the HLA allotype-specific binding motif. Due to this polymorphism antigen-specific CD4+ T cells exist at a very low frequencies in patient peripheral blood, frequently in the range of 1:6,000 to 1:100,000 (Nepom, Buckner et al. 2002). To identify and analyse these cells the traditional functional assays, such as antigen-induced proliferation or cytokine secretion, can be used. New techniques using MHC tetramers can now be added to the immunological toolbox to expand these types of assays, improving sensitivity and quantitation. Indeed different studies have used this technology for the enumeration of CD4+ T cells specific for viral, bacterial and auto-antigens (Novak, Liu et al. 1999; Meyer, Trollmo et al. 2000; Reijonen, Novak et al. 2002).

Nevertheless this technology of tetramers can necessitate different approaches due to the complexity of the molecule. Class II molecules are non-covalent dimers of α and β chains, which have a variable range of stability and solubility in solution. Detection of individual CD4+ T-cells based on their antigen specificity has been hampered by 2 significant barriers: the low frequency of such CD4+ T cells in periphery and the low avidity of binding between the TCR and the MHC-peptide complex (Nepom,

Buckner et al. 2002). A third difficulty results in the production of the molecule, indeed three strategies can be used are: the expression of the α/β chains by *E. coli* followed by the folding of the molecule in a buffer containing the peptide, the synthesis of the molecule associated with the peptide by insect cells or the production of the HLA class II by mammalian cells. All these techniques have different steps and the solubility of the molecule or the avidity of the peptide to the TCR, are as many difficulties in the production of a HLA class II associated with a peptide.

2-2-5-The T cell receptor (TCR)

The TCR is an antigen-specific membrane-bound molecule present on the surface of T-cells. Between 90 and 99% of peripheral blood T cells express TCR αβ chains, whilst the remaining population express TCR γδ chains. The TCR β-chain is encoded by four gene segments termed D (diversity), J (joining) and V (variable), which combine with the more conserved C (constant) region (Kronenberg, Siu et al. 1986) to make up the full β-chain under the influence of recombination activation genes (RAG1 & 2) and terminal deoxynucleotidyl transferase (TdT).

The DJ genes recombine first, involving the joining of the D_β gene segment to one of six J_β genes. There are two possible pools of genes that encode these segments (1 and 2) from each chromosome pairing, so recombination can occur from either set. A V_β gene then recombines to the $D_\beta J_\beta$ segment to encode the variable (V) region of the β-chain. This $V_\beta D_\beta J_\beta$ gene is then incorporated with the C_β segment to encode the full β-chain. Due to the presence of additional D_β and J_β segments upstream of the C_β segment, unsuccessful $V_\beta D_\beta J_\beta$ rearrangements, i.e. those that do not encode a functional β-chain,

can be rescued by further rearrangements which increase the chance of successful β-chain expression. The β-chain then associates with a surrogate α-chain, pTα, to form a pre-TCR, which is transported to the cell surface (Figure 9B). During this maturation stage, T cells express both CD4 and CD8 (double positive). The α-chain forms in an identical manner to the β-chain, except that it lacks the D region. Therefore, $V_\alpha J_\alpha$ recombination occurs first to encode the V domain, followed by $V_\alpha J_\alpha C_\alpha$ recombination to encode the full α-chain (Krangel, Hernandez-Munain et al. 1998). Similarly to the β-chain, the α-chain may undergo rescue gene rearrangements if the initial recombination is unsuccessful. In fact, due to the presence of multiple V_α and J_α genes spread over some 80 kb of DNA, the number of α-chain rearrangements that can occur is virtually endless and invariably results in either the production of a successful α-chain or the prolonged lack of an activation signal leads to T cell death.

The V, D, J genes are clustered together at specific loci on chromosome 9. In order to splice these genes together during recombination, recombination signal sequences (RSSs) are required. These signals are composed of seven conserved nucleotides that reside next to the gene encoding sequence. These RSSs, present on the 3', or 5' end of adjoining gene segments, are recognised by RAG1 and RAG2, which initiate the recombination. These segments are spliced together as an mRNA transcript to allow translation of the full length protein (Figure 9C). The two complete chains (α and β) combine, via disulphide bonds, to form a TCR capable of binding to peptide/self major histocompatibility complexes (pMHCs), presented by cortical epithelial cells, in the thymic stroma (Fink and McMahan 2000). Successfully binding T cells receive survival signals from these cells in a process called positive selection. Thymic nurse cells then

present the naïve T cells, now single positive for either CD4 or CD8, with self-antigens acquired during embryonic development in a process termed "central tolerance". T cells that express TCRs that bind too strongly to these self-antigens are clonally deleted in the thymus. Negatively selected T cells must bind adequately weakly to these self antigens before migrating into the periphery (Sprent and Kishimoto 2002). Thus, a peripheral population of T cells is generated with the ability to respond to foreign antigen, whilst generally remaining non-reactive to self-antigen (Figure 9D). The resulting T cells are responsible for identifying a range of antigens and responding to them appropriately.

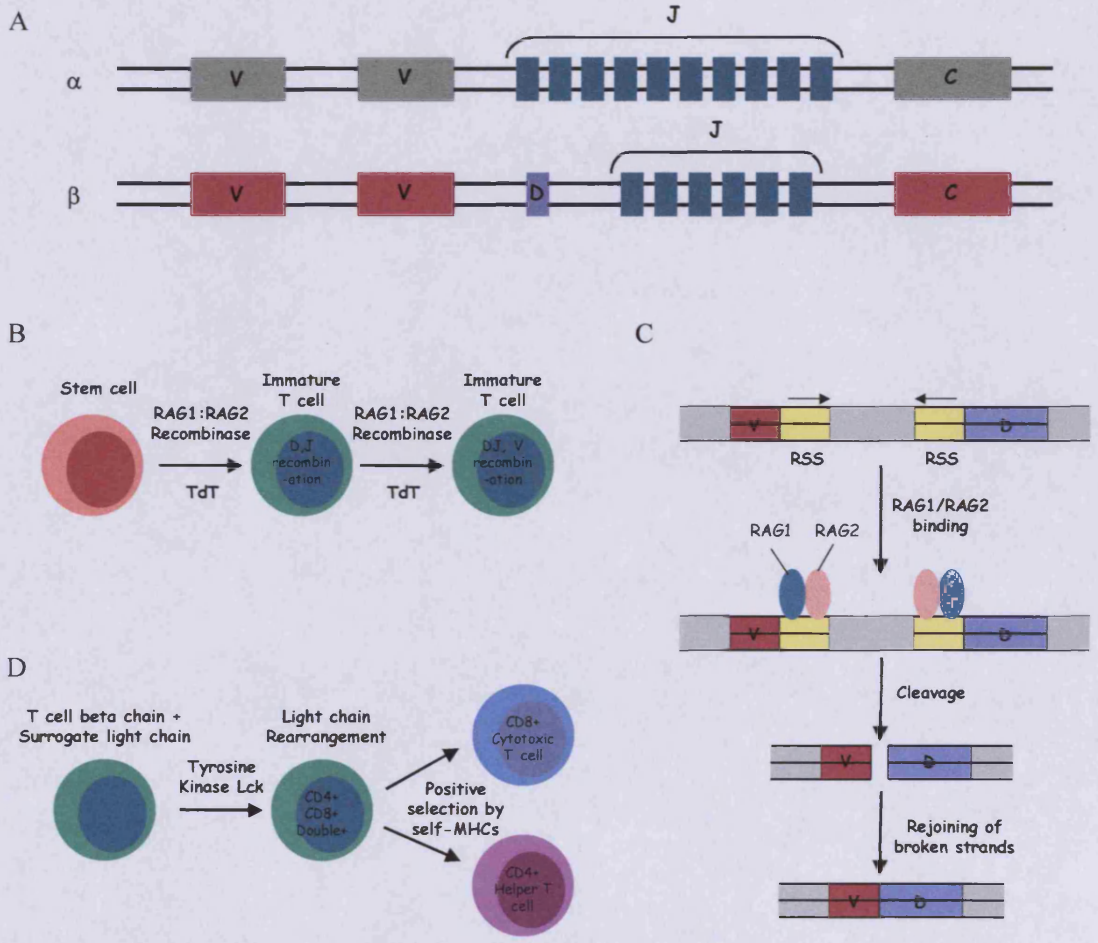


Figure 9: A-Gene locus of the TCR α and β-chains. The α-chain V domain is encoded by the V and J gene clusters, and the C domain is encoded by the C gene. The β-chain genes are similar to α-chain, except that the β-chain contains D gene. Furthermore, the α-chain contains a large number of V and J genes that can be used to increase the number of possible V domain rearrangements. B-T cells first express a β chain, which occurs *via* the rearrangement of the D_β and J_β genes encoding the variable domain, involving the activities of RAG1/RAG2 recombinase. Joining of these segments requires the activities of TdT. Successful rearrangement of the variable (V_β) domain precedes TdT mediated joining to the C_β region, which encodes the constant domain. The β-chain then associates with a surrogate α-chain (pTα) and CD3, creating a pre-T cell receptor, which is transported to the cell surface. C- RAG1 and RAG2 enzymes recognise RSSs that flank the gene encoding DNA segments at the 3' and 5' ends of adjoining gene segments. The broken strands are then rejoined under the influence of TdT to produce full α or β-chain encoded by the spliced genes. D- T cells, under the influence of tyrosine kinase Lck, start to express CD4 and CD8 ($CD4^+CD8^+$ double-positive). Multiple α-chains are expressed until positive selection occurs *via* the binding of the TCR to self-pMHC, presented by cortical epithelial cells in the thymic stroma. After positive selection, the developing T cells loose either CD4, or CD8 expression to become single positive adapted from Garland and Geoffrey.

2-2-6-T-cells

The interaction between the TCR and a class I or class II MHC associated with a peptide result in T cell proliferation and differentiation into a variety of cell fates that determine the class of immune response (Lanzavecchia, 2000). The CD4+ T-cells can polarize toward T helper 1 (T_h1) or T helper 2 (T_h2). The CD8+ T-cells differentiate into cytotoxic cells (CTL) capable of killing virus-infected cells.

CD4+ T helper:

T-cell responses are initiated in the T-cell areas of secondary lymphoid organs, where naïve T-cells encounter antigen-loaded DCs. The duration of TCR stimulation represents the driving force for T-cell activation, differentiation, and ultimately death (Lanzavecchia, Lezzi et al. 1999). Naïve T-cells require stimulation for approximately 20 hours in order to be committed to proliferate, if the cells receive excessive stimulation they die by activation-induced cell death, whereas cells receiving insufficient stimulation may remain non-fit and die by neglect. The fittest cells survive and enter the memory pool as central and effector memory T-cells (Lanzavecchia and Sallusto 2000). In presence of pathogen many DC precursors are continuously recruited into inflammation site and from there continuously migrate to the draining lymph nodes where they arrive endowed with T cell-stimulatory and –polarizing capacity (Robert, Fuhlbrigge et al. 1999). This stimulation of the naïve CD4+ T-cells influences their differentiation in T_h1 or T_h2 cells, indeed low doses of antigen favour T_h2 development while higher doses favour T_h1 development. Peptides that bind with high affinity to the TCR induce a predominant T_h1 phenotype in subsequent effectors, while low affinity peptides induce

T_h2 cells (Noble 2000). Associated with these interactions between the TCR and the peptide/MHC complex, the cytokine micro-environment plays an important role in this polarization. Schematically, the APCs in response to the antigen type secrete IL-12, which leads to differentiate in T_h1 or IL-6, which leads to differentiate in T_h2 (Kidd 2003). The T_h1 cells drive the type-1 pathway ("cellular immunity") to fight viruses and other intracellular pathogens, eliminate cancerous cells, and stimulate delayed-type hypersensitivity (DTH) skin reaction. T_h2 cells drive the type-2 pathway ("humoral immunity") and up-regulate antibody production to fight extracellular organisms; type 2 dominance is credited with tolerance of xenografts and of the foetus during pregnancy.

CD8+ T cells:

The development of cytotoxic T lymphocyte (CTL) responses is necessary for the control of a variety of bacterial and viral infection. The stimulation of naïve CD8+ T-cells by a peptide/class I MHC in the lymph nodes induce their expansion and their migration to the infection site where these cells clear the pathogen by killing infected cells and secreting effector cytokines. After the resolution of the infection most of effector CTL die, leaving 5-10% of the original burst size as long-lived memory cells (Williams and Bevan 2007).

There is a great deal of evidence that during the stimulation of the naïve CD8+ T-cells, CD4+ T-cell help plays an extremely important role in the generation of effective CD8+ T cell immunity (Clarke 2000). Keene and Forman (Keene and Forman 1982) proposed that the purpose of this three cells interaction was to facilitate delivery of short-range cytokines, such as IL-2, secreted by the CD4+ helper T cells to the antigen-specific

CD8+ T-cell. A second hypothesis, that help for CD8+ T-cell activation is supplied indirectly, *via* CD4+ T cell-mediated up-regulation of APC co-stimulatory activity. Indeed the interaction between CD40L on activated CD4+ T-cells with the CD40 on APCs has been shown to induce a variety of co-stimulatory molecules (Clarke 2000).

The functions of the CTL are to kill infected cells or cancer cells, the interaction between the class I MHC associated with the restricted epitope induce a re-activation of the CTL which result in the death of the infected cell. The principal mechanism through which a CTL kills is release of pre-formed cytotoxic granules containing perforin and granzymes (Podack, Konigsberg et al. 1985). Perforin creates pores in the plasma membrane of the infected cell that firstly encourages osmotic lysis of the cell, and secondary permits the entrance of proteolytically active granzymes leading to apoptosis. CTL can also induce apoptosis of the target cells *via* the FAS/FAS-L pathway which activates the caspases (Rouvier, Luciani et al. 1993).

Regulatory T-cells:

In addition to the T_h1 and T_h2 populations there is another CD4+ T-cell population named regulatory T-cells (Treg). These cells are considered to play a central role in self-tolerance (Pakravan, Hassan et al. 2007). They have been categorized into two major subgroups based on their ontogeny: the naturally Treg (FOXP3+, CD4+, CD25+), which develop in the thymus and are present in a naïve mice and healthy individuals from birth, and the inducible Treg, which are generated in the periphery under various tolerogenic conditions.

The natural Treg recognize self antigen *via* their TCR and their principal function is to suppress the activation of auto-reactive T_h1, T_h2 and CD8+ T-cells by cell-cell contact. The inducible Treg appear after encounter with antigen in presence of IL-10 and they have a very low proliferative capacity following activation (Roncarolo and Battaglia 2007). These cells regulate immune responses through the secretion of TGFβ and IL-10, thy will suppress both naïve and memory T-cell responses and down-regulate the expression of co-stimulatory molecules and inflammatory cytokines by APCs (Roncarolo, Gregori et al. 2006).

γδ T-cells:

The majority of mature T cells expresses an $\alpha\beta$ TCR which recognizes peptides presented on the surface of antigen-presenting cells (APC) to $CD8^+$ T cells by MHC class I or to $CD4^+$ T cells by MHC class II molecules. In addition, a sizeable subset of $CD3^+$ T cells carries an alternative TCR heterodimer composed of γ and δ chains. In the peripheral blood of healthy adult individuals, $\gamma\delta$ T cells account for 1–10% of circulating T cells, whereas $\gamma\delta$ T cells comprise a much higher proportion of T cells in other anatomical localizations, notably in the epithelia of the small intestine (Hayday, 2000). Two features are fundamentally different between conventional $\alpha\beta$ T cells and $\gamma\delta$ T cells. In contrast to $\alpha\beta$ T cells, human $\gamma\delta$ T cells do not (or only rarely) recognize peptides processed from complex protein antigens by professional APC but rather see unconventional antigens such as phosphorylated microbial metabolites and lipid antigens. These characteristics explain their role in innate immunity and not in adaptive immunity (Beetz, 2008).

2-2-7-B-cells and humoral responses

Humoral immunity is mediated by the production of antibodies by activated B-cells. This is the contact between the T-cells and the B-cells *via* CD40/CD40L, which induce the activation of the B-cells, and this is enhanced by antigen-receptor engagement and by interleukines such as IL-4 (Hodgkin and Basten 1995). This interaction undertakes a tightly regulated proliferation and differentiation program in which antigen-specific memory B cells and effector plasma cells are generated. The plasma cells secrete large amounts of antibodies, which are soluble but otherwise identical versions of the membrane-bound B cell receptor. In the first encounter with antigen, a primary antibody response is generated; later, a re-encounter with the same antigen causes a more rapid secondary response, producing high levels of antibodies with a high binding affinity for the target antigen (Bernard 2005).

3-IMMUNE SYSTEM AND HPV

Epidemiologic and natural history studies strongly suggest that the human immune response to HPV infection follows a similar pattern (Schiffman and Kjaer 2003). Virtually all studies show that genital HPV infection is extremely common in young sexually active women, with a prevalence as high as 80% in certain adolescent populations (Moscicki, 2007). For most of these HPV infections DNA for a specific HPV type can no longer be detected. The time for this is between 8 and 14 mouths for HPV16 infection and 5 to 6 months for low-risk HPV.

3-1-Immunity against HPV

The cervical epithelium contains Langerhans cells (LC), which are a type of DC and act as the principal APCs into this site. LCs express HLA class I and HLA class II on their surface and react positively to HLA-DR antigens. They capture the HPV antigen and migrate to the the lymph nodes, where they present the antigen associated with a class I MHC or a class II MHC to the lymphocytes (Goncalves and Donadi, 2004). The role of T-cell immunity in controlling infection by various HPV types is further indicated by the increase incidence of HPV infections, HPV-associated warts, CIN lesions and cancers in immunocompromised patients such as transplant patients or HIV seropositive subjects (van der Burg, de Jong et al. 2002).

Furthermore different studies investigating the spontaneous regression of genital warts and cervical lesions have provided a more detailed picture of the immune response during the lesions clearance. Indeed Coleman *et al.* showed that regressing warts contained more T lymphocytes and macrophages than non-regressing control. They characterised these T cells and observed a predominance of CD4-positive lymphocytes with a greater expression of activation marker in regression lesions (Coleman, Birley et al. 1994). It was shown that pro-inflammatory molecules, such as TNFα and IFNγ, dominated the cytokine environment. Recent studies observed a correlation between a spontaneous regression of established HPV16 high-grade squamous intraepithelial lesions of the cervix, and a HPV16 E7 peptide-specific CD4+ T cell response (Peng, Trimble et al. 2007). Studies observed in HPV patients treated for CIN showed that recurrence-free survival is associated with a cellular immune response specific to synthetic peptides from the E6 and E7 proteins (Sarkar, Tortolero-Luna et al. 2005).

3-1-1-CD4+ T-cell responses

Immunohistological studies on chronological series of biopsies from beagles experimentally infected with canine oral papillomavirus (Nicholls, Moore et al. 2001), indicate clearly that regression of infected lesions is associated with a T_h1 response but the viral antigens, which provoke this response, are not known (Stanley 2001). The T_h1 response is associated with a cytokine environment dominated by pro-inflammatory cytokines such as IL-12, TNFα or IFNγ, and an up-regulation of the adhesions molecules required for lymphocytes trafficking on the endothelium of the wart capillaries (Bernard 2005). Nevertheless there is no clear understanding regarding the relationship between the responses detected and the clearance or persistence of HPV disease, however some patterns are emerging.

Indeed different studies in healthy donors show a spontaneously CD4+ immunity against HPV18 E6 epitopes or HPV16 E6 epitopes (Welters, de Jong et al. 2003, Facchinetti, 2005, de Jong, 2004) which could be a memory response and associated with a clearance of the infection. Inversely the patients infected by HPV18 or HPV16 fail to demonstrate a T_h1 response against the E6 protein (Welters, van der Logt et al. 2006) or have response less high than in healthy donors (de Jong, van Poelgeest et al. 2004). These studies suggest that the absence of effective E6-specific CD4+ T-cells in early stage of HPV infection could contribute to the progression to cancer. Other studies observed a correlation between the tumour-infiltrating lymphocytes (TIL) in cervical cancer and the regression or progression of the tumours (Sheu, Chang et al. 2007). Clinically, a decrease

in the number of infiltrated CD4+ T cells is highly correlated with a rapid tumour growth and lymph node metastasis in cervical carcinoma (Sheu, Hsu et al. 1999).

Cross-sectional analysis of CD4+ T-cell responses against HPV16 E7 protein, show exactly the inverse profile. Indeed T_h1 responses against E7 are not observed or rarely in healthy donors, but frequently occur in HPV16 positive patients with cervical disease (van der Burg, Ressing et al. 2001). Longitudinal studies have provided evidence that T_h responses against E7 could be correlated with progression to cancer or clearance of the infection (de Gruijl, Bontkes et al. 1998). It was proposed that the responding T_h cells were generated in response to an increase in antigen exposure, which could arise from either lesion progression (increased burden with E7 expressing infected or transformed cells), or as result of detection and clearance of the HPV infected cells.

3-1-2-CD8+ T-cell responses

The profiles of the CTL responses against the E6 and E7 proteins seem contradictory between various studies, that is why the relationship between CTL responses and disease clearance or progression has not been resolved.

In some studies, CTL reactivity against both HPV16 E6 and E7 was predominantly found in patients that cleared infection (Nakagawa, Stites et al. 2000). In other studies, especially patients with persistence infection or progressive disease displayed CTL reactivity (van der Burg, de Jong et al. 2002). Another study of our group has demonstrated previously that short-term *in-vitro* culture with the HPV16 E7 $_{(11\text{-}20)}$ peptide, expanded E7-specific CD8+ T-cells to a greater extent in the PBMC from CIN3 patients than from healthy donors (Youde, Dunbar et al. 2000). In contrast, Gurski et al.

were able to detect HPV16 $E7_{(11-20)}$-specific CTL responses in 3/6 healthy women but not in cervical cancer patients (Gurski, Schirmer et al. 1997). All these studies demonstrate the need for longitudinal studies using standardised immunological assays and containing larger cohorts of women with varying severity of cervical disease, to gain a better understanding of the role of HPV-specific CTL in protection from viral persistence and disease progression.

3-1-3-B-cell responses

The role of the humoral immune response in HPV infections has started to be clarified recently. Antibodies specific for HPV proteins L1 and L2 can be detected from HPV experienced individuals using virus like particle (VLP) based ELISAs. IgG is the most abundant antibody isotype detected in serum, follow by IgA (Onda, Carter et al. 2003). The presence of IgG antibodies to HPV L1-capsids is known to be a type-specific biomarker of past and present HPV infection (Matsumoto, Yasugi et al. 2006). A correlation between antibody responses to L1-capsids induced by E6/E7 specific B cells, and the viral clearance has been observed in a longitudinal study of HPV infection (Nakagawa, Viscidi et al. 2002). In animal models, antibody responses to HPV L1-capsid prevent animals from new infection, but do not induce tumour regression (Kirnbauer, Chandrachud et al. 1996). The high level of IgG antibody to HPV16 VLP has been reported to be associated with reduced risk for subsequent infection with HPV16 and also in HPV31, -33, -35, -52 and –58 genetically related types (Sheu, Chang et al. 2007). Some studies show the presence of antibody against early proteins without evidence of pronostic significance (Stanley 2001).

In summary antibody responses against HPV are the basis of the current prophylactic vaccines but the natural antibody response is weak, takes a long time and only 46% of women have a seroconvertion (Viscidi, Kotloff et al. 1997). Therefore detection of serum antibodies is not a reliable indication of HPV infection.

3-2-Immune escape

Even if the immune system is really efficient against HPV infection, in rare cases the infection progress to cancer and it almost always involves accidental integration of the viral genome in the cellular DNA. This kind of rare event implies that the virus is able to escape from immune system at various steps.

3-2-1-Low profile

One of the first mechanisms of immune-evasion resides in the life cycle of the virus. Indeed the replication of viral DNA in undifferentiated cells and subsequent particle formation in external differentiated epithelial cells reduces exposure to the host immune system. In addition, the virus cycle is non-lytic and therefore does not elicit any pro-inflammatory signals that activate the LCs (Kupper and Fuhlbrigge 2004). Furthermore the virus encodes only non-secreted proteins, expressed at low levels and primarily in the nucleus of infected cells (Kanodia, Fahey et al. 2007). At the end the production of highly immunogenic capsid proteins is limited to the terminally differentiated outer layer, which is shed from the epithelium (Cason, Patel et al. 1989). Finally a third way used by the virus to escape immune system is the different codon usage of HPV and human genes. Indeed the extensive third-position degeneracy of the

genetic code permits ample variation in the set of codons preferred by viruses with respect to that prevalent in human cells. By this codon bias HPV prevent compromising viability of the host cell by excessive viral early protein expression, as well as to evade the immune system (Cid-Arregui, Juarez et al. 2003).

Thus the first strategy used by the virus to escape from immune system is to maintain a very low profile.

3-2-2- Modulation of antigen presentation

It is now known that HPV mediates deregulation of antigen processing machinery, which results in down-regulation of peptide-MHC complexes on the surface of infected cells and protects them from immune attack. Immunogenic peptides from both E6 and E7 proteins are not efficiency processed and presented by HPV+ tumour cells and it is correlated with reduced expression of human leukocyte antigen (HLA) class I and the TAP 1 and 2 in cervical carcinoma cell lines (Evans, Borysiewicz et al. 2001). Then the E7 protein of HPV is mostly found in the nucleus of infected cells and in the case of HPV16 and 18 it represses the promoter for MHC class I heavy chain expression (Kanodia, Fahey et al. 2007). Furthermore Connor *et al.* have observed changes at 2 alleles which were at both HLA-A and –B loci and which permit the loss of class I MHC expression in cervical carcinoma (Connor and Stern 1990). The HPV16 E5 mediates alkalinization of the Golgi complex and endosomes, inhibits endosome acidification, leads to disruption of the exocytic and endocytic trafficking. Furthermore the Ii chain of the class II MHC needs an acidic endocytic compartment for its sequential degradation, and the alkalinization of endosomes by E5 blocks the formation of mature MHC class II

complex with the antigenic peptide. This suggests that as well as interfering with CTL and NK cell recognition of infected cells, HPV may inhibit CD4+ T-cell recognition through down-regulation of MHC class II molecules and thereby evade the immune system. The HPV16 E5 protein was recently shown to selectively down-regulate the cell surface expression of HLA-A and HLA-B due to its interactions with the HLA I heavy chain (Ashrafi, Haghshenas et al. 2006), thereby potentially decreasing viral peptide presentation to CD8+ T-cell (Ashrafi, Haghshenas et al. 2005). These interactions between HLA I heavy chain and E5 protein, which result in the retention of HLA-A and –B in the Golgi apparatus, do not occur with HLA-C and –E the natural killer cell inhibitor ligands (Ashrafi, Haghshenas et al. 2005). The absence of pro-inflammatory signals associated with HPV infection, together with the immune evasion strategies employed by the viral proteins, permit the virus to establish infection by temporarily evading the immune system.

3-2-3-Interference with IFN

Barnard and McMillan (Barnard and McMillan 1999) showed that the E7 protein of HPV16 inhibits the induction of IFNα-inducible genes, therefore interfering with the effective elimination of HPV. Indeed this IFN is synthesized by infected cells, which combat the replication of the viruses in the host cells. It acts by stimulating the synthesis of a protein-kinase that blocks the initiation of translation of viral proteins by activating endoribonuleases and allows the degradation of the viral and cellular mRNA. It also activates the synthesis and expression of MHC class I molecules (Goncalves and Donadi 2004). The inhibition of this IFN is one of the immune escape mechanisms of the HPV.

3-2-4-Inhibition of cytokines and chemo-attractants

Identified as one of the first chemokines, MCP-1 attracts a variety of cells types including monocytes, memory T-cells and NK cells. A study shows that after infection the expression of MCP-1 is suppressed (Rosl, Lengert et al. 1994). The mechanism by which HPV suppresses MCP-1 production is not known, but E6 and E7 from "high-risk" HPV have been shown individually and together to suppress MCP-1 expression in primary epithelial cells derived from the female genital tract (Kanodia, Fahey et al. 2007). The E6 and E7 proteins are able to inhibit the transcription of IL-8, which is a chemo-attractant for neutrophils, basophils and T-cells (Huang and McCance 2002).

Finally HPV modulate the expression of IL-18, an interleukin, which helps to prime CD8+ T-cells, indeed the E6 protein binds IL-18 and might degrade it *via* the ubiquitin pathway (Cho, Kang et al. 2001). Alternatively E6 and E7 proteins competitively bind to IL-18 receptor and thus may inhibit IL-18 induced IFNγ production (Cho, Kang et al. 2001).

Therefore the modulation or inhibition of cytokines or chemo-attractants is one of the mechanisms used by HPV against immune attack.

3-2-5-Other mechanisms used by HPV

Studies have shown other mechanisms used by the HPV to evade immune system but their understanding remains vague. A switch to T_h2 polarity has been associated with a progression to cancer (Kanodia, Fahey et al. 2007). A modulation of adherence molecules on the surface of APCs such as LCs, decreases the contact between these cells

and infected cells. There is an inhibition of APC migration in lymph nodes dues to a reduction of MIP-3a production induced by E6 and E7 (Guess and McCance 2005).

4-VACCINATION AGAINST HPV

The causal link between HPV infection and malignant transformation in cervical neoplasia provides a unique opportunity for both prophylactic and therapeutic vaccination (Stern 2005).

4-1-Prophylactic vaccination

Prevention of persistent infection with HR HPVs should have a major impact on the prevalence of malignant cervical lesions and it is from this observation that the first studies on VLPs have started. These VLPs are morphologically similar to infectious virions, present L1 and L2 epitopes that are highly immunogenic, but lack viral DNA (Stern 2005). Safety and immunogenicity trials in healthy volunteers have been done with HPV 16 L1 VPLs produced from baculovirus infected insect cells (Harro, Pang et al. 2001) and from yeast (Koutsky, Ault et al. 2002). These have established that the vaccines are well tolerated and generated strong immune responses that are several orders of magnitude higher than those exhibited by naturally infected population. Following studies which included 25, 000 women several efficacy trials demonstrated protection against persistent infection with HPV 16 and 18 and against the development of precursor lesions to cervical cancer (Gissmann and Muller 2007). All these studies have permitted the production of prophylactic vaccines against HPV, which reduce the incidence of genitals warts and the risk of developing cervical cancer. But the vaccine can only be

used for systemic vaccination of young girls aged 9-15 years before the first infection by HPV and can not be used in infected population (Garcia Carranca and Galvan 2007). Furthermore, even if this vaccine is a very important advance against HPV infection, it is a prophylactic vaccine, which cannot be used in patient already infected by HR HPV, only therapeutic vaccines can be used in this case.

4-2-Therapeutic vaccines

The ideal HPV therapeutic vaccine would appropriately stimulate or redirect the immune response so as to eradicate infected tissue or mediate regression of malignant lesions. The chosen targets in therapeutic vaccines depend of the grade of infection, indeed immunisation with specific early proteins, such as E1 or E2, are promising for benign or low grade HPV induced disease (Stanley 2002). Vaccines encoding E1 or E2 genes modified to increase antigen expression, protect against challenge in dog model with live virus (Stanley 2006).

In cervical cancer and most CIN 2/3, viral gene expression is deregulated and the E6 and E7 genes are constitutively expressed because they are essential for maintenance of the malignant phenotype, making them ideal targets for different immunotherapies. Furthermore E6 and E7 are the only viral proteins that will be expressed in all high grades of infection (Stanley 2002). Therapeutic vaccines against HPV are summarised in the table 3.

4-2-1-Peptides or recombinant proteins

The goal of this strategy is to predict immunodominant or subdominant peptides of viral antigens that would associate with particular HLA allelic products and are recognized by T-cells. The advantages of this approach are cost effectiveness, as peptide vaccines are cheap to produce and immunological responses following vaccination are relatively straightforward to measure (Stern 2005). Studies using a HLA-A2 associated with HPV16 E7 peptide in phase I or phase II clinical trial have shown an immunogenic role of these molecules which are able to enhance the CTL response against HPV 16 (Steller, Gurski et al. 1998; Campo 2002).

Recombinant proteins have the advantage over peptide approaches in delivery of all potential epitopes to the antigen processing cells of the immune system. The challenges involve the production and purification of the viral proteins including fusions with molecules like heat shock proteins and their formulation with appropriate adjuvants in order to elicit the necessary processing and subsequent activation of T cells (Goldstone, Palefsky et al. 2002).

Different assays have been done with various fusion proteins. TA-GW fusion protein (HPV6L2E6) shows a good protection against recurrence of warts (Lacey, Thompson et al. 1999). TA-CIN fusion protein (HPV16 L2/E6/E7) induces in mice models E7 specific CD8+ T-cell immune response and tumour protection (de Jong, O'Neill et al. 2002). Various studies with heath shock protein fused to E7 (hspE7), show a protection in mice against challenge with an E7-expressing murine tumour cell line and in phase II of clinical trial a protection was observed too (Chu, Wu et al. 2000, Mahdavi, 2005).

Overall the experience from the various polypeptide vaccines is that they are apparently safe, but immunogenicity has only been shown in a fraction of patients and does not correlate with limited clinical outcome data (Stern 2005).

4-2-2-Plasmid DNA-vaccines

Due to the weak intrinsic potency of naked DNA vaccines, various strategies have been developed to enhance their immunogenicity, one of them is to use an encapsulated HPV DNA vaccine. Garcia et *al.* (Garcia, Petry et al. 2004) reported on the use of encapsulated plasmid DNA-encoding fragment derived from E6 and E7 of HPV16 and 18 in biodegradable particle (ZYC101a). This study design was double-blinded, randomised, and placebo-controlled (saline placebo) and was carried out in multiple centres in U.S. and Europe. Patients received three injections at 0, 3 or 6 months of either 100μg or 200μg of the vaccine. The results showed a statistically significant higher rate of CIN2 or CIN3 resolution in the <25 years of age group (placebo, 23%; 100μg, 67%; and 200μg, 72%). However there was no difference in resolution rates between vaccine and placebo in the group>25 years old. Now this vaccine has been acquired by MGI Pharmaceuticals and further studies are planned (Mahdavi and Monk 2005). A recent study showed that a DNA vaccine encoding a fusion of IL-2 and E7 proteins induces a strong E7-specific CTL response (Lin, Tsai et al. 2007).

Nevertheless this kind of strategy has two disadvantages: the first one is the low immunogenicity of the DNA, and the other one a possible insertion of the HPV DNA which could enhance the expression of oncogenes.

4-2-3-DC-based vaccines

A study demonstrated that recombinant, full-length, E7-pulsed, autologous DCs could elicit a specific CD8+ CTL response against autologous tumour target cells in three patients with HPV16 or 18 positive cervical cancer (Santin, Hermonat et al. 1999). Another group has pulsed autologous monocyte-derived DCs with recombinant HPV16 E7 or HPV18 E7 and used its for the treatment of 15 patients (Ferrara, Nonn et al. 2003). Specific cellular immune responses were detected in four of the 15 patients.

4-2-4-Viral Vector Vaccine

This strategy has the advantage of being highly immunogenic and having different immunogenic properties of viruses. Several studies have shown that immunotherapy targeting E6 and/or E7 using vaccina vector generates strong CTL activity and tumour responses in preclinical studies. Indeed a phase I/II trial in 8 patients with late stage cervical cancer using a recombinant vaccinia virus expressing the E6 and E7 proteins of HPV16 and 18 (TA-HPV), has permitted the development of HPV-specific antibody response in each patient and HPV-specific CTL response in one patient (Borysiewicz, Fiander et al. 1996). A 2002 study on the same TA-HPV vector confirmed the safety and immunogenicity of the vaccine, with 4 patients who develop HPV-specific CTL response (Kaufmann, Stern et al. 2002).

Another recombinant vaccine composed of modified vaccinia virus Ankara (MVA) expressing the E2 of bovine papillomavirus has been recently tested to treat flat condyloma lesions associated with oncogenic HPV in men. After 4 weeks of treatment,

93% of the patients showed no lesion or presence of papillomavirus as diagnosed by coloscopy and brush examination (Albarran, de la Garza et al. 2007).

4-2-5-"prime-boost" strategies

Prime-boost strategies use a priming immunisation (e.g. DNA plasmid or viral protein) followed by a heterologous boost with a different viral vector encoding the immunogenic epitope (Mahdavi and Monk 2005). Different assays have been done with this kind of strategy and the most impressive was the assay of Baldwin et *al.* (Baldwin, van der Burg et al. 2003). They vaccinated with a fusion protein of HPV16 L2E6E7 12 women with high grade HPV positive vaginal or vulval intraepithelial neoplasia. In 42% of the cases a decrease in lesion size, average 40% reduction, was observed. Whilst this result is promising, the absence of placebo control group complicates interpretation, as the naturally occurring oscillations in lesion size have not been account for.

Table 3: Published clinical trials of therapeutic HPV vaccines (adapted from (Winters, Roden et al. 2006).

Delivery system		Antigen	Participants	Key findings	Phase
Viral vector	(Smith, Tristram et al. 2005)	TA-HPV, Xenova E6-E7 fusion protein	11 patients CIN3, VIN3, VAIN	5 patients made novel HPV specific T cell responses after vaccination. No clinical responses	Phase I/II
	(Corona Gutierrez, Tinoco et al. 2004)	MVA E2 recombinant vaccinia	36 women CIN1, 2 and 3	34/36 complete responses	Phase I/II
	(Hallez, Simon et al. 2004)	Fusion protein PD-E7. E7 linked to Hemophilus influenza	7 patients with CIN1 and 3	5/7 CTL responses	Phase I/II
	(Davidson, Boswell et al. 2003)	TA-HPV, Xenova, E6-E7 fusion protein	18 women with high grade VIN	8 women had partial clinical responses, 13 women had HVP specific immune responses after vaccination. Correlation between response and pre-vaccination local immune status	Phase II
	(Kaufmann, Stern et al. 2002)	TA-HPV, Xenova, E6-E7 fusion protein	29 women stage 1b or 2 a cervical cancer prior to radical hysterectomy	HPV specific CTL in 4 women Serological response in 8 women	Phase I/II
Peptide	(Chu, Wu et al. 2000)	HPV16 E7 peptides	15 women with advanced cervical cancer	T helper responses in 4 patients, No specific CTL responses	Phase I/II
	(Frazer, Quinn et al. 2004)	Protein/Iscomatrix adjuvant E6E7-IMX, CSL	31 women with CIN randomized to vaccine or placebo	Most developed antibody responses, about half developed new CTL responses	Phase I
	(de Jong, O'Neill et al. 2002)	TA-CIN L2, E6, E7 Fusion protein, Xenova	40 healthy volunteers randomized to vaccine or placebo	TA-CIN specific IgG antibody and T cell proliferative responses in majority	Phase I
Prime boost	(Smyth, 2004)	TA-HPV + TA-CIN, Xenova	29 women with high grade VIN	Antibody and CTL responses, no simple correlation with the clinical responses	Phase I/II
DNA	(Garcia, Petry et al. 2004)	Plasmid DNA encoding fragments derived from E6 and E7 proteins in microparticules	161 women with CIN2/3 randomized to vaccine/placebo prior to cone	43% of vaccinated women cleared disease versus 27% of placebo recipients (not statistically significant).	Phase I/II
DC	(Ferrara, Nonn et al. 2003)	Autologus DC pulsed with recombinant HPV16 E7 and HPV18 E7	15 women stage IV cervical cancer	Specific cellular responses in 4/11	Phase I/II

Abbreviations: CIN, cervical intraepithelial neoplasia; IFA, incomplete Freund's adjuvant; LEEP, loop electrosurgical excision procedure; MVA, modified vaccinia Ankara; PD, hemophilus influenza protein D.

PROJECT AIMS

Infection with HPV, in particular HPV16 and 18, is the causative factor in the development of cervical cancer because the immune system fails to eliminate the virus. Our understanding of the mechanisms which prevent the clearance of HPV remains poor. Some studies on the T-cell responses against HPV show a relation between the failure to clear the infection and a weak E6-specific Th1 response. Indeed some healthy donors seem to induce a better CD4 immunity against HPV-18 E6 epitopes than the patients (Facchinetti, Seresini et al. 2005). In the infection by HPV-16, the failure of CD4+ T-cell function in E2- and E6-specific immunity seems to be associated with cervical disease (de Jong, van Poelgeest et al. 2004). Conversely lymphoproliferative responses to specific HPV-16 E6 and E7 peptides appear to be associated with the clearance of HPV infection and the regression of CIN (Kadish, Ho et al. 1997). Therefore these data suggest the necessity of a better understanding of the immune response against HPV16 E6 antigen. One of the ways, which could be explored, is the activation of the CD4+ T-cells by this antigen, and then the molecular interaction between the TCR and the class II MHC associated with a peptide derived from the E6 protein. Indeed these interactions are the basis of an efficient immune response against HPV. In this goal the laboratory has already identified a target in the E6 protein (HPV16 $E6_{127-141}$: DKKQRFHNIRGRWTG), which can induce some CD4+ responses in healthy donors. Furthermore it has been demonstrated HLA-DR*0101 presents $E6_{127-141}$ (Gallagher and Man 2007), and two clones (CD4+), specific for this peptides have been generated, belx1 and belx2. These two clones have two different TCR; indeed they express the same α chain but not the same β chain. belx1 expresses the TRBV14 and belx2 expresses the TRBV4-2. The responses of these clones to HPV16 $E6_{127-141}$ in terms of IFNγ secretion are not similar,

indeed belx2 secretes more IFNγ than belx1. The explanation for these variations in the functional characteristics of these two clones could be due to their different TCRs. Indeed the interaction between the TCRs and the HLA-DR*0101-HPV16 $E6_{127-141}$ complex could be dissimilar. In order to understand a part of the mechanisms which regulate this kind of events it is necessary to construct the soluble molecules and to study their interactions.

Therefore the principal objectives of this project were:

-The construction of soluble HLA-DR1 protein associated with HPV16 $E6_{127-141}$ to allow biophysical studies.

-Investigate the functional importance of changes in the peptide structure using T-cell clones against HPV16 $E6_{127-141}$.

-Investigate the functional effect of natural HPV type variation of peptide sequences on T-cell responses.

MATERIALS AND METHODS

1-CONSTRUCTION OF THE HLA-DRB*0101 GENES

To produce HLA-DRB*0101 associated with peptide $E6_{127-141}$ several steps were necessary. First we have generated the DNA sequence of the α chain by amplification of cDNA (provided by Dr J. Boulter) corresponding to the extracellular domain and the wild type signal peptide of HLA-DRA*0101 from a B cell line (figure 10). In parallel we amplified the *fos* leucine zipper attached to the biotinylation tag. Finally we have proceded to the amplification of all the chain corresponding to the extracellular domain of HLA-DRA*0101 attached to the *fos* leucine zipper and the biotinylation tag for α. The construction of the DRB*0101 chain was generated in four steps. First the cDNAs corresponding to the extracellular domain of HLA-DRB*0101 from B cell line were amplified and attached to the linker by overlapping PCR. Secondly the amplification of the *jun* leucine zipper sequence was performed, following by the amplification of the wild type signal peptide for the secretion of the molecule associated with the HPV peptide, for β. Thirdly the amplification of the complete chain has been done. Finally the sites of the restriction endonuclease BglII were added to the DRA*0101 and the restriction endonuclease sites, BamH1, were added to the DRB*0101. A similar construct with the CLIP peptide instead of the HPV16 $E6_{127-141}$ peptide was also generated.

All these amplifications have been done by Polymerase Chain Reaction (PCR), or overlapping PCR. The final concentrations of reagents in PCR mixture (50µl) were: 0.3 mM dNTPs (Bioline, London, UK), 1 mM $MgCl_2$ (Bioline), 25 pmol of forward and reverse primers, and 2.5 U of Taq DNA polymerase (Bioline) in PCR buffer (Bioline, 16mM $(NH_4)_2So_4$, 0.01% Tween-20, 67 mM Tris-HCl pH 8.8). Typical cycling conditions are shown in table 4. All PCRs were performed using a Biometra Tpersonal

thermocycler (Glasgow, UK). PCR products were purified by DNA-binding columns (QIAquick® PCR purification kit, Qiagen, Valencia, California, USA) and following the manufacturer's instructions. Briefly, the PCR products were purified on a column with different buffers, provided by the kit, and several steps of centrifugation at maximum speed with a bench centrifuge. The samples were eluted with the buffer provided in the kit and the quantity of DNA was directly measured by spectrometry at ratio 260/280 nm.

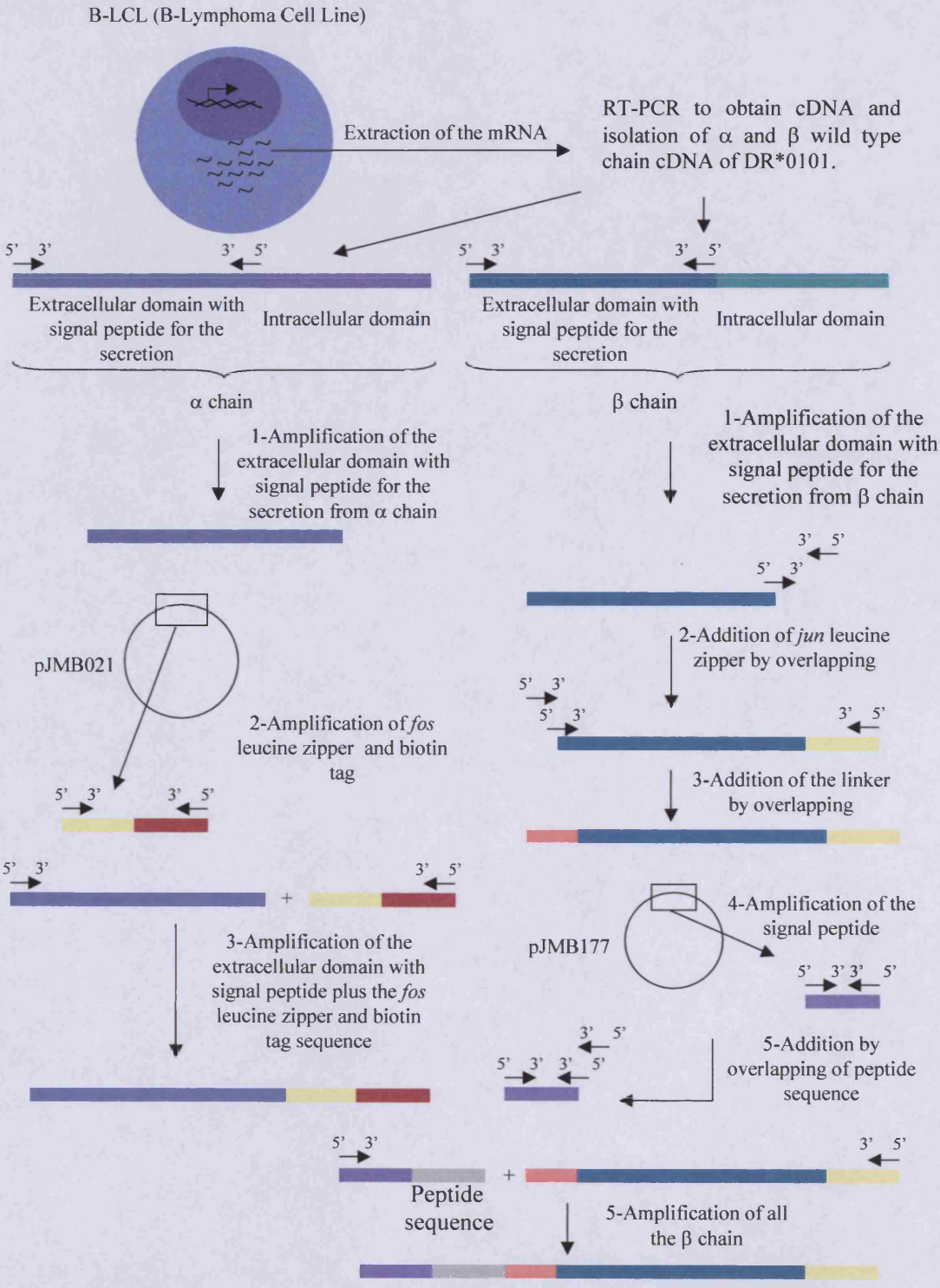


Figure 10: Schema of the construction of α and β chains to express a soluble HLA-DR*0101 associated with HPV16 $E6_{127-141}$ peptide.

Table 4: Typical cycling conditions

Steps	T°C	Time	Number of cycles
Initial denaturation	95°C	5 min	1
Denaturation	94°C	40 sec	
Annealing	[a]55-68°C	40 sec	30
Extension	72°C	[b]1-3 min	
Final extension	72°C	10 min	1

[a] The annealing temperature depends on the melting T°C of primers.
[b] The extension time depends on the size of the PCR product.

The different primers used for these constructions and for the sequencing of the DNA are presented in the table 5.

Table 5: List of the primers used for the construction

Name	Sequence	Use
485F	5'-gaagatctatggccataagtggagtccctg-3'	Extracellular domain of α-chain plus wild type signal peptide for the secretion.
487R	5'-agcatcaaactcccagtgcttgag-3'	
487F	5'-ctcaagcactgggagtttgatgctcccgggggtctg-3'	*fos* leucin zipper.
486R	5'-gaagatcttaatgccattcgattttctgagcttc-3'	
488F	5'-cgggatccatggtgtgtctgaagctccctg-3'	Wild type signal peptide for the secretion and the E6 $_{127-141}$ HPV peptide or CLIP peptide.
493R	5'-ttatgaaatctttgttttttatcagccaaagccagt-3'	
494R	5'-gtccatcgtccacgaatattatgaaatctttgt-3'	
495R	5'-gtgagccaccaccagatccagtccatcgtccacgaa-3'	
23F	5'-gtagtccggaccagttagtaagatgcgaatggcaactcctctgct-3'	
24R	5'-gtagtccggatgcttgcataagcagaggagttgccat-3'	
491F	5'-cggggctctggaggtgggtccggggacacccga-3'	Extracellular domain of the β-chain associated with a linker.
490R	5'-tgctctccattccactgtgagag-3'	
492F	5'-tggatctggtggtggctcactagtgccacggggctc-3'	
490F	5'-ctctcacagtggaatggagagcacccgggggtagaa-3'	*jun* leucine zipper
489R	5'-cgggatccttaccatggatcctagtagtccatg-3'	
M13F	5'-tcgtgactgggaaaac-3'	sequencing
M13R	5'-caggaaacagctatgac-3'	
ASbasicprom	5'-tgcatcgtcgcaaaacgg-3'	
1629 DWN	5'-ctgtgcgttgttgatttacag-3'	
485F	5'-gaagatctatggccataagtggagtccctg-3'	
488F	5'-cgggatccatggtgtgtctgaagctccctg-3'	

2-CLONING OF THE α AND β HLA-DRB*0101 IN THE PCR®2.1-TOPO®

Purified PCR products were subcloned into the pCR®2.1-TOPO® vector (TOPO TA Cloning ® kit, Invitrogen, Paisley, UK). The pCR2.1-TOPO cloning vector (figure 11) is a linear plasmid with single 3'-thymidine (T) overhangs, allowing the direct cloning of PCR products due to the production of single 3'-adenosine (A) overhangs by Taq DNA polymerase. The selection of transformed bacteria is based on ampicillin resistance introduced by the vector.

The ligation mixture (10 µl final volume) contained 10 ng of purified PCR product, 50 ng of pCR2.1-TOPO vector, 1 µl of 10X ligation buffer (300mM Tris-HCl (pH 7.8 at 25°C), 100mM $MgCl_2$, 100mM DTT and 10mM ATP), 1-3U of T4 DNA ligase (Promega, Southampton, UK) and sterile water to a total of 10 µl. The mixture was incubated at 16°C overnight. This product of ligation was used to transform E. coli cells, strain DH5α. One to ten ng of DNA (ligation product) were added to 50 µl of Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen) and incubated on ice for 30 minutes. The cells were heat shocked for 20 seconds at 42°C in a water bath without shaking and placed immediately on ice for 2 minutes. After transformation 950 µl of SOC medium (2% (w/v) Bacto-Tryptone, 0.5% (w/v) Yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM Glucose, pH7.0) were immediately added, and the cells were incubated at 37°C for 1 hour at 225 rpm in an orbital shaker (Sanyo, UK). After 1 hour 20 µl to 200µl from the transformation were spread on Luria Bertani (LB)/ agar plates (1% (w/v) Bacto-Tryptone, 0.5% (w/v) Yeast extract, 172 mM NaCl, pH 7.7/ 1.5% (w/v) Agar) containing 100 µg/ml ampicillin (Sigma, Poole, UK) and incubated at 37°C. The following day, the

clones were screened by PCR (see tables 4 and 5). Then the DNA sequence of the positive clones were checked by sequencing.

3-DNA SEQUENCING OF POSITIVE CLONES

After the PCR screening the positive clones were collected from petri dishes and grown in 5 ml of LB medium plus ampicillin in 25ml tubes (Sterilin, Staffordshire, UK) (100 μg/ml, Sigma) at 37°C overnight. Plasmid extraction from each clone was carried out by using the QIAprep® spin miniprep kit (Qiagen) according to the manufacturer's instructions. Each DNA insert was checked by PCR sequencing using the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq® Polymerase FS (Perkin and Elmer, Waltham, Massachusetts, USA). The PCR mixture (15 μl) contained 700 ng of each plasmid, 5 pmol of primer and 3 μl of Terminator mixture, containing the enzyme, the buffer, the dNTPs and the Big Dye Terminators. Thermal cycling (25 cycles) conditions were: denaturation, 96°C for 30 seconds; annealing, 50°C for 15 seconds; extension, 60°C for 4 minutes. Following thermal cycling the PCR products were precipitated by adding 1.5 μl of 3 M CH_3COONa, pH 5.4 and 45 μl of ethanol (Fisher, London, UK) and incubating for 20 minutes at –70°C. The samples were then centrifuged at 13,000 rpm for 20 minutes at room temperature, and precipitated DNA was washed with 75% (v/v) ethanol. DNA sequencing was carried out in our DNA sequencing facility (Central Biotechnology Services, CBS) by using an automated ABI 377 DNA sequencer (Applied Biosystems, Warrington, UK).

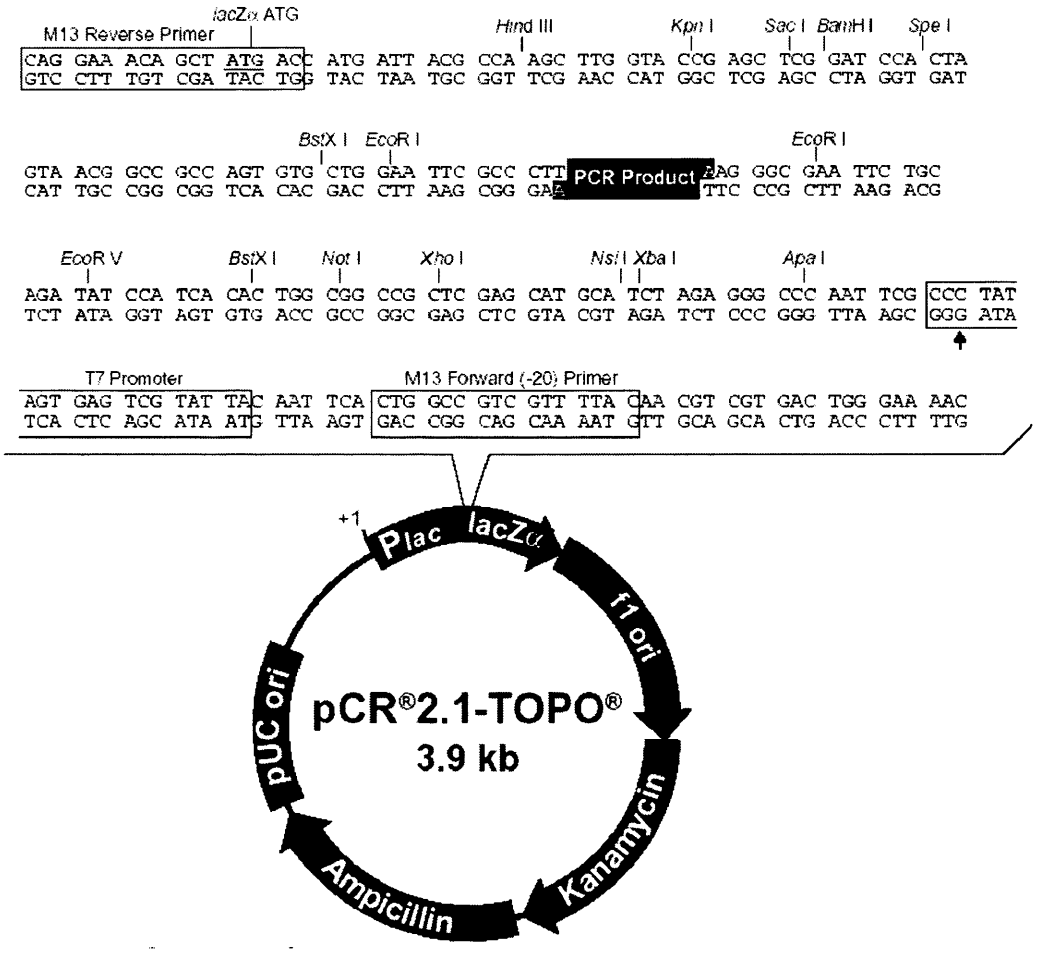


Figure 11: Map of the circularized vector, pCR®2.1. The arrow indicates the start of transcription for the T7 RNA polymerase (from Invitrogen).

4-CLONING INTO TRANSFER VECTOR PACAB3

After analysing and checking of the sequence the HLA-DR*0101 α and β chains were cloned in pAcAB3 (kindly provided by Dr N. Pumphrey, Avidex, Oxon, UK), which is a multiple plasmid transfer vector used for expression of up to three proteins of interest. This vector is an AcNPV (Autographa californica nuclear polyhedrosis virus) polyhedrin locus-based vector, which contains an *E. coli* origin of replication, an ampicillin resistance marker, a copy of the AcNPV polyhedrin promoter, two copies of the AcNPV p10 promoters, a cloning region downstream from each promoters and a large tract of AcNPV sequence to facilitate homologous recombination (figure 12).

Before cloning of the two different chains into pAcAB3, each TOPO vector containing the α and β chains was digested by the endonuclease Eco RI (Invitrogen). 100 ng of vector was digested by adding 30 U of Eco RI in reaction buffer 4 (Invitrogen) (final volume 15 µl) and placing the reaction at 37°C for 1 hour.

The products of these digestions were resolved on a 1% (w/v) agarose (Invitrogen) gel in TAE buffer (40mM Tris Acetate pH 7.2, 1 mM EDTA). Agarose was dissolved in TAE buffer by boiling. The solution was allowed to cool to approximately 55°C before ethidium bromide (Sigma, dorset, UK) was added at a final concentration of 50ng/ml. The solution was poured into the gel casting tray of an Electro-4 gel tank (Hybaid Ltd, Hampshire, UK), and left for at least 30 minutes to set. The gel was transferred to the gel tank and TAE buffer was added. Before loading, DNA products were mixed with DNA loading buffer (0.25% (w/v) bromophenol blue, 30% glycerol in dH_2O) at a final DNA product: loading buffer ratio of 10:1. Electrophoresis was carried out at constant voltage (100V) until separation of the DNA fragments. The SmartLadder

(Bioline) was used as a DNA size marker. DNA products of digestion were cut out from the gel and visualized using an UV transilluminator lamp. DNA was purified using a DNA-binding column (QIAquick® gel extraction kit, Qiagen) and following the manufacturer's instructions. Briefly the agarose with the DNA was dissolved at 52°C in presence of a buffer provided bin the kit. After this step the DNA was purified on a column as for the PCR products (see part 1).

A second digestion was performed for each chain: the α chain was digested by the endonuclease Bgl II (NEB, London, UK) and the β chain by the endonuclease Bam H1 (NEB). For each digestion 100ng of DNA was digested by adding 30 U of enzyme in the buffer provided by the manufacturer and left 90 minutes at 37°C and each product of digestion was purified using QIAquick® PCR purification kit (Qiagen) and stored a – 20°C.

In parallel the digestion of pAcAB3 by Bgl II was performed, and followed by a dephosphorylation of its 5' phosphorylated ends produced by the endonuclease digestion. In this goal an alkaline phosphatase (Promega) was used to catalyse the hydrolysis of 5'-phosphate groups from DNA, which prevents re-circularization and religation of linearized cloning vector DNA. This was performed by adding 1U of alkaline phosphatase to the digestion products and leaving for 1 hour at 37°C. After the reaction the linearized pAcAB3 was purified using QIAquick® PCR purification kit (Qiagen), and a ligation was performed overnight with the α chain. Following the ligation, DH5α cells were transformed with product of ligation and spread on LB/agar plates containing 100μg/ml ampicillin. On the following day, the bacteria colonies were screened for inserts and their orientation to the promoter by PCR screening.

The same experiments were performed with the β chain. The construct amplified in TOPO vector was digested by EcoRI, purified, digested by BamH1 and purified. In parallel the pAcAB3, containing the α chain, was digested by BamH1 and a ligation between the β chain and the vector was performed overnight. DH5α cells were transformed by product of ligation and spread on LB/agar plates. The following day, the bacteria were screened by PCR screening, to check the presence of the insert and the orientation of the insert compared with the promoter.

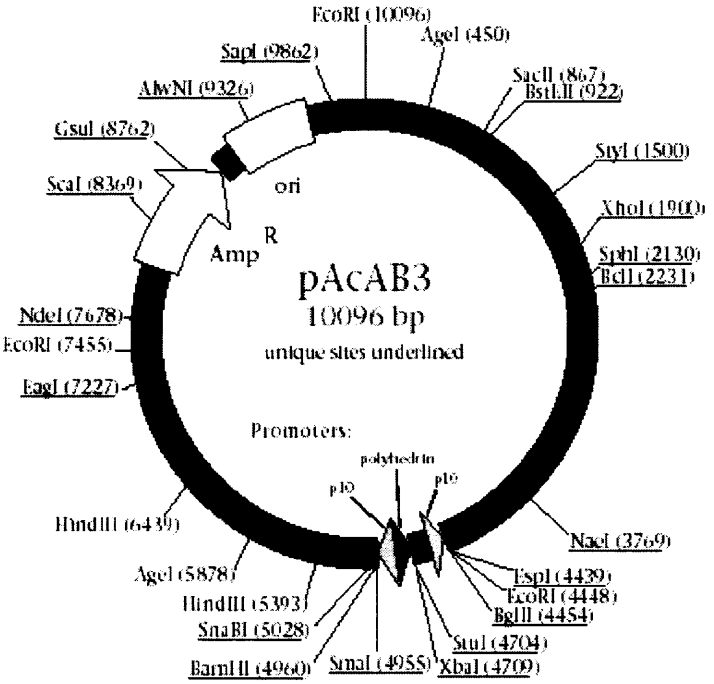


Figure 12: The pAcAB3 is a 10.0 kb AcNPV polyhedrin locus-based vector that contains a copy of the AcNPV polyhedrin promoter and two AcNPV p10 promoters. Dwonstream of the first p10 promoter are a Sma I and a Bam HI cloning site, followed by polyhedrin locus-derived terminal sequences. Upstream of this, an inverted polyhedrin promoter has been inserted containing a Xba I and a Stu I cloning site, followed by a second p10 promoter, a Bgl II insertion site and an SV40 terminator. The two p10 promoters are in opposite orientations. Using pAcAB3, three foreign genes can be simultaneously expressed during the very late phase of the Baculovirus infection cycle (from PharMingen, London, UK).

5-CLONING INTO TRANSFER VECTOR PBACGUS4X-1

pBACgus4x-1 is a baculovirus transfer plasmid (Novagen, Nottingham, UK) designed for cloning and co-expression of up to four target genes or multiple copies of the same gene in insect cells. The plasmid contains two polyhedrin promoters and two p10 promoters, each of which is upstream of unique cloning sites for sequential insertion of target genes. The plasmid also carries the *gus* gene encoding β-glucuronidase under control of the late basic protein promoter, which serves as a reporter to verify recombinant viruses by staining with X-gluc (figure 13).

The protocol is exactly the same as cloning in pAcAB3, we first digested the vector by Bgl II, carried out dephosphorylation, performed a ligation between the α chain and the vector, checked the direction of the insert by PCR screening and sequencing, and proceeded to the insertion of the β chain.

6-EXPRESSION OF HLA- DR*0101 IN INSECT CELLS

6-1-Generating recombinant baculoviruses by co-transfection with BD Biosciences system

The principle of this technique is shown in the figure 14. Basically baculogold is an Autographa californica nuclear polyhedrosis virus (AcNPV) baculovirus DNA, which contains a lethal deletion and does not code for viable virus. Co-transfection of the baculogold DNA with a complementing baculovirus expression vector rescues the lethal deletion by homologous recombination.

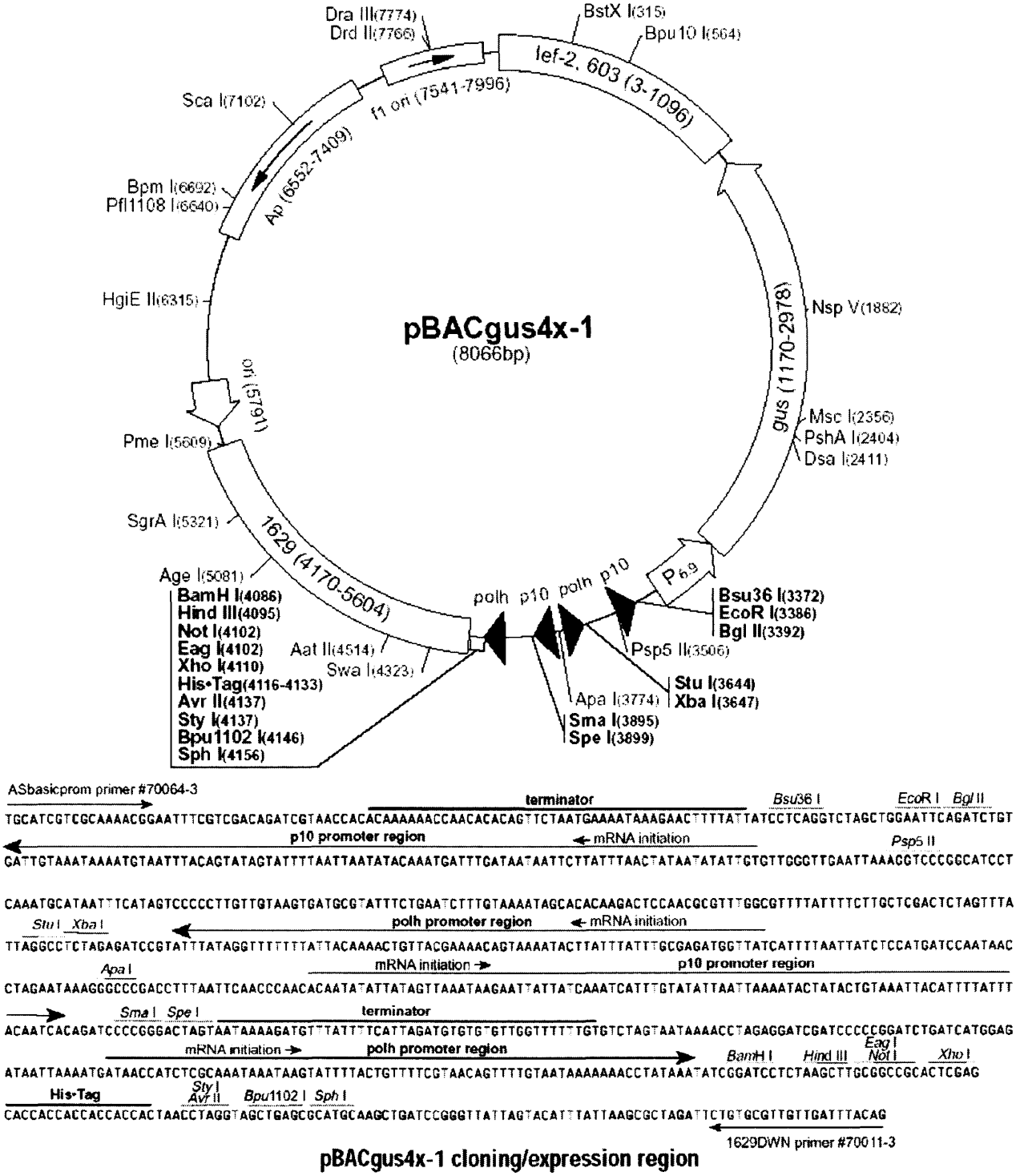


Figure 13: Map of the pBACgus4x-1 transfer plasmid (from Novagen)

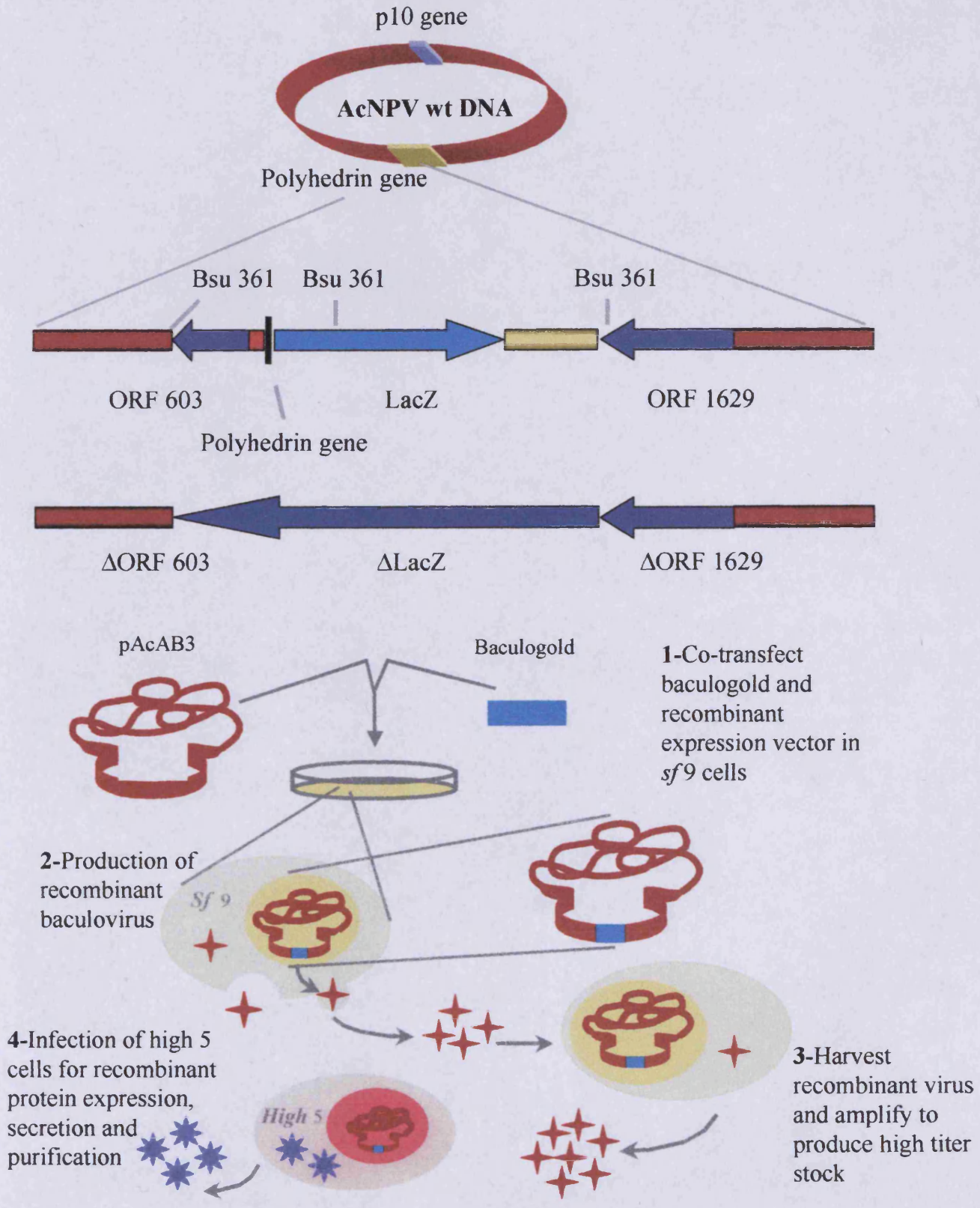


Figure 14: Principle of the co-transfection of baculovirus and recombinant transfer vector

The co-transfection of the BD BaculoGold™ (BD Biosciences, Oxford, UK) linearized baculovirus DNA and the complementing transfer vector construct, pAcAB3, was done in *Spodoptera frugiperda* Sf9 cells (Novagen). Sf9 cells were seeded at $2x10^6$ cells per well in a 6 wells plate (Falcon, Loughborough, UK) in BacVector® Insect Cell Medium (Novagen) with an initial cell density which should be 50-70% confluent, and left approximately 15 minutes at 27°C to allow the insect cells to attach firmly to plate. Subsequently the medium was removed from the plate and 1ml of Transfection buffer A (BD Biosciences) was added. In parallel, 0.5μg of BD BaculoGold™ (BD Biosciences) and 2μg of recombinant pAcAB3 were mixed in a sterile 1.5 ml tube and left for 5 minutes at room temperature before adding 1 ml of Transfection buffer B (BD Biosciences). Then the mixture was added drop-by-drop to the insect cells on the tissue culture plate, and the plate was incubated 4 hours at 27°C. Following incubation the transfection solution was removed and 3 ml of insect cell medium were added and the plate was rocked back and forth several times, before once again removing the medium and adding 3 ml of fresh medium. Finally the plate was incubated for 4 to 5 days at 27°C and the supernatant was collected and used for amplification of virus. The control vector pVL1392-XylE (BD Biosciences) was used as positive control for the co-transfection and the negative control was a co-transfection without recombinant vector.

6-2-Amplification of recombinant baculovirus with BD Biosciences system

To obtain a high titre stock solution of recombinant baculovirus we had to amplify the recombinant virus after co-tranfection. To this end, freshly seeded insect cells should be infected at a multiplicity of infection (MOI) of >1. This was done by infecting $5x10^6$

Sf9 cells per 10 cm plate (approximately 60% confluent) with 500µl of transfection supernatant in 15ml of insect cells medium. The cells were incubated for 3 days at 27°C before harvesting the medium.

6-3-Plaque assay to titre recombinant virus with BD Biosciences system

Sf9 cells, 70-80% confluent, were seeded at $2x10^6$ cells per well in 6 wells plate (Falcon) and left 5 minutes at room temperature to allow the cells to attach. At the same time serial dilutions (10^{-4}-10^{-7}) of our amplified co-transfection virus supernatant were made and 1 ml of each serial dilution was added per well. We left the infection occur for 4 hours at room temperature on a rocker and prepared a 2% solution of plaque assay agarose (Novagen) in sterile water by heating mixture in a microwave oven to 60°C and keeping it at 42°C. Following infection the agarose solution was mixed with Sf9 medium 1% Foetal Calf Serum (FCS) (Invitrogen) to obtain a final agarose concentration of 1%. The medium was removed and the cells were overlaid with 2ml of the 1% agarose solution and left for approximately 10-15 minutes at room temperature until the agarose was completely hardened. The plate was incubated at 27°C during 5 to 7 days in a humid atmosphere. After 7 days 1ml of neutral red solution (25% neutral red diluted in PBS, Sigma) was added to each well and plate was incubated at 27°C for 2 hours. Following incubation the neutral red solution was removed; the plate was inverted, wrapped in foil and left overnight at room temperature. The plaques were counted the day after under a microscope with a magnification of 20X.

6-4-Generating recombinant baculoviruses by co-transfection with Nogaven system

The principle is exactly the same as BD Biosciences system but with another transfer vector, pBACgus4x-1. Insect cells were seeded at $1x10^6$ cells per well in 6 wells plate (Falcon) and incubated at 27°C for 1 hour. Co-tranfection mix was prepared during incubation by adding in the following order; 1ml of insect cell medium, 5µl of insect genejuice transfection reagent (Novagen), 5 µl of BacMagic™ DNA (100 ng total) and 5µl of pBACgus4x-1 (500 ng) in a sterile 6 ml polystyrene tube. The co-transfection mix was incubated for 15-30 minutes at room temperature to allow complexes to form. After incubation of insect cells the medium was removed and 1ml of transfection mixture was added dropwise to center of well. The plate was placed in humidified container at 27^0C overnight. After 1 day 1ml of medium was added and the plate was incubated for 5 days in total at 27°C. Supernatant was then collected in the dark at 4°C. The transfection control vector (Novagen) was used as a positive control and the negative control was a co-transfection without recombinant vector.

6-5-Amplification of recombinant baculovirus with Novagen system

100 to 200 ml of cultures of Sf9 cells, at an appropriate cell density in log phase growth (e.g., $2x10^6$ cells /ml), were infected at a low MOI (<1pfu/cell) in an 1 l flask (Corning, Schiphol-Rijk, The Netherlands) with 0.5 ml of recombinant virus. The flask was incubated for 4-5 days at 27°C in an orbital shaker at 150 rpm. Then cell culture medium was collected and centrifuged at 1000g for 20 minutes at 4°C. The supernatant was removed aseptically and stored in the dark at 4°C.

6-6-Titration of the recombinant virus with FastPlax™ Titer from Novagen

Sf9 cells resuspended in 2ml of medium containing a total of $1x10^6$ cells were added to a single well and left for about 30 minutes at 27°C to allow the cells to attach to the plate. During this step the virus dilutions (10^{-5}-10^{-7}) were made. After 30 minutes of incubation the medium was removed from the well and 1ml of virus dilution was added. The plate was incubated for 60 minutes at room temperature (RT) and 2ml of fresh medium was added to each well. Subsequently the plate was incubated at 27°C for 26-27 hours in humid atmosphere. After this incubation the medium was removed, and the well was washed twice by adding 2ml per well of PBS. Cells were fixed by adding 2 ml of 3.7% formaldehyde solution (Sigma) diluted in PBS for 10-15 minutes. Each well was washed twice with PBS and 2ml of 1% gelatin solution in 1X TBST (Novagen) was added to block the cells for 30 minutes. The gelatin solution was removed and cells were washed twice with 2 ml PBS. Following this, 1 ml of FastPlax™ antibody diluted 1:10,000 in 1X TBST (Novagen) was added to the well for 60 minutes with gentle rocking at RT. The well was washed three times with 2 ml of 1X TBST and each wash was incubated for 10 minutes. Then 1 ml of goat anti-mouse β-gal conjugate antibody (Novagen) diluted 1:100 in 1X TBST (Novagen) was incubated for 1 hour with gentle rocking, the well was washed three times with 2 ml 1X TBST (Novagen) and each wash was incubated for 10 minutes. After the last wash 2ml of developing solution (60µl X-Gal Novagen, 60µl NBT Novagen in 15ml PBS, 5mM $MgCl_2$) was added and the plate was incubated at 37°C for 15 to 60 minutes. To stop colour development two more washes with 2ml of 1X TBST were done. The infected cells and foci were counted

immediately using a dissecting microscope and the titer was calculated using the following formula:

$$\text{Pfu / ml (MOI)} = 10 \times (\#\text{ infected cells or foci}) \times (\text{dilution factor})$$

This formula was used for each well and an average was done to determine the final titer.

6-7-Expression of HLA-DRB*0101 in High 5 cells

We used another insect cell line for the expression of the HLA-DRB*0101, the High 5 cells, due to their ability to express molecules at higher rates than *sf*9 cells. For the expression, cells had to be infected at a high MOI to ensure all cells were infected simultaneously and the culture was synchronous. After optimization of the expression, 200 ml culture of High 5 cells in Express Five® SFM medium (Invitrogen) were prepared at an appropriate cell density in log phase growth (2.5 x 10^6 cells/ml). This culture was infected by the recombinant baculovirus at a MOI of 10 and incubated at 27°C in shake incubator for 5 days. Following incubation the culture was centrifuged at 453g for 20 minutes at 4°C, the supernatant was harvested, filtered using a 0.45μm filter in a first instance, then a 0.22μm filter and the purification of the HLA-DRB*0101 was done.

6-8-Purification of HLA-DR*0101

HLA-DRB*0101 was first purified by passing the filtered supernatant on anion exchange column. A Poros50HQ™ column was equilibrated first in buffer B (10mM Tris, 1M NaCl) and secondly in buffer A (10mM Tris), the supernatant was loaded onto the column and eluted using the buffer A. Different fractions were collected, concentrated

to 0.5 ml, using centrifugal concentrators (Sartorius, Stedim, Australia), and purified using gel filtration column. A Superdex200HR™ (GE Healthcare, UK) was equilibrated in PBS, the sample was loaded onto the column using a 2 ml injection loop and eluted using PBS. The factions were analysed by gel (SDS-Page, Invitrogen), concentrated and stored at -80°C. For the gel, 8 µl of sample were mixed to 2 µl of 5X loading buffer (0.313M Tris-HCl, pH 6.8 at 25°C, 10% SDS, 0.05% bromophenol blue and 50% glycerol) or 2 µl of 5X reducing loading buffer (loading buffer plus 0.5M DTT), the 10 µl were loaded on a NuPAGE® Gel (Invitrogen), 5µl of SeeBlue® Plus2 Pe-stained Standard (Invitrogen) were used as marker of molecular weight protein. The electrophoresis was carried out at constant voltage (150V) during 1 hour, the gel was washed twice in distilled water, stained in SimplyBlue™ SafeStain (Invitrogen) for 30 minutes and de-stained overnight with water and 10mM NaCl.

6-9-Biotinylation of HLA-DR*0101

Briefly, HLA-DRB*0101 with a biotinylation sequence tag, was treated with 400 µM biotin, 5 µM ATP, 200 µM PMSF, 1 µM leupeptin, 1 µM pepstatin and 15 µl of BirA enzyme. The mixture was incubated overnight at room temperature and gel filtered using a Superdex200HR™ column to remove free biotin (see paragraph 6-8).

6-10- Surface Plasmon Resonance (SPR)

The binding analysis was performed using a BIAcore 3000™ equiped with a CM5 sensor chip. Briefly, chip coupling solutions containing 100 µl of 100mM NHS and 100 µl of 400mM EDC were used to activate the CM5 sensor chip prior to streptavidin

binding (110 µl of 200 µg/ml in 10mM acetate pH 4.5). 100 µl of 1M ethanolamine hydrochloride was used to deactivate any reactive groups. Biotinylated HLA-DRB*0101 at ~1 µM was then coupled to the streptavidin coated chip. For MHC coupling, approximately 600-3000 RU of protein was attached to the CM5 sensor chip. L243 antibody (anti-HLA-DR) or soluble TCR samples were concentrated to around 1mM. For equilibrium analysis, ten serial dilutions were carefully prepared in triplicate for each sample, which were injected over the relevant sensor chips at 25°C. All experiments were carried out in triplicate using the same chip on the same day. Results were analysed using BIAevaluation 3.1™ (BIAcore) and Microsoft Excel™. The equilibrium binding constant (K_D) values were calculated using a nonlinear curve fit ($y = (P_1x)/(P_2 + x)$).

7-PRODUCTION OF HLA-DRB*0101 IN ECHERICHIA COLI (E.COLI)

7-1- Inclusions bodies production and purification

Between 200 and 500 µg of expression plasmid pGMT7 (kindly provided by Dr J Boulter) containing the sequence of either the α chain or the β chain of HLA-DR*0101, were used for the transformation of Rosetta BL21 (DE3) competent cells (Novagen). The bacteria were mixed with the plasmid and incubated at 42°C for 45 seconds and incubated for 2 minutes at 4°C, the transformed cells were cultured in LB medium for 1 hour at 37°C and spread on LB/agar plates. The day following the transformation a single colony was used to inoculate 1 L of warm TYP media (16 g tryptone, 16 g yeast extract, 5 g NaCl, 2.5 g K_2HPO_4) containing 100µg/ml ampicillin, the culture was incubated at 37°C, 220 rpm. When the cells were in exponential phase (OD reached approximately 0.5 at 600nm) the inclusion bodies were inducted by adding 0.5ml of 1M IPTG (Sigma) in

culture media for 3 hours. Following this, the transformed cells were harvested by centrifugation (1510g for 20 minutes at 4°C), the supernatant was discarded. The pellet was dissolved in 40ml of lysis buffer (10mM TRIS pH 8.1, 10mM $MgCl_2$, 150mM NaCl, 10% glycerol). The dissolved pellet was sonicated (Sonicator W-385 heat system, ultra sonics, UK) on ice at 20% power for 20 minutes using a 2 second interval, following 200µl of 20mg/ml DNase I (Invitrogen) was added and shaken at RT for 30 minutes. The sonicated pellet was then treated with 100 ml of triton wash buffer (0.5% w/v Triton X100, 50mM TRIS pH 8.1, 100mM NaCl, 10mM EDTA) and centrifuged for 20 minutes at 3775g, this step was repeated 3 times. The pellet was then treated with 100ml of re-suspension buffer (50mM TRIS pH 8.1, 100mM NaCl, 10mM EDTA) and centrifuged for 20 minutes at 3775g. Finally, the inclusion bodies (IBs) were dissolved in the smallest possible volume of guanidine buffer (6M guanidine, 50mM TRIS, 2mM EDTA, 100mM NaCl) and stored in 15ml falcon tube at -80°C. Prior to refolding, protein concentration was estimated using a Coomassie Plus reagent kit™ (Pierce™) using BSA as a standard. Furthermore a NuPAGE® Gel (Invitrogen) was run to check the expression of recombinant proteins.

7-2-Refolding of HLA-DRB*0101

For refolding of HLA-DR*0101, 60mg of α chain IBs and 30mg of β chain IBs were diluted to a final concentration of 10mg/ml in guanidine buffer (6M guanidine, 50mM TRIS, 2mM EDTA, 100mM NaCl). This was incubated at 37°C for 15 minutes with 10mM DTT, and added to cold refold buffer (50mM TRIS, pH 8, 2mM EDTA, pH 8, 2.5M urea), the oxydo-reduction couple was added at the last minute (0.74g/l

cysteamine, Sigma, and 0.83g/l cysteine, Sigma). Each refold reaction was incubated with mixing at 4°C for at least 1 hour before dialysis. Dialysis was performed at 4°C over two days with 20 volumes of dialysis buffer (changing the dialysis buffer once) until the conductivity of the refolds was under 2000μS/cm. The HLA-DRB*0101 refolds were filtered and ready for the purification steps as it is described in the section 6-8.

8-PRODUCTION OF TCR IN ECHERICHIA COLI (E.COLI)

8-1- Inclusions bodies production and purification

Between 200 and 500 μg of expression plasmid pGMT7 (kindly provided by Dr J Boulter) containing the sequence of either the α chain or one of the two β chain of TCR, were used for the transformation of Rosetta BL21 (DE3) competent cells (Novagen). The bacteria were mixed with the plasmid and incubated at 42°C for 45 seconds and incubated for 2 minutes at 4°C, the transformed cells were cultured in LB medium for 1 hour at 37°C and spread on LB/agar plates. The day following the transformation a single colony was used to inoculate 1 L of warm TYP media (16 g tryptone, 16 g yeast extract, 5 g NaCl, 2.5 g K_2HPO_4) containing 100μg/ml ampicillin, the culture was incubated at 37°C, 220 rpm. When the cells were in exponential phase (OD reached approximately 0.5 at 600nm) the inclusion bodies were inducted by adding 0.5ml of 1M IPTG (Sigma) in culture media during 3 hours. Following this, the transformed cells were harvested by centrifugation (1510g for 20 minutes at 4°C), the supernatant was discarded. The pellet was dissolved in 40ml of lysis buffer (10mM TRIS pH 8.1, 10mM $MgCl_2$, 150mM NaCl, 10% glycerol). The dissolved pellet was sonicated (Sonicator W-385 heat system, ultra sonics, UK) on ice at 20% power for 20 minutes using a 2 second interval, following

200µl of 20mg/ml DNase I (Invitrogen) was added and shaken at RT for 30 minutes. This sonicated pellet was then treated with 100 ml of Triton wash buffer (0.5% w/v Triton X100, 50mM TRIS pH 8.1, 100mM NaCl, 10mM EDTA) and centrifuged for 20 minutes at 3775g, this step was repeated 3 times. The pellet was then treated with 100ml of re-suspension buffer (50mM TRIS pH 8.1, 100mM NaCl, 10mM EDTA) and centrifuged for 20 minutes at 3775g. Finally, the inclusion bodies (IBs) were dissolved in the smallest possible volume of guanidine buffer (6M guanidine, 50mM TRIS, 2mM EDTA, 100mM NaCl) and stored in 15ml falcon tube at -80°C. Prior to refolding, protein concentration was estimated using a Coomassie Plus reagent kit™ (Pierce™) using BSA as a standard. Furthermore a NuPAGE® Gel (Invitrogen) was run to check the expression of recombinant proteins.

8-2-Refolding of TCR

For refolding of TCR, different mixtures of α, β chains and redox couples have been tested (see table 8 chapter 3) . Each refold reaction was incubated with mixing at 4°C for at least 1 hour before dialysis. Dialysis was performed at 4°C over two days with 20 volumes of dialysis buffer (changing the dialysis buffer once) until the conductivity of the refolds was under 2000µS/cm. The HLA-DRB*0101 refolds were filtered and ready for the purification steps as it is described in the section 6-8.

9-TISSUE CULTURE

9-1-Tissue culture media

For the culture of cells, which were not CD4+ T cells 10% FCS-RPMI media was used. RPMI 1640 (Invitrogen) was supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin (Invitrogen), 25 mM of HEPES (Sigma), 2mM of L-glutamine (Invitrogen), and 10% of foetal calf serum (FCS, Invitrogen). For the culture of the clones bexl1 and belx2 CD4+ T cells AB-RPMI media 5% of heat inactivated human AB serum (Welsh Blood Transfusion Service) was used. For the clones HC3 and HC6 RPMI X-VIVO 15 media (Lonza, Tewkesbury, UK) 5% of heat inactivated human AB serum (Welsh Blood Transfusion Service) was used. RPMI 1640 (Sigma) was supplemented with 100U/ml of penicillin, 100 μg/ml of streptomycin (Invitrogen), 25 mM of HEPES (Sigma), 2mM of L-glutamine (Invitrogen).

For all washes during the isolation of peripheral blood mononuclear cells (PBMC), thawing, and when the APC were pulsed with peptides, a serum-free RPMI media was used. It was the RPMI 1640 (Invitrogen) supplemented as above but without adding FCS.

All the cells were cryopreserved using a freezing mix with 10% dimethyl sulphoxide (DMSO, Invitrogen), 40% RPMI 1640 (Invitrogen) and 50% FCS (Invitrogen). Cells were thawed rapidly using a 37°C water bath. After thawing, cells were washed in serum-free RPMI media, centrifuged at 425g for 5 minutes and resuspended in the appropriate serum containing RPMI media (10% FCS or 5% AB-serum). After thawing a T-cell line, the cells were plated out in AB-RPMI media supplemented with 10ng/ml of human recombinant IL-7 (Genzyme, Oxford).

9-2-Tissue culture vessels

All standard plasticware were obtained from either Nunc (Loughborough), Greiner (Stonehouse), Fisher (Loughborough) and Corning (Loughborough), with the exception of 48 well plates from Falcon (Loughborough).

10-BLOOD SAMPLES, PBMC CULTURE AND EXPANSION OF T-CELLS

10-1-Blood donors

The blood used in the screening for p37 (HPV16 E6 peptide) responses was collected by the Welsh Blood Transfusion Service (WBTS). All the buffy-coats came from female donors and were tested for HIV, HBV and HCV by the WBTS. Due to the anonymous character of the donation it was impossible to know the age of the donors, or whether they had history of cervical dysplasia or neoplasia.

The blood used as feeders was collected from laboratory staff (into 50 ml Falcon tubes containing 10U/ml of heparin) without any restriction of age or gender. Blood was taken with informed consent. Since donors were healthy volunteers, no ethical approval was required.

10-2-Isolation of PBMC from whole blood

PBMC were enriched from whole blood by density gradient centrifugation. Briefly, blood was layered upon an equal volume of Ficoll-Hypaque (Histopaque-1077, Sigma) in a 50ml Falcon tube. Blood was centrifuged with the brake inactivated for 20

minutes at 755g. This resulted in the formation of a distinct monolayer, enriched with PBMC and platelets at the surface between Histopaque and plasma. This monolayer was harvested with a pastette (Alpha, Hampshire, UK) into a 50 ml Falcon tube and serum-free RPMI media was added until 50 ml. Enriched PBMC were centrifuged at 612g for 10 minutes and serum-free RPMI media was then used to wash them, with the first 2 washes centrifuged at 425g for 5 minutes and the final spin at 272g for 5 minutes so as to reduce the platelet content of pellet.

10-3-Stocks of peptides

All the peptides were obtained from Mimotopes (Heswall, UK), dissolved in DMSO (Sigma) to give a stock of 100mg/ml. From this, aliquots of 10μg/μl were done by dilution in DMSO (Sigma) and stored at –20°C.

10-4-Expansion of Belx1 and Belx2 clones using allogenic feeder system

Belx1 and belx2 clones, CD4+ T-cells specific for HPV16 $E6_{127-141}$ peptide, were expanded in a T75 flask. $2x10^7$ irradiated PBMC feeders mixture (from 3 donors) were cultured in 50ml AB-RPMI medium supplemented with 20U/ml of IL-2, 0.5μg/ml Purified Phytohaemagglutinin (PHA). 1 x 10^6 T-cells were added to the flask which was placed into a 37°C incubator tilted at 45° so as to enhance cell-cell contact. On day 5 of expansion, half of the medium was removed and replaced with the same volume of AB-RPMI medium supplemented with 20U/ml of IL-2. Cells were re-suspended and cultured for further 2 days upright. Cells were then harvested and plated out in fresh AB-RPMI medium supplemented with 20U/ml of IL-2, at $2x10^6$ cells/well, in 24 well plates. Every

2-3 days when media turned yellow, half of the media was replaced by AB-RPMI medium supplemented with 20U/ml of IL-2.

10-5-Expansion of HC3 and HC6 clones using allogenic feeder system

HC3 and HC6 clones, CD4+ T-cells specific for influenza peptide (HA100 and HA107), were expanded in a T75 flask. $2x10^7$ irradiated PBMC feeders mixture (from 3 donors) were cultured in 50ml X-VIVO 15 RPMI medium supplemented with 20U/ml of IL-2, 0.5μg/ml PHA. $1x10^6$ T-cells were added to the flask which was placed into a 37°C incubator tilted at 45° so as to enhance cell-cell contact. On day 5 of expansion, half of the medium was removed and replaced with the same volume of X-VIVO 15 RPMI medium supplemented with 20U/ml of IL-2. Cells were re-suspended and cultured for further 2 days upright. Cells were then harvested and plated out in fresh X-VIVO 15 RPMI medium supplemented with 20U/ml of IL-2, at 2 x 10^6 cells/well, in 24 well plates. Every 2-3 days when medium turned yellow, half of the medium was replaced by X-VIVO 15 RPMI medium supplemented with 20U/ml of IL-2.

11-DETECTION OF PEPTIDE SPECIFIC T-CELLS RESPONSE BY IFNγ ELISA ASSAY

To measure the IFNγ secretion induced by peptides, some co-cultures of T-cells in presence of APC and peptides were done. T-cells were plated out at $5x10^4$ cells/well in 48 well plates. B-LCL cells were added at a ratio of 2:1 in a final volume of 0.5 ml of AB-RPMI. Cells were cultured in the presence of different concentrations of peptides (0μg/ml to 10μg/ml). Cells were incubated at 37°C overnight, 100μl of supernatant was

collected from each well and used for ELISA and 300µl of supernatant was harvested and stored at –80°C.

For ELISA, a 96 well flat-bottom Maxisorp plate (Nunc) was coated with 50µl of anti-human IFNγ antibody 1-D1K (diluted with coating buffer (0.04M Na_2CO_3, 0.06M $NaHCO_3$ pH 9.6) to 2µg/ml, Mabtech, Nacka Strand, Sweden) and incubated overnight at 4°C. The plate was washed twice with 200µl/well of PBS and 100µl/well of blocking buffer (PBS 1% FCS) was added and incubated for 1 hour at room temperature (RT). Plate was then washed 5 times with ELISA wash buffer (PBS 0.05% Tween) and the collected supernatants were added in triplicate at 100µl/well. For each experiment the IFNγ standard (Mabtech) was used according to manufacturer instructions. Briefly, IFNγ standard was reconstituted to give 10µg/ml stock solution, which was then aliquoted and stored at –20°C. One aliquot of 1µl was thawed and diluted 1 in 10,000 with dilution buffer to give a final solution at 1000pg/ml, 200µl of the standard solution was added in triplicate to the first wells and 100µl of this was used to generate $^1/_2$ serial dilutions of IFNγ using dilution buffer. The plate with the supernatants and the standard was incubated at RT for 2 hours and then washed 5 times with 200µl/well ELISA wash buffer. The antibody 7-B6-1-biotin (Mabtech) was diluted in dilution buffer (PBS, 0.1% BSA) at 1µg/ml, added at 50µl/well and incubated for 1 hour at RT. After the incubation the plate was washed 5 times and 50µl/well of substrate mix (equal volume of substrate reagent A and substrate reagent B mixed at RT, BD biosciences) were added and incubated in the dark for 10 to 30 minutes, until the standard had clearly developed. The reaction was stopped by adding 50µl/well of ELISA stop reagent (1M phosphoric acid).

The optical density was then analysed using a Bechenmark Microplate reader (Bio-Rad, Watford, UK) at 450nm. A standard curve was created with Graphpad Prism© using the serially diluted IFNγ standard, allowing the determination of the IFNγ concentration in the collected samples.

12-PROLIFERATION ASSAY

To compare the cellular activation induced by the different peptides proliferation assays were performed. $1x10^5$ BLCL cells were loaded with 10μg/ml of HVP16 $E6_{127-141}$ in 1ml of RPMI 1640 (Invitrogen) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin (Invitrogen), 25 mM of HEPES (Sigma), 2mM of L-glutamine (Invitrogen) for 1 hour at 37°C. Following this incubation BLCL cells were washed once and 15 ml of 10% FCS-RPMI (Gibco) was added. The BLCL were gamma-irradiated at 3000 rads. BLCL were plated in a 96 well plate and belx1 or belx2 cells were added at $5x10^4$cells/well. The co-culture was incubated in 10% FCS-RPMI for 3 days at 37°C. At day 2, 1μCi of [methyl-^{3}H]Thymidine (GE healthcare) was added to each well. The following day the cells were harvested on a paper filter using a cell harvester (Tomtec, UK), the filter was wet with scintillant (Fisher) and the radioactivity was counted with a liquid scintillation and luminescence counter (Wallac, UK). PMA was used as a positive control and the BLCL/belx1 or BLCL/belx2 was used as background of the experiment.

13-CALCIUM SIGNALLING

13-1-Calcium signalling of a cellular population

BLCL cells were incubated at a concentration of $1x10^6$/ml with 10µg/ml of HPV16 $E6_{127-141}$ for 1 hour at 37°C. In parallel, belx2 cells were incubated at $1x10^6$ cells/ml with 2µM of acetoxymethyl ester of Fura-2.AM (Calcium sensitive fluorescent probe), for 1 hour at 37°C in the dark. Following the incubations each type of cell was washed and resuspended in RPMI 1640 without phenol red (Invitrogen), 10% FCS. 100µl of each type of cell were collected in a round bottom 96 well plate and centrifuged at 735g for 5 minutes. Following this centrifugation the cells were re-suspended in 25 µl of 0.1% Triton X-100 (Fisher) in RPMI without phenol red (control for the maximum of fluorescence), or 0.1% Triton X-100 plus 5mM EDTA (Fisher) in RPMI without phenol red (control for the minimum of fluorescence), or RPMI without phenol red. The belx2 cells, loaded with Fura-2.AM, were plated in a black 96 well plate (Greiner) and the plate was placed in the plate reader rack. BLCL were injected 20 seconds after the beginning of reading with Fluostar™ fluorescence reader with excitation at 340 and 380 nm.

13-2-Calcium signalling of one cell

As previously the BLCL and belx2 were loaded with respectively HPV16 $E6_{127-141}$ (10µg/ml) and Fura-2.AM (2µM). The cells were incubated on ice after loading and washed in 10% FCS-RPMI. With the kindly help of Professor Hallett, BLCL cells were set down on a slide and left under a fluorescence microscope. After a few seconds, when the BLCL stopped moving, the belx2 cells were added and the calcium flux was observed on the camera connected to the microscope.

14-FLUORESCENCE CYTOMETRY

14-1-Staining with the ultimer from pro-immune

To observe the binding of the HLA-DR*0101/HPV16 $E6_{127-141}$ ultimer to its TCRs and its specificity various cell lines or clones have been stained. $1x10^5$ cells were incubated at 37°C with 10µl of the ultimer (for 1, 2 or 3 hours). Following this incubation cells were washed and stained with a 2µl of mouse anti-human CD4-FITC antibody (Serotec, Kidlington, UK) for 20 minutes at 4°C. The cells were washed twice in PBS 0.1% FCS and re-suspended in 200µl of PBS 0.1% FCS. The samples were directly acquired and analysed on FASCcalibur (BD biosciences), using the software CellQuestPro™ and analysed using the software CellQuestPro™.

14-2-Staining of the TCR

To observe the down-regulation of the TCR on belx2 cell clone a staining of the TCR was performed. $3x10^5$ BLCL cells were loaded with different concentration of peptides (0.001 to 10µg/ml) for 1 hour at 37°C in 10% FCS-RPMI. BLCL cells were washed and incubated with $1.5x10^5$ belx2 cells at 37°C for 4 hours in FACS tubes (Falcon). Following this incubation the reaction was stopped by addition in excess of cell wash buffer (BD biosciences) and the cells were washed and co-stained with 2µl of mouse anti-human TCR PAN ALPHA/BETA:RPE antibody (Serotec) and 2µl of mouse anti-human CD4-FITC antibody (Caltag, Buckingham, UK) for 20 minutes at 4°C. The cells were washed twice in PBS 0.1% FCS and re-suspended in 200µl of PBS 0.1% FCS. The samples were directly acquired and analysed on FACScalibur (BD biosciences), using the software CellQuestPro™.

RESULTS

1-SOLUBLE HLA-DRB*0101 AND SOLUBLE TCR

The E6 and E7 proteins are constitutively expressed in both high grade CIN and cervical cancer, making them ideal tumour-specific antigens for investigation. Previous studies from our laboratory have shown that CD4+ T cell responses against HPV16 peptides were detected in nearly 50% of healthy young women. These T-cell responses were tested against different pools of HPV16 E6 peptides and in two healthy donors the E6 response was mapped to HPV16 $E6_{127-141}$. Then two clones, belx1 and belx2, were generated from the PBMC of one of these donors (Gallagher and Man 2007). These clones could be phenotypically differentiated based upon their expression of either TRBV 14 or TRBV 4-2 (β chain of their TCR). Furthermore the two lines were different in their capacity to secrete IFNγ in response to HPV16 $E6_{127-141}$; belx1 demonstrated an inferior IFNγ response to HPV16 $E6_{127-141}$ compared to belx2, but both of them recognized HPV16 $E6_{127-141}$ presented by HLA-DR1.

One of the explanations for the functional variation observed between these two clones against the HLA-DR1 HPV16 $E6_{127-141}$ complex could be due to differences in their TCR expression.

To investigate this at a molecular level, recombinant soluble TCR and MHC-peptide complexes would be generated. The affinity of interactions between soluble TCR and MHC-peptide complexes could then be measured using surface plasmon technology (BIAcore™) (Cole, Rizkallah et al. 2006). From these biophysical studies it was hoped that:

-Critical peptide residues could be identified that influenced TCR, MHC interactions or both.

-Optimised peptides could be designed as vaccine candidates.

-Stable MHC-peptide complexes could be generated in order to produce fluorescent multimeric molecules suitable for detecting HPV specific T-cells directly from blood.

1-1-Construction of the HLA-DR*0101

Dr J. Boulter defined the cloning scheme for the construction of the α and β chains, according to literature (Gauthier, Smith et al. 1998). The DRA1*01 chain construct was generated in three steps: (1) amplification of complementary DNA strands corresponding to the extracellular domain and the wild type secretion signal peptide of HLA-DRA1*01 from a B cell line, (2) the amplification of the *fos* leucine zipper attached to the biotinylation tag, (3) the amplification of all the domains corresponding to the extracellular domain of HLA-DRA1*01 attached to the *fos* leucine zipper and the biotinylation tag, figure 15.

The construction of the DRB*01 chains were generated in four steps: (1) amplication of the complementary DNA strands, corresponding to the extracellular domain of HLA-DRB1*01 which was then attached to a linker by overlapping PCR, (2) the amplification of the *jun* leucine zipper sequence, (3) the amplification of the wild type signal peptide for the secretion of the molecule associated with the HPV peptide by overlapping PCR, (4) the amplification of the complete chain. The sites of the restriction endonuclease BglII was added to the DRA1*01 construct and the restriction endonuclease site, BamHI, was added to the DRB1*01 construct, figure 15. The results of these constructs are showed in figure 16. In parallel to this construct another one was

generated with the CLIP peptide sequence instead of the HPV16 $E6_{127-141}$ sequence.

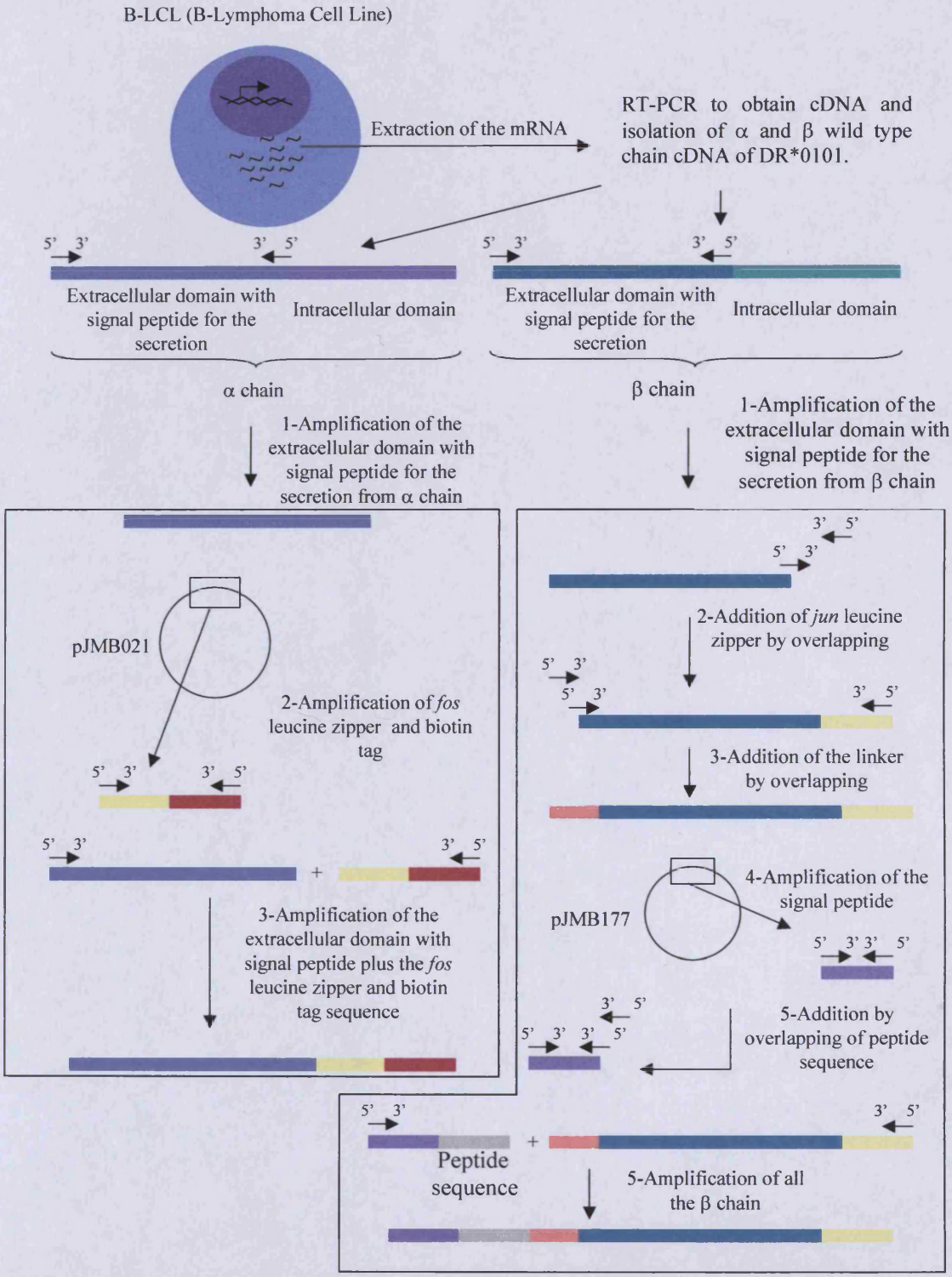


Figure 15: Schema of the construction of α and β chains to express a soluble HLA-DR*0101 associated with HPV16 $E6_{127-141}$ peptide.

HLA-DRA*0101

AGATCTATGGCC……..CCCGGGGGTCTGACTGAT……TCCGGTGGT

M A P G G L T D S G G

Bgl II ◄--------- DRA*0101 ------► ◄---------- *fos* Leucin zipper ----------►

GGTCTGAAC……..TGGCATTAAGATCT

G L N W H .

◄---------- Biotin tag ----------► Bgl II

HLA-DRB*0101

GGATCCATGGTGTGT……..GCTTTGGCTGATAAAAAA……..TGGACTGGATCTGGTGG

M V C A L A D K K W T G S G G

BamH1 ◄------ Signal peptide for secretion ----► ◄---------- Peptide ----------► ◄----------

T……..GGGTCCGGGGACACC……..GAATGGAGAGCACCCGGGGGTAGA……A

G S G D T E W R A P G G R

Linker ----------► ◄---------- DRB*0101 ----------► ◄---- *jun* Leucin

ACTACTAGGATC

N Y .

Zipper ----► BamH1

Figure 16: Construction of α and β chains of the HLA-DR*0101

At each step of the construction, PCR screening was implemented to check the presence of the insert in the vector, and sequencing was performed in order to check the sequence of the insert for both α and β chain constructs. An example of PCR screening confirming that cloning was correct is presented in figure 17.

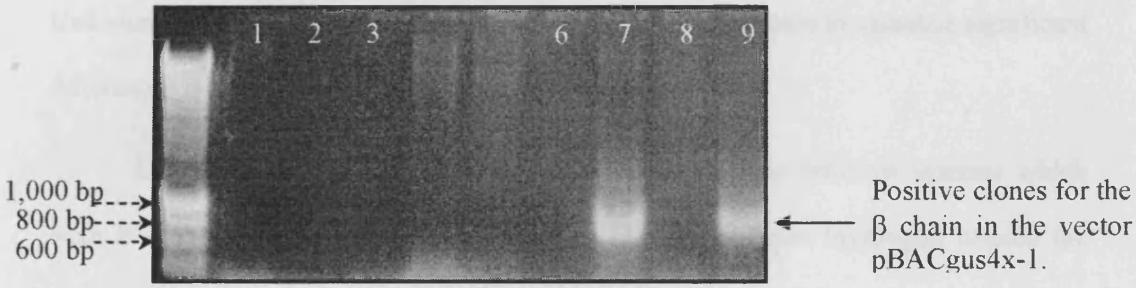


Figure 17: An example of screening: screening of different DH5α colonies after their transformation by the product of ligation between the β chain and pBACgus4x-1.

1-2-Production of recombinant virus in insect cells

Initially, recombinant baculovirus was produced using a BaculoGold kit from BD bioscience. The three constructions of the α and β chains (with HPV16 $E6_{127-141}$ or CLIP peptide) were cloned in the transfer vector pAcAB3, using the BglII and BamH1 endonuclease restriction sites respectively. After checking the correct orientation of each insert in the transfer vector, recombination with the BaculoGold was performed at room temperature, using the kit provided by BD bioscience. Following this step, sf9 cells were infected by the recombinant virus, the virus was amplified and an assay of virus titration was carried out. We used neutral red staining to count the plaques of cellular lysis,

because neutral red stains the lysomes of the living cells (figure18 A). Nevertheless, it was difficult to distinguish a true lysis plaque from a false positive just by the staining with neutral red alone. In order to identify the true lysis plaques and to define the viral titre of the initial stock, 6 well plates sf9 were infected by various dilutions of the initial viral stock from 1 to 10^{-7}, and each infection was performed between 3 and 10 days. Unfortunately, after coloration with neutral red it was not possible to visualise significant differences in lysis plaque formation between each well.

Due to these difficulties, it appeared necessary to use different systems which were available from Invitrogen and Novagen. The system from Invitrogen offered the expression of one gene, therefore the co-expression of two chains (α and β) was not possible. The system from Novagen offered co-expression of up to four genes and staining of the infected cells due to expression of β-glucuronidase, which could then be stained with X-gluc. The cloning of the α and β (with HPV16 $E6_{127-141}$ or CLIP) chains was performed in the transfer vector pBACgus4X-1, using the BglII and BamHI endonuclease restriction sites, and the recombination was performed using the BacMagic DNA at room temperature, using the buffer from the kit provided with the BacMagic and the transfer vector. In order to test if there was any recombinant virus in the media, a staining with X-gluc was performed. Indeed, recombinant gus-containing virus expressed β-glucuronidase (gus) and after contact with the X-gluc the media stained blue (data not shown). The positive supernatant was collected, used for an amplification of the recombinant virus and a viral titre assay was done with FastPlax™ Titer Kit, figure 18B. FastPlax Kit takes advantage of the appearance of detectable levels of AcNPV gp64 glycoprotein, the major envelope glycoprotein of the baculovirus, on the cell surface, as

early as 8 to 24 hours post-infection. Detection is based on a high affinity monoclonal antibody against gp64, which is added directly to fixed cells. Antibody binding is detected with Goat Anti-Mouse IgG β-Galactosidase conjugate followed by enhanced colour development with X-Gal/NBT substrates. This system was more effective than the BD bioscience system because only the cells infected could express the AcNPV gp64 protein and there were no false positives. Different assays were done to observe the specificity of the technique showing that the staining of the cells was proportional to the time of infection. After 24 hours of infection, only a few isolated cells were stained, compared to 48 hours post-infection, in which the staining of groups of infected cells, could be observed, figure 18B.

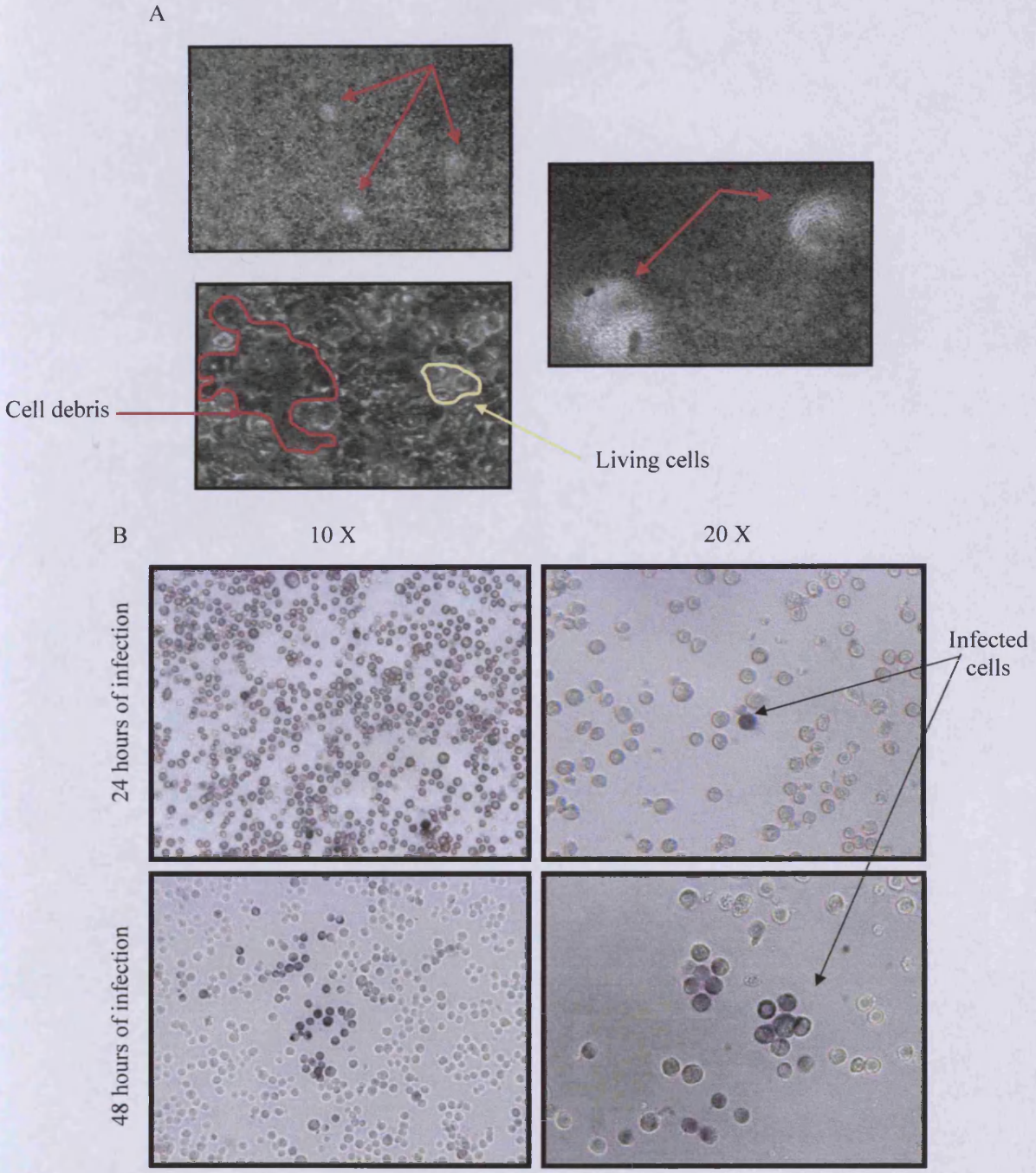


Figure 18: Detection of sf9 cells infected by recombinant virus. A-Staining using neutral red after infection during 5 days by recombinant virus using the BD system. We distinguished some light plaques and with a higher magnification we could observe cellular debris (red line) near living cells (yellow line). Nevertheless we could not be sure this represented a real lysis plaque caused by the virus. B-Staining of sf9 cells expressing viral AcNPV gp64, the envelope glycoprotein of the baculoviruses, to their surface using FastPlax™ Titer Kit. The cells were infected for 24 or 48 hours, fixed using 3.7% formaldehyde solution and stained directly. Positive cells appeared purple to black and the negative cells stayed white.

1-3-Expression of HLA-DR*0101 in insect cells

Following the previous experiments the viral titre was defined by using the formula:

Pfu/ml = 5 x (# infected cells and foci) x (dilution factor)

An average of 3 dilutions, in duplicate, were used to obtain the final viral titre.

Two viral amplifications were performed to obtain a viral stock with a good viral titre: $1.36x10^8$ pfu/ml, although after the recombination of the transfer vector with the baculovirus and the first infection the viral titre was too low to be detected by the FastPlax™ Titer Kit. The amplified viral stock was then used to infect High5 cells to obtain good expression of HLA-DR*0101- HPV16 $E6_{127-141}$ or HPV-DR*0101-CLIP. Different assays were performed using various multiplicity of infection (MOI) and time points, the following table recapitulate the various conditions for the optimisation of the expression of the HLA-DR*0101 in High5 cells.

Table 6: setting of the expression of the HLA-DR*0101 in High5 cells

MOI 2	2 ml of supernatant/66 ml of media/$170x10^6$ cells (samples taken at 48, 72, 96 hours and 5 days)
MOI 5	5.2 ml of supernatant/62.8 ml of media/$170x10^6$ cells (samples taken at 48, 72, 96 hours and 5 days)
MOI 10	10 ml of supernatant/58 ml of media/$170x10^6$ cells (samples taken at 48, 72, 96 hours and 5 days)

Each condition was tested and each supernatant was collected, filtered with 0.45µl filter and purified by ion exchange. The harvested fractions from the purification were

tested on a SDS-PAGE gel and the positive ones (60 kD) were further purified by gel filtration. These experiments showed that a MOI of 2 was not sufficient to induce the production of soluble HLA-DR*0101, indeed no peaks were detectable after purification on ion exchange column of the supernatant (data not shown). A MOI of 5 was sufficient to produce a very small amount of protein after an infection of 5 days, figure 19. The best conditions for good expression of HLA-DR*0101 were infection with a MOI of 10 for 96 hours, the infection of 5 days induced the production of less soluble HLA-DR*0101 and more cellular proteins (data not shown). Following these experiments, an infection of High5 cells in a bigger volume was implemented and after 96 hours the supernatant was collected and purified by ion exchange and gel filtration, figure 20.

In order to test the conformation of the soluble HLA-DR*0101 associated with HPV16 $E6_{127-141}$ or CLIP obtained from High 5 culture, surface plasmon resonance assays were performed. Soluble HLA-DR*0101-HPV16 $E6_{127-141}$ or HLA-DR*0101-CLIP was immobilised on a CM5 chip via the interaction between its biotin tag and the streptavidin on the chip, and L243 antibody (specific for the α chain of HLA-DR1) was injected, figure 21. A biotinylated HLA-DR*0101-HA (with influenza peptide) was used as positive control on the flow cell number 2 (Fc 2), a class I MHC (A2) was used as negative control, Fc 1, and the biotinylated HLA-DR*0101-CLIP was on Fc3. Unfortunately no binding was observed after different assays with different concentration of L243 antibody or soluble HLA-DR*0101, the figure 21A shows the best observed response. Nevertheless low amounts and impure HLA-DR*0101-CLIP was generated, figure 21B. The results shown low amount of class II MHC binding the L243 antibody

compared to the positive control with HLA-DR*0101-HA (kindly provided by Dr Cole, and generated from CHO cells).

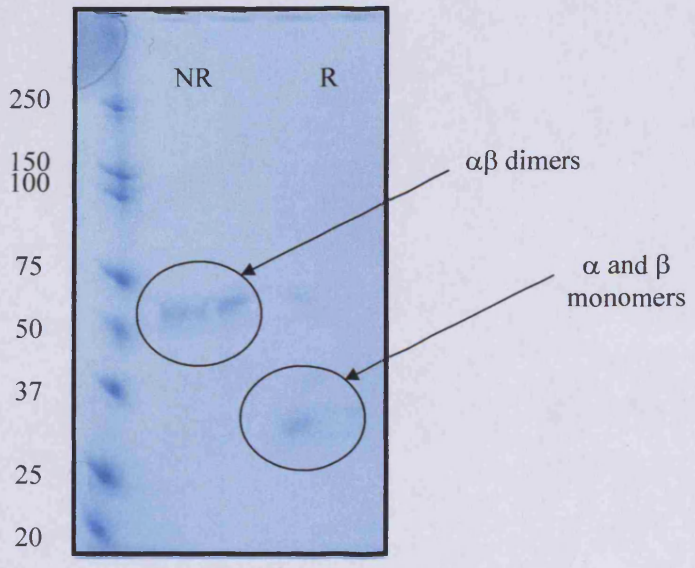


Figure 19: Expression of HLA-DR*0101 in High 5 cells after infection with a viral stock of MOI 5. The cells were cultured at $2.5x10^6$ cells/ml and virus was added to obtain a MOI of 5. Following 5 days of infection the supernatant was collected and filtered at 0.45µl. The sterile solution was purified by anion exchange and gel filtration. The positive fractions were collected and a SDS-PAGE gel was performed. The sample was loaded on the gel with a non-reducing buffer (NR), band at 60kD and with a reducing buffer (R). Due to the low expression of DR1, other MOIs and times of infection were tested.

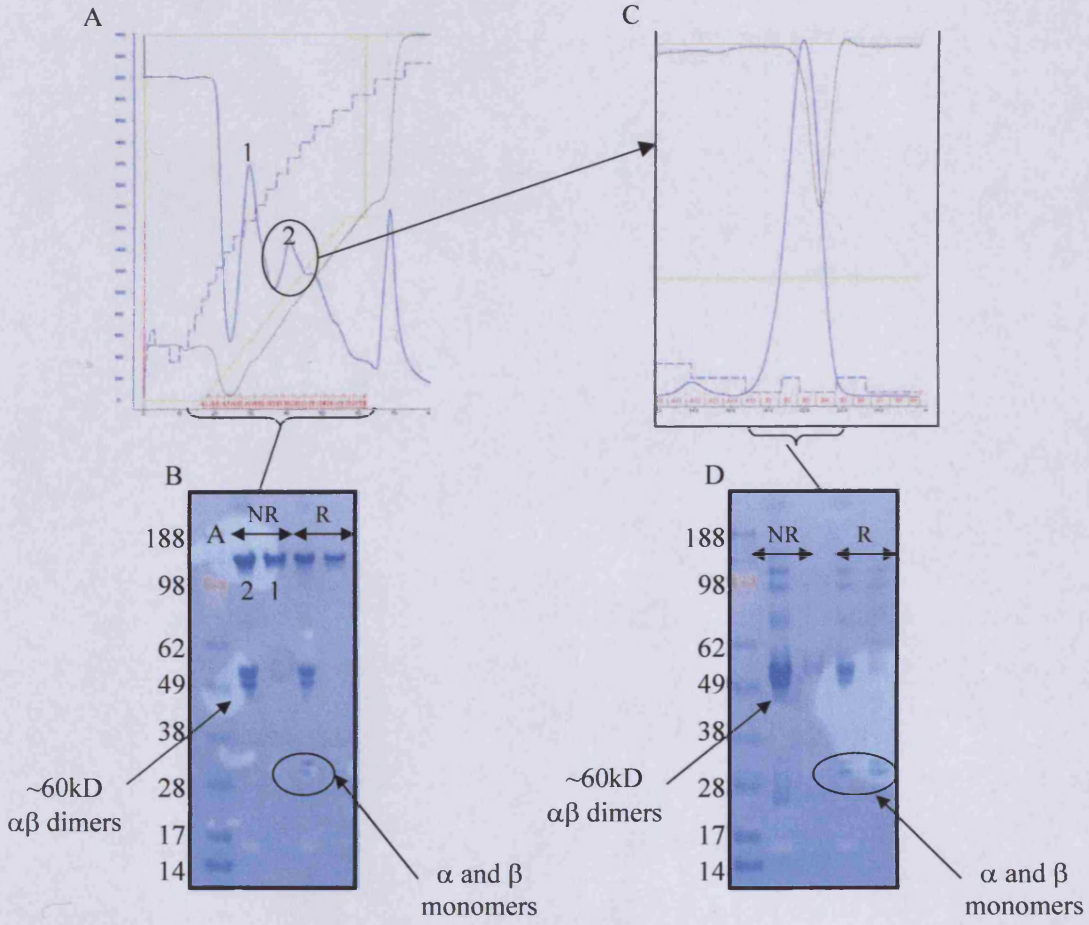


Figure 20: Expression of the HLA-DR*0101 in High5 cells. A-Purification using an anion exchange column of supernatant from High5 cells infected during 96 hours at MOI 10. B-SDS-PAGE of the two fractions chosen due to their molecular weight (~60kD), after purification by anion exchange column, in non-reduced conditions (NR) and reduced conditions (R). C-Purification using a gel filtration column (S200) of the two fractions. D-SDS-PAGE of the fractions collected after the gel filtration purification in non-reduced conditions (NR) and reduced conditions (R).

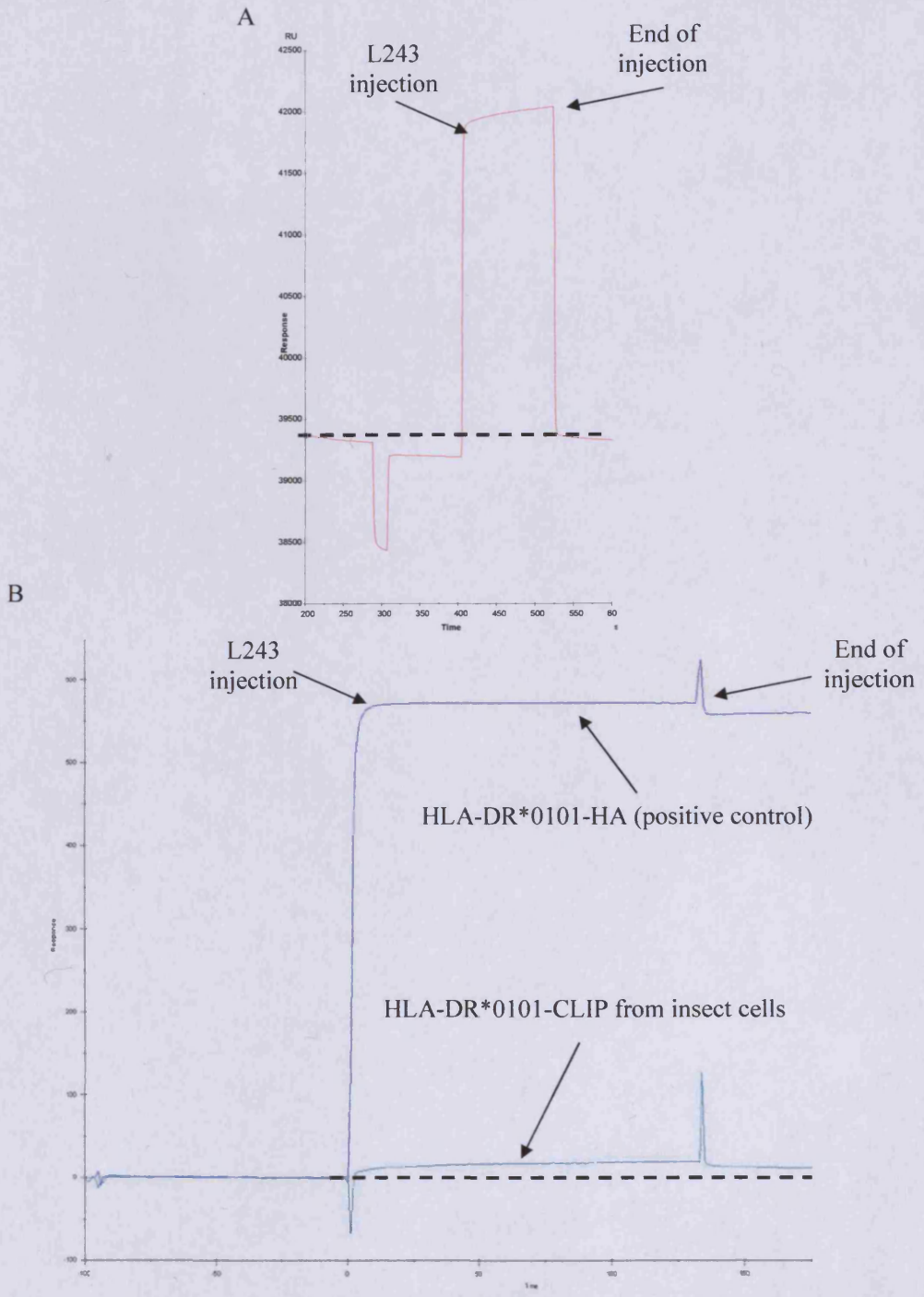


Figure 21: BIAcore assays. A-Assay with HLA-DR*0101-HPV16 $E6_{127-141}$. The biotinylated HLA-DR1 (0.25mg/ml) was immobilised on the CM5 chip via the interaction between its biotin tag and the streptavidin which was immobilised on the chip. L243 antibody was injected at 0.4mg/ml. B-Assay with HLA-DR*0101-CLIP. The biotinylated HLA-DR1 (0.25mg/ml) was immobilised on the CM5 chip via the interaction between its biotin tag and the streptavidin which was immobilised on the chip (green line). L243 antibody was injected at 0.4mg/ml. HLA-DR*0101-HA (0.25mg/ml) was used as positive control (blue line). Some specific binding was observed.

1-4-Expression of HLA-DR1*0101 and its TCR in *E.coli*

The benefits of protein expression with baculovirus can be summarized as: eukaryotic post-translational modification (proper proteolysis, N- and O-glycosylation, acylation, amidation, carboxymethylation, phorphorylation, and prenylation), proper protein folding and function, high expression levels (up to 30% of the total cell protein), high-density suspension culture and safety. Despite these potential advantages, particular patterns of post-translational processing and expression must be empirically determined for each construct. Different problems can explain the poor results obtained for few proteins. Indeed it is possible to have an inefficient secretion, poor refolding of the molecule due to intracellular aggregates caused by the infection cycle, or species-specific or tissue-specific modifications that do not occur. These disadvantages could explain why no binding between the soluble HLA-DR*0101-HPV16 $E6_{127-141}$ and the L243 antibody was observed.

A number of other protein expression systems exist including *E.coli*, yeast and mammalian expression systems, table 6. The mammalian system is generally expensive and produces smaller yields, although it has the potential to provide all the post-translational modification required for the production of soluble protein. The yeast expression, which could be a good alternative to the insect cell expression system is time consuming and is not a technique used by the laboratory. The *E.coli* expression system is robust, low cost and produces high yields of proteins. Furthermore this expression system was already used in the laboratory. In this system target genes were cloned into pGMT7 plasmids under the control of T7 transcription signals. The *E. coli* bacteria, BL21 DE3, were transformed by either α-pGMT7 (DR1α) or β-pGMT7 (DR1β). BL21 have the

advantage of being deficient in both *lon* and *ompT* proteases and DE3 indicates that the host is a lysogen of λDE3, and therefore carries a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter. Such strains are suitable for production of protein from target genes cloned in pGMT7 vectors by induction with isopropyl-β-D-1-thiogalactopyranoside (IPTG). IPTG binds to repressor of the *lac* operon and inactivates it, but is not a substrate for β-galactosidase and it cannot be metabolized by *E. coli.* Its concentration remains constant and the rate of expression of *lac p/o*-controlled genes, is not a variable in the experiment. In addition, IPTG is transported efficiently, independent of whether the *lacY* gene is functional.

The α and β chains of HLA-DR*0101 or the α or β chains of the TCRs specific for the recognition of the HPV16 $E6_{127-141}$-HLA-DR*0101 were cloned in the expression vector pGMT7, and BL21 DE3 *E.coli* cells were transformed with the constructs for the production of each chain (DR1α, DR1β, TCRα and TCRβ). Following induction by IPTG, the inclusion bodies were collected purified and the *in vitro* refolding of the different molecules was performed. The denaturated inclusion bodies were purified by anion exchange and different fractions were collected, concentrated and purified by gel filtration. Fractions were analyzed by SDS-PAGE (figure 22). From this gel, fractions 11 to 13 for the α chain and 11 to 14 for the β chain were chosen due to their purity and the high concentration of soluble molecules. Different refolding conditions (table 7) were tested and analysed by SDS-PAGE (figure 23) but no *in-vitro* refolding attempt generated a molecule with the correct molecular weight which was 60kD, an example of the refolding result is presented figure 23.

Table 6: Comparison of Expression Systems (adapted from Genway).

Characteristics	*E. coli*	Yeast	Insect cells	Mammalian cells
Cell Growth	Rapid (30 Min)	Rapid (90 Min)	Slow (18-24 H)	Slow (24 H)
Complexity of Growth Medium	Minimum	Minimum	Complex	Complex
Cost of Growth Medium	Low	Low	High	High
Expression Level	High	Low - High	Low - High	Low - Moderate
Extracellular Expression	Secretion to Periplasm	Secretion to Medium	Secretion to Medium	Secretion to Medium
Protein Folding	Refolding Usually Required	Refolding May Be Required	Proper Folding	Proper Folding
N-linked Glycosylation	None	High Mannose	Simple, No Sialic Acid	Complex
O-linked Glycosylation	No	Yes	Yes	Yes
Phosphorylation	No	Yes	Yes	Yes
Acetylation	No	Yes	Yes	Yes
Acylation	No	Yes	Yes	Yes
gamma-Carboxylation	No	No	No	Yes
Yield (mg) (per liter culture)	50-500	10-200	10-200	0.1-100
Success Rate (%) (soluble or functional	40-60	50-70	50-70	80-95
Project Cost	Low	Low	Middle	High
Recommended Use	Antigen protein, Protein standards, Functional proteins	Antigen protein, Protein standards, Functional proteins	Proteins with glycosylation, Assay standards, Secreted form.	Functional study, PTM study, Assay standards, Characterization
Advantage	Simple, robust, lowest cost, highest yield	Simple, low cost, good for certain proteins	Relatively higher yield, better PTM	Natural protein configuration, best PTM
Disadvantage	Least PTM[a]	Longer time, less PTM	Longer time, higher cost	Highest cost, lower yield

[a] PTM = Post-Translational Modification such as glycosylation

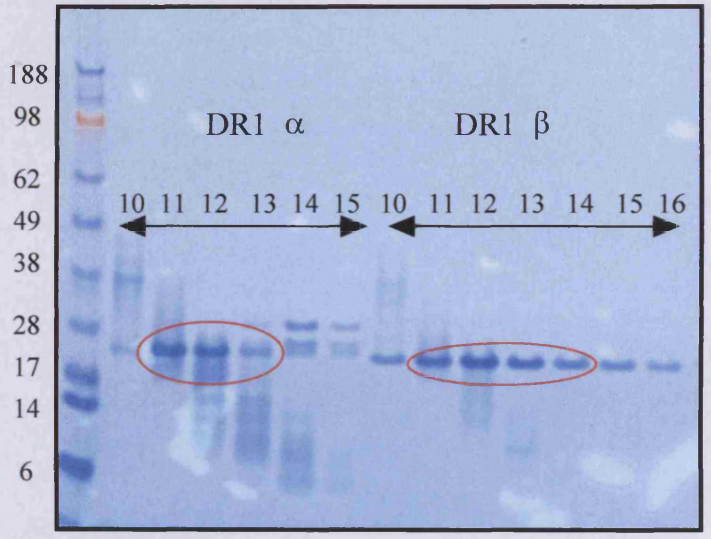


Figure 22: Expression of the α (33kD) and β (28kD) chains of HLA-DR*0101 in BL21/DE3. Inclusion bodies were induced by addition of IPTG for 4 hours. After different washes with lysis and denaturating buffer the α and β chains were purified by anion exchange and by gel filtration. Different fractions were collected and a SDS-PAGE analysis was performed.

Table7: Different refolding conditions used to test the production of soluble HLA-DR*0101-HPV16 $E6_{127-141}$ complex

TCEP Glycerol	GSH/GSSG Metals	TCEP Metals	GSH/GSSG Glycerol	TCEP Glycerol	EDTA GSH/GSSG GuHCl	TCEP Metals	GSH/GSSSG PEG6000
TCEP L-arg	EDTA GSH/GSSG EDTA	Cyclodexdrin TCEP Glycerol	EDTA Cyclodextrin GSH/GSSG L-arg	Cyclodexdrin TCEP Metals	Cyclodextrin GSH/GSSG Glycerol	Cyclodextrin TCEP GuHCl	Cyclodextrin GSH/GSSG EDTA
Cyclodexdrin TCEP	100mM NaCl EDTA GSH/GSSG	100mM NaCl TCEP PEG6000	100mM NaCl GSH/GSSG	100mM NaCl TCEP	100mM NaCl GSH/GSSG PEG600	100mM NaCl TCEP GuHCl	100mM NaCl EDTA GSH/GSSG GuHCl
Cyclodexdrin TCEP GuHCl	100mM NaCl Cyclodextrin GSH/GSSG GuHCl	100mM NaCl TCEP GuHCl	100mM NaCl Cyclodextrin GSH/GSSG	100mM NaCl Cyclodextrin TCEP	100mM NaCl Cyclodextrin GSH/GSSG Glycerol	100mM NaCl Cyclodextrin TCEP L-arg	100mM NaCl Cyclodextrin GSH/GSSG Metals
100mM NaCl TCEP PEG6000	250mM NaCl GSH/GSSG PEG6000	100mM NaCl Cyclodextrin TCEP Metals	250mM NaCl GSH/GSSG EDTA	100mM NaCl Cyclodextrin TCEP GuHCl	250mM NaCl GSH/GSSG EDTA	100mM NaCl Cyclodextrin TCEP PEG6000	100mM NaCl EDTA GSH/GSSG Cyclodextrin
100mM NaCl Cyclodexdrin TCEP	250mM NaCl Cyclodextrin GSH/GSSG Glycerol	250mM NaCl TCEP PEG6000	250mM NaCl Cyclodextrin GSH/GSSG GuHCl	250mM NaCl TCEP GuHCl	250mM NaCl GSH/GSSG EDTA PEG6000	250mM NaCl TCEP	250mM NaCl GSH/GSSG Glycerol
250mM NaCl TCEP metals	250mM NaCl Cyclodextrin GSH/GSSG EDTA	250mM NaCl Cyclodextrin TCEP L-arg	250mM NaCl Cyclodextrin GSH/GSSG metals	250mM NaCl Cyclodextrin TCEP	250mM NaCl GSH/GSSG EDTA Cyclodextrin	250mM NaCl Cyclodextrin TCEP	250mM NaCl Cyclodextrin GSH/GSSG PEG6000
L-arg EDTA	250mM NaCl Cyclodextrin	Cyclodextrin L-arg	250mM NaCl Metals	L-arg	250mM NaCl L-arg	Glycerol	250mM NaCl
100mM NaCl PEG6000	250mM NaCl Cyclodextrin GSH/GSSG GuHCl	Cyclodextrin EDTA	250mM NaCl Glycerol	Cyclodextrin EDTA	250mM NaCl PEG6000 Metals	L-arg EDTA	250mM NaCl L-arg
100mM NaCl Glycerol	250mM NaCl Cyclodextrin L-arg	100mM NaCl GuHCl	250mM NaCl Cyclodextrin PEG6000	Cyclodextrin GuHCl	250mM NaCl Cyclodextrin Glycerol	Cyclodextrin Metals	250mM NaCl Cyclodextrin Metals
100mM NaCl Cyclodextrin Metals	Buffer only	100mM NaCl EDTA	Buffer only	100mM NaCl Metals	Buffer only	100mM NaCl	Buffer only
250mM NaCl	ddH_2O	100mM NaCl Cyclodextrin Glycerol	ddH_2O	100mM NaCl Cyclodextrin PEG6000	Empty	100mM NaCl Cyclodextrin GuHCl	Empty
pH 7.0		pH 7.5		pH 8.0		pH 8.5	

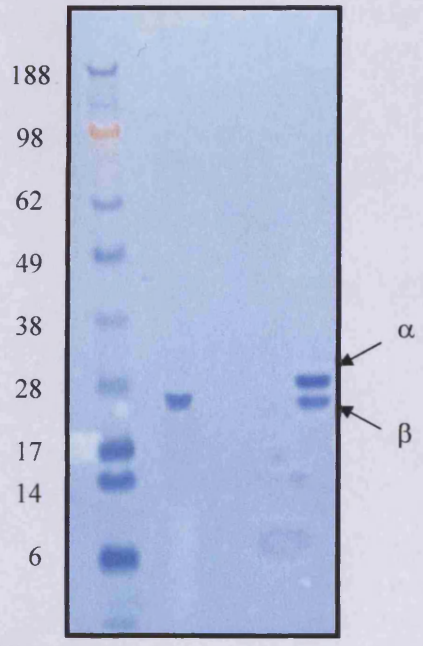


Figure 23: Different refolding conditions were analysed by SDS-PAGE for identification of bands at size corresponding to the HLA-DR*0101 (~60kD). Only bands corresponding to the α and β chains complexed could be observed (33kD for α chain and 28kD for the β chain).

The same experiments were performed with the α and β chains of the TCRs specific for the HLA-DR*0101-HPV16 $E6_{127-141}$. Dr J. Boulter performed the amplification, sequencing of the TCR α and β chains from belx1 and belx2 T cells, and cloning in pGMT7. He found three different TCR chains, one α (plasmid: pJMB154 with the α chain TRAV17) and two β (pJMB155 with the β chain TRBV14 with corresponded to belx1 and pJMB156 with the β chain TCRBV4-2 which corresponded to belx2). The *E. coli* protein expression system was used to produce the different chains, using the expression vector pJMB154, pJMB155 and pJMB156 to transform BL21DE3 *E. coli* bacteria. After induction, the inclusion bodies were washed, solubilized, and the expression of recombinant proteins was checked by SDS-PAGE gel, figure 24. The expression of the α chain from pJMB154 and of the β chain from pJMB156 was successful, but no expression of the β chain from the vector pJMB155 was observed. Different refolding conditions were used in order to produce soluble TCR, which were purified by anion exchange and gel filtration, table 8. Each refold was checked by SDS-PAGE for species with the correct molecular weight for the TCR (50 kD). However, no potential bands with the correct molecular size were observed.

Despite several attempts, no refolded HLA-DR*0101-HPV16 $E6_{127-141}$ peptide complex or TCR was produced. Therefore to move the project forward, an HLA-DR1*01/HPV16 $E6_{127-141}$ ultimer was ordered from Pro-immune (Oxford, UK). This company has successfully synthesized other HLA-DR1 ultimers e.g. HLA-DR1 complexed to a hemagglutin peptide.

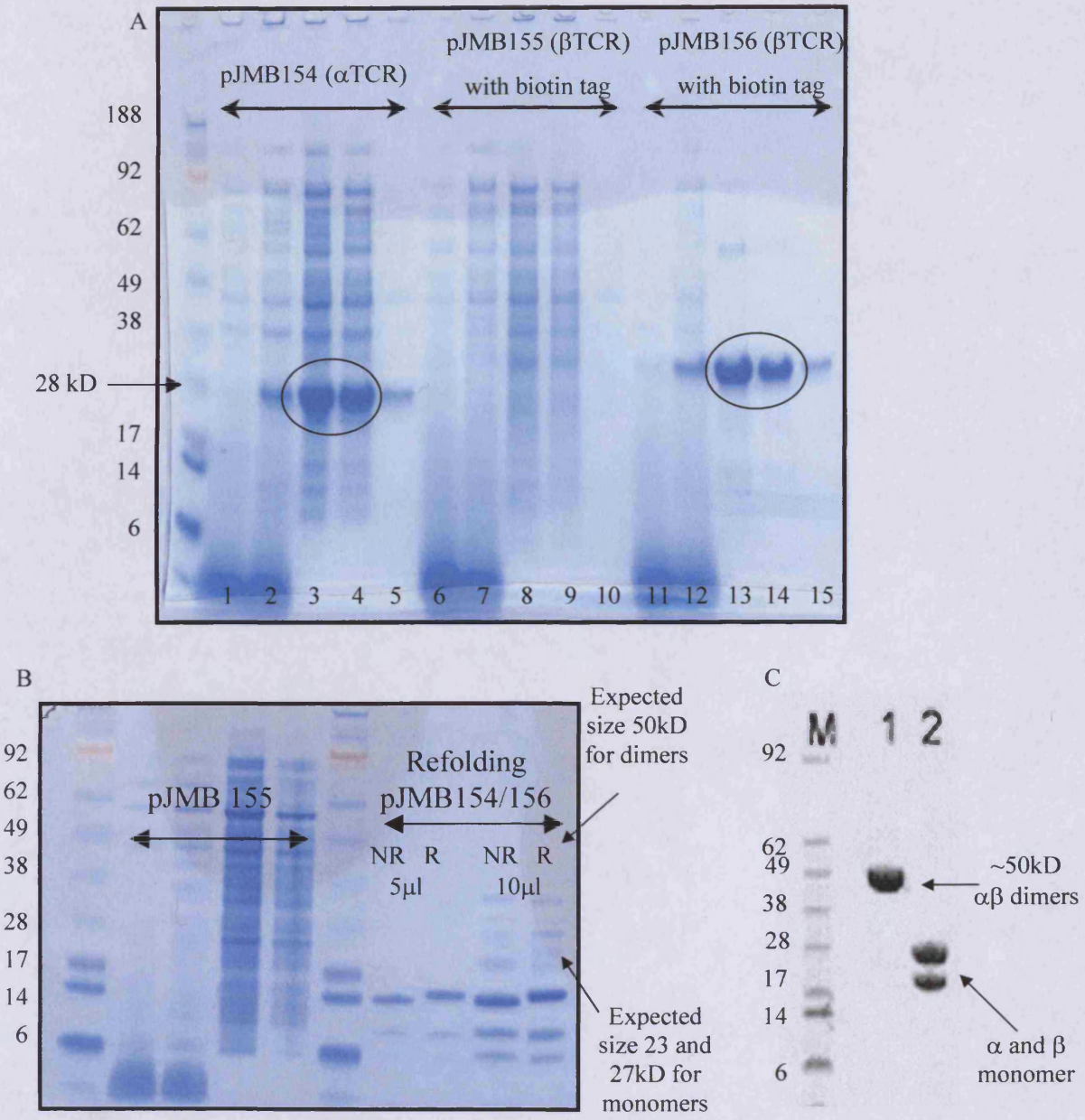


Figure 24: Expression and refolding of recombinant proteins. A-Expression of the recombinant TCRs α and β chains after purification of inclusion bodies from BL21 DE3, the expected size for the α chain was 23kD and for the β chain 27kD (due to its extra loops). B-Left part is another assay for the expression of the pJMB155 vector in the BL21DE3, on the right is the incorrect refolding of the TCR and we can observe that the bands do not correspond to the expected size of the TCR dimer, or α or β chains. C-Refolding of a TCR specific for influenza peptide, the αβ dimer of TCR was observed at ~50kD and the α and β monomer were observed at 23kD and 27kD, correct refolding of a TCR specific for influenza peptide is presented to show the expected results (SDS-gel profited by D. Cole).

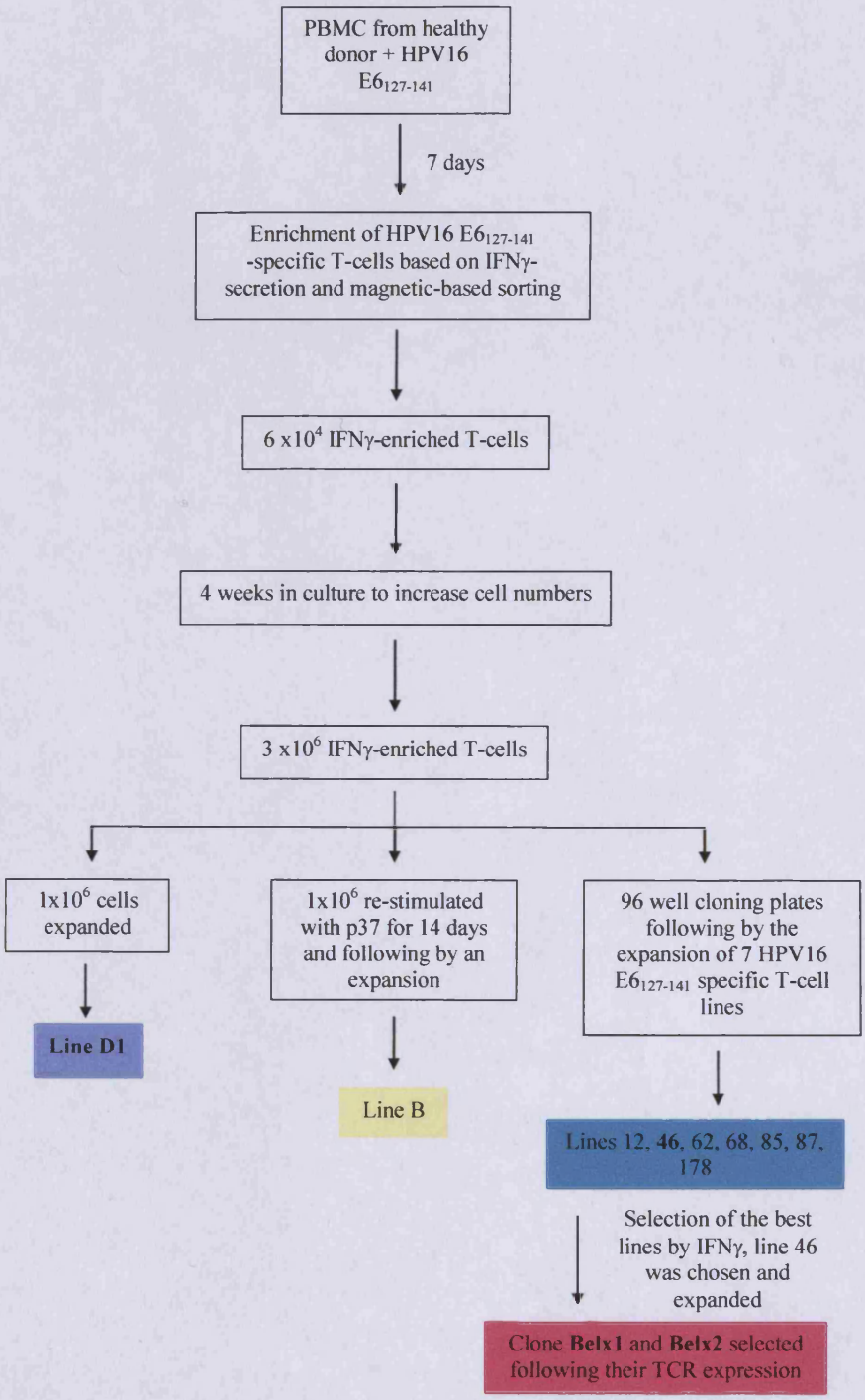
Table 8: Conditions tested for the refolding of TCRs specific of the HLA-DR*0101-HVP16 E6127-141

pH	8	8	8	8	8.5	8.5	8.5
Tris	50mM	50mM	50mM	50mM	50mM	50mM	50mM
EDTA	2mM	2mM	2mM	2mM	2mM	2mM	2mM
Urea	2.5M	2.5M	2.5M	2.5M	2.5M	2.5M	2.5M
Cysteamine	3.7g/l	3.7g/l	3.7g/l	3.7g/l	3.7g/l	3.7g/l	3.7g/l
Cystamine	0.83g/l	0.83g/l	1.245g/l	0.83g/l	0.83g/l	0.83g/l	1.245g/l
L-arginine	none	none	none	200mM	none	200mM	none
α chain	60mg/l	30mg/l	60mg/l	60mg/l	60mg/l	60mg/l	60mg/l
β chain	30mg/l	30mg/l	30mg/l	30mg/l	30mg/l	30mg/l	30mg/l

1-5-Staining of Belx1 and Belx2 by HLA-DR*0101 ultimer

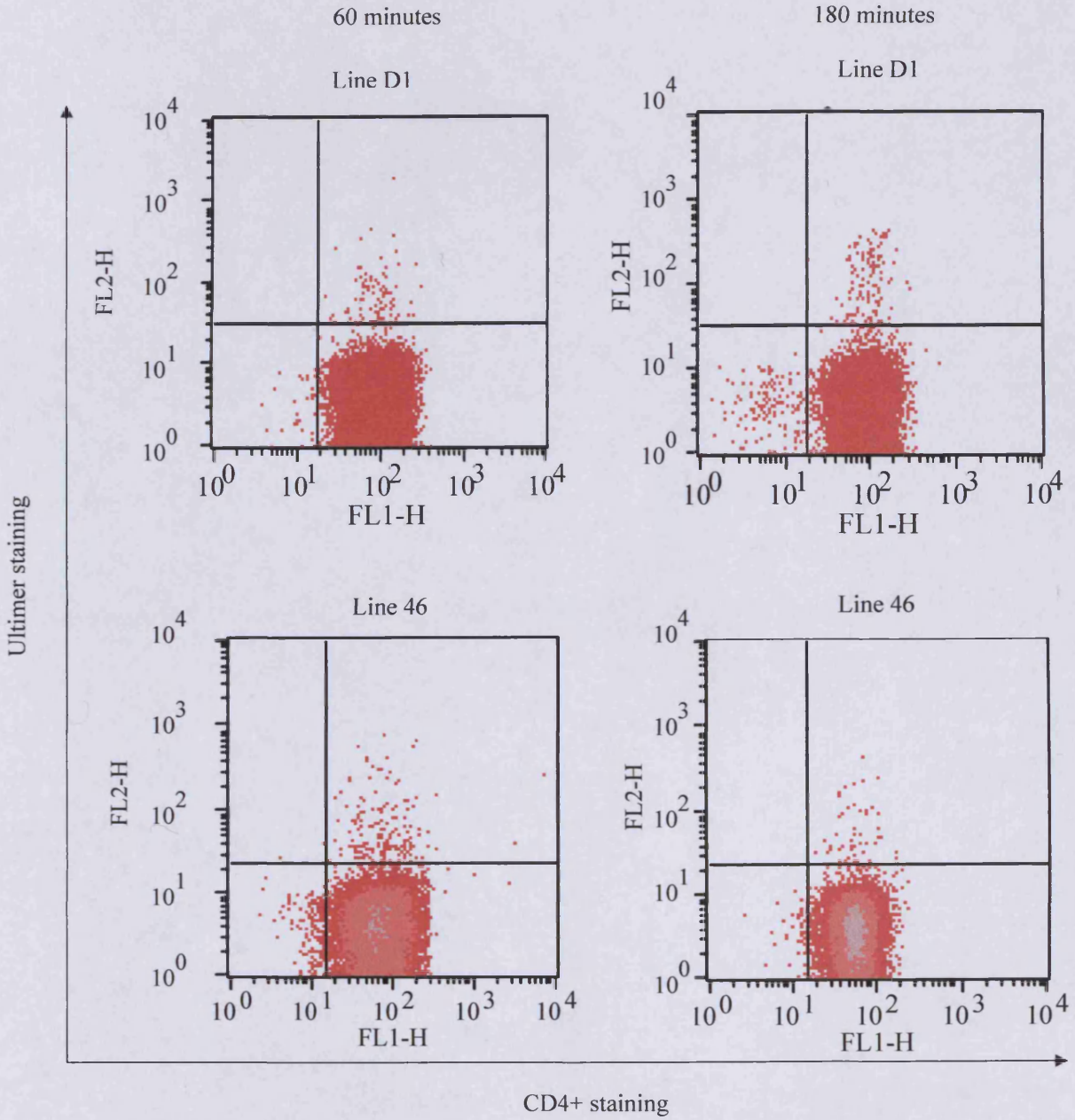
Soluble HLA class II peptide ultimers are predicted to bind to the TCR of peptide specific T-cells. Since these complexes are fluorescent, binding can be detected by flow cytometry. To test the specificity of the HLA-DR*0101-HPV16 $E6_{127-141}$ ultimers made by Proimmune, the ultimers were used to stain two HPV16 $E6_{127-141}$ specific T-cell clones (belx1 and belx2), and the parental T-cell lines (figure 25). It was predicted that the line D1 would not have a high percentage of stained cells, line 46 would have a better staining, and the belx1 and belx2 clones would have the highest staining (between 90 and 100%). A first batch sent by Proimmune was tested using several different conditions (figure 26). The staining was done at 37°C for 120 or 180 minutes, nevertheless no staining by the HLA-DR*0101-HPV16 $E6_{127-141}$ ultimer was observed. We have tested a second batch from Proimmune, also with negative results (data not shown).

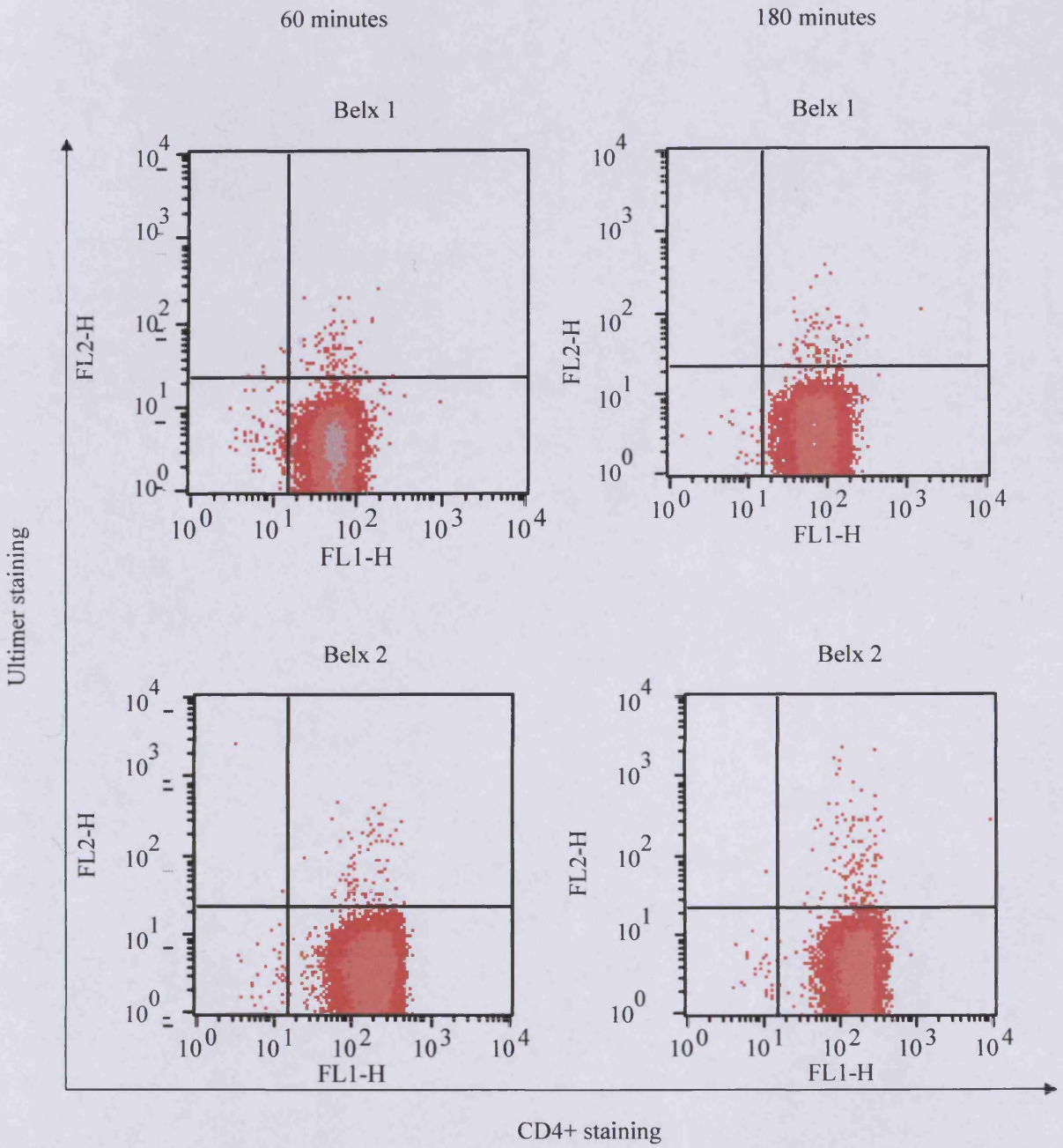
In order to test the functionality of the cells an IFNγ ELISA was performed with belx1 and belx2. B-LCL cells, expressing HLA-DR1*01, were loaded with the different concentration of HPV16 $E6_{127-141}$ peptide at 37°C for 1 hour, washed and co-cultured with either belx1 or belx2 overnight. The IFNγ ELISA was performed the following day, figure 27. This assay demonstrated the specificity and the response of each clone for the peptide. This response was dependant on the concentration of the peptide and was characteristic for each clone; indeed belx2 showed a better response than belx1 with a higher secretion of IFNγ and a response with smaller concentration of peptide than belx1.



Figur 25: Overview of the process by which peptide HPV16 $E6_{127-141}$-specific T-cell lines were generated from healthy donor (adapted from K.Gallagher thesis).

Figure 26: Staining of the different lines or clones by an antibody against CD4 and the ultimer from pro-immune (first batch). The cells were incubated at 37°C during 60 or 180 minutes with the ultimer, washed and incubated 20 minutes at 4°C with the CD4+FITC antibody. The cells were not fixed and directly used for Facs.




60 minutes
180 minutes
Belx 1
Belx 1
Belx 2
Belx 2
FL2-H
FL1-H
Ultimer staining
CD4+ staining

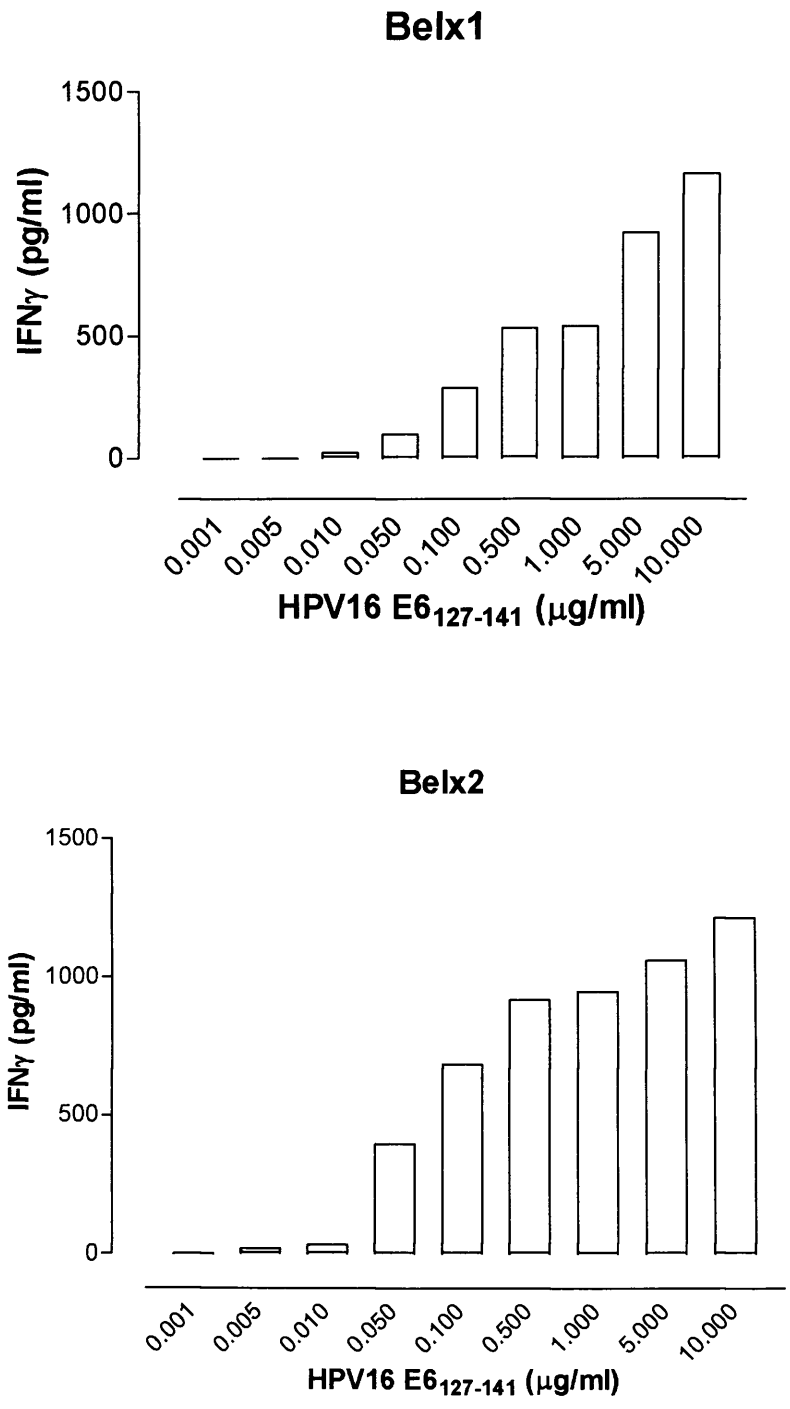


Figure 27: Titration of the specific response against HPV16 $E6_{127-14}$ generated by the clones Belx1 and Belx2. The CD4+ T-cells were co-cultured with B-LCL loaded with peptide at 37°C overnight and the supernatant were collected and tested by IFNγ ELISA.

1-7-Discussion

It was proposed that the functional variation between belx1 and belx2, was caused by different TCR avidity or affinity. To investigate this, at a molecular level, attempts were made to generate recombinant soluble TCR and MHC peptide complexes. Nevertheless no soluble TCR or HLA-DR*0101-HPV16 $E6_{127-141}$ molecules were obtained only a low amount of soluble HLA-DR*0101-CLIP was produced.

A variety of expression systems were used to prepare recombinant class II MHC-peptide complexes (table 9). Each method has its advantages and its disadvantages. *E. coli* expression is the method of choice for the production of class I MHC proteins, and can provide a large quantities of highly purified protein. However, the folding conditions for class II MHC proteins need to be optimized individually for each species and allotypes and at present only four different class II MHC proteins have been produced using *E.coli* system (Vollers and Stern 2008). The most common recombinant expression system for class II MHC proteins is the co-expression in baculovirus infected insect cells. However, each generated DR1 molecule, in *E. coli* or insect cells systems, was associated with an influenza peptide, which is known to promote a high stability of the molecule. The stability between the HPV16 $E6_{127-141}$ peptide and HLA-DR*0101 is not known and it is possible that it is much lower than for influenza peptide. Furthermore it is important to know that the choice of methodologies for the production of class II MHC-peptide complexes will depend on the particular alleles of interest and previous experience in protein expression (Cameron, Norris et al. 2002).

Table 9: Class II MHC proteins used in MHC tetramer staining of antigen-specific CD4+ T-cells in human.

MHC protein	Allotype	Expression system	Modification	References
DR1	DRB1*0101	*E. coli*		(Cameron, Norris et al. 2002)
		E. coli	Covalent peptide	(Cunliffe, Wyer et al. 2002)
		Insect		(Cameron, 2002)
		Insect	Zipper	(Danke and Kwok 2003)
		Mammalian	Covalent and zipper	(Day, Seth et al. 2003)
DR2	DRB1*1501	Mammalian	Covalent and zipper	(Day, Seth et al. 2003)
	DRB5*0101	Mammalian	Covalent and zipper	(Day, Seth et al. 2003)
DR3	DRB1*0301	Insect	Zipper	(Bronke, Palmer et al. 2005)
DR4	DRB1*0401	Insect	Covalent peptide	(Meyer, Trollmo et al. 2000) (Kotzin, Falta et al. 2000)
		Insect	Zipper	(Novak, Liu et al. 1999)
		Mammalian	Covalent and zipper	(Day, Seth et al. 2003)
	DRB1*0402	Insect		(Veldman, Eming et al. 2007)
	DRB1*0404	Mammalian		(Gebe, Novak et al. 2001)
DR5	DRB1*1101	*E. coli*	Covalent peptide	(Cunliffe, Wyer et al. 2002)
		Insect	Zipper	(Moro, Cecconi et al. 2005)
DR6	DRB1*1302	Insect	Zipper	(Laughlin, Miller et al. 2007)
DR7	DRB1*0701	Insect	Zipper	(Danke and Kwok 2003)
DQ6	DQA1*0102 DQB1*0602	Insect	Zipper	(Kwok, Liu et al. 2000)
DQ2	DQA1*0501 DQB1*0201	Insect	Zipper	(Quarsten, McAdam et al. 2001)
DP4	DPA1*0103 DPB1*0401	Insect	Covalent and zipper/Ig	(Zhang, Renkvist et al. 2005)

We were able to produce the recombinant baculovirus as observed by the colouration of the cellular supernatant by X-gluc. The staining of the cells expressing the viral gp64 after amplification showed that the sf9 cells were sensitive to the infection by the recombinant virus. The purification of the soluble class II MHC by anion exchange and gel filtration showed that the High 5 cells seemed to produce intact molecules. However the BIAcore assay demonstrated that no binding was observed between HLA-DR*0101-HPV and L243 (anti-HLA-DR antibody), although a binding of a low amount of HLA-DR*0101-CLIP was observed. The expression of each unit for HLA-DR*0101 in *E. coli* was successfully performed, but no correct refolding was observed. One explanation for these results could be the weak stability of the class II MHC in presence of the HPV peptide, compared to the CLIP peptide which is naturally used by the cell to stabilise the class II MHC before loading of exogenous peptides.

Studies featuring CD4+ T cell staining by class II tetramers has became more common but there are still very few studies compared to the thousands done with class I MHC tetramers (Vollers and Stern 2008). Production of class II MHC tetramers remains complicated, and there are several different methods dependant on the class II molecule studied. Nevertheless there are other limits to the staining of the CD4+ T cells by tetramers including: the low frequency of antigen specific CD4+ T-cells and complications potentially caused by low TCR/pMHC II affinity. Lucas et al. overcame the low frequency obstacle, by using microbeads to enrich the target population (Lucas, Day et al. 2004). Before enrichment, no distinct population of HA-specific CD4+ cells could be detected in fresh PBMCs, although a population of $CD4^+$ DR1-HA^+ cells was clearly visible after enrichment with anti-PE microbeads. Another strategy chooses to

detect cells after class II MHC tetramer staining is the *ex-vivo* expansion of the target cells (Lemaitre, Viguier et al. 2004). However highly enriched (including clonal) populations of antigen specific T-cells were used in the current study. Therefore the failure of the commercially produced ultimers to bind must be due to other factors. It is possible that antigen specific T-cells are not stained by class II MHC tetramers because the TCR affinity for soluble MHC/peptide complexes is too weak (Vollers and Stern 2008). By contrast, TCR avidity for MHC-peptide complexes on live antigen specific cells may be influenced by other cell surface molecules. This might explain the lack of binding of the Proimmune ultimer, despite clear functional specificity of the tested cells.

Despite using several different conditions, no binding of the Proimmune ultimer to T-cells could be detected. The technical team at Proimmune could not offer any explanation for the results. It is possible that either unstable or incorrectly folded molecules were produced but without knowledge of the exact composition of the commercial product it was not possible to assess this with protein biochemistry techniques. The company would not provide any technical details about manufacture of the ultimer, but did offer a full refund.

No biophysical measurements for TCR/MHC/peptide interactions were obtained in this project (figure 28). It was not possible to make either soluble MHC/HPV16 $E6_{127-141}$ complexes or TCRs, and no binding could be detected for commercially produced MHC/peptide complex. Therefore to move the project forward it was decided to investigate the functional difference between belx1 and belx2 at a cellular level using in the first instance truncated versions of HPV16 $E6_{127-141}$ and in a second instance some peptide variants derived from other HPV types.

Figure 28: Summary of problems solving for protein production

2-STUDY OF THE RESPONSES INDUCED BY TRUNCATED VERSION OF HPV16 $E6_{127-141}$.

Previous studies in the laboratory defined HPV16 $E6_{127-141}$ as a new epitope that may be useful in investigating protective T-cell responses against HPV. Two clones belx1 and belx2 were generated and defined which HLA presented the peptide; HLA-DRB1*01. From this data Dr. Istvan Bartok, Imperial College, London carried out molecular modelling. The HPV16 $E6_{127-141}$ epitope was superimposed onto the crystallised structures of HLA-DRB1*01-$HA_{306-318}$ (Stern, Brown et al. 1994), of HLA-DRB1*01-HIV1 gag_{35-47} (Zavala-Ruiz, Strug et al. 2004) and of HLA-DRB1*01-Triose Phosphate $Isomerase_{23-27}$ (Sundberg, Sawicki et al. 2002), figure 29. From these models it appears that HPV16 $E6_{127-141}$ "sat" in the binding groove of the HLA-DR molecule in a similar way to the known and well-characterised epitopes. The epitope HPV16 $E6_{127-141}$ (DKKQRFHNIRGRWTG) was aligned based upon the HLA-DRB1*01 binding motif with P1=F, P4=I and P6=G. In figure 30A the epitope is shown with the HLA-DR molecule removed for clarity. The amino acids at positions 1, 4, 6 and 9 face down and are the anchor residues that bind in HLA-DRB1*01 specificity pockets. The three arginine residues (R, positively charged at positions P-1, P5 and P7), the glutamine residue (Q, position P-2), the tryptophan residue (W, position P8) and the glycine residue (G, position P10) protrude on the water accessible surface of the bound peptide. It is possible that one or more of these residues could form contacts with CDR3 loops of the TCR structure, and therefore be relevant to the recognition of the epitope by the T-cell, figure 29B. The CDR3 region of the α chain (TRAV 17: CATDAGTGNQFYFGTGTSLTVIP) identified from the belx1 and belx2 T-cell clones,

contains a negatively charged aspartic acid (D), which could in theory form electrostatic interactions with a positively charged arginine. At the end molecular modelling of HPV16 $E6_{127-141}$-HLA-DRB1*01 complex suggests that this epitope binds the HLA-DR molecule in similar fashion to other well characterised HLA-DRB1*01-restricted peptides. The anchor residues predicted by binding motif for this HLA-DR allotype appear to fit well in the binding groove. This model identified several amino acid residues that could be involved in TCR recognition and provides a basis for our study investigating the effect of amino acid truncation or substitutions in HPV16 $E6_{127-141}$ on T-cell recognition.

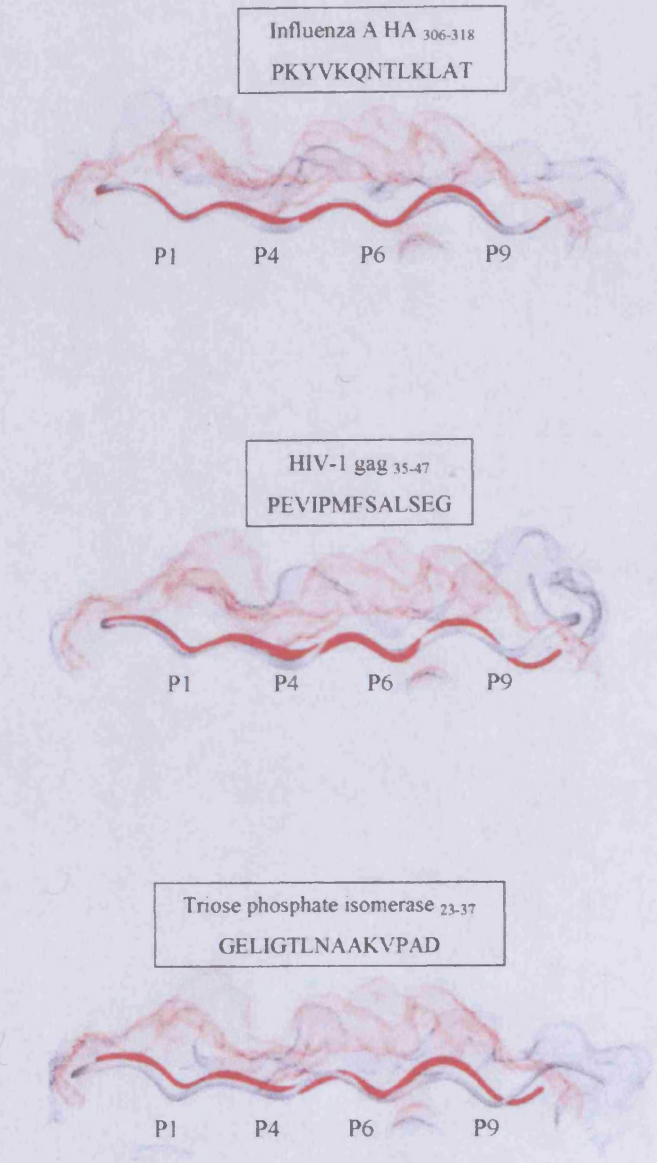
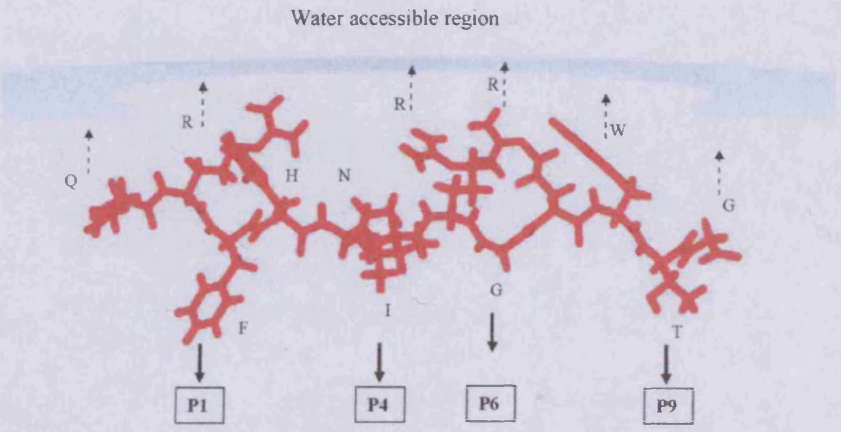


Figure 29: HPV16 $E6_{127-141}$ epitope superimposed onto the models of $HA_{306-318}$, HIV1 gag_{35-47}, and triose phosphate $isomerase_{23-37}$ peptides complexed with HLA-DRB1*01 (defined from Xray crystallisation studies). The E6 peptide is shown in red ribbon whilst the reference peptides are shown in grey ribbon.

A



B

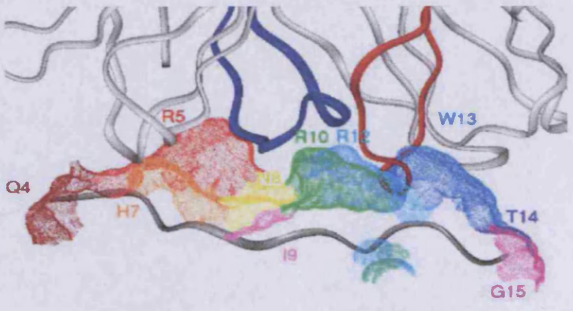


Figure 30: A-HPV16 $E6_{127-141}$epitope depicted in "ball and stick" with the HLA-DR molecule excluded for clarity. The anchor residues proposed to sit in the specific pockets are labelled as P1, P4, P6 and P9. Water accessible residues which may potentially contact the TCR are highlighted with dashed arrows. Models were constructed on a silicon graphics octane 2 worstation using insight II modelling suite (MSI/Accelrys). The first three amino acid residues DKK were excluded from analysis to simplify the modelling. B-3D structure of the interaction between HPV16 $E6_{127-141}$ peptide and TCR. The red loop shown in ribbons represents the CDR3 region of the α chain and the blue loop represents the CDR3 region of the β chain. This model was created by Istvan Bartok, Imperial College, London.

2-1-Effect of amino acid truncation in HPV16 $E6_{127-141}$ on belx1 T-cell recognition

In both class II and class I MHC, polymorphism pockets in the binding site provide the basis for the preference of different alleles or different peptide sequence motifs, but at least two more pockets accommodate peptide side chains in class II than in class I molecules (Stern, Brown et al. 1994). These differences allow the binding of peptide with different size for each MHC. Indeed in class II MHC, the groove is open at either end, and the peptide termini are not fixed so that bound peptides are usually significantly longer than in class I MHC. In class II MHC, the peptide termini, particularly the N-terminal extension (P-4 to P-1), can play a major role in the TCR interaction (Rudolph, Stanfield et al. 2006).

In order to study the impact of HPV16 $E6_{127-141}$ termini, different truncated peptides were designed according to their putative TCR contact residues or putative MHC anchor residues from HPV16 $E6_{127-141}$ (table 10). These peptides were tested in functional assays (IFNγ release) with two T-cell clones (belx1 and belx2).

Table 10: List of the truncated peptide designed from the HPV16 $E6_{127-141}$ epitope.

Peptides	Sequences																	
HPV16 $E6_{127-141}$	D	K	K	Q	R	F	H	N	I	R	G	R	W	T	G			
1		K	K	Q	R	F	H	N	I	R	G	R	W	T	G			
2			K	Q	R	F	H	N	I	R	G	R	W	T	G			
3				Q	R	F	H	N	I	R	G	R	W	T	G			
4					R	F	H	N	I	R	G	R	W	T	G			
5	D	K	K	Q	R	F	H	N	I	R	G	R	W	T				
6	D	K	K	Q	R	F	H	N	I	R	G	R	W					
7	D	K	K	Q	R	F	H	N	I	R	G	R						
8				Q	R	F	H	N	I	R	G	R	W	T	G	R	C	M

Putative TCR contact residues

Putative MHC anchor residues

2-1-1-Effect of the $N_{terminal}$ truncation of HPV16 $E6_{127-141}$ on T-cells

The effect of removing 1 to 4 amino acids from the N terminus of HPV16 $E6_{127-141}$ was tested using belx1 T-cells. These T-cells recognized the HPV16 $E6_{127-141}$ peptide in a dose dependent manner (figure 31) from 0.05μg/ml to 10μg/ml, confirming previous results from the laboratory.

Four peptides were studied for the truncation of the N terminus end; the first designed peptide was deleted of the aspartic acid (D) in position P-5, peptide 2 was deleted of aspartic acid plus the lysine (K) in position P-4, peptide 3 was deleted of the first three amino acids; the aspartic acid, and the two lysines (D=P-5, K=P-4 and K=P-3) and peptide 4 was deleted of four amino acids, the D (P-5), the two K (P-4 and –3) and the glutamine (Q) in position P=-2.

The first three peptides induced similar responses. A dose dependent secretion of IFNγ was observed from 0.05μg/ml to 10μg/ml, which was similar to the wild type peptide, nevertheless this secretion was lower in term of quantity of IFNγ. The profile of the response for peptide 4 was different from the previous responses. Indeed the IFNγ secretion was detectable from 1μg/ml to 10μg/ml and lower than 500pg/ml (figure 31).

The impact of the deletion of amino acids from the $N_{terminal}$ end did not seem to have a big impact on the IFNγ secretion by belx1, indeed the profiles of the responses induced by the peptides 1, 2 and 3 were similar to the response against HPV16 $E6_{127-141}$ peptide. Only peptide 4 had a different profile with a decreased response.

2-1-2-Effect of the $C_{terminal}$ truncation of HPV16 $E6_{127-141}$ on T-cells

The next couple of truncations were the deletion of amino acids from the $C_{terminal}$ end. The peptide 5 was deleted of the glycine (G) in position P10. The peptide 6 was deleted of the G plus the threonine (T) in position P9 and the peptide 7 was deleted of G, T and tryptophan (W) in position P8. Peptide 5 induced a response from 0.5μg/ml to 10μg/ml (the response observed at 0.1μg/ml was not considered positive due to the high standard deviation), which was very late compared to the wild type peptide (0.05μg/ml). Furthermore the IFNγ secretion was lower than for HPV16 $E6_{127-141}$, the maximum value was 601.67pg/ml (figure 31). The deletion of this G seemed to induce a response less specific and with an intracellular signalling lower than wild type peptide. The profiles of the response for peptides 6 and 7 were exactly the same; no responses were observed for any concentration.

Contrary to the responses observed for the $N_{terminal}$ end, the $C_{terminal}$ seemed to have greater effect on T-cell recognition by belx1.

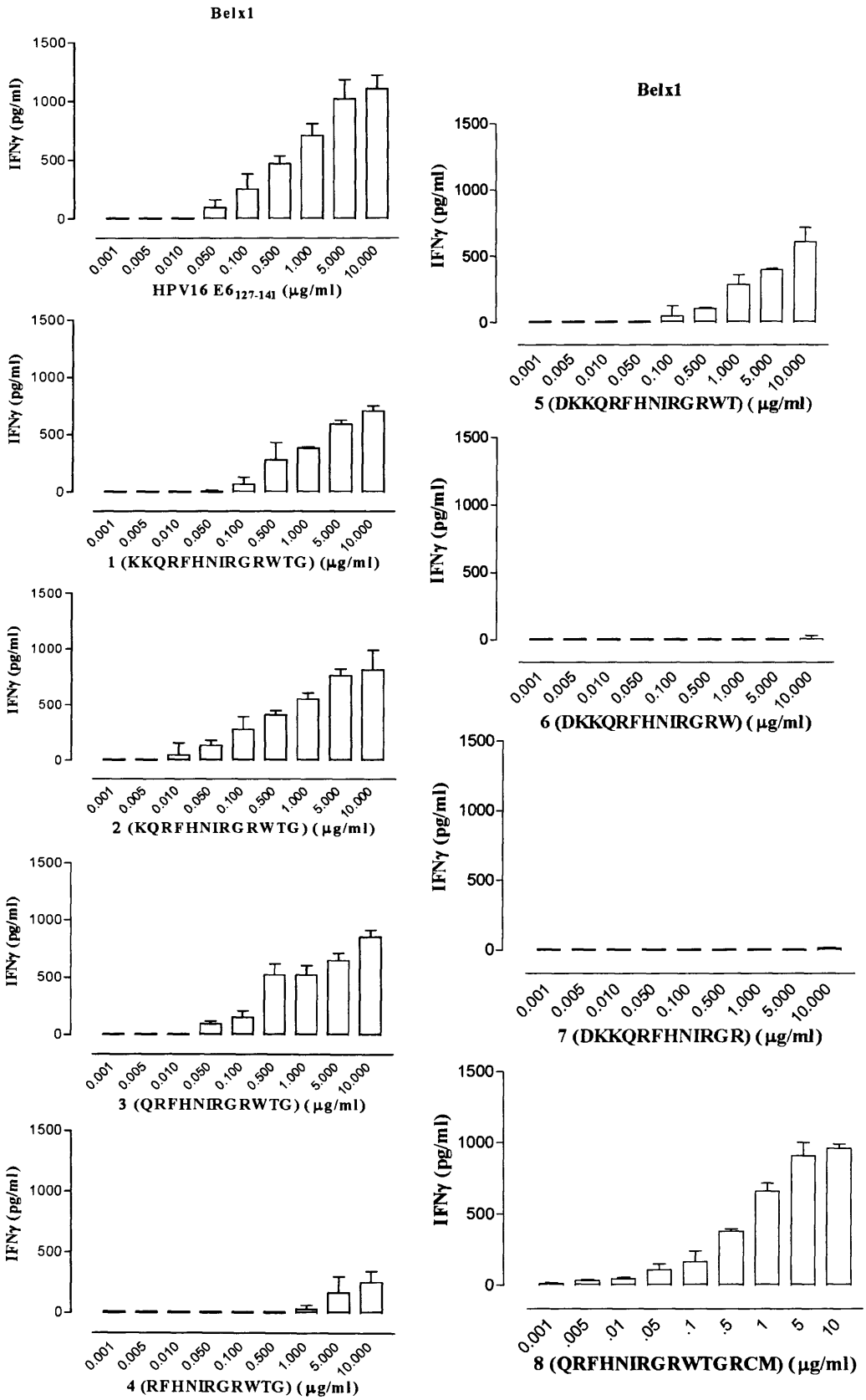
In order to study the impact of a longer $C_{terminal}$ end to the response by belx1, peptide 8 was tested. This peptide was truncated of the first three amino acid at the $N_{terminal}$ end (D, K, K), and three residues were added; an arginine R, a cysteine C and a methionine M. The results showed a secretion of IFNγ from 0.005μg/ml to 10μg/ml (the secretion of IFNγ for the wild type peptide was observed from 0.05μg/ml). It seemed that the addition of these three residues could affect the response generated by belx1, indeed the response seemed better with peptide 8 than the peptide HPV16 $E6_{127-141}$.

2-2-Impact of the deletions on T-cell in term of % of response

There was variation in the amount of IFNγ produced by belx1 T-cell clone in response to the peptide variants. Therefore to allow comparison with response to the wild type peptide the responses were standardised to the maximum response for each peptide or the maximum response for HPV16 $E6_{127-141}$ (figure 31). For this standardisation each experiment was performed three times using triplicates. Furthermore the concentration (T_{50}) of peptide necessary to obtain 50% of the maximum response for each peptide was determinated (table 11).

Four groups could be defined from these analyses; group one with the peptides 2 and 3, group two with peptides 1, 4 and 5, group three with peptides 6 and 7 and the last group with peptide 8. In the first group the peptides reached their T_{50} at concentration lower than HPV16 $E6_{127-141}$ which could indicate these peptides are better binders than the wild type. Nevertheless these peptides were not able to induce the same level of IFNγ secretion as the wild type, indeed the amount of IFNγ secreted did not reach 50% of the quantity secreted in response to the wild type peptide. Group two, contained the peptides 1, 4 and 5, these peptides had similar T_{50} compared to HPV16 $E6_{127-141}$ but induced secretion of lower amounts of IFNγ. These results indicated these three peptides could interact in the same manner as the wild type peptide but they were less efficient at inducing T-cell activation. No T_{50} could be calculated for peptide 6 and 7 because they did not induce IFNγ secretion. These data confirmed the major role of the $C_{terminal}$ amino acids for the interaction of the TCR and pMHC complex and for the activation of the T cells. Peptide 8 had a T_{50} smaller than HPV16 $E6_{127-141}$ and reached 50% of maximum

secreted IFNγ induced by HPV16 $E6_{127-141}$ at lower concentration, the peptide seemed to be a better binder or cellular activator than the wild type.


Belx1
IFNγ (pg/ml)
1500
1000
500
0
0.001
0.005
0.010
0.050
0.100
0.500
1.000
5.000
10.000
HPV16 E6127-141 (μg/ml)
1 (KKQRFHNIRGRWTG) (μg/ml)
2 (KQRFHNIRGRWTG) (μg/ml)
3 (QRFHNIRGRWTG) (μg/ml)
4 (RFHNIRGRWTG) (μg/ml)
Belx1
5 (DKKQRFHNIRGRWT) (μg/ml)
6 (DKKQRFHNIRGRW) (μg/ml)
7 (DKKQRFHNIRGR) (μg/ml)
0.001
.005
.01
.05
.1
.5
1
5
10
8 (QRFHNIRGRWTGRCM) (μg/ml)

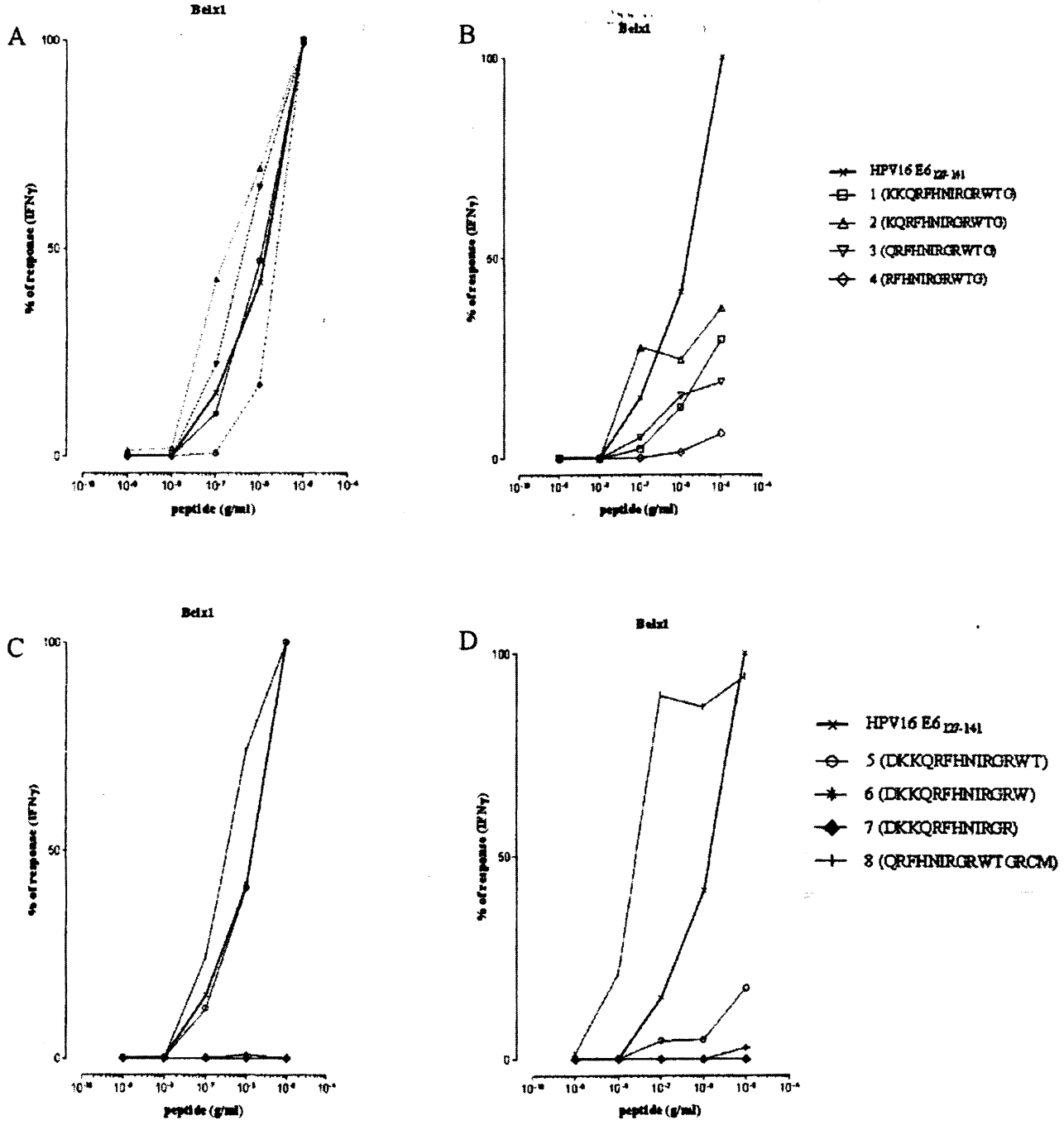


Figure 31: IFNγ ELISA performed on Belx1. B-LCL cells were loaded with the peptide at different concentrations during 1 hour at 37°C, and then the cells were washed and co-cultured with Belx1 CD4+ T cells overnight at 37°C. The supernatants were collected and tested by ELISA. Each ELISA was performed in triplicate three times, from these experiments a % of response has been calculated compared to the maximum response of each peptide (A and C) or compared to the maximum response of HPV16 $E6_{127-141}$ (B and D).

2-3-Effect of amino acid truncation in HPV16 $E6_{127-14}$ on belx2 T-cell recognition

In order to study the impact of HPV16 $E6_{127-141}$ termini, different truncated peptides were designed according to their putative TCR contact residues or putative MHC anchor residues from HPV16 $E6_{127-141}$ (table 10). These peptides were tested in functional assays (IFNγ release) with the T-cell clone belx2. Previous studied have demonstrated that belx2 has higher "avidity" for HPV16 $E6_{127-141}$. Furthermore belx2 expresses a different TCR to belx1. Each peptide was tested at different concentration, 0.001μg/ml to 10 μg/ml in triplicates, to obtain a dose/response and to compare the specificity and the affinity of each peptide.

2-3-1-Effect of the $N_{terminal}$ truncation of HPV16 $E6_{127-141}$ on T-cells

The effect of removing 1 to 4 amino acids from the N terminus of HPV16 $E6_{127-141}$ was tested using belx2 T-cells. These T-cells recognized the HPV16 $E6_{127-141}$ peptide in a dose dependent manner (figure 32) from 0.005μg/ml to 10μg/ml, confirming previous results from the laboratory.

Four peptides were studied for the truncation of the N terminus end; the first designed peptide was deleted of the aspartic acid (D) in position P-5, peptide 2 was deleted of aspartic acid plus the lysine (K) in position P-4, peptide 3 was deleted of the first three amino acids; the aspartic acid, and the two lysines (D=P-5, K=P-4 and K=P-3) and peptide 4 was deleted of four amino acids, the D (P-5), the two K (P-4 and –3) and the glutamine (Q) in position P=-2.

The first three peptides induced a similar response, indeed for all of them belx2 recognized the peptide in a dose dependent manner from 0.005μg/ml to 10 μg/ml. The profile of IFNγ secretion in response to these three peptides was similar to the response induced by the wild type peptide. The profile of the response for peptide 4 was not similar to the wild type, indeed a dose/response could be observed but there was a big shift of this titration to the high concentration. The secretion of IFNγ appeared from 0.1μg/ml and was less than to 500pg/ml (for the wild type peptide a response inferior to 500pg/ml was obtained for a concentration of 0.005μg/ml).

As for belx1 the deletion of amino acids from the $N_{terminal}$ end did not seem to have a big impact on the IFNγ secretion by belx2, indeed the profiles of the responses induced by peptides 1, 2 and 3 were similar to the response against HPV16 $E6_{127-141}$ peptide. Only peptide 4 had a different profile with a decreased response compared to HPV16 $E6_{127-141}$ (figure 32).

2-3-2-Effect of the $C_{terminal}$ truncation of HPV16 $E6_{127-141}$ on T-cells

The next couple of truncations were the deletion of amino acids from the $C_{terminal}$ end. Peptide 5 was deleted of the glycine (G) in position P10. Peptide 6 was deleted of the G plus the threonine (T) in position P9 and peptide 7 was deleted of G, T and tryptophan (W) in position P8. For peptide 5 a response appeared from 0.05μg/ml to 10μg/ml and a plateau was reached at 1μg/ml. In response to peptide 6 a secretion of IFNγ was observed from 1μg/ml to 10μg/ml. As for belx1 no IFNγ secretion was observed in response to the peptide 7 (figure 32).

Contrary to the responses observed for the $N_{terminal}$ end, the $C_{terminal}$ seemed to have a predominant role in the interaction of the peptide with the class II MHC and/or the TCR, then in the activation of the cell and at the end in the secretion of IFNγ.

In order to study the impact of a longer $C_{terminal}$ end to the response by belx2 peptide 8 was tested. This peptide was truncated of the first three amino acid at the $N_{terminal}$ end (D, K, K), and three residues were added; an arginine R, a cysteine C and a methionine M. The results showed a secretion of IFNγ from 0.005μg/ml to 10μg/ml. The plateau was reached at 0.05μg/ml, which was the same concentration as the wild type peptide (figure 32).

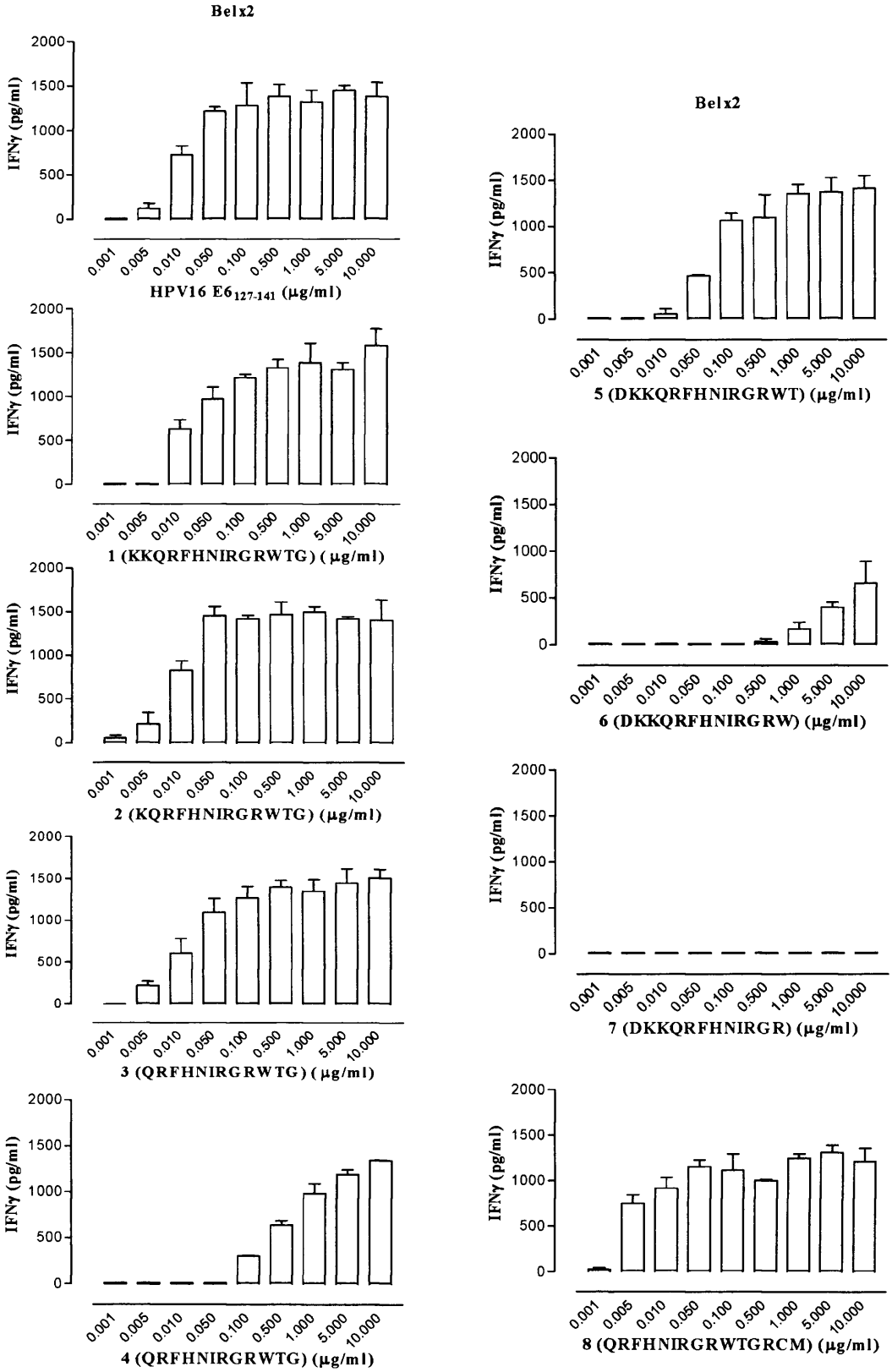
2-4-Impact of the deletions on T-cell in term of % of response

There was variation in the amount of IFNγ produced by belx2 T-cell clone in response to the peptide variants. Therefore to allow comparison with response to the wild type peptide the responses were standardised to the maximum response for each peptide or the maximum response for HPV16 $E6_{127-141}$ (figure 32). For this standardisation each experiment was performed three times using triplicates. Furthermore the amount of peptide (T50) necessary to obtain 50% of the maximum response for each peptide was determined (table 11).

From this standardisation three groups could be defined for belx2; one group with peptides 1, 3, 4, 5, and 6 which had a higher T_{50} to the T_{50} of the wild type peptide (1.6-20 fold higher). These results indicated than HPV16 $E6_{127-141}$ was a better binder or cellular activator than these 5 peptides. Another group, containing peptides 2 and 8, had a T_{50} lower than the wild type (4.5-12 folder lower). It could implicate these peptides are

better binders or more efficient at activation than the wild type. Nevertheless neither peptide 2 nor peptide 8, were able to induce a higher level of IFNγ secretion than the wild type. Peptide 7 did not have a calculated T_{50} because it did not induce IFNγ secretion.

Summarising the results in terms of % response (Table 11) demonstrated the differential effect of peptide truncation on belx1 and belx2, with belx1 being less sensitive to peptide truncation. Furthermore it appeared that the $C_{terminal}$ end was more important that the $N_{terminal}$ end for both belx1 and belx2. Deleting residues decreased the response (peptide 7) while adding residues increased the response (peptide 8).


Belx2
IFNγ (pg/ml)
2000
1500
1000
500
0
0.001
0.005
0.010
0.050
0.100
0.500
1.000
5.000
10.000
HPV16 E6127-141 (μg/ml)
1 (KKQRFHNIRGRWTG) (μg/ml)
2 (KQRFHNIRGRWTG) (μg/ml)
3 (QRFHNIRGRWTG) (μg/ml)
4 (QRFHNIRGRWTG) (μg/ml)
Belx2
5 (DKKQRFHNIRGRWT) (μg/ml)
6 (DKKQRFHNIRGRW) (μg/ml)
7 (DKKQRFHNIRGR) (μg/ml)
8 (QRFHNIRGRWTGRCM) (μg/ml)

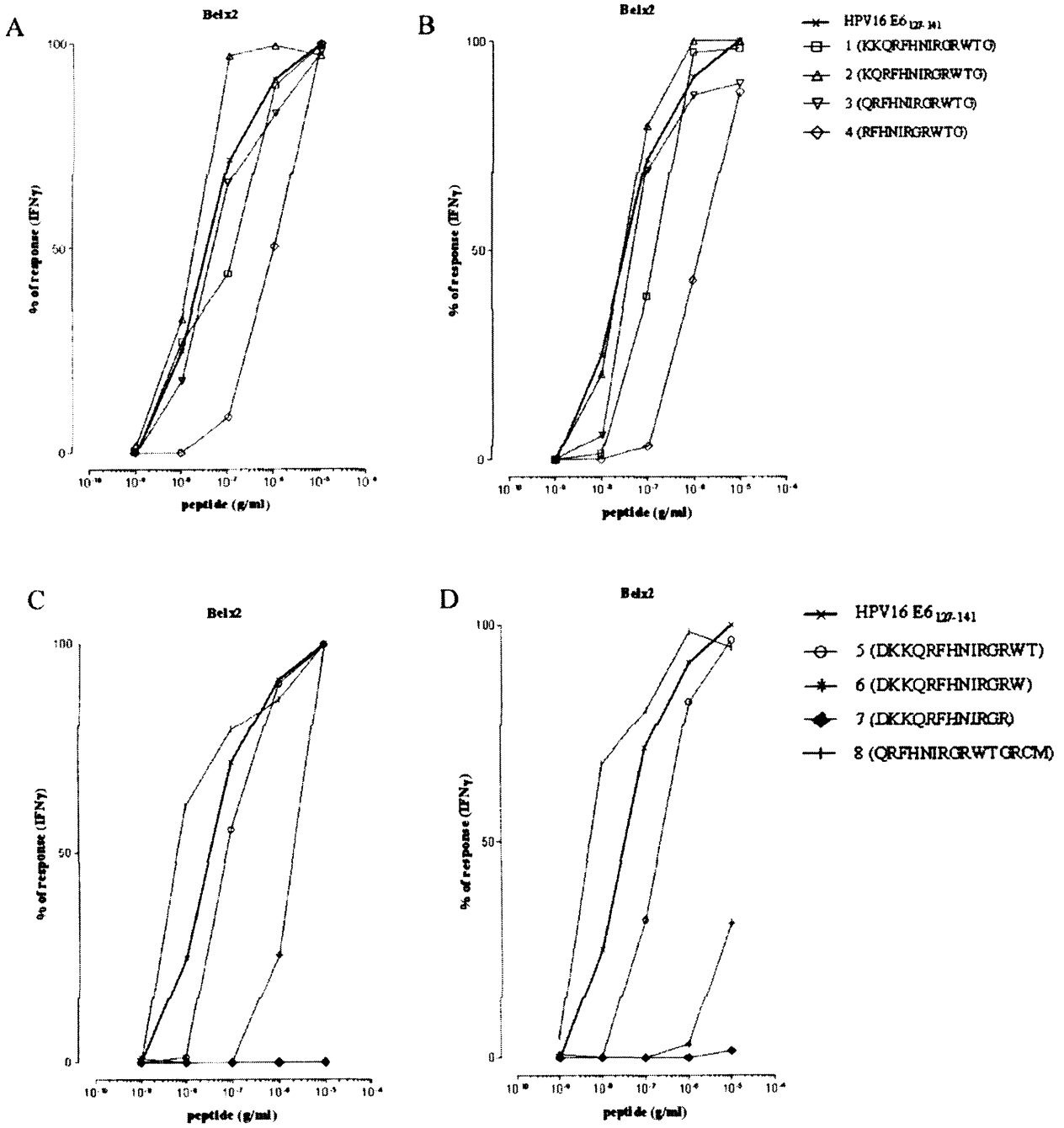


Figure 32: IFNγ ELISA performed on Belx2. B-LCL cells were loaded with the peptide at different concentrations during 1 hour at 37°C, and then the cells were washed and co-cultured with Belx2 CD4+ T cells overnight at 37°C. The supernatants were collected and tested by ELISA. Each ELISA was performed in triplicate three times, from these experiments a % of response has been calculated compare to the maximum response of each peptide (A and C) or compare to the maximum response of HPV16 $E6_{127-141}$ (B and D).

Table 11: T_{50} of HPV16 $E6_{127-141}$ and peptides 1 to 8.

Peptides	T_{50} (μg/ml) Belx1	Belx 2
HPV16 $E6_{127-141}$	1	0.052
1	1	0.10
2	0.11	0.011
3	0.5	0.073
4	1.5	1
5	1.07	0.083
6	none	1.01
7	none	none
8	0.3	0.0042

2-5-Discussion

In this chapter the terminal ends of the HPV16 $E6_{127-141}$ peptide were modified by truncation (or addition) and the impact on the T cell response was studied. The molecular modelling predicted that the first three residues of the $N_{terminal}$ (127-129; DKK) were not crucial for MHC binding. It was hoped that by truncating peptides at both ends of the peptide, some insight into the role of individual residues in TCR or MHC binding interactions could be gained.

For the two clones, belx1 and belx2 it was demonstrated that the $C_{terminal}$ end had a greater impact important on the IFNγ secretion than the $N_{terminal}$ end. Indeed the deletion of first three amino acids of the HPV16 $E6_{127-141}$ peptide did not affected the response; similar profiles of response and quantities of IFNγ were observed in each case. These results were consistent with the molecular modelling. Peptide 4 (deletion of 4 residues from $N_{terminal}$ end) induced different responses for the two clones; for belx1 an abolishment of the response was observed but for belx2 the response was reduced compare to wild type peptide response. A first explanation for these responses was that peptide was too short to bind the class II MHC. The deletion of four amino acids generated a 11mer which is smaller than the majority of the loaded peptides on a class II MHC (12 to 18 mer). However the belx2 clone could respond, proving that the peptide could bind. A second explanation was the impact of the deletion of the Q in P-2, this residue was a putative TCR contact residue. The major difference in belx1 and belx2 was their TCRs, indeed the two clones had the same α chain but not the same β chain. One of the characteristic of the TCRs is their ability to differ in their αβ chain pairing, such that the pseudo Vα/Vβ twofold angle, which currently varies from 166° to 188°, can

contribute to the variation in TCR orientation on the pMHC (Rudolph and Wilson 2002), and potentially could have relevance to TCR signalling if the pairing changes propagate to the constant domains or are tranduced to accessory proteins (Garcia 1999). Furthermore few studies have demonstrated that the V_β chain of the TCR recognizes the $N_{terminal}$ end of the peptide with CDR1 and 3 (Garcia 1999) (Garcia, Scott et al. 1996) (Garboczi, Ghosh et al. 1996) (Garcia and Teyton 1998) (Garcia 1999) (Reinherz, Tan et al. 1999) (Tsurui and Takahashi 2007). The deletion of the Q could generate a change in the twofold angle when the pMHC interacted with the TCR, only in presence of the $V_\beta 16$, and it could affect the intracellular signalling. It is possible that the $V_\beta 7$ chain was less affected by the lost of residue. In general the peptide truncations had less effect on belx1 compared to belx2, which is consistent with the idea that the respective TCRs have a different orientation on the pMHC complex.

By contrast the deletion on the $C_{terminal}$ end of the HPV16 $E6_{127-141}$ peptide had an important impact on the response of each clone. Each deletion was associated with either a putative TCR contact residue or a putative MHC anchor residue. The deletions generated peptides that potentially were not able to bind HLA-DR*0101 or to not interact with the TCR. Furthermore the P10 position has been identified as minor or auxiliary anchor residues in several pMHC (Ferrante and Gorski 2007).

Finally the deletion of the first three residues and the addition of the three following amino acid; R, C, M had an impact on belx1 and belx2. Peptide 8 was a better cellular activator for the lower concentrations, indeed its T_{50} is lower than the T_{50} observed for HPV16 $E6_{127-141}$ peptide. It is possible that this tail which does not bind the MHC or the TCR could provide a peptide conformation that stabilises the interactions

between the $C_{terminal}$ end of the peptide and the MHC or the TCR. It would be interesting to perform molecular modelling with this peptide, and also to test whether it would be more effective in the generation of soluble MHC:peptide complexes.

In summary the $N_{terminal}$ of HPV16 $E6_{127-141}$ peptide does not seem crucial for the interactions with the MHC or the TCR, which was predicted by the molecular model. By contrast the $C_{terminal}$ end seems crucial for the interactions, due to its contact with the MHC, or the TCR or both, and this is also consistent with predictions from the molecular model. A minimum peptide epitope of HPV16 $E6_{130-141}$ was defined that provided the basis for further experiments looking at the effect of amino acid substitutions at individual positions within the core of the epitope.

3-RESPONSES OF T-CELL CLONES AGAINST PEPTIDE FROM OTHER HPV TYPES

Previous results have showed that the $C_{terminal}$ end of the HPV16 $E6_{127-141}$ peptide had an important impact on the response of each T-cell clone but the deletions at $N_{terminal}$ end did not seem to have an effect. The T cell clones were able to recognise a minimum epitope comprising HPV16 $E6_{130-141}$ (QRFHNIRGRWTG). In order to have a better understanding of the molecular mechanisms which govern the interactions between peptide, MHC and TCRs, the presentation of peptides from other HPV types was studied. Indeed women are often infected by several HPV types including HPV16, HPV18, HPV 33 (Hoory, Monie et al. 2008). All these variants are found in histology tests of women with cell carcinoma, adenocarcinoma or normal cytology in various percentages (Bosch and de Sanjose 2003) or detected by PCR (Hoory, Monie et al. 2008). The immunological effects of infection with multiple HPV types have not been well-studied. For example, can cross-reactive T-cell responses be generated against closely related HPV types? Alternatively, could infection by HPV type A have a negative effect on immune responses against HPV type B? In order to study, *in-vitro*, these kinds of events different peptides were designed (table 12). The chosen strategy was to compare the sequence of HPV16 $E6_{127-141}$ to the E6 sequence of other HPV types and define which types of HPV were most closely related to the HPV16 $E6_{127-141}$ sequence. Five peptides from five different HPV types have been chosen; all of them are "high-risk" viruses and are able to induce cancer. The first difference in the sequence of these peptides, compared to HPV16 $E6_{127-141}$, was the substitution of the Q in position P-2 to a K. In order to check if this substitution affected the response generated by belx1 and belx2 peptide p9 was designed.

From the previous results it was observed that truncations at the $N_{terminal}$ end did not have a major effect on the T-cell response, so peptide p10 was designed to see if it was the case for a peptide with the substitution in P-2. From these first set of data the other peptides were designed. Comparison of the aligned peptide sequences suggested that the amino acid variation might have an impact on TCR recognition (table 12).

Table 12: Sequences of the variant peptides

Peptides	Sequences														
HPV16 $E6_{127-141}$	D	K	K	Q	R	F	H	N	I	R	G	R	W	T	G
9	D	K	K	**K**	R	F	H	N	I	R	G	R	W	T	G
10				**K**	R	F	H	N	I	R	G	R	W	T	G
HPV31				**K**	R	F	H	N	I	**G**	G	R	W	T	G
HPV52				**K**	R	F	H	N	I	**M**	G	R	W	T	G
HPV58				**K**	R	F	H	N	I	**S**	G	R	W	T	G
HPV33				**K**	R	F	H	N	I	**S**	G	R	W	**A**	G
HPV67				**K**	R	F	H	N	I	**S**	**N**	R	W	T	G

Putative TCR contact residues

Putative MHC anchor residues

3-1-Effect of amino acid substitutions on T-cell recognition:

Each peptide was tested, by IFNγ ELISA, at different concentration, 0.001μg/ml to 10 μg/ml in triplicates. To obtain a dose/response and to compare the specificity and the affinity of each peptide two T-cell clones (belx1 and belx2) with different avidities for HPV16 $E6_{127-141}$ were tested.

3-1-1-Belx1

9 and 10 (DKKKRFHNIRGRWTG and KRFHNIRGRWTG):

The responses induced by these peptides had similar dose/response profiles to that generated by HPV16 $E6_{127-141}$ (figure 33). Furthermore the quantity of secreted IFNγ was similar to the quantity induced by the wild type peptide for the higher concentrations, nevertheless for the lower concentrations such as 0.05μg/ml the quantity of secreted IFNγ was inferior. These results provided the basis for the next experiments with different substitutions because they showed that neither the substitution of the Q to a K nor the truncation had an impact on the response of the T-cells.

HPV31, 52, 58, 33, 67:

All the responses induced by these peptides had exactly the same profile; a complete abolishment of the IFNγ secretion. No IFNγ secretion was observed even for the highest concentration of peptide which was 10μg/ml. It seemed belx1 was very sensitive to the substitutions generated in the designed peptides and there was no evidence of cross-reactivity.

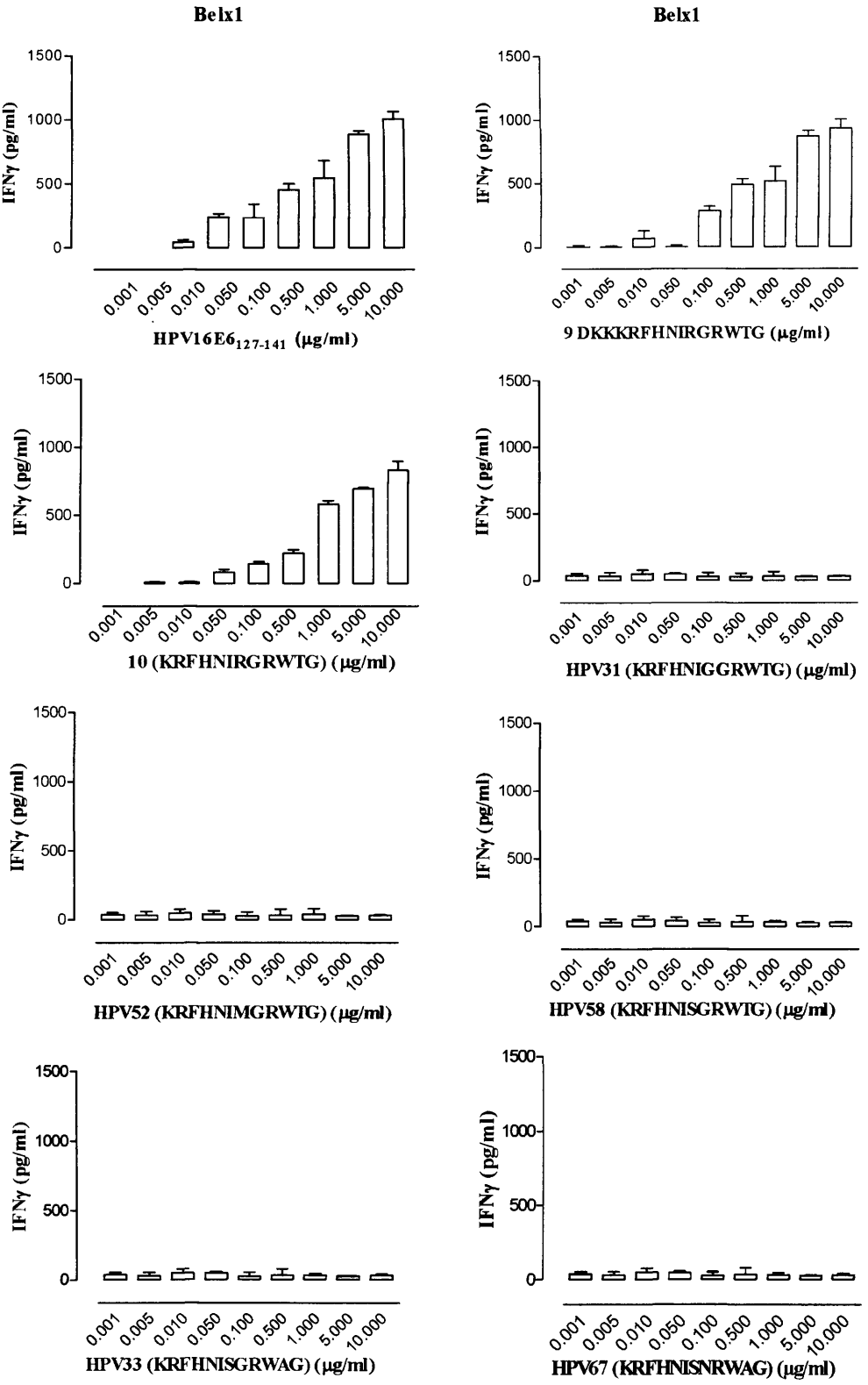


Figure 33: IFNγ ELISA performed on belx1. B-LCL cells were loaded with the peptide at different concentrations of peptide during 1 hour at 37°C, then the cells were washed and co-cultured with belx1 CD4+ T-cells overnight at 37°C. The supernatants were collected and tested by IFNγ ELISA.

3-1-2-Belx2

9 and 10 (DKKKRFHNIRGRWTG and KRFHNIRGRWTG):

The T-cell responses induced by these peptides had similar dose/response profiles to those generated by HPV16 $E6_{127-141}$ (figure 34). Nevertheless T-cells responded better against HPV16 $E6_{127-141}$; 362.98pg/ml of IFNγ was secreted in presence of BLCL loaded with 0.010 μg/ml, whereas no secretion or a very low secretion was observed for the two other peptides. As for belx1 these results gave the basis for the next experiments with peptides from different HPV types.

HPV31, 52, 67:

All the responses induced by these peptides had exactly the same profile; a complete abolishment of the IFNγ secretion. No IFNγ secretion was observed even for the highest concentration of peptide.

HPV58 and 33:

The responses of belx2 to these two peptides were different from the previous peptides from other HPV types, in that they induced weak responses. Activation of T cells only occurred at peptide concentrations of 1μg/ml and above, and the magnitude of the responses was much lower (30% of the HPV16 peptide).

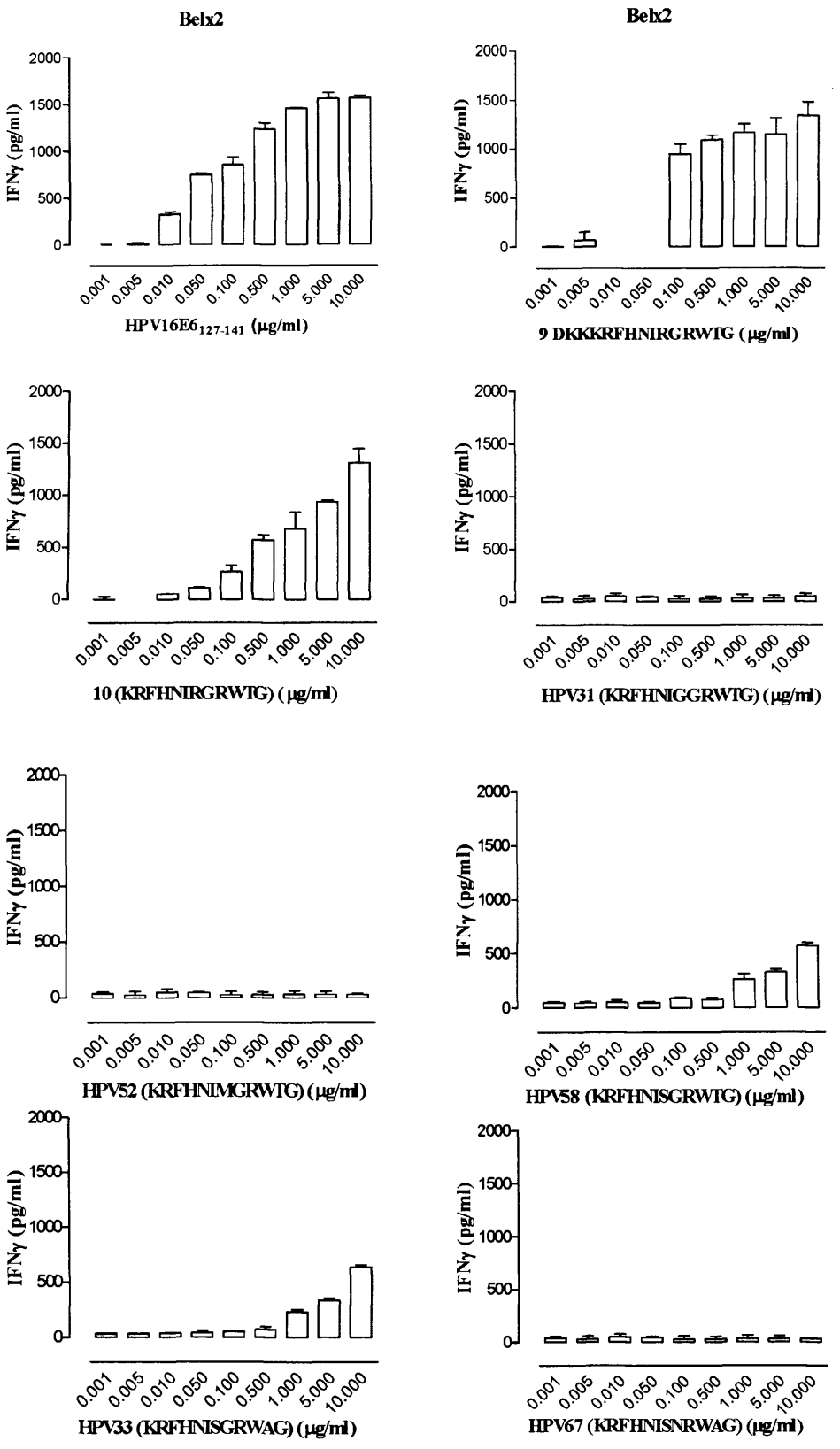


Figure 34: IFNγ ELISA performed on belx2. B-LCL cells were loaded with the peptide at different concentrations of peptide during 1 hour at 37°C, then the cells were washed and co-cultured with belx2 CD4+ T-cells overnight at 37°C. The supernatants were collected and tested by IFNγ ELISA.

3-2-Impact of the substitutions in term of % of response:

In order to evaluate and compare the impact of each substitution on the response, IFNγ ELISA were performed in triplicate and repeated three times. From these data a percentage of the response was calculated in comparison to the maximum response for each peptide, figure 34, and the T_{50} (concentration of peptide necessary to obtain 50% of the response) was determinated for each peptide, table 13.

The figure 36 summarizes the responses induced by each peptide; indeed for belx1 and belx2 the peptides 9 and 10 had a similar profile to HPV16 $E6_{127-141}$. Furthermore a later response could be observed for HPV33 and 58 nevertheless this response was lower than either the wild type or peptides 9 or 10, indeed the T_{50} for these two peptides were respectively 2.83 and 5.27 (table13). As the results were similar for peptide 10 and the wild type peptide and as all the study was started with the HPV16 $E6_{127-141}$, it was decided to use the wild type peptide as reference.

These results demonstrated the amino acids substitutions in the peptides had an effect on T-cell clone responses, different hypotheses could explain these results:

-the substitutions could induce some radical changes in the conformation, which could induce a different binding to MHC.

-the substitutions would allowed the binding of the peptide to the MHC, but this new complex could not be recognized by the TCR

-the peptide could bind the MHC and the complex could be recognized by the TCR but no positive intracellular signalling would be generated.

In order to address these different hypotheses a strategy was developed using to assess peptide binding to HLA-DR*0101 based on the functional inhibition of an influenza specific T cell clone (HC3).

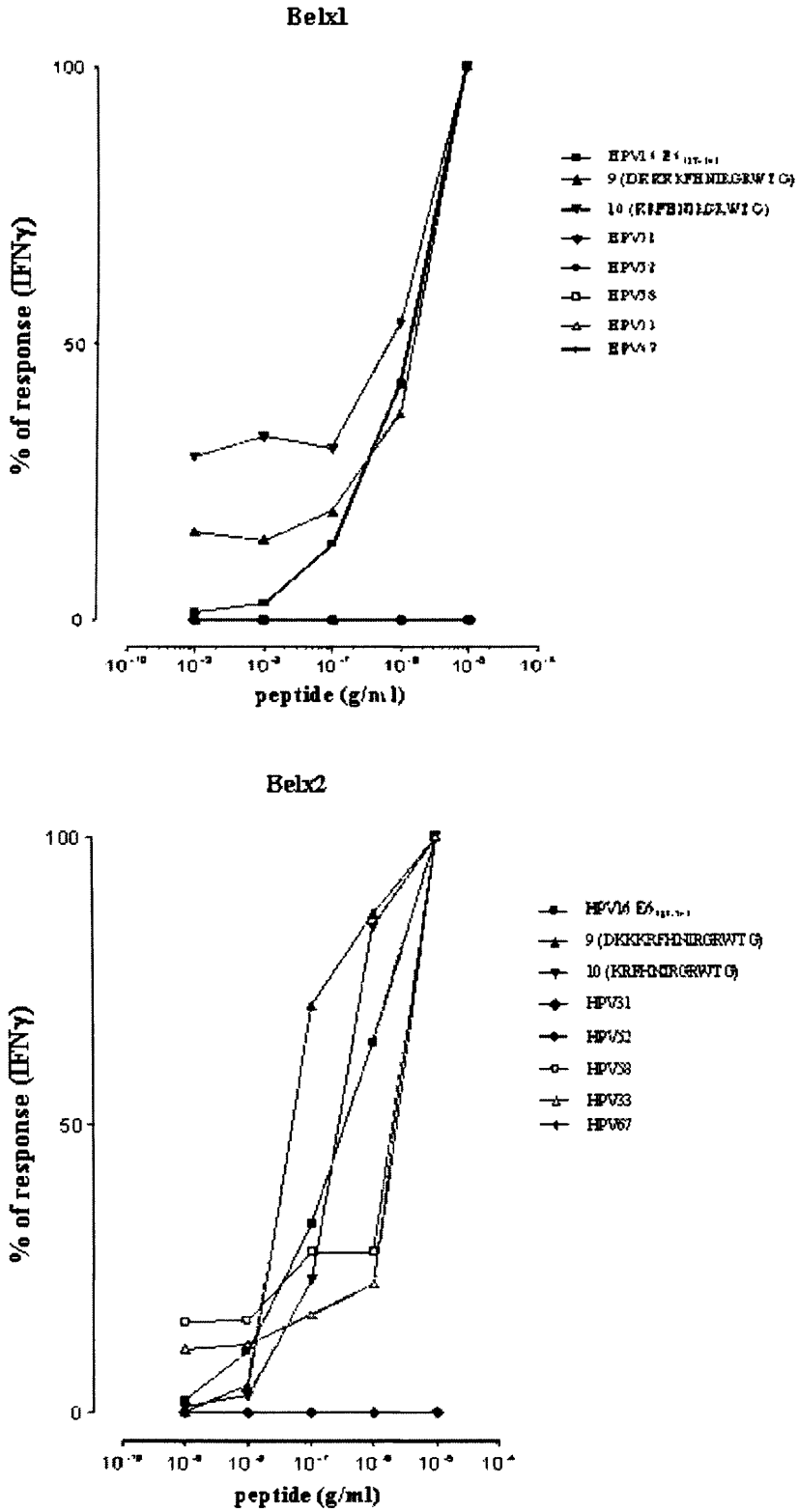


Figure 35: T-cell responses against the different variant peptides. Each peptide was tested at different concentrations by IFNγ ELISA in triplicate. Each ELISA was performed three times and the % of response was calculated in function of the higher value of secreted IFNγ for each peptide.

Table 13: T_{50} of HPV16 $E6_{127-141}$, peptides 9, 10 and HPV variants.

Peptides	T_{50} (μg/ml) Belx1	Belx 2
HPV16 $E6_{127-141}$	**0.95**	**0.057**
9	0.95	0.011
10	**0.95**	**0.01**
HPV31	none	none
HPV52	**none**	**none**
HPV58	none	2.83
HPV33	**none**	**5.27**
HPV67	none	none

3-3-Binding of variant HPV peptides to HLA-DR1:

This assay was used to indirectly measure peptide binding to HLA-DR*0101. The principle of this assay was to load the BLCL with the HPV peptide at different concentrations. After 1 hour at 37°C the APC were washed and a constant concentration of influenza HA peptide, specifically recognised by the HC3 clone, was loaded on the same BLCL during 1 hour at 37°C. The cells were washed, co-cultured with the clone and an IFNγ ELISA was performed on the supernatants.

An influenza HA (YDVPDYASLRSLVASS) specific T-cell clone was used for the experiment, indeed two flu clones (kindly provided by Dr Giovanna Lombardi and Saskia Yates, Kings College, London) were generated in London and each of them were known to recognise the Flu peptide via HLA-DR*0101. The first step was to test if these clones were able to secrete IFNγ after stimulation by their peptides, in this goal a titration assay was performed (figure 36). BLCL were loaded with different concentrations of peptide during 1hour and co-cultured with the clone HC3 or HC6. The results showed HC3 secreted IFNγ in response to its peptide loaded on BLCL, and had high avidity with a plateau reached for 0.1μg/ml of peptide. The HC6 clone demonstrated very weak IFNγ secretion against peptide (figure 36), and this could not be increased by stimulation with PMA. In parallel of this titration an IFNγ ELISA assay was performed using HPV16 $E6_{127\text{-}141}$ peptide on HC3 and HC6 clones. No IFNγ secretion was observed (figure 36), this confirmed the specificity of the clone for its peptide and the no cross-reactivity of the flu clone against HPV16 $E6_{127\text{-}141}$. From these results it was decided to use the HC3 clone for the inhibition assay, testing peptides for HPV16 $E6_{127\text{-}141}$, HPV31, HPV52, HPV58, HPV33 and HPV67.

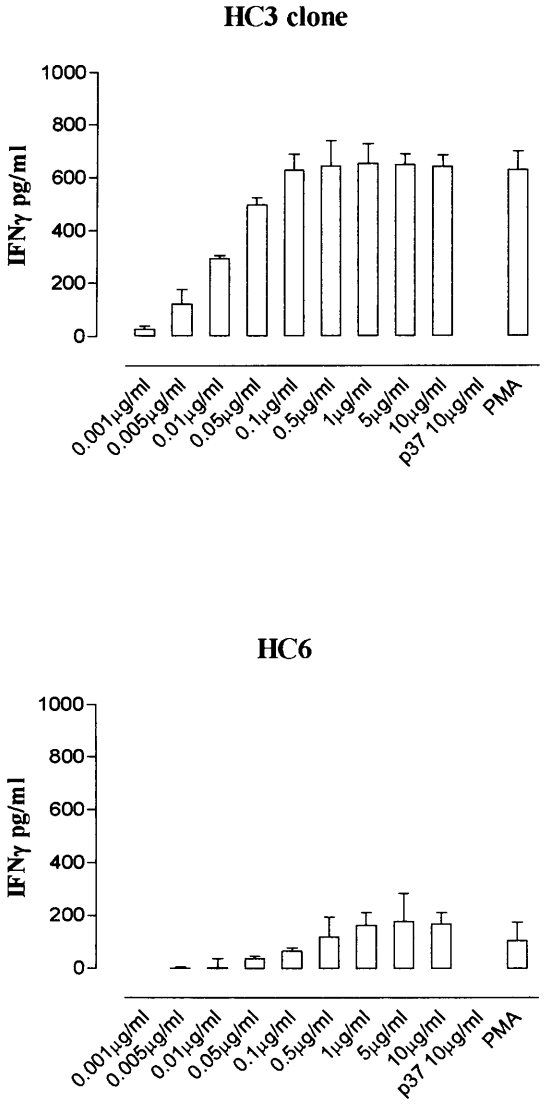


Figure 36: IFNγ ELISA performed on HC3 and HC6 clones. B-LCL cells were loaded with the peptide at different concentrations during 1 hour at 37°C, then the cells were washed and co-cultured with HC3 or HC6 CD4+ T cells overnight at 37°C. The supernatants were collected and tested by ELISA. The clones were tested against HPV16 $E6_{127-141}$ (p37) to check there was no cross-reactivity with the wild type peptide.

From the dose/response experiments, a sub-optimum concentration of 0.05μg/ml was chosen for the flu peptide to compete against the binding of test HPV peptides from 10 to 0.001μg/ml. All the peptides could inhibit at the top dose (10μg/ml) suggesting that could all bind. On the basis of the dose/response results two groups of peptides could be defined.

Weaker binders (HPV16, HPV31, HPV52 and HPV67) that could inhibit the HC3 T-cell response but only at high peptide concentration (superior or equal to 1μg/ml). Stronger binders (HPV33 and HPV58) that could inhibit the HC3 T-cell response at lower peptide concentration (lower than 1μg/ml) (figure 37). This was seen more clearly when the results were summarized in term of % of inhibition (figure 38).

Since all the HPV peptides inhibit the HC3 response to some degree, it indicated that all the HPV peptides could bind. Therefore the failure of HPV peptides to induce IFNγ secretion by belx1 or belx2 T-cells, was not due to problems in peptide binding.

3-4-Activation assays

The results in 3.3 suggested that the largely negative response against variant HPV peptides was due to interaction of the peptide with TCR and not MHC. To have a better understanding of the interaction between the peptides and the TCRs and potential effects in the cellular activation it was necessary to carry out some activation assays. The engagement of the TCR induces the activation of the cell (Geginat, Campagnaro et al. 2002), which leads cells in the cellular cycle. These cellular activations can be measured by a proliferation assay using incorporation of thymidine.

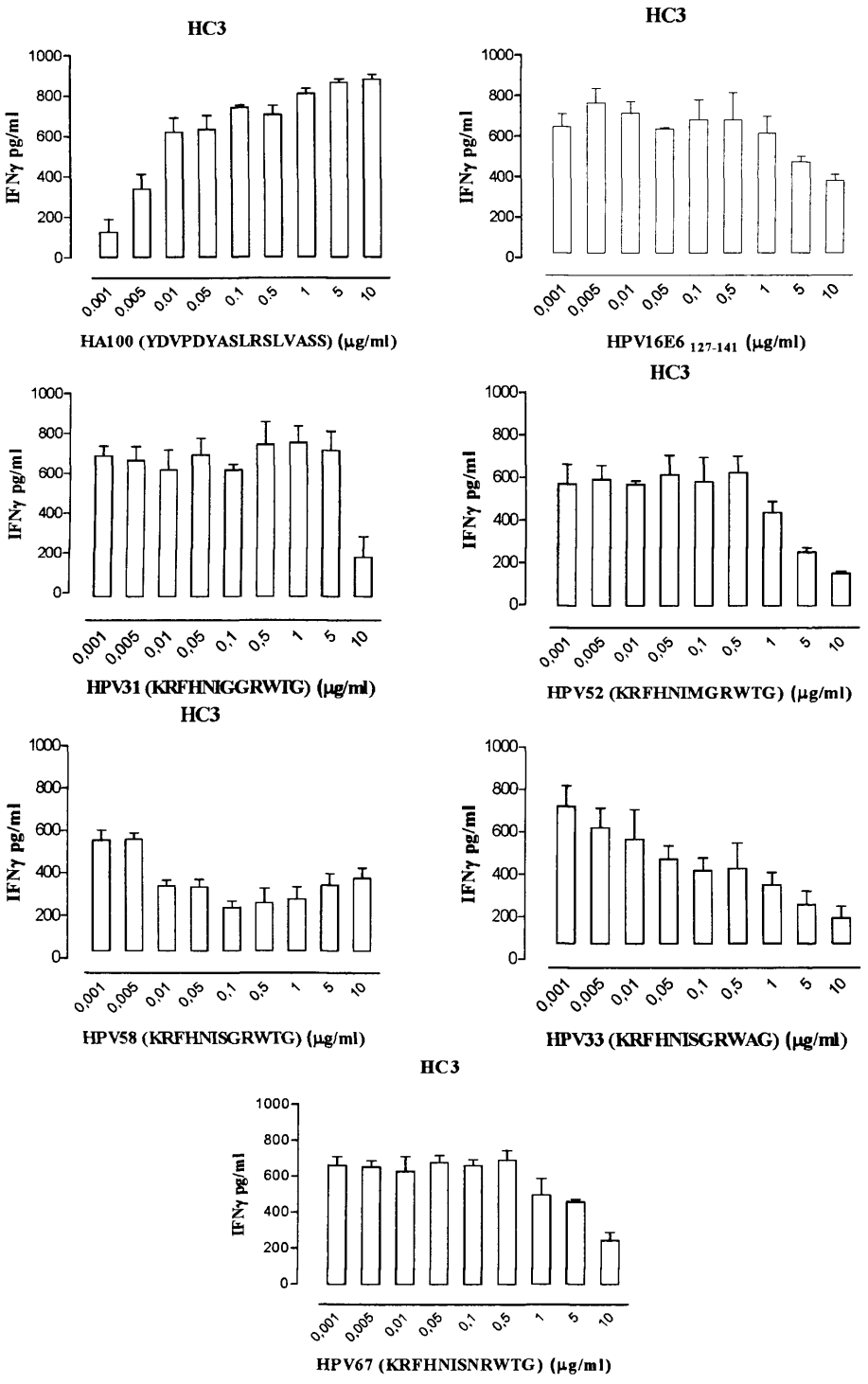


Figure 37: Inhibition assay with HC3 clone. B-LCL cells were loaded with different concentrations of peptide during 1 hour at 37°C. After this incubation cells were washed and loaded with 0.05μg/ml of influenza peptide (HA100) during 1 hour at 37°C. Finally loaded B-LCL were co-cultured with HC3 clone overnight at 37°C. The supernatants were collected and tested by IFNγ ELISA.

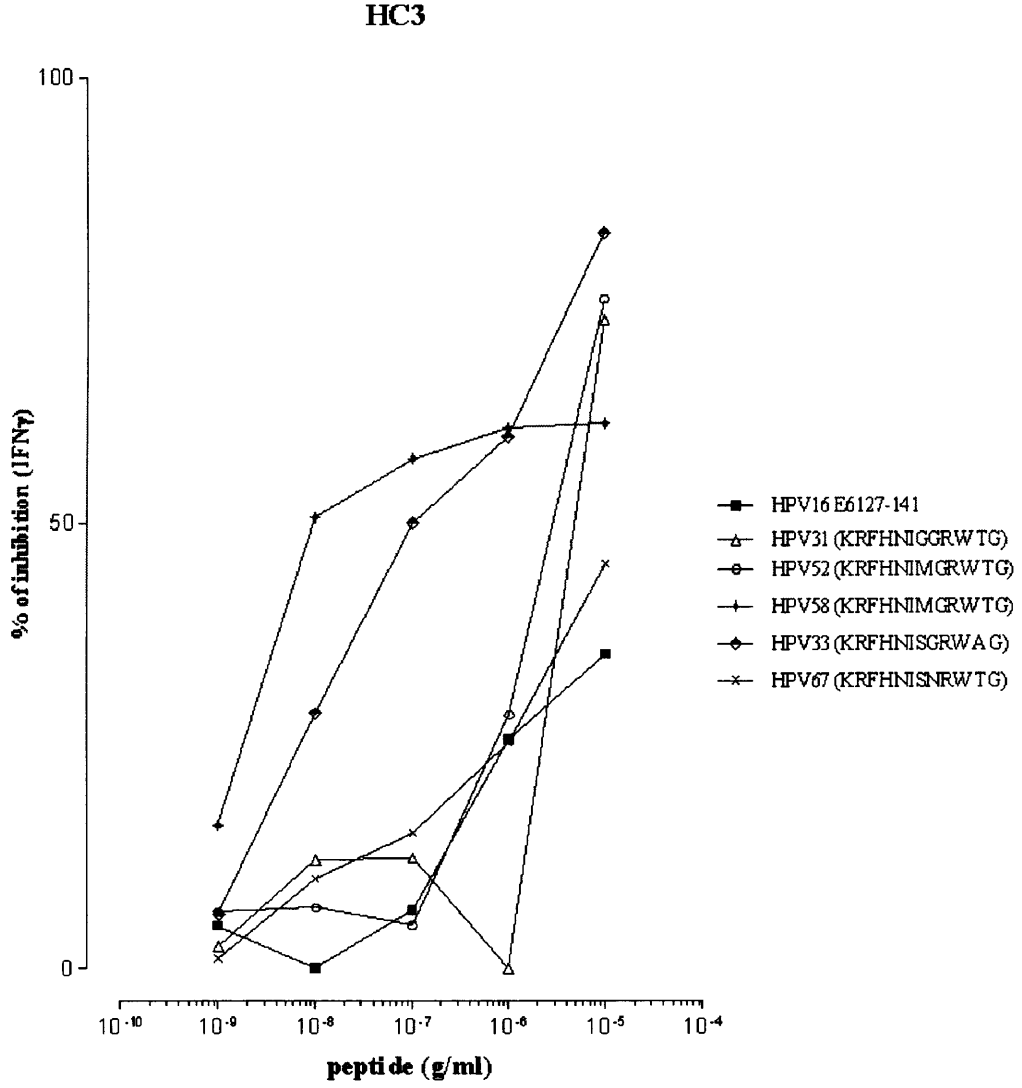


Figure 38: % of inhibition of the different HPV variants. Each peptide was tested at different concentrations by IFNγ ELISA in triplicate. Each ELISA was performed three times and the % of inhibition was calculated in function of the higher value of secreted IFNγ for each peptide.

3-4-1-Proliferation assays

Belx1 or belx2 cells were co-cultured with irradiated BLCL and a peptide at 0.1μg/ml or 10μg/ml, then tested for proliferation (figure 39). Unfortunately no significant differences were observed between each peptide or each concentration; all the peptides induced low level of proliferation of T-cell clones. It seemed this technique was not sensitive enough to determine differences in functional activation between peptides (several experiments were performed all gave the same result).

3-4-2-Calcium signalling assays

Calcium (Ca^{2+}) signals in cells of immune system participate in the regulation of cell differentiation; gene transcription and effector functions (figure 40). An increase in intracellular levels of Ca^{2+} ions results from the engagement of immunoreceptors, such as the TCR (Feske 2007). Several studies have demonstrated that the engagement of the TCR with various pMHC was able to induce different increases of intracellular Ca^{2+} (Feske 2007). The goal of these experiments was to study the variations of intracellular Ca^{2+} after engagment of the TCR by various peptides loaded on BLCL.

Ca^{2+} signalling on a cellular population:

In the first instance a model permitting the study of the variations of intracellular Ca^{2+} in a cellular population was used. The T-cells were loaded with a Ca^{2+} sensitive fluorescent probe Fura-2. Following this loading the T-cells were stimulated by the BLCL loaded with HPV16 $E6_{127-141}$ at 10μg/ml and the variation of intracellular Ca^{2+} was measured straight away using the Fluorostar reader (figure 41).

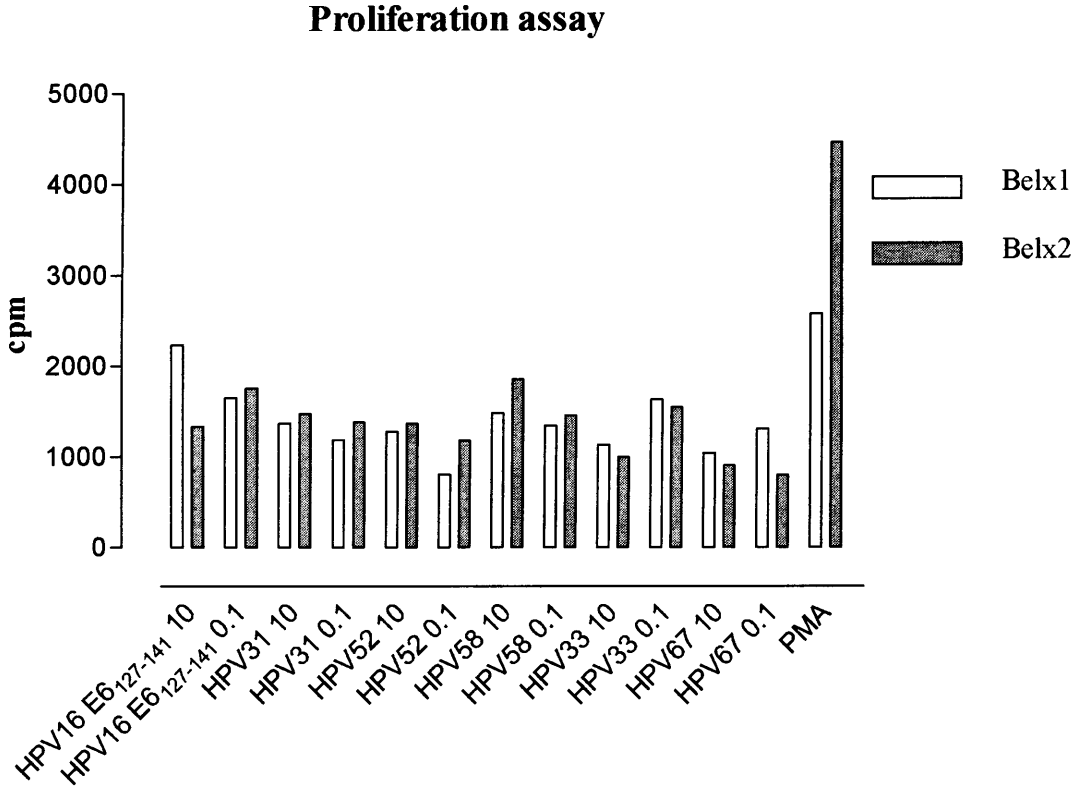


Figure 39: One representing proliferation assay. BLCL were loaded with different concentration of peptide during 1 hour and irradiated. After 3 washes the cells were co-cultured, in 96 well plates, with belx1 and belx2 during 2 days. At 48 hours 1μC of [methyl-^{3}H]Thymidine was added in each well and the day after the cells were harvested and the radioactivity was measured. PMA was used as positive control for the proliferation assay. A negative control with the cells plus media (average of 200 cpm) was subtracted to each value.

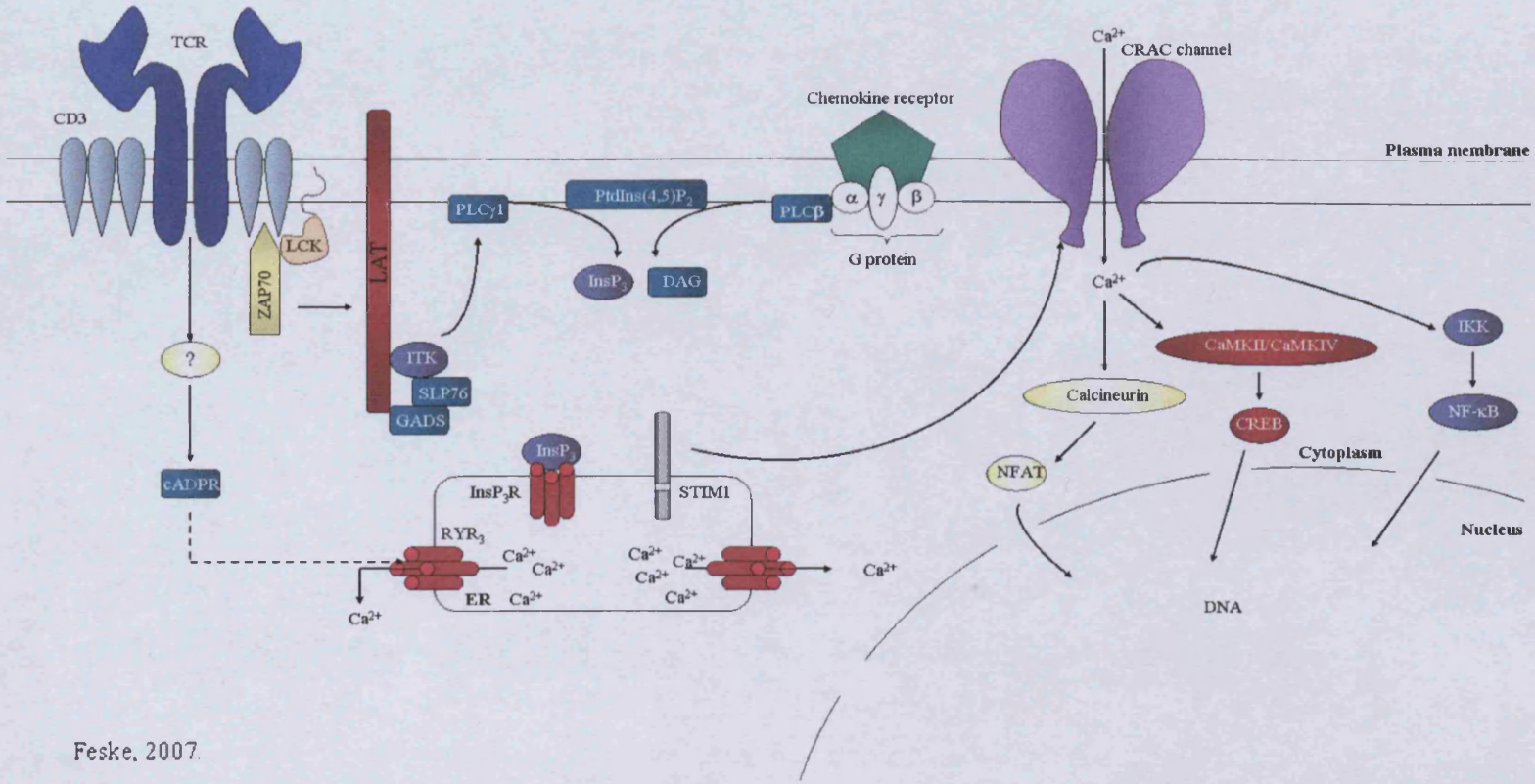


Figure 39: In resting T-cells, a steep gradient in Ca^{2+} concentrations exists between the cytoplasm and the extracellular space, as well as between the cytoplasm and the lumen of the endoplasmic reticulum (ER). Antigen recognition through the TCR results in the activation of protein tyrosine kinases, such as LCK and ZAP70 (ζ-chain-associated protein kinase of 70 kDa), which initiate phosphorylation events of adaptor proteins, such as SPL76 (SRC-homology-2-domain-containing leukocyte protein of 76 kDa) and LAT (linker for activation of T-cells). This lead to the recruitment and activation of the TEC kinase ITK (interleukin-2-inducible T-cell kinase) and phospholipase Cγ1 (PLC γ1). Similarly, binding of G-protein-coupled chemokine receptors results in the activation of PLCβ. PLCβ and PLCγ1 catalyse the hydrolysis of the membrane phospholipid phosphadylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) to inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ to and opens $InsP_3$ receptors ($InsP_3Rs$) in the membrane of the ER, resulting in the release of Ca^{2+} from intracellular Ca^{2+} stores. A decrease in Ca^{2+} content of the ER is "sensed"by stromal interaction molecule 1 (STIM1), which in turn activates calcium-release-activated calcium (CRAC) channels in the plasma membrane. Ca^{2+} influx though CRAC channels and elevated intracellular Ca^{2+} concentration activate Ca^{2+}-dependent enzymes, such as calcineurin, and thereby transcription factors, such as NFAT (nuclear factor of activated T cells), NF-κB (nuclear factor-κB) and CREB (cyclic-AMP-responsive-element-binding protein). cADPR (cyclic ADP ribose), CaMK (calmodulin-dependent kinase), GADS (growth-factor-receptor-bound-protein-2-related adaptor protein), IKK (inhibitor of NF-κB kinase), RYR_3 (ryanodine receptor 3).

Unfortunately no significant variations were observed from these preliminary experiments, it appeared this technique was not sensitive enough to detect any variation. Nevertheless the controls showed that the cells were loaded with the probe and that the cells could be stimulated by ionomycin (figure 41). Indeed in presence of Triton X-100, a detergent, the cells generated a release of calcium (figure 41-A), which demonstrated that the cells were correctly loaded with the fluorescent probe. Furthermore this release of calcium was abolished in presence of EDTA, a chelater (figure 41-B). The stimulation by the ionomycin demonstrated that the T-cells were viable and could be stimulated, nevertheless the observed calcium wave was very small (figure 41-C). All these results suggested the signal was too small in magnitude to allow measurement of differences between peptides , indeed neither potent mitogens such as ionomycin nor activators such as OKT3 (anti-CD3 antibody which induces the activation of the cell by cross-linking to its target) could induce a strong calcium release. The stimulation of the T-cells by pMHC is likely to induce a smaller signal than the ionomycin or OKT3, so it may be below the threshold of detection for this assay system.

This technique allowed studying a T-cell population but it was likely that not all the population was stimulated in at same time by the ionomycin, the OKT3 or the pMHC. In order to continue the investigation on calcium release of the T-cells after stimulation it was decided to use a technique observing one cell.

Ca^{2+} signalling on a cell :

After these assays another model was used. The BLCL cells were loaded with 10µg/ml of HPV16 $E6_{127-141}$ and belx2 cells were loaded with a Ca^{2+} sensitive fluorescent probe Fura-2. With the help of Professor Hallett (Department of Surgery, Cardiff

University) the BLCL were dropped on a slide and after few minutes, when the BLCL had adhered to the slide, the belx2 T-cells were added and the cellular interaction was observed under a fluorescent microscope, figure 41. The results showed a variation of the intracellular Ca^{2+} during the stimulation of belx2 by HPV16 $E6_{127-141}$ loaded BLCL, nevertheless the intensity was too low and could not permit the study of intracellular Ca^{2+} signal difference induced by various peptides. Furthermore the chance to see an event was very low, indeed quite a few assays were necessary to obtain one cell in contact with another one. It was also very difficult to observe this type of signalling event because the activation of the cell was very fast (155 sec, figure 42).

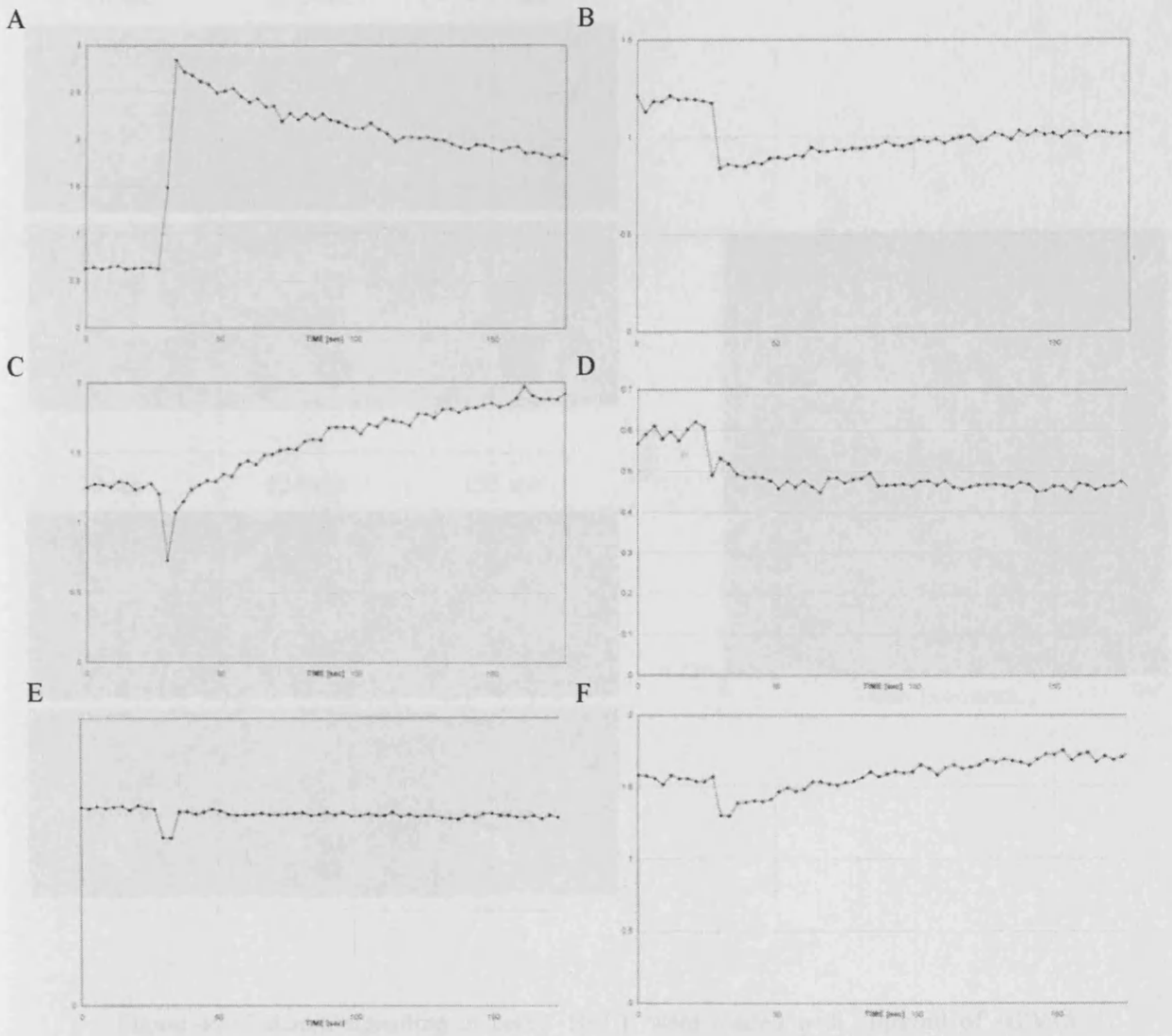


Figure 41: Calcium signalling in Belx2. BLCL were loaded with 10μg/ml of HPV16 $E6_{127-141}$ and added to belx2 cells preloaded with Ca^{2+} sensitive fluorescent fura-2 probe. A-Positive control for loading of the probe, the cells were in triton X-100 buffer. B-Cells in triton-X100 buffer plus EDTA, which is a chelater of calcium. C-The cells were stimulated by ionomycin 1M. D-The cells were stimulated by ionomycine and EDTA was added in the buffer. E-Belx2 plus BLCL loaded with 10μg/ml of HPV16 $E6_{127-141}$. F-Belx2 plus OKT3 antiboody, which is an anti-CD3 antibody.

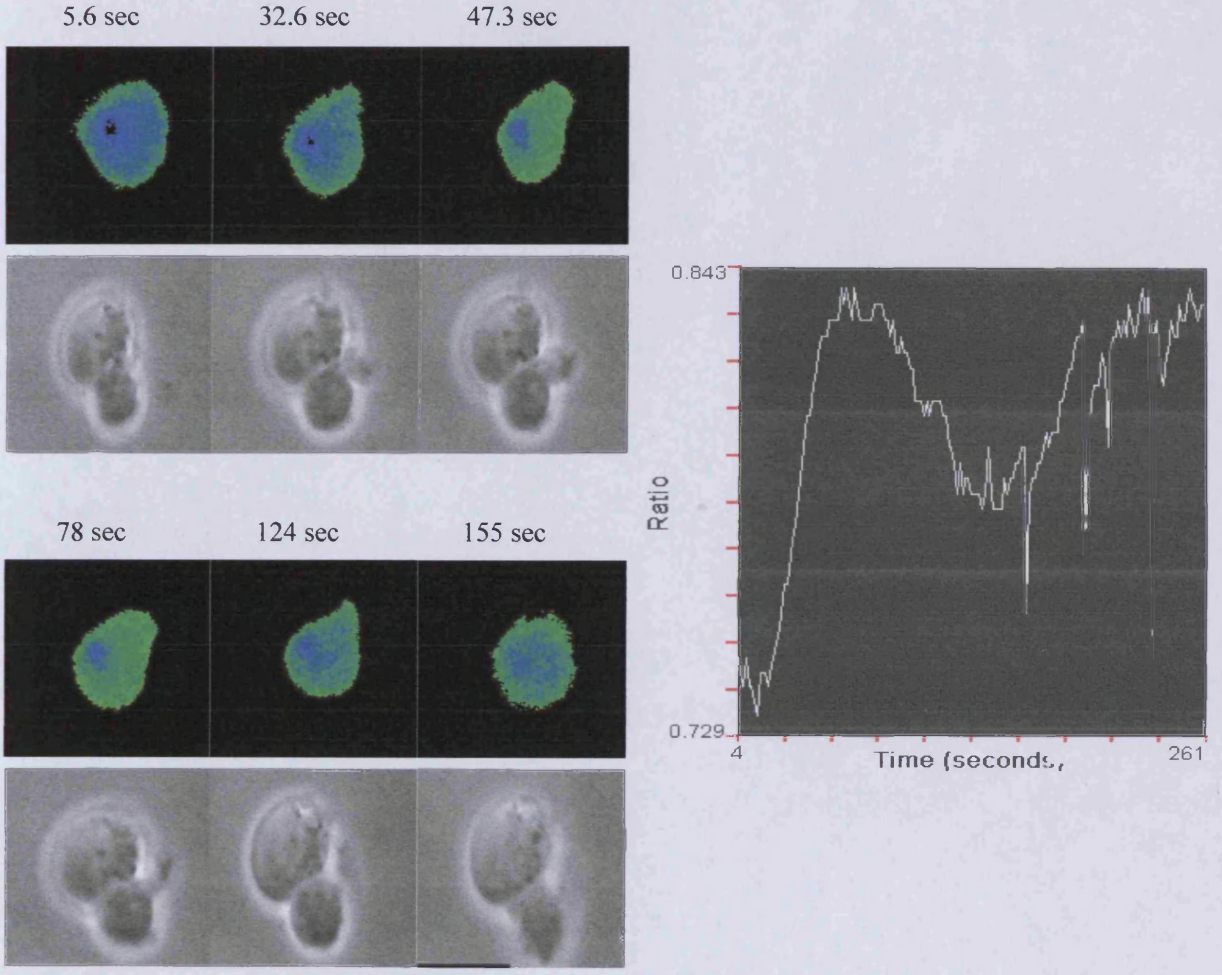


Figure 42: Calcium signalling in belx2. BLCL were loaded with 10μg/ml of HPV16 $E6_{127-141}$ and added to belx2 cells preloaded with Ca^{2+} sensitive fluorescent fura-2 probe. BLCL were put on a slide. Few seconds later belx2 cells were added and contacting cells that were in contact were observed under fluorescent microscope at a magnification of 200X.

3-4-3-Study of down-regulation of the TCR

The previous results showed that it was very difficult to observe directly the activation of the T-cells by measuring proliferation or calcium signalling, therefore an indirect way had to be chosen to observe the activation of T-cells. The number TCR-CD3 complexes, on the surface of the T-cell, is maintained by an equilibrium between synthesis and secretion of new polypeptides, their internalization, recycling, and degradation. T-cell activation by antigen, anti-CD3 antibody or pharmacological activators of protein kinase C, results in increased TCR-CD3 internalization, followed by the down-regulation of TCR-CD3 complexes number on the surface (Alcover, 2000). The chosen strategy was to measure cell surface expression of the TCR after stimulation of the cells, BLCL were loaded with different concentration of HPV16 $E6_{127-141}$ (0.001μg/ml to 10μg/ml) and co-cultured with belx2 during 4 hours. Following this incubation a staining with an-CD4+ FITC antibody and an anti-TCR-PE antibody was performed, figure 42. The results of this preliminary experiment showed a down-regulation of the TCR for the higher concentrations of peptide (Figure 43). Following this first experiment the effect of different HPV peptides was studied, BLCL cells were loaded with different peptides: HPV16 $E6_{127-141}$, HPV31, HPV52, HPV58, HPV33 and HPV67 at various concentration (0.05μg/ml to 10μg/ml). After a co-culture of 4 hours the cells were stained with the same antibodies as previously, figure 44. It was decided to perform this experiment with belx2 because these cells had higher avidity than belx1, and could recognize soluble E6 protein (Gallagher, 2007). The lower concentrations of peptides were not tested because no difference in TCR down regulation was observed in the preliminary study. From these results two groups could be defined; one with HPV16

$E6_{127-141}$, HPV58, HPV33 and HPV67 with induced a down-regulation of the TCR from the cell surface, the second one which did not induced a significant down-regulation of the TCR. The second group contained the peptides from HPV31 and HPV52. In the first group HPV67 and HPV16 $E6_{127-141}$ induced a down-regulation of the TCR with low peptide concentrations suggesting a strong interaction between the TCR and pMHC. HPV58 and HPV33 induced a down-regulation of the TCR only for the higher concentrations of peptide suggesting a weak interaction between the TCR and the pMHC complex. The second group, which contained HPV52 and HPV31, did not induce a down-regulation of the TCR. This could suggest either there was not binding between the TCR and the pMHC or that there was a binding between the two molecules but this did not induce an activation of the T-cell.

To confirm the previous results an inhibition assay was performed using belx2 to investigate potential antagonistic activity. The APC were loaded with 10 μg/ml of variant peptides during 1 hour at 37°C. Following this incubation the cells were washed and loaded with various concentrations of HPV16 $E6_{127-141}$ (0.001μg/ml to 10μg/ml). The APC were co-cultured with belx2 clone overnight and an ELISA was performed on the supernatant. (figure 45). The results demonstrated that peptides HPV31, HPV52, HPV58 and HPV33 were able to inhibit the T cell response against HPV16 $E6_{127-141}$ in a dose dependent manner. This confirmed that all the peptides were able to bind to HLA-DR1*0101, and this would be predicted if the peptides had no antagonistic activity. By contrast the HPV67 peptide could induce a strong inhibition of the HPV16 $E6_{127-141}$ response even in the presence of much higher concentrations of the HPV16 $E6_{127-141}$ peptide. This profile is consistent with the HPV67 peptide having antagonistic properties.

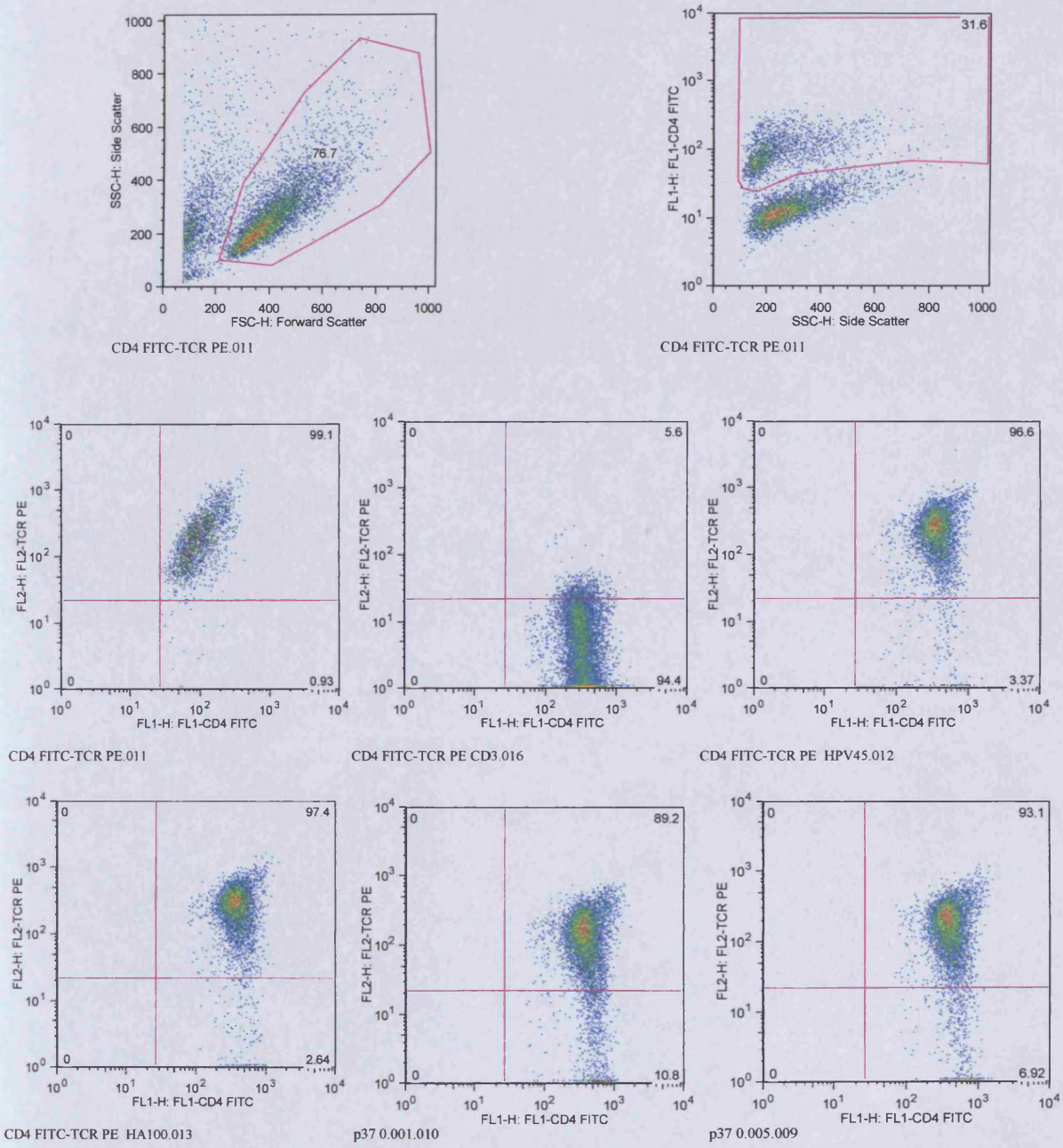


Figure 43: BLCL were loaded with HPV16 $E6_{127-141}$ at different concentration (0.001µg/ml to 10µg/ml) and co-cultured during 4 hours with belx2 cells. The cells were stained with an anti-CD4+ antibody and an anti-TCR antibody. BLCL were loaded with OKT3 (anti-CD3) and co-cultured with belx2 as positive control, two negatives controls were used loading with the HA100 (flu peptide), which known to bind HLA-DR1*01 but did not stimulate belx2 cells and a peptide from HPV45 which is not bind HLA-DR1*01.

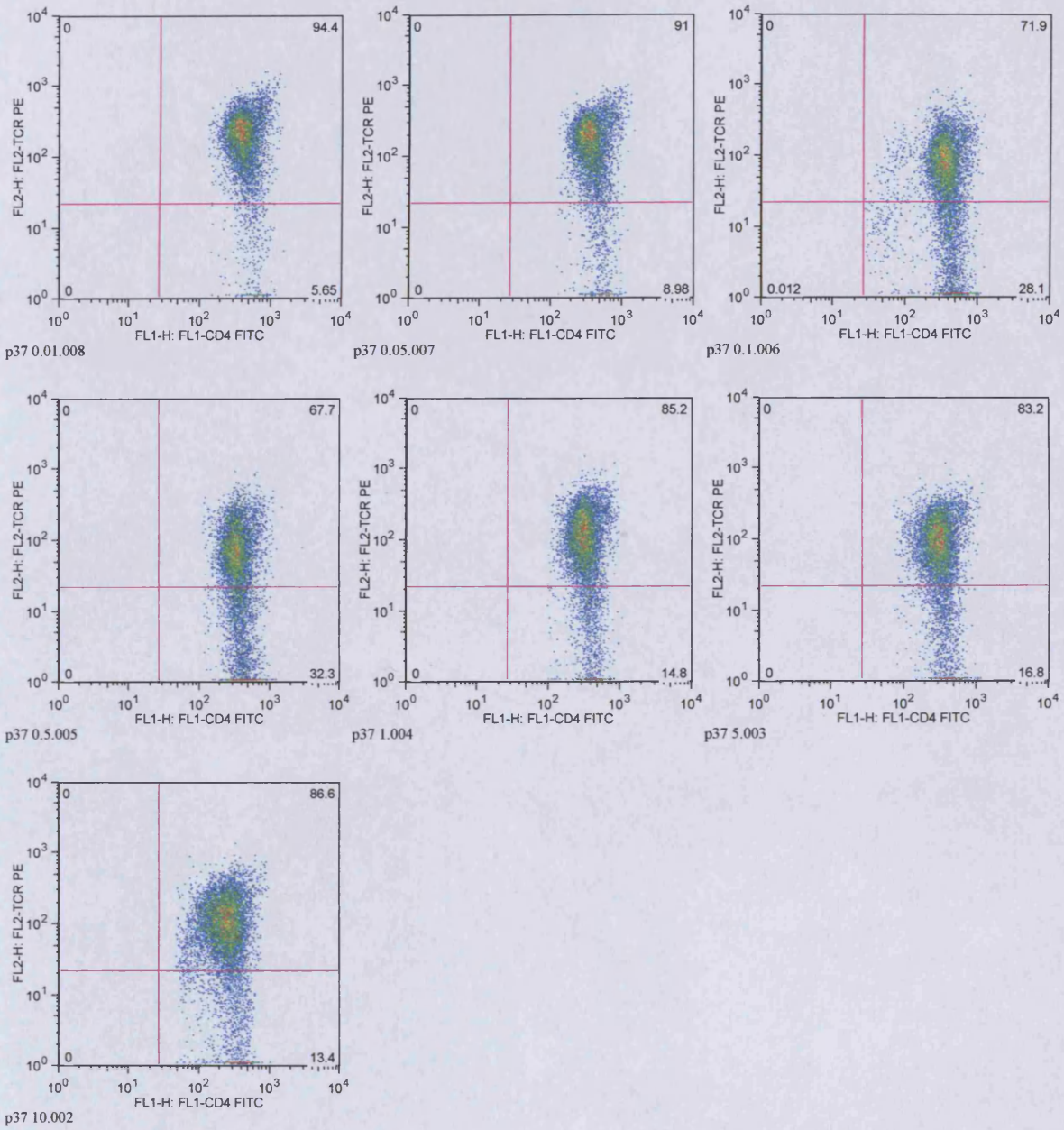
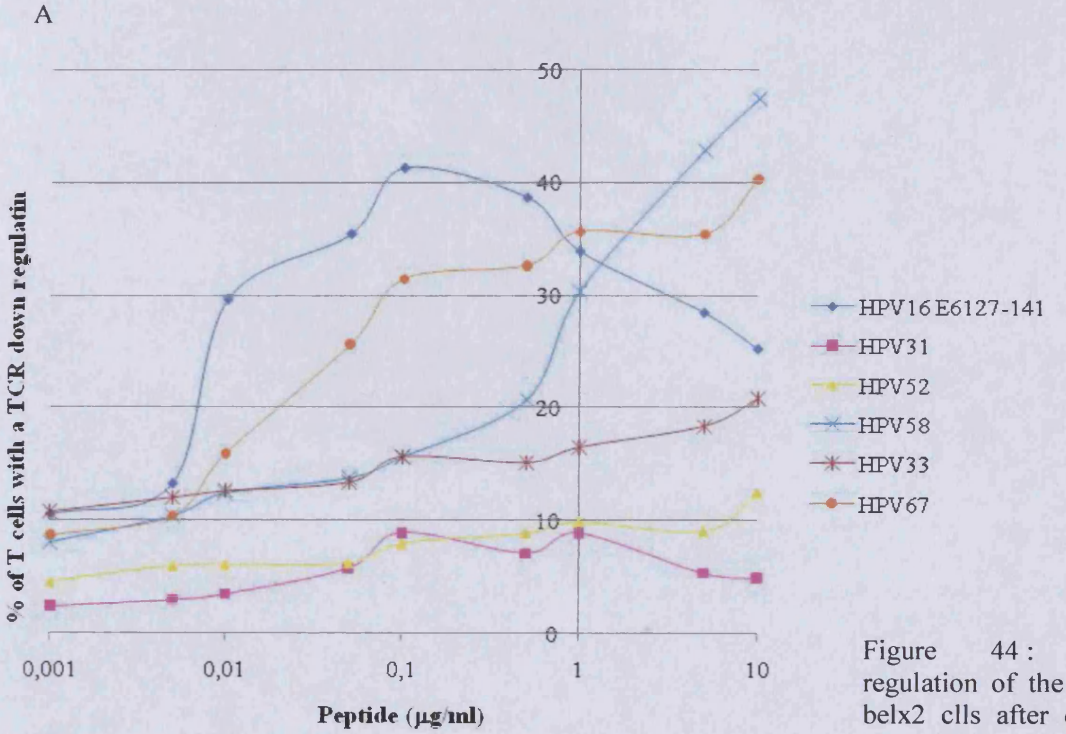


Figure 43: BLCL were loaded with HPV16 $E6_{127-141}$ at different concentration (0.001µg/ml to 10µg/ml) and co-cultured during 4 hours with belx2 cells. The cells were stained with an anti-CD4+ antibody and an anti-TCR antibody. BLCL were loaded with OKT3 (anti-CD3) and co-cultured with belx2 as positive control, two negatives controls were used loading with the HA100 (flu peptide), which known to bind HLA-DR1*01 but did not stimulate belx2 cells and a peptide from HPV45 which is not bind HLA-DR1*01.



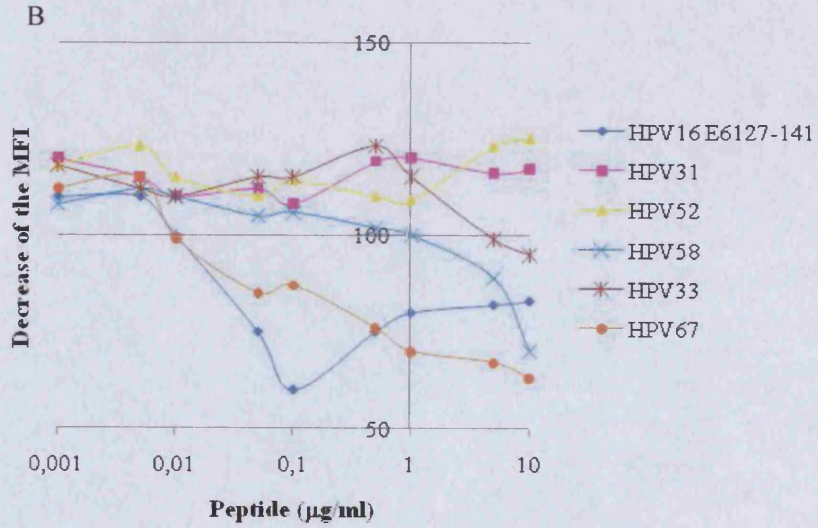


Figure 44 : Down-regulation of the TCR in belx2 clls after exposure to peptide antigens. B-LCL cells were washed after this incubation and co-cultured with belx2 during 4 hours at 37°C. Folliwing this incubation belx2 cells were stained with a mouse anti-human PAN TCR-PE antibody and a mouse anti-human CD4+-FITC antibody. The results were analysed in term of % of T-cells with a TCR down-regulation (A) or in term od decrease of MFI (Mean Fluorescence Cells) (B). Analysis of the flow cytometry results were performed using FlowJo software.

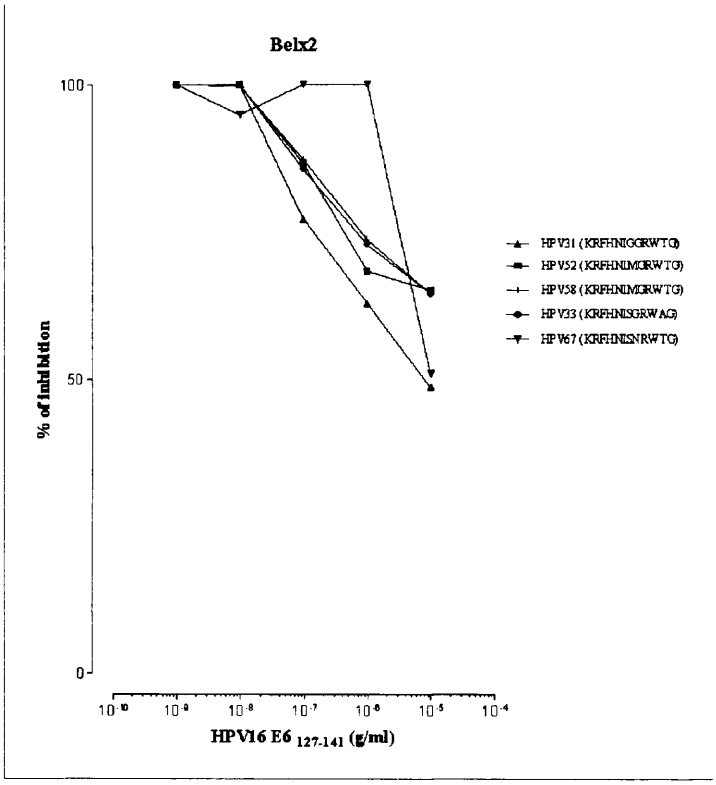


Figure 45: % of inhibition induced by different HPV variants. B-LCL were loaded with 10μg/ml of HPV variants during 1 hour at 37°C, washed and loaded with 10 to 0.001μg/ml of HPV16 $E6_{127-141}$. The cells were washed and co-cultured with belx2 overnight at 37°C. The supernatants were collected and tested by IFNγ ELISA. Each ELISA was performed three times and the % of inhibition was calculated in function of the higher value of secreted IFNγ for each peptide.

3-5-Discussion

Initially, T-cell activation was thought to be an all or none event, i.e., recognition by T-cells either did or did not occur (Salzmann and Bachmann 1998). More recently, it became evident that T-cell activation is more complex than initially thought, indeed various observations suggested that signal transduction via the TCR may vary qualitatively, depending upon the nature of the ligand (Sette, Alexander et al. 1994). Some altered peptide ligands failed to stimulate the full spectrum of T-cell responses but triggered only a fraction of them, they were called T-cells agonists or partial T-cell agonists (Evavold, Sloan-Lancaster et al. 1993). In contrast to these T-cell agonists or partial T-cell agonists, some altered peptide ligands completely failed to activate T-cells and were able to inhibit responses triggered by full T-cell agonists. These inhibitory peptides were called T-cell antagonists or partial T-cell antagonist peptides (Sette, Alexander et al. 1994). In order to have a better understanding of the interactions between HPV16 $E6_{127-141}$ peptide, HLA-DR*0101 and TCR, different substitutions were performed in the peptide, the substitutions corresponding to peptide from E6 protein of other HPV types. Furthermore all these substitutions included amino acids which were either a putative TCR contact residues or putative MHC anchor residues.

In the goal to define if the substitution of amino acids could induce agonist, partial agonist, antagonist or partial antagonist responses and to understand how the molecular interactions between the peptide, MHC and TCR could interfere in the responses a strategy has been defined (figure 46). The first experiments, which were the titration of the IFNγ secretion by belx1 and belx2 in response to these peptides, provided a first level of screening. Indeed if the peptides induced IFNγ they were considered as agonist or

partial agonist depending of the level of secreted IFNγ. The second set of experiments used an inhibition assay involving an influenza T-cell clone. In the case of no IFNγ secretion these experiments determined whether the peptide was able to bind the HLA-DR*0101. The third set of experiments had to be an activation assay to determine any potential effect on the TCR. Different strategies have been used in this goal such as calcium signalling assays and TCR down regulation assays. Only the TCR down regulation assays were sensitive enough to provide usable data. These experiments generated information on the interactions between the pMHC and the TCR, indeed the engagement of the TCR with the pMHC complex induces an activation of the T-cell and a down regulation of the TCR. The substitution of one or two amino acids may be sufficient to inhibit the binding to MHC, nevertheless the pMHC complexes with altered peptides could change the kinetic of signal transduction, the formation of immunological synapse, or induce negative signal (Ryan, McNeil et al. 2004) (Sette, Alexander et al. 1996). Then the third set of experiments provided information on the cellular activation after contact with the TCR, and permitted to know if the peptide was a null peptide (no TCR down regulation) or a antagonist (down regulation or partial down regulation).

Two peptides were able to induce a secretion of IFNγ, HPV58 and HPV33. This production of IFNγ was observed for the higher concentrations of peptides and only for the clone belx2 that is why these peptides were named partial agonist (figure 47). Interestingly both peptides appeared to have stronger binding to HLA-DR*0101 than HPV16 peptides, based on the HC3 inhibition assays (figure 38). Therefore the reduction in functional activity appears to be influenced by peptide/TCR interactions.

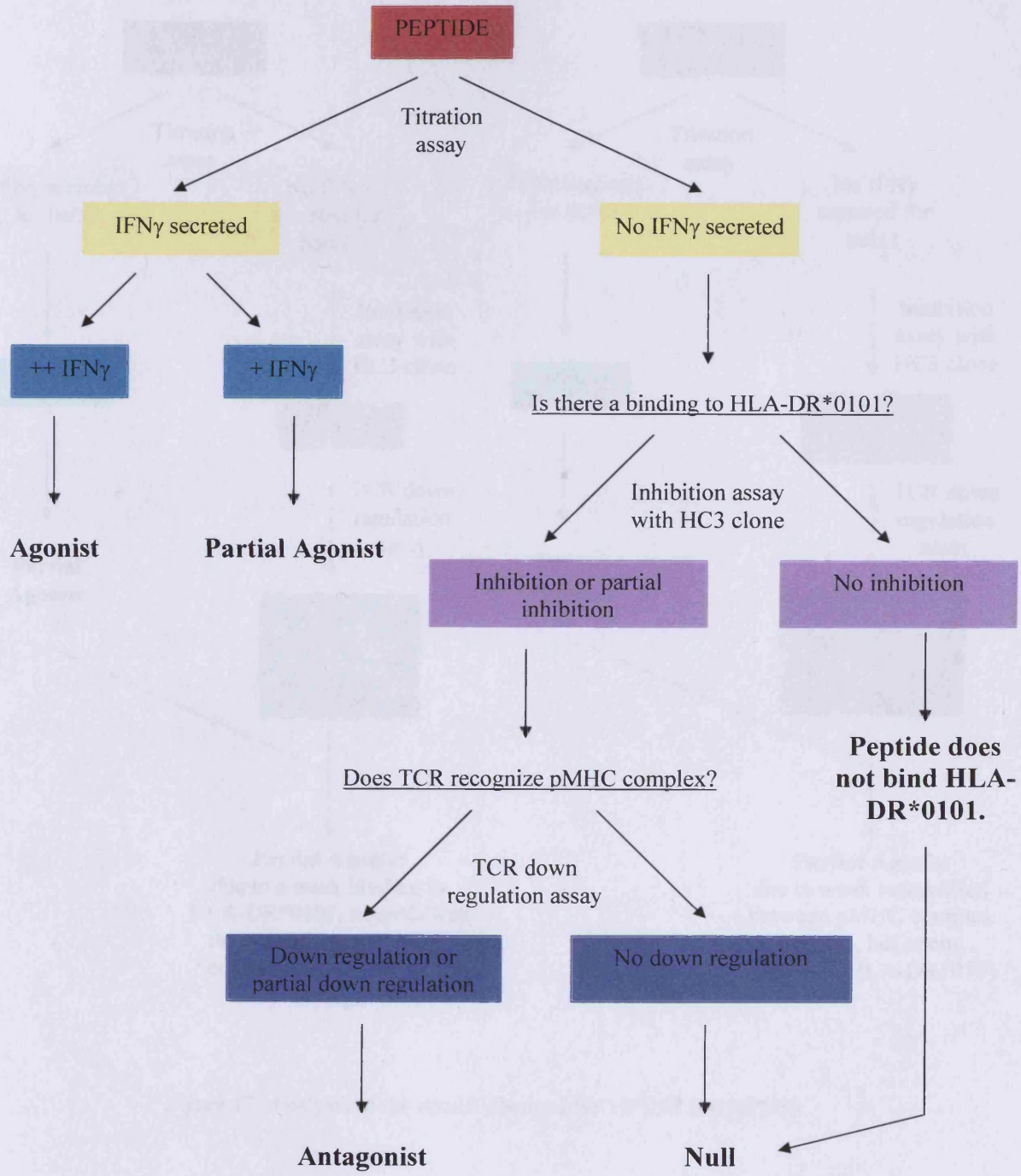


Figure 46: Strategy used to determinate if the HPV peptides were agonist, partial agonist, antagonist or partial antagonist of HPV16 $E6_{127-141}$

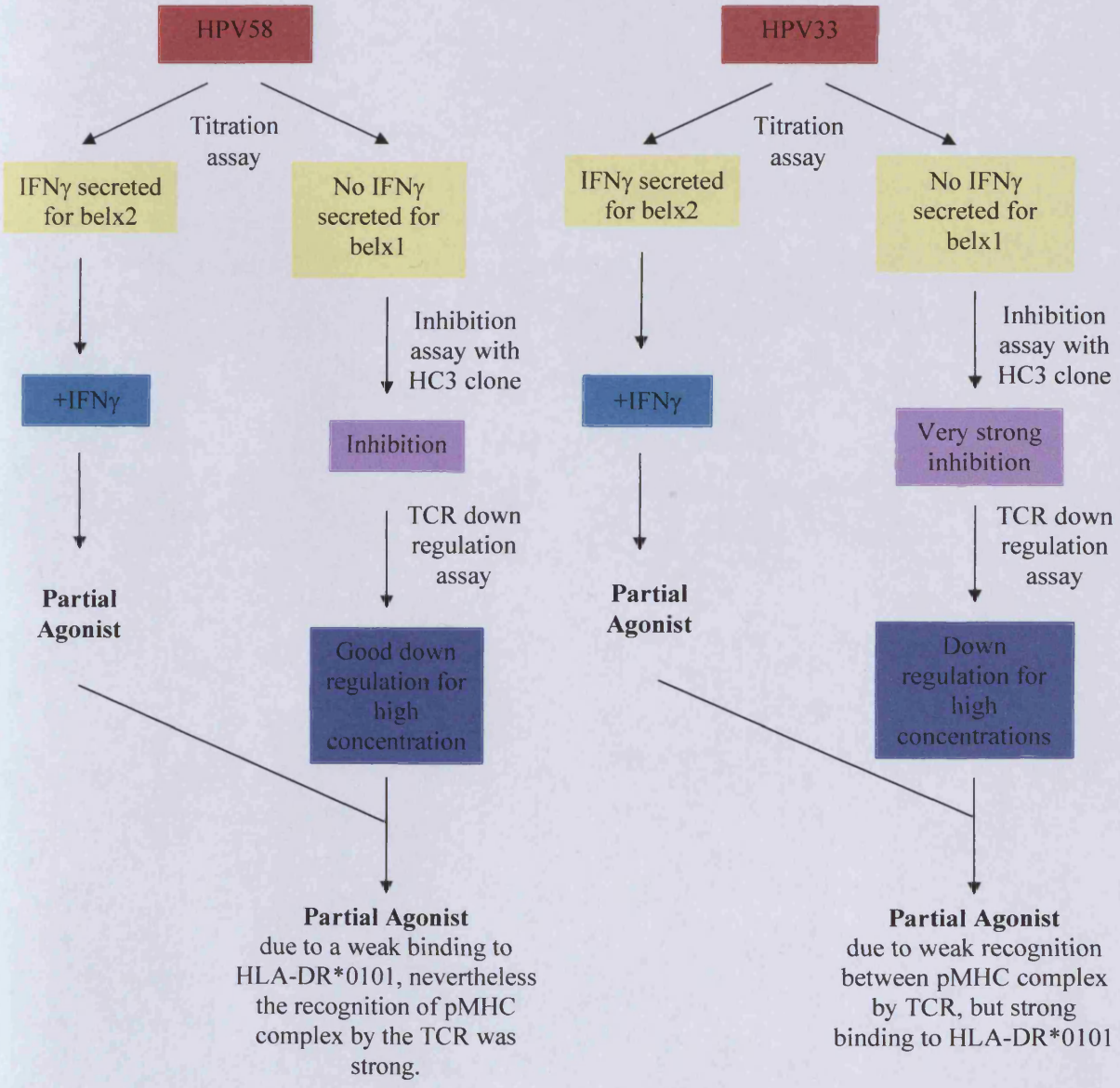


Figure 47: Analysis of the results obtained for HPV58 and HPV33

Both HPV33 and HPV58 have a R→S change at putative TCR residue (table 12). It is possible that the loss of a positive charge could reduce the affinity of TCR/peptide interactions. As might be predicted for weak/partial agonists, high concentrations of peptide were required to down-regulate the TCR compared to HPV16 $E6_{127-141}$ peptide (figure 44).

The three other peptides, HPV31, HPV52 and HPV67 did not induce the production of IFNγ neither for belx1 or belx2 (figure 47). All of them inhibited the binding between HA100 (influenza peptide) and HLA-DR*0101. This inhibition was only for the highest concentration of peptide (10μg/ml) in the case of HPV31 and HPV67, whereas inhibition could be seen for range of peptide concentrations for HPV52. This suggests that HPV31 and HPV67 were relatively weak binders to HLA-DR*0101. Only HPV67 induced a down regulation of TCR in the absence of any functional activation, which is a major characteristic of the antagonist peptides (Sette, Alexander et al. 1994). The other peptides induced minimal down regulation of the TCR suggesting that these peptides are "null peptides", capable of binding to MHC but not able to activate or antagonise a functional response (figure 48). HPV31 has a R→G change in a putative TCR contact residue. The change of a positively charged basic amino acid to an uncharged polar amino acid appears to have abolished T-cell activation. Similarly HPV52 has a R→M change, at the same location; here the change from a positively charged basic amino acid to a hydrophobic amino acid has also abolished T-cell activation. The effect of substitutions in that location is dependent on the amino acid, because the R→S change for HPV58 and 33, still allow partial TCR activation. HPV67 was a very interesting peptide, because it had potent TCR down-regulation activity, similar to the HPV16 $E6_{127-}$

$_{141}$ peptide (figure 44), but weak binding to MHC (figure 38). However the original HPV16 peptide would also be classified as a weak binding peptide according to the same assay (figure 38). The HPV67 peptide has R→S change, similar to HPV33 (partial agonist) and a G→N change at a putative MHC binding residue (table 12). The HPV67 peptide is able to interact with the TCR (figure 44) to cause down-regulation, but this is insufficient to induce IFNγ response of belx2, in the presence of high concentrations of HPV16 $E6_{127-141}$ peptide (figure 45). These results suggest that HPV67 is an antagonistic peptide.

All these data are summarized in the table 14. It is possible that by only assaying for IFNγ secretion and TCR down-regulation, that other T-cell effector functions could have been missed. Therefore these peptides have been tested for their capacity to induce other types of cytokines, IL-4 and IL-10 ELISA were performed on belx1 and belx2 (data not shown), but no secretion was observed. These data suggest that these peptides were not able induce changes in the cytokine profiles (Th1 to Th2) of the specific T-cell clones.

The results in this chapter demonstrate that amino acids changes in putative TCR contact residues can have several effects; they can abolish (null peptide) or reduce T-cell activation (partial agonist). Alternatively they can cause an inhibition of T-cell responses by down-regulation of the TCR (antagonist). To investigate the interactions between the peptides and the TCR and the effect on the cellular activation it would be interesting to use a tetramer of HLA-DR*0101 associated with each peptide and to carry out binding assays. Earlier work had demonstrated that recombinant soluble HLA-DR*0101-CLIP complexes could be generated, and these could be used as the source material to make

tetramers containing different variant peptides. Flourescently labelled tetramers could then be used for staining of HPV16 $E6_{127-141}$ specific T cells (table 15 summuraries all the data).

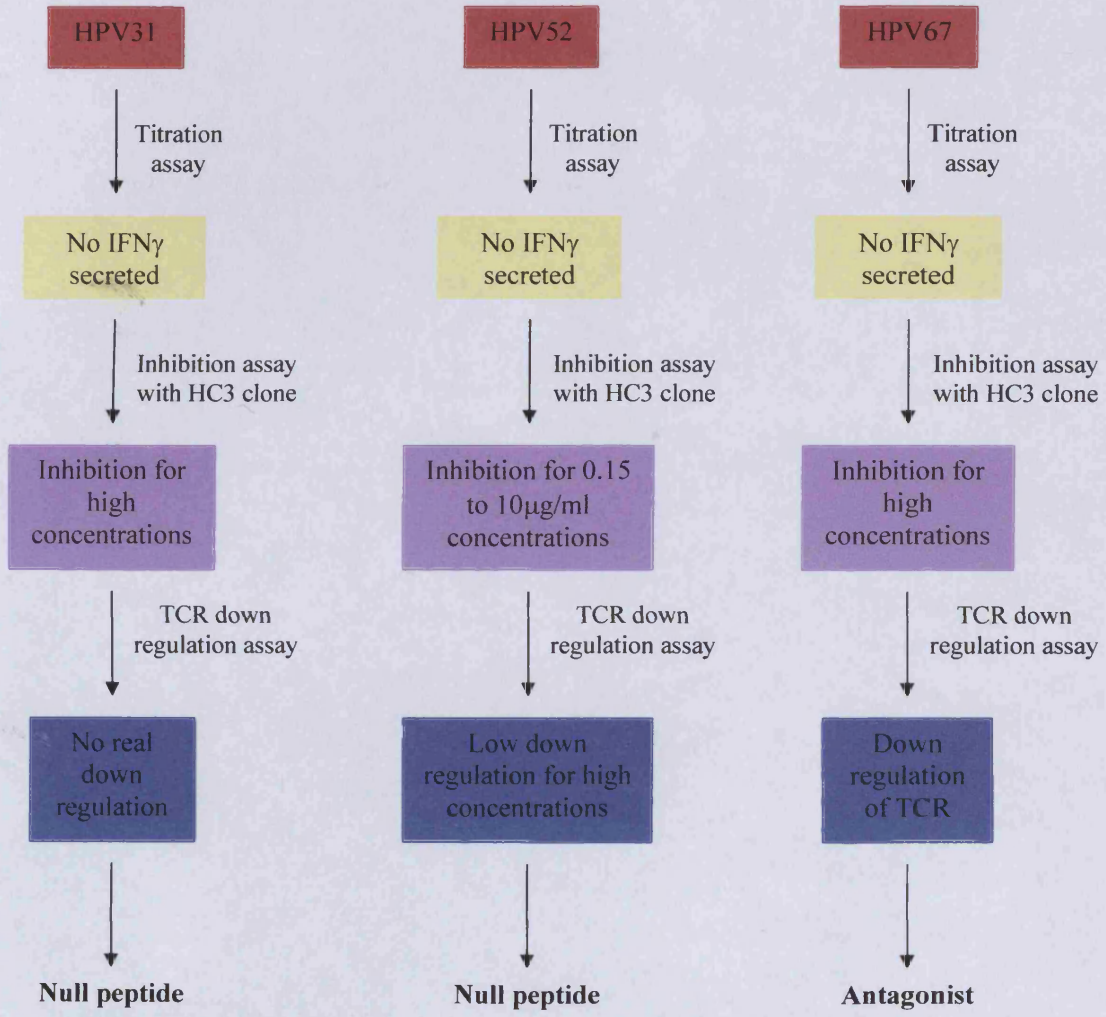


Figure 48: Analysis of the results obtained for HPV31, HPV52 and HPV67

Table 14: List of the role of each variant peptide.

Peptides	Agonist	Partial agonist	Antagonist	Null
HPV16 $E6_{127-141}$	+++	-	-	-
HPV31	-	-	-	+
HPV52	-	-	-	+
HPV58	-	+	-	-
HPV33	-	+	-	-
HPV67	-	-	++	-

Peptides	IFNγ secretion belx1	IFNγ secretion belx2	Peptide binding to the HLA-DR1 (HC3 assay)	Down regulation of the TCR	Characteristic of the peptide
HPV16 $E6_{127-141}$	+++	++++	+/-	++	NA
1	+++	++++	NA	NA	NA
2	+++	++++	NA	NA	NA
3	+++	++++	NA	NA	NA
4	+	++	NA	NA	NA
5	++	+++	NA	NA	NA
6	-	+	NA	NA	NA
7	-	-	NA	NA	NA
8	+++	+++++	NA	NA	NA
9	+++	++++	NA	NA	NA
10	+++	++++	NA	NA	NA
HPV31	-	-	+/-	-	Null peptide
HPV52	-	-	+	-	Null peptide
HPV58	-	+	++	+	Partial agonist
HPV33	-	+	++	+	Partial agonist
HPV67	-	-	+	+++	Antagonist

Table 15: Summary of peptide activities

DISCUSSION

The main causative agent in the development of cervical cancer is HR HPV. However, the progression from persistent HPV infection to CIN and cervical cancer is a rare event and in nearly all cases the virus is effectively cleared. An important role for T-cell mediated immunity in the control of HPV infection has been inferred by a number of observations from immunocompromised patients and studies of naturally regressing genital warts (Coleman, Birley et al. 1994) (Petry, Scheffel et al. 1994), and also animal studies (Nicholls, Moore et al. 2001). A number of studies have investigated the immune responses to HPV16 and HPV18 proteins in patients with CIN or cervical cancer (Bontkes, de Gruijl et al. 1999) (Bontkes, de Gruijl et al. 2000) (Bontkes, de Gruijl et al. 1999) (de Jong, van Poelgeest et al. 2004) (Steele, Mann et al. 2005) (Welters, van der Logt et al. 2006) and more recently in healthy donors (de Jong, van der Burg et al. 2002) (Facchinetti, Seresini et al. 2005) (Welters, de Jong et al. 2003; Welters, van der Logt et al. 2006). The E6 and E7 proteins are constitutively expressed in both high grade CIN and cervical cancer, making them ideal tumour-specific antigens for study. In healthy women, memory T-cell responses tend to be directed at the E6 protein, with responses to the E7 protein being relatively rare (van der Burg, Ressing et al. 2001), (Welters, de Jong et al. 2003). As these women have successfully cleared viral infection, it is feasible that the epitopes against which the anti-viral immune response was generated could correlate with protection from persistent infection. The identification of such epitopes could potentially be useful in the development of T-cell based immunotherapeutic strategies. In this goal the previous studies of the laboratory have generated two T-cell clones specific for a peptide from HPV16 E6 protein. Indeed ten healthy female donors were screened for T-cell responses against HPV16 and HPV18 E6 and E7 peptide pools. Five donors

made HPV E6 specific responses (as detected by IFNγ ELISpot assay). Two T-cell lines were generated from one individual against the HPV16 $E6_{(127-141)}$ epitope. These lines exclusively express either TCR TRBV14 (belx1) or TCR TRBV4-2 (belx2) and TRAV17, and demonstrate differential cytokine secretion profiles in response to peptide re-stimulation. Furthermore it has been demonstrated HPV16 $E6_{127-141}$ was exclusively presented by HLA-DR*0101 (Gallagher and Man 2007). It appeared that the differential cytokine secretion profiles observed for belx1 and belx2 could be due to their TCR.

A central aim in this project was to study the interactions between the HLA-DR*0101-HPV16 $E6_{127-141}$ complexes and TCR. In order to evaluated the type of molecular interactions involved in this mechanism it was necessary to generate a HLA-DR*0101-HPV16 $E6_{127-141}$ tetramer, soluble TCRs and study their binding using BIAcore™. In order to produce HLA-DR*0101-HPV16 $E6_{127-141}$ tetramer different technologies have been used; baculovirus expression system and *E. coli* cells expression system. Unfortunately after severals attempts with the two expression systems and various conditions no tetramer could be obtained, and commercially produced tetramers did not appear to bind to specific T cells.

One explanation for this failure to produce soluble class II MHC associated with HPV peptide could be the stability of the complex. Class II molecules are non-covalent dimers of α and β chains, which have a variable range of stability and solubility in solution. Peptides bind into α/β chain groove of class II based on specific interactions with amino acid side chains, which confer specificity, determining which peptides will bind which class II molecules (Nepom, Buckner et al. 2002). The stability of the molecule is dependant of the interaction between three factors; the α chain, the β chain

and the peptide; indeed peptide-free class II molecules are relatively unstable (Jensen, Weber et al. 1999). Even if HPV16 $E6_{127-141}$ peptide could be loaded on cells expressing stable HLA-DR*0101, and these complexes are able to activate specific T-cells via engagement of the TCR, the interactions and the stability of the HLA-DR*0101 and HPV16 $E6_{127-141}$ stay unknown. Indeed if the peptide is not able to stabilise the molecule during its synthesis and refolding *in-vivo* (baculovirus expression system) or *in-vitro* (*E. coli* expression system) there will not be production of the molecule. Nevertheless previous studies in the laboratory have demonstrated the processing and presentation of this epitope by BLCL. APC have been incubated with soluble full-length E6 protein and co-cultured with belx2 T-cell clone overnight and an ELISpot assay has been carried out. Strong positive responses were detected against the epitope, which clearly indicated that the full length E6 protein could be processed and the epitope presented to belx2 (Gallagher and Man 2007). These results indicated that the stability of the complex was sufficient for its formation and the processing mechanism occurs in the BLCL. However, it was not possible to assess the processing of this epitope in cervical carcinoma cells; there are no readily available $HPV16^+$ cervical cancer cell lines expressing HLA-DR*0101, and previous attempts to transfect HLA-DR*0101 into $HPV16^+$ CaSki cells were unsuccessful.

An alternative explanation for the failure to produce soluble HLA:HPV peptide complexes may be that there was inadequate assembly in the insect cells. Soon after synthesis in the ER, three α/β MHC class II dimers associate with a trimer of invariant (Ii) chains, which ensure that ER derived proteins do not bind to the ER membrane anchored MHC II molecule (Cresswell 1996) (Romagnoli, Layet et al. 1993). This

nonameric complex exits the ER and pass through the Golgi apparatus before being transported to the endocytic pathway, under the influence of transport signals present in the cytoplasmic region of the Ii chain (Guermonprez, Valladeau et al. 2002). During the formation of these complexes, calnexin is present to add stability to the newly formed molecule (Anderson and Cresswell 1994). Ii has a second function, which starts with the dissociation of MHC II from calnexin and results in the transport of the MHC II molecule from the ER to endosomal compartments for peptide loading. In order for peptide loading to occur, Ii must be removed from the MHC II peptide-binding groove. This is mediated through the activities of cathepsin S (Villadangos, Bryant et al. 1999), which cleaves Ii in a number of steps. Ii is then released from the MHC II molecule through the actions of the class II associated invariant-chain peptide (CLIP), which performs the final Ii cleavage. This also releases the MHC II molecule from the membrane. In order for MHC II to bind to endosomally derived proteins, an MHC-like molecule called DM must first associate. This association leads to the dissociation of CLIP from the MHC II peptide-binding groove, allowing endosomally processed peptide fragments to bind. MHC DM (Kropshofer, Hammerling et al. 1999) also facilitates a process termed "peptide-editing", which ensures that only stable MHC II peptide complexes are expressed and transported to the cell surface for potential TCR interactions (Busch, Doebele et al. 2000). All these mechanisms occur in the BLCL which are human cells even if they are transformed. In the insect cells it is not the similar system, indeed the expression and secretion of the soluble molecule is due to the infection by the cell of a recombinant virus which carries the correct DNA for the molecule. Indeed another explanation of the failure could be a major trouble in the DNA construction, nevertheless similar construction was used to

produce HLA-DR*0101-CLIP and a low quantity was secreted. These results demonstrated that the DNA construction and cloning strategy were adequate, even if this soluble molecule was produced in low quantity. It is possible that the production of HLA-DR*0101-CLIP was easier in insect cells than HLA-DR*0101-HPV16 $E6_{127-141}$ due to a complexity of the molecule or a better stability in insect cell environment.

Therefore to move the project forward, an HLA-DR*0101-HPV16 $E6_{127-141}$ ultimer was ordered from Pro-immune (Oxford, UK). This company has successfully synthesized other HLA-DR1 ultimers e.g. HLA-DR1 complexed to an Influenza hemagglutin peptide. Despite using several different conditions, no binding of the Proimmune ultimer to T-cells belx1 or belx2 could be detected. It is possible that antigen specific T-cells are not stained by class II MHC tetramers because the TCR avidity for soluble MHC/peptide complexes is too weak (Vollers and Stern 2008). By contrast, TCR avidity for MHC/peptide complexes on live antigen specific cells may be influenced by other cell surface molecules. This might explain the lack of binding of the Proimmune ultimer, despite clear functional specificity of the tested cells. Another explanation was the production by Proimmune of unstable or incorrectly folded molecules due to an unstable binding between HLA-DR*0101 and HPV16 $E6_{127-141}$. It was decided to investigate the functional difference between belx1 and belx2 at a cellular level using in a first instance truncated versions of HPV16 $E6_{127-141}$ and in a second instance variants from other HPV types.

The MHC-restricted recognition of antigen peptides by TCRs is a central event in the cellular immunity against pathogens and in the immune surveillance of cancer cells. In class II MHC polymorphic pockets in the binding site provide the basis for the

preference of different alleles for different peptide sequence motifs (Stern, Brown et al. 1994). Indeed class II molecule consists of an α-chain forming α1 and α2 domains and a β-chain forming β1 and β2 domains. The MHC molecule firmly holds a peptide in a groove formed with two α-helices and a β-sheet floor and presents the peptide to T-cells (Tsurui and Takahashi 2007). These complexes class II MHC-peptide are recognized by the TCR, the Vα domain recognizes the $C_{terminal}$ side of the peptide with CDR1 and 3, and the α-helix of the β1 domain with CDR2; and the Vβ domain recognizes the $N_{terminal}$ side of the peptide with CDR1 and 3, and the α helix of the α1 domain with CDR2 (figure 49). Belx1 and belx2 are T-cell clones specific for HPV16 $E6_{127-141}$ which differs by their TCR. In order to study the impact of the $C_{terminal}$ and $N_{terminal}$ ends of the peptide to the TCR and MHC binding various truncated peptides have been designed (table 16).

For the two clones, belx1 and belx2, it was showed that the $C_{terminal}$ end had a greater impact on T-cell IFNγ secretion than the $N_{terminal}$ end. Indeed the deletion of first three amino acids of the HPV16 $E6_{127-141}$ peptide did not affect the response, similar profile of response and quantities of IFNγ were observed in each case. The peptide 4 did not induce the same type of response for the two clones, for belx1 the response was abolished, for belx2 the response required a higher concentration of peptide than the wild type. An explanation could be the impact of the deletion of the Q in P-2. Indeed following the identification of HLA-DR*0101 as the allotype responsible for HPV16 $E6_{127-141}$ (Gallagher and Man 2007), the peptide sequences was compared to the corresponding binding motifs (www.syfpeithi.de). Peptide binding motifs for HLA-DR*0101 (Rammensee, Bachmann et al. 1999) have been defined but are often imprecise (Marsh 2000). The motifs are based upon the sequences of peptides that are known to

bind, but do not form an exhaustive list of potential anchor residues for a given allotype. It is therefore not uncommon for peptide epitopes to exhibit partial or no alignment with a binding motif (Marsh 2000). For HLA-DR*0101 restricted peptides, 4 key anchor positions (within the 9 amino acid core region) have been identified; P1, P4, P6 and P9. The potential amino acids for each of the anchor residues are listed below (Rammensee, Bachmann et al. 1999).

P1 =Y, V, L, F, I, A, M, W
P4 =L, A, I, V, M, N, Q
P6 =A, G, S, T, C, P
P9 =A, I, V, N, F, Y

The sequence of HPV16 $E6_{127-141}$ can be aligned to complement P1, P4 and P6 as highlighted in bold and underlined in the following; D K K Q R **F** H N **I** R **G** R W T G. Based on this alignment it appears that HPV16 $E6_{127-141}$ is a conventional HLA-DR*0101 restricted peptide. Using the SYFPEITHI predictive database (Rammensee, Bachmann et al. 1999) HPV16 $E6_{127-141}$ is predicted to bind to HLA-DR*0101 molecules.

The residue in P-2 (Q) was a putative TCR contact residue. The major difference in belx1 and belx2 was their TCRs, indeed the two clones had the same α chain but not the same β chain. One of the characteristics of the TCRs is their ability to differ in their αβ chain pairing, such that the pseudo Vα/Vβ twofold angle, which currently varies from 166° to 188°, can contribute to the variation in TCR orientation on the pMHC (Rudolph and Wilson 2002), and potentially could have relevance to TCR signalling if the pairing

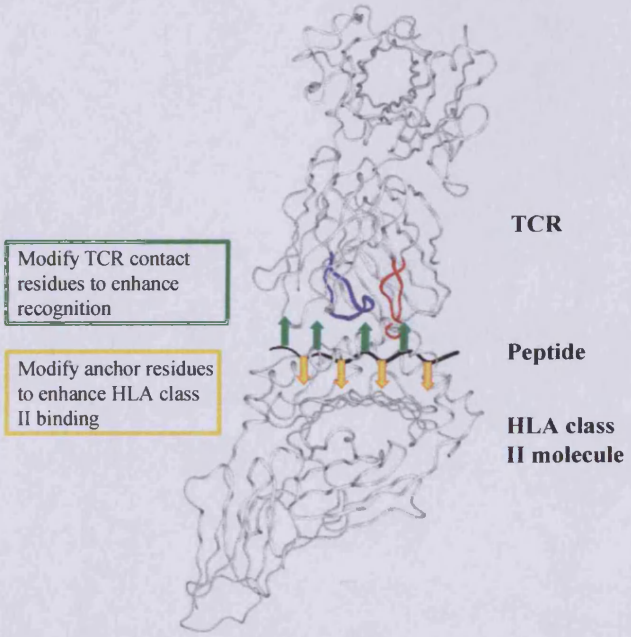


Figure 49 Interactions of TCR with an HLA-DR*0101-peptide complex. The model depicts the interaction of a TCR with HL-DR*0101-endogen peptide complex. The red loop shown in ribbons represents the CDR3 region of the α chain and the blue loop represents the CDR3 region of the β chain. The amino acids of the peptide which potentially interact with the TCR are shown in green. The anchor residues involved in HLA class II binding are highlighted in yellow. This model was created by Dr. Istvan Bartok, Imperial College, London.

Table 16: List of all the peptides designed from the HPV16 $E6_{127-141}$ epitope.

Peptides	Sequences																
	P-5	P-4	P-3	P-2	P-1	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
HPV16 $E6_{127-141}$	D	K	K	Q	R	F	H	N	I	R	G	R	W	T	G			
1		K	K	Q	R	F	H	N	I	R	G	R	W	T	G			
2			K	Q	R	F	H	N	I	R	G	R	W	T	G			
3				Q	R	F	H	N	I	R	G	R	W	T	G			
4					R	F	H	N	I	R	G	R	W	T	G			
5	D	K	K	Q	R	F	H	N	I	R	G	R	W	T				
6	D	K	K	Q	R	F	H	N	I	R	G	R	W					
7	D	K	K	Q	R	F	H	N	I	R	G	R						
8				Q	R	F	H	N	I	R	G	R	W	T	G	R	C	M
9	D	K	K	**K**	R	F	H	N	I	R	G	R	W	T	G			
10				**K**	R	F	H	N	I	R	G	R	W	T	G			
HPV31				**K**	R	F	H	N	I	**G**	G	R	W	T	G			
HPV52				**K**	R	F	H	N	I	**M**	G	R	W	T	G			
HPV58				**K**	R	F	H	N	I	**S**	G	R	W	T	G			
HPV33				**K**	R	F	H	N	I	**S**	G	R	W	**A**	G			
HPV67				**K**	R	F	H	N	I	**S**	**N**	R	W	T	G			

Putative TCR contact residues

Putative MHC anchor residues

changes propagate to the constant domains or are translated to accessory proteins (Garcia 1999). Furthermore few studies have demonstrated that the V_β chain of the TCR recognizes the $N_{terminal}$ end of the peptide with CDR1 and 3 (Garcia 1999) (Garcia, Scott et al. 1996) (Garboczi, Ghosh et al. 1996) (Garcia and Teyton 1998) (Garcia 1999) (Reinherz, Tan et al. 1999) (Tsurui and Takahashi 2007). The deletion of the Q could generate a change in the twofold angle when the pMHC interacted with the TCR, only in presence of the $V_\beta 16$, and it could affect the intracellular signalling. It is possible that the $V_\beta 7$ chain was less affected by the loss of the Q residue. At the end, to answer to these questions it would be necessary to be able to produce the soluble molecules (TCRs and MHCs) and observe their interactions and affinity using BIAcore™ technology; nevertheless it was not possible to do this during the current study.

By contrast the deletion on the $C_{terminal}$ end of the HPV16 $E6_{127-141}$ peptide had an important impact on the response of each clone. Each deletion was associated with either a putative TCR contact residue or a putative MHC anchor residue. The deletions generated peptides that potentially were not able to bind HLA-DR*0101 or to interact with the TCR. Furthermore the P10 position has been identified as minor or auxiliary anchor residues in several pMHC complexes (Ferrante and Gorski 2007).

Alignment of the HPV16 $E6_{127-141}$ sequence with the E6 protein sequences from five different HR HPVs revealed that residues in the core region involved in HLA-DR binding are relatively well conserved. In particular, the HPV16 $E6_{127-141}$ anchor residues (at positions 1, 4, 6 and 9) are very well conserved in the E6 proteins from all the HR HPVs assessed. Furthermore, the putative TCR contact residues at P-1, P7 and P8 are

also well conserved (Table 15). TCR recognition of pMHC is a highly sensitive ligand interaction whereby subtle changes in peptide can cause drastic variations in T-cell effector function (Ryan, McNeil et al. 2004). Indeed antagonist peptide can inhibit T-cell activation, and, similarly, engagement with a partial agonist elicits some, but not all, of the responses that characterize T-cell activation by fully agonistic ligand (Rudolph, Stanfield et al. 2006). In order to have a better understanding of the interactions between HPV16 $E6_{127-141}$ peptide, HLA-DR*0101 and TCR, it was interesting to test the E6 proteins from the five HR HPVs. Different substitutions were performed in the wild type peptide, and all of them included amino acids which were either a putative TCR contact residues or putative MHC anchor residues. To define if a peptide could be an agonist, a partial agonist, an antagonist, a partial antagonist or a null peptide several experiments including ELISA, inhibition assays and TCR down regulation were performed. The results showed the peptides from HPV58 and HPV33 were partial agonist, peptides from HPV31 and HPV52 were null peptides and peptide from HPV67 was an antagonist. Many models of T-cell activation have been proposed to explain the differential effects of altered peptides. But no specific model is completely validated, few explanations are proposed. One possiblility is that lowering peptide affinity reduces antigen dose by decreasing the number of potential pMHC complexes (Ryan, McNeil et al. 2004). An alternative explanation is that substitution at MHC contact positions shift the orientation of TCR-exposed side chains, thus affecting the recognition of ligand by TCR. Indeed the TCR itself seems to adapt to small changes in the pMHC ligand by small conformational changes or rearrangements of its central CDR loops (Rudolph and Wilson 2002). The dissociation rate of pMHC complexes could also prove to be a critical variable in

dictating T-cell responses levels. It is possible too that those substitutions at MHC-contact positions shift the orientation of TCR-exposed side chains, thus affecting the recognition of ligand by the TCR. It is also formally possible that alteration of MHC anchor residues could change the conformation of the MHC α-helices. Nevertheless the mechanism by which antagonist peptides inhibit T cell activation remains incompletely understood and few studies have been performed, and even the crystal structures of several agonist and antagonist complexes, could not predict their biological behaviour because such similar structure can not resolve the subtle differences in kinetic and thermodynamic properties that lead to such different signalling outcomes (Rudolph and Wilson 2002).

These results suggested that it is important to evaluate the therapeutic potential of altered peptide ligands. Indeed no therapeutic vaccine exist for the moment, although clinical trials involving viral vectors, peptides, prime boost, DNA or DC have been performed (see table 3). In the chapter 5 it was demonstrated that the substitution of two amino acids in the wild type peptide was enough to produce an antagonist peptide which could inhibit the response induced by HPV16 $E6_{127-141}$.

This finding illustrates a potential mechanism for turning off a T cell response against a particular peptide epitope and suggests that this should be investigated for other peptide epitopes. It also suggests that care must be taken when modifying peptide sequences for vaccines because modification might result in a peptide that turns off the response.

It has been demonstrated that different factors are necessary for full malignant transformation (Campo 1998) and the mechanism of escape by the virus could be one of

them. HPV infects the keratinocytes within the cervical epithelium. Within the cervical epithelia, the primary cell population responsible for antigen presentation is the LC. These cells are thought to endocytose HPV antigen within the epithelia and upon activation, migrate to the lymph nodes where they present the processed peptide in the context of HLA class II molecules to the CD4+ T-cells (Kupper and Fuhlbrigge 2004). Subsequent to T-cell priming, the HPV-specific activated CD4+ T-cell population may then migrate from the lymph node and enter the infected cervical tissue. Local re-stimulation of the T-cells either by infected keratinocytes or other APC such as DCs and macrophages presenting the cognate peptide, stimulates the T-cells to produce cytokines such as IL-2 and IFNγ. The local production of these immunomodulatory cytokines could have several beneficial effects. Firstly IFNγ is a potent anti-viral cytokine which could limit viral spread and also activate macrophages resulting in the production of potent cytotoxic compounds such as NO and reactive oxygen species. The production of IL-2 would positively effect the development of the CD8+ CTL response, which in other systems is known to play a crucial role in the clearance of virally infected or transformed cells. The principles of this model are based on a recent report which described a vital role for CD4+ T-cells (through collaboration with macrophages) in the control of a primary immune response against a HLA class II negative tumour in mice (Corthay, Skovseth et al. 2005). The presentation of an antagonist peptide by the APC to the T-cells could induce a tolerance of the organism to the viruses. Indeed an antagonist peptide could either down-regulate the TCR (as peptide variant from HPV67) or bind the TCR and leave less site free for the binding of agonist peptides. Furthermore if a person, infected by HPV16, is infected by HPV67 this could result in the abolishment of the

responses against HPV16 due to a tolerance induced by the new infection. Indeed a model could be established in HPV infection or co-infection (figure 50). This model could take place in young women who are more likely to regularly encounter different HPV types as a result of high levels of sexual activity. Young women could be infected by a first type of HPV, such as HPV16, in this case either the immune system will eradicate the infection in the 3 following years (about 80% of the infected population) or there will be a persistence of the infection which could drive to lesions and cancer. In the case of a clearance a naïve immune response takes place and is followed by a adaptive immune response. During the establishment of the immune response if a co-infection takes place with a virus, such as HPV67, this co-infection could inhibit the response induced by HPV16 and then allows a persistence of the first virus, which could generate a persistent infection that could lead to cancer. These kinds of events could be an explanation for the evasion of a virus type from the immune system. Furthermore it has been demonstrated that HPV67 is a HR HPV, and associated with HPV31 is able to induce the development of squamous cell carcinoma, an explanation could be the antagonistic role of the peptide from one virus which allow the evasion of the other virus.

In the future various studies could help a better understanding of the responses against HPVs. The purification of HLA-DR*0101-CLIP, from transfected insect cells, needs to be improved, using a column carrying L243 antibody to obtain a higher yield and realise a peptide exchange. Indeed a few studies have already used this technique which consists in the production of a class II MHC tetramer associated with CLIP peptide and the exchange of the CLIP peptide by the target peptide (Day, Seth et al. 2003). The technique has the advantage to allow the production of several different HLA:peptide

complexes starting from the same HLA:CLIP source. From this it could be possible to exchange CLIP peptide by the wild type or the variant peptides and to study the interaction between pMHC and T-cells. Animal studies could be another aspect of the future studies.Using HPV16 $E6_{127-141}$ and the variants it could be interesting to observe if the *in-vitro* results could be correlated to *in-vivo* results. Indeed it could be interesting to test the responses induced by HPV16 $E6_{127-141}$ on T-cells from severals samples of DR1+ve tumour at different grades of infection. These observations could allow to have a better understanding of the immune responses against HPV16 and to observe, in the case of co-infection, if there is a potential inhibition of the T-cell response.

This study was based on two T-cell clones generated from one healthy donor, it could be interesting in the future to generate other clones, against HPV16 $E6_{127-141}$, from other DR1 healthy donors in order to generalize these data. Previous data (Gallagher and Man, 2007) showed that different epitope from HPV16 E6 could be presented by other HLA-DR (for example HPV16 $E6_{19-33}$ is potentially presented by HLA-DR*0104 or *0115). Based on these results it could be interesting to generate other T-cell clones against different epitopes from HPV16 E6 and to compare the responses against the different clones, this kind of studies could help the understanding of molecular interaction between TCR and pMHC. Finally, study the responses against HPV18 $E6_{127-141}$ by belx1 and belx2 could permit to observe if there is a cross-presentation or reactivity between the two viruses, which contribute to a significant proportion of disease worldwide.

Over the last few years severals studies have demonsrated the efficacy of the prophylactic vaccines against HPV such as Gardasil (Govan 2008), nevertheless these vaccines are not therapeutic vaccines and they cannot be used in patient already infected

with HPV. In the future it seems likely that the most effective immunotherapeutic approaches will need to combine several different strategies in order to overcome many of the problems presented by the cervical cancer environment. As an example, the provision of additional pro-inflammatory molecules such as IFNγ or TNFα, along with an HPV vaccine construct could facilitate the generation of an appropriately polarised and effective T-cell response. Recent advances in soluble TCR technology (Laugel, Boulter et al. 2005) may soon permit the targeting of pro-inflammatory cytokines specifically to the infected/transformed tissue by attaching them to viral-specific soluble TCRs (which have been modified to increase their avidity). Such an approach could also help to decrease the occurrence of unpleasant side effects that are suffered as a result of systemic injection with these cytokines. In animal tumour models, the depletion of T-regulatory cells has yielded some positive results (Jones, Dahm-Vicker et al. 2002), and it is possible that such an approach may be effective in the treatment of HPV-mediated disease (van der Burg, Piersma et al. 2007). Thus the understanding of the activation of HPV specific T-cells is still important for development of immunotherapy and the current study shows the potential for poorly designed peptide vaccines, to make disease worse rather than better.

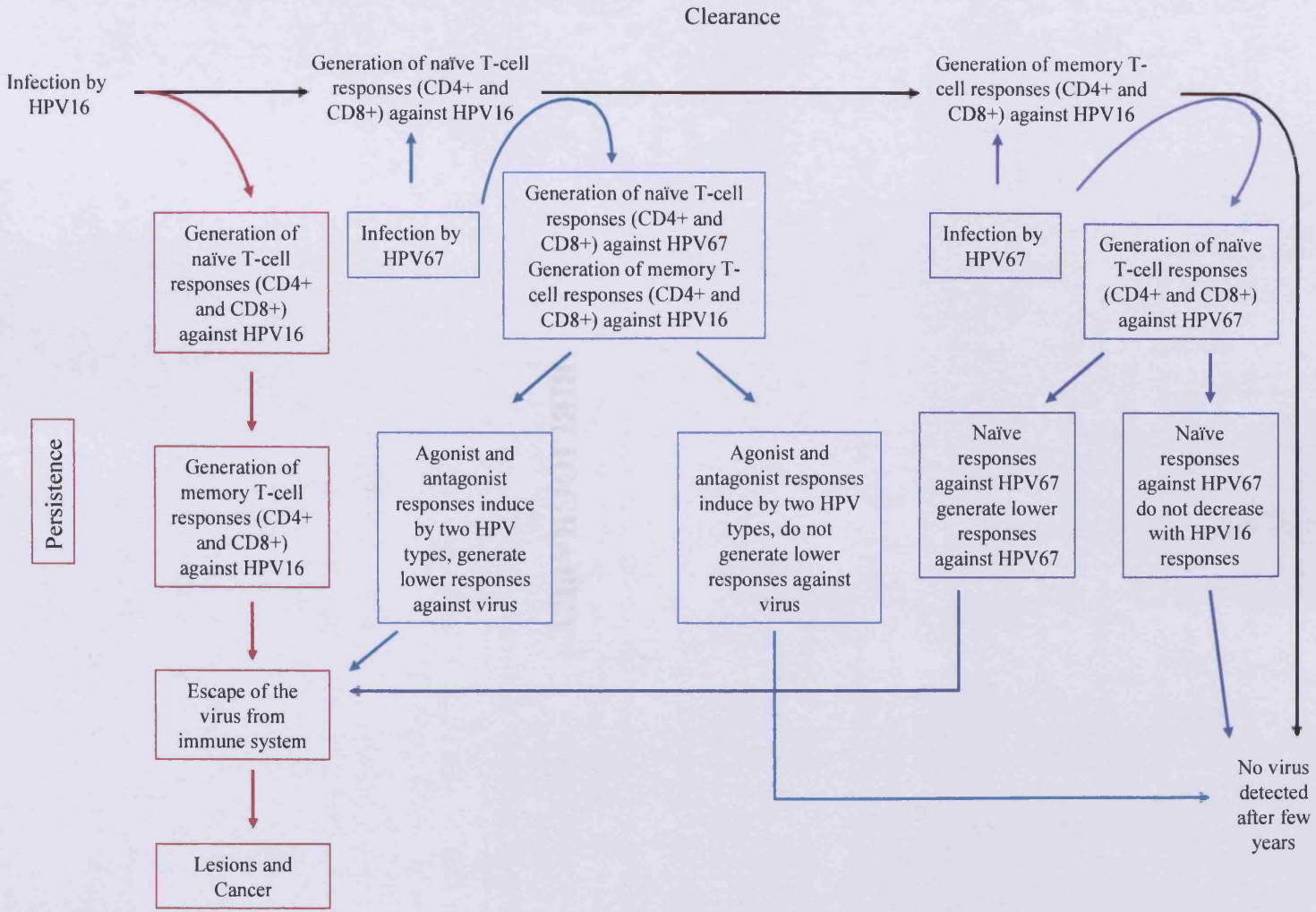


Figure 50: Model of co-infection in young femals

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