Characterisation of the CD4+ T-cell response against oncogenic human papillomavirus from healthy volunteers

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A dissertation submitted to Wales College of Medicine, Cardiff University in candidature for the degree of Doctor of Philosophy

November 2005
Summary

Infection with the human papillomavirus (HPV), particularly types 16 and 18, is causally associated with the development of cervical cancer. The major transforming proteins of high risk (HR) HPV's are E6 and E7. These proteins are constitutively expressed in cervical cancer cells and are therefore attractive targets for T-cell based immunotherapy. CD4+ T-cells are an important component of both anti-viral and anti-tumour immune responses, yet their role in the clearance and control of HPV infection has been poorly characterised. The majority of HPV-derived CD4+ T-cell epitopes have been defined in patients with either established pre-malignant or malignant cervical disease and are therefore unlikely to correlate with protection from disease progression. In this study the principal aim was to investigate the HPV E6 and E7 specific T-cell responses in healthy women, from which novel T-cell epitopes could be defined and characterised.

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), and 6 CD4+ T-cell epitopes were defined; HPV16 E6(19-33, 85-99, 121-135, 127-141) and HPV18 E6(43-57, 49-63). The four epitopes investigated further were all HLA-DR restricted and peptides HPV16 E6(127-141) and HPV18 E6(43-57) were shown to be presented by HLA-DRB1*01 and HLA-DRB1*15 respectively. Two T-cell lines were generated from one individual against the HPV16 E6(127-141) epitope. These lines exclusively express either TCR Vβ16 (Belx1) or TCR Vβ7 (Belx2), and demonstrate highly differential cytokine secretion profiles in response to peptide re-stimulation. The endogenous processing and presentation of the HPV16 E6(127-141) epitope from a full length recombinant HPV16 E6 protein was demonstrated with a range of different antigen presenting cells and the Belx2 T-cell line.

Overall this study demonstrates a powerful approach for defining novel CD4+ T-cell epitopes that may be useful in investigating protective T-cell responses and how HPV oncoproteins are naturally presented to the immune system. The Belx1 and Belx2 T-cell lines are valuable immunological tools that can be used further investigate the HPV16 E6(127-141) response. The epitopes defined in this study could potentially be useful in an immunotherapeutic context, either indirectly through their use in monitoring vaccine-induced or natural immune responses, or directly through their inclusion in a therapeutic vaccine construct.
Acknowledgments

I would like to thank Dr. Steve Man for giving me the opportunity to work on this project and also for his guidance and encouragement over the last 3 years.

I am also grateful to the girls in the lab- Claudia, Karen, Kelly and Sarah, who have helped me out so much along the way, and also to the other members of the department who have been a great source of help as well as fun. I would like to thank everyone who donated blood, as it would not have been possible to do this study without it!

Thanks to all my friends for putting up with my useless attempts to keep in touch for the last few months, and to Brian for all his help and support and for keeping me sane. Also thanks to my mum, dad and Andy and Tim for all their advice and encouragement!

This research was supported by the Medical Research Council.
Abbreviations

aa                 Amino acid
APC                Antigen presenting cell
bp                 Base pairs
BLCL               B-cell line (EBV immortalised)
BPV                Bovine papillomavirus
BSA                Bovine serum albumin
CBA                Cytometric bead array
CDR                Complementarity determining region
CIN                Cervical intraepithelial neoplasia
CM                 Central memory (T-cell)
CMV                Cytomegalovirus
COPV               Canine oral papillomavirus
CRPV               Cottontail rabbit papillomavirus
CsA                Cyclosporin A
CTL                Cytotoxic T-lymphocyte
DC                 Dendritic cell
DNA                Deoxyribonucleic acid
DMSO               Dimethyl sulfoxide
ds                 Double stranded
E6-AP              E6-associated protein
EDTA               Ethylenediaminetetra acetic acid
EBV                Epstein-Barr virus
ELISA              Enzyme-linked immunoabsorbent assay
ELISpot            Enzyme-linked immunospot assay
EM                 Effector memory (T-cell)
ER                 Endoplasmic reticulum
EV                 Epidermodyplasia verruciformis
FCS                Foetal calf serum
FITC               Fluorescein isothiocyanate
HIV                Human immunodeficiency virus
HLA                Human leukocyte antigen
HCV                Hepatitis C
HPV                Human Papillomavirus
HR                 High risk (Human papillomavirus)
HSV                Herpes simplex virus
ICAM               Intercellular adhesion molecule
ICS                Intracellular cytokine staining assay
IL                 Interleukin
IFA                Incomplete Freunds adjuvant
IFN                Interferon
Ig                 Immunoglobulin
ITAM               Immunoreceptor tyrosine-based activation motif
kb                 Kilobase
LAT                Linker for activation of T-cells
LAMP               Lysosomai-associated membrane protein
LCR                Long control region
LC                 Langerhans cell
LFA  Lymphocyte function associated antigen
LMP  Low molecular weight protein
LR   Low risk (Human Papillomavirus)
MHC  Major histocompatibility complex
MIP  Macrophage inflammatory protein
MOI  Multiplicity of infection
NK   Natural killer cell
OD   Optical density
OR   Origin of replication
ORF  Open reading frame
PAF  Parafomaldehyde
PBMC Peripheral blood mononuclear cell
PI3K Phosphatidylinositol 3-kinase
PLC  Phospholipase C
PBS  Phosphate buffered saline
PCR  Polymerase Chain Reaction
PE   Phycoerythrin
PFA  Phytohaemagglutinin
pfu  Plaque forming unit
PMA  Phorbol myristate acetate
PPP  Positive peptide pool
PV   Papillomaviruses
Rb   Retinoblastoma protein
RNA  Ribonucleic acid
RRP  Recurrent respiratory papillomatosis
TAP  Transporter associated with antigen processing
TCR  T-cell Receptor
TF   Transcription factor
TGF-β Transforming growth factor-β
Th   T-helper
TNF  Tumour necrosis factor
Tr   T-regulatory cell
TT   Tetanus toxoid
VLP  Virus-like particles
VIN  Vulval intraepithelial neoplasia
VAIN Vaginal intraepithelial neoplasia
VEGF Vascular endothelial growth factor
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Chapter 1

Introduction

1.1 Papillomaviruses

Papillomaviruses (PVs), until recently classified as members of the papovavirus family, have now been reclassified as the family papillomaviridae (de Villiers et al., 2004). PVs have been identified from over 20 different animal species (Doorbar, 2005). These viruses are grouped according to the host they infect e.g. human papillomavirus (HPV), and are highly species specific.

To date, ninety six different HPV types have been fully characterised (de Villiers et al., 2004). To qualify as a distinct HPV type, the nucleotide sequence of the L1 gene must differ from all other HPV types by at least 10%. This criteria was agreed upon at the International Papillomavirus Workshop, Quebec 1995 (de Villiers et al., 2004). HPVs exhibit restricted tissue tropism, infecting only cutaneous (skin), or mucosal (genital and respiratory tract) epithelia and can be grouped accordingly (Stanley, 2001). Within each group, viruses can be further classified as either ‘high risk’ or ‘low risk’ based upon their oncogenic potential. Examples of these viruses and the lesions they induce are shown in Table 1.1.

Infection of cutaneous epithelia with a low risk HPV (LR HPV) leads to the generation of hyperproliferative lesions (warts). For example cutaneous warts found on the hand can be caused by infection with HPV types 2, 7 or 27 (Bernard, 2005). Infection of cutaneous epithelia of the feet with HPV 1 leads to the formation of verrucas and plantar warts (Bernard, 2005). However, not all cutaneous HPVs cause benign lesions. Patients suffering from a rare skin condition epidermodysplasia verruciformis (EV), will often develop squamous cell carcinomas of the skin (Harwood et al., 2004). These cancers most commonly contain DNA of the high risk HPV (HR HPV) types 5 or 8
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(Sterling, 2005). Studies examining immuno-compromised patients have also implicated infection with cutaneous HPV in the development of non-melanoma skin cancers (Shamanin et al., 1996).

Table 1.1 Low and high risk HPVs and the lesions they induce (Bernard, 2005).

<table>
<thead>
<tr>
<th>HPV types</th>
<th>Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low Risk</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-Plantar warts</td>
</tr>
<tr>
<td>2, 27</td>
<td>-Common warts</td>
</tr>
<tr>
<td>3, 10</td>
<td>-Flat warts</td>
</tr>
<tr>
<td>6, 11</td>
<td>-Laryngeal papillomas, benign genital warts</td>
</tr>
<tr>
<td><strong>High Risk</strong></td>
<td></td>
</tr>
<tr>
<td>16, 18, 31, 45, 52, 58</td>
<td>-CIN, VIN, VAIN* and cervical cancer</td>
</tr>
<tr>
<td>5, 8</td>
<td>-Skin cancer in epidermodysplasia</td>
</tr>
<tr>
<td></td>
<td>-verruciforms patients</td>
</tr>
<tr>
<td>57</td>
<td>-Non melanoma skin cancer</td>
</tr>
</tbody>
</table>

There are at least 30 mucosal HPV types capable of infecting the genital tract (Stanley, 2001). Infection with mucosal LR HPV induces the formation of genital warts. Over 90% of genital warts (condyloma accuminata) are caused by HPV types 6 and 11 (Dartmann et al., 1986; Gissmann and zur Hausen, 1980; Greer et al., 1995). These HPV types can also infect oral mucosa causing recurrent respiratory papillomatosis (RRP) (Dartmann et al., 1986; Gissmann et al., 1982; Sekine et al., 1989). Although these lesions are benign, due to their anatomical location frequent surgery is required to maintain the airways.

2
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There are 15 HR HPVs that infect the genital mucosa (Baseman and Koutsky, 2005). Infection with these HR HPVs is causally associated with the development of high grade cervical intraepithelial neoplasia (CIN) and cervical cancer (Walboomers et al., 1999; Zur Hausen, 1991; zur Hausen, 1996). HR HPV DNA can be detected in 99.7% of cervical carcinomas (Walboomers et al., 1999). The most prevalent HR HPV type is HPV16, which is detected in over 50% of all cervical cancer cases worldwide (Bosch et al., 1995). HPV18 is the second most prevalent HR HPV associated with cervical cancer (14%), closely followed by HPV types 31, 45, 52 and 58 (Bosch et al., 1995). These HR HPVs (particularly type 16 and 18) are also implicated in the development of a number of head and neck cancers (Gillison et al., 2000; Syrjanen, 2005). Most strikingly, HR HPV DNA is detected in 51% of tonsillar carcinomas (Syrjanen, 2004).

1.2 Structure of Human papillomaviruses

Human papillomaviruses are small double stranded DNA viruses approximately 55nm in diameter. They are non-enveloped viruses with an icosahedral outer capsid consisting of the structural proteins L1 and L2. The circular DNA genome is approximately 8 kilobase (kb) pairs in length which can be divided into two main components. Firstly, a non-coding long control region (LCR) of around 1kb, containing the origin of replication (OR) and specific enhancer elements to which both viral and cellular regulatory factors can bind (Munger et al., 2004). The remaining 7kb contains eight open reading frames (ORF) encoding the nonstructural early proteins E1, E2, E4, E5, E6 and E7, and the outer capsid late proteins L1 and L2 (depicted in Figure 1.1). The early proteins function to coordinate viral DNA replication, transcription and virion assembly, as well as demonstrating immunomodulatory activities. The individual functions of each protein are briefly summarized in Table 1.2.
Table 1.2 High risk HPV protein functions.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Proposed function</th>
<th>Mode of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Viral genome replication</td>
<td>-DNA helicase and ATPase activity, necessary to unwind DNA at the OR allowing DNA replication</td>
<td>(Chiang et al., 1992; Hughes and Romanos, 1993)</td>
</tr>
<tr>
<td>E2</td>
<td>Viral genome replication</td>
<td>-DNA binding transcription factor interacting with motifs within LCR</td>
<td>(McBride et al., 1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Acts as either transcriptional repressor or activator</td>
<td>(Bernard et al., 1989; Demeret et al., 1997)</td>
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<tr>
<td></td>
<td></td>
<td>-Interaction with E1 protein vital for OR recognition.</td>
<td>(Chiang et al., 1992)</td>
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<tr>
<td>E4</td>
<td>Induces cytoskeletal changes</td>
<td>-Interacts with the keratin cytoskeleton causing collapse of the cytotkeratin matrix which facilitates virion release</td>
<td>(Doorbar et al., 1991)</td>
</tr>
<tr>
<td>E5</td>
<td>Stimulates cell growth</td>
<td>-Interacts with epidermal growth factor receptor</td>
<td>(Crusius et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Immune evasion</td>
<td>-Inhibits endosome acidification</td>
<td>(Straight et al., 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Down regulates HLA class I and interferes with HLA class II expression</td>
<td>(Ashrafi et al., 2005)</td>
</tr>
<tr>
<td>E6</td>
<td>Inhibition of apoptosis and keratinocyte immortalisation</td>
<td>-Interacts with p53 leading to its degradation</td>
<td>(Scheffiner et al., 1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Induces telomerase activity</td>
<td>(Klingelhutz et al., 1996)</td>
</tr>
<tr>
<td>E7</td>
<td>Inhibition of cell cycle arrest and keratinocyte immortalisation</td>
<td>-Binds to Rb resulting in the loss of cell cycle control at the G1/S-phase transition</td>
<td>(Boyer et al., 1996)</td>
</tr>
<tr>
<td>L1</td>
<td>Viral capsid protein</td>
<td>-Major coat protein (360 copies per capsid)</td>
<td>(Doorbar and Gallimore, 1987; Florin et al., 2002)</td>
</tr>
<tr>
<td>L2</td>
<td>Viral capsid protein</td>
<td>-Minor coat protein (12 copies per capsid)</td>
<td>(Doorbar and Gallimore, 1987; Florin et al., 2002)</td>
</tr>
</tbody>
</table>
required for virion assembly. The linear scale is in kilobase pairs.
early genes that encode non-structural proteins, and 2 late genes encoding structural proteins. There are 6 the relative positions of the ORFs and the non-coding long control region (LCR). A schematic representation of the HPV16 genome depicting Figure 1.1 A simplified overview of the HPV16 Genome (adapted from Longworth and Limins, 2004).
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1.3 The HPV life cycle
The life cycle of HPV is directly coupled to the differentiation pathway of the infected keratinocyte (Munger et al., 2004). Normal epithelial basal cells migrate into the suprabasal layers and are exfoliated from the surface and replaced by new cells. During their migration the cells exit cell cycle and undergo terminal differentiation. During natural infection the HPV virion gains access to and infects epithelial stem cells of the basal epithelia. This presumably occurs as a result of microabrasions of the stratified epithelium (Frazer, 2004). Viral attachment to the cell surface is believed to be mediated by heparin sulphate (Giroglou et al., 2001; Joyce et al., 1999), however the precise mechanism by which HPV enters the cell is unknown. One possible mode of entry has recently been proposed involving the internalisation of the HPV virion via clathrin-mediated endocytosis (Day et al., 2003).

Following internalization, the virus sheds its capsid, releasing the viral DNA which is maintained episomally in the infected cell and its progeny (termed transit amplifying cells). During this non-productive phase, the virus uses the host DNA replication machinery to replicate its own viral DNA genome (McMurray et al., 2001). In the infected basal epithelia the viral genome is maintained at a relatively low copy number (50-100 copies per cell) (McMurray et al., 2001). As infected cells divide, the viral DNA genome is partitioned into daughter cells. Infected daughter cells leave the basal layer and migrate distally towards the epithelial surface, whilst the infected stem cell remains in the basal layer, acting as a reservoir of HPV DNA which can persist for long periods of time (Stubenrauch and Laimins, 1999). Within the differentiating cell the virus enters its productive phase involving genome amplification. For this to happen, the cell must be forced to enter S-phase of the cell cycle and this is brought about primarily by the interactions of E6 and E7 proteins with host regulatory factors. HPV DNA is packaged and virions are assembled in the uppermost layers of the epithelium. The mature HPV virions are released into the genital tract from the infected epithelial cells (Longworth and Laimins, 2004).
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The cascade of viral gene expression is linked to the migration and differentiation of the infected cell. In the first stages after infection the immediate early proteins E1, E2 and E5 are expressed in the basal and parabasal layers. E1 and E2 expression are required for replication of the viral DNA genome (Munger et al., 2004). As the infected differentiating cells reach the suprabasal and mature epithelial layers, levels of E1 and E2 increase and the E6 and E7 proteins are expressed. The E6 and E7 proteins hijack cell cycle control most notably through their respective interactions with p53 and the retinoblastoma protein (Rb) which is vital for viral replication. The E4 protein and viral capsid proteins L1 and L2 are only expressed in the terminally differentiated squamous cells where the virions assemble (Tindle, 2002). The HPV life cycle is summarized in Figure 1.2.
Figure 1.2 The life cycle of the Human Papillomavirus (adapted from Frazer, 2004). The architecture of normal stratified squamous epithelia is depicted on the right. In this tissue, basal epithelial cells migrate distally, during which they undergo terminal differentiation. Upon reaching the most superficial squamous layer, these cells are exfoliated from the surface and replaced by new cells. HPV infects stem cells in the basal layer of the cervical epithelia, presumably through microabraisions of the tissue (depicted on the left side). Following infection, the viral genome is maintained episomally. The infected cell divides, and its' progeny (transit amplifying cell) migrates vertically. The expression of HPV proteins is closely coupled to the differentiation pathway of the infected epithelial cell. In the parabasal and suprabasal layers the early proteins are abundantly expressed. These are involved in the replication of viral DNA (E1 and E2) and the disruption of the cell cycle (E6 and E7). In the upper layers of the epithelium, E4 and the structural proteins L1 and L2 and are expressed. HPV DNA is encapsidated and the virion is assembled in the most superficial epithelial layers. The mature HPV virions are then released from the infected cells.
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1.4 E6 and E7 oncogenic proteins

E6 and E7 are the major transforming proteins in HPV. Their principal function is to maintain the differentiating keratinocyte in a 'pseudo' S-phase of the cell cycle, thereby permitting high levels of viral DNA replication (Ruesch and Laimins, 1998).

The expression of E6 or E7 can in some systems be sufficient to immortalise certain cell lines (Band et al., 1991; Wazer et al., 1995). However, for efficient immortalisation of human keratinocytes the expression of both E6 and E7 is required (Hawley-Nelson et al., 1989; Munger et al., 1989).

E6 is a small protein of 158 amino acids. E6 of HR HPVs is expressed in the nucleus and cytoplasm (Guccione et al., 2002). It contains Cys-x-x-Cys motifs believed to form two Zn binding domains (Cole and Danos, 1987). E6 has been shown to interact with a large number of different host proteins such as E6-BP (Chen et al., 1995) and IFN regulatory factor 3 (Ronco et al., 1998). However, its interaction with the tumour suppressor protein p53 is particularly relevant when considering the transforming properties of E6. Normally p53 expression is up-regulated in response to DNA damage, resulting in cell cycle arrest or apoptosis (Adams and Kaelin, 1998). This mechanism is in place to prevent cells containing damaged DNA from proliferating and is important in the prevention of tumour development. In keratinocytes expressing the E6 protein (from HR HPV) the half life of p53 is greatly reduced (Hubbert et al., 1992). In high risk HPV types the E6 protein interacts with both p53 and a ubiquitin ligase E6-AP (E6-associated protein) (Huibregtse et al., 1993). The subsequent ubiquitination of p53 targets this host protein for proteasomal degradation, thereby allowing DNA replication and cellular proliferation to proceed unchecked.

High risk E6 also contributes to immortalization of epithelial cells through the initiation of telomerase activity, thereby extending the life span of the infected cell (Klingelhutz et al., 1996). It should be noted that the E6 proteins from HR and LR HPVs differ significantly in their oncogenic potential (LR E6 possess almost no transforming
activity), most likely a result of differential interactions with the host target proteins (Elbel et al., 1997).

Overall, the primary amino acid sequence of the E6 protein from HR HPV is highly conserved within specific types. However, variations in the coding sequence of E6 of both HPV16 and HPV18 have been detected, some of which result in changes in the amino acid sequence (Hildesheim and Wang, 2002). A greater degree of amino acid variability has been detected for HPV16 E6 than HPV18 E6, which is possibly related to the fact that far more studies have examined variation in the HPV16 E6 coding region. The amino acids which have been found to vary in the HPV16 and HPV18 E6 protein are shown in Figure 1.3 (Chen et al., 2005; De Boer et al., 2005; Matsumoto et al., 2000; Schlecht et al., 2005; Swan et al., 2005).

**HPV16 E6**

MHQKRTAMFQ DPQERPRLP QLCTELQTTI HDILECVY 40
KQQLLREVY DFADFRLCVY YRDGNPYAVC DKLKFSK 80
SEYRHYCYSY LGTTLQQYNY KPLCDLLIRC INCQKPLCP 120
EQRHLDKQKQ RFHNIRGRWT GRCSGCCRSS RTRRETQL 158

**HPV18 E6**

MARFEDPTRR PYKLPDLCTE LNTSLQDIEI TCVYCKTVLE 40
LTEVEFEAFK DLFVVYRDSI PHAACHKCID FYSRIRELRH 80
YSDSVYGDTL EKLNTGLYN LLIRCLRCQK PLNPAEKLRLH 120
LNEKRFHNI AGHYRQQCHS CCNRAQRERL QRRRETQV 160

Figure 1.3 Amino acid variations defined in HPV16 and HPV18 E6 proteins. The amino acid sequences of the E6 protein from HPV16 and HPV18 are shown in single letter code. Those amino acids that have been shown to vary are highlighted in bold type.
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The E7 protein consists of 98 amino acids and is expressed in the nucleus of the infected cell (Longworth and Laimins, 2004). It contains a Zn binding domain that is thought to mediate a number of interactions with cellular proteins such as the AP-1 family of transcription factors (TF) (McMurray et al., 2001). Most notable is the interaction of E7 with the retinoblastoma tumour suppressor protein (Rb) and other Rb-related proteins such as p130 and p107 (Dyson et al., 1989). Rb is a transcriptional regulator which acts as a negative regulator of the cell cycle at the G1-S-phase border (Adams and Kaelin, 1998). The Rb protein binds to a TF called E2F, preventing transcription of the E2F responsive genes (Weintraub et al., 1995). E2F is important in the expression of numerous growth-promoting genes such as DNA polymerase and thymidine kinase. In dividing cells the Rb becomes phosphorylated by cyclin-dependent kinases (Senderowicz, 2000). This causes a structural change in Rb, allowing the release of E2F, thereby lifting the inhibition of transcription of the growth-promoting genes. The E7 protein mimics this process by binding to the Rb protein, causing it to disassociate from E2F (Chellappan et al., 1992). As a result, cell cycle control at the G1-S-phase boundary is disrupted, resulting in uncontrolled proliferation.

During initial infection with HPV, the viral genome is maintained episomally (Stubenrauch and Laimins, 1999). In most cases of HPV-associated CIN III and cervical cancer the viral genome has become integrated into the host chromosome. The integration event often leads to a disruption of the HPV genome, resulting in a situation where expression of E6 and E7 proteins is no longer regulated (zur Hausen, 1996). The E2 ORF is commonly disrupted resulting in loss of E2 expression. As this protein is thought to be involved in the regulation of E6 and E7 transcription, it was thought feasible that the absence of E2 could contribute to the overexpression of E6 and E7 in transformed cells (Demeret et al., 1997). This model may not be entirely accurate as it has also been shown that the E2 protein does not regulate E6 and E7 expression in cells containing episomally maintained viral DNA. This suggests other disruptions in the HPV genome following integration could be contributing to the constitutive expression of E6 and E7 (Bechtold et al., 2003).
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Whilst constitutive expression of E6 and E7 is required for the immortalisation of cervical epithelia, it is not sufficient for cellular transformation to occur (Hawley-Nelson et al., 1989; Munger et al., 2004). It can take several years for cervical cancer to develop after infection with high risk HPV, during which secondary oncogenic mutations in the host genome must be acquired. The continued expression of E6 and E7 increases the instability of the host genome, a fact supported by the chromosomal abnormalities commonly found within cervical carcinoma cells (Duensing and Munger, 2004). In particular, the absence of p53 in the uncontrollably dividing cells allows DNA replication to go unchecked, resulting in the accumulation of genetic abnormalities that could ultimately lead to the development of cancer (Munger et al., 2004).

1.5 Epidemiology of HR HPV infections
Infection of the genital tract with HR HPV types is very common. This is especially true among sexually active young women. In England the cumulative risk of HR HPV infection over three years was shown to be 27.3% among women aged 15-19 years. For HPV types 16 and 18 alone the cumulative risk was 17% (Woodman et al., 2001). Recently Cuschieri et al., 2004 investigated the prevalence of HPV infection in women (mean age 36.6 years), using samples from a routine cervical screening clinic based in Edinburgh (Cuschieri et al., 2004). Overall 20% of samples tested were positive for HPV DNA and 76.6% of this subset were positive for HR HPV. Infection with multiple HR HPVs was also shown to be common, especially among women aged between 16 and 25 (20% were positive for >1 HR HPV). Table 1.3 summarises the overall prevalence of HR HPV types among HPV infected women from nine countries in IARC studies (Baseman and Koutsky, 2005; Munoz et al., 2003). Studies examining the association between HPV infection rates and age have shown an interesting trend. The prevalence of HPV infection has been observed to peak in women under the age of 25 and then tends to decrease with increasing age. However some studies have also identified a second peak of HPV detection in women aged ~55 and above (Herrero et al., 2000).
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Table 1.3 Prevalence of type-specific HR HPV DNA within the HPV-infected female population (adapted from Munoz et al., 2003).

<table>
<thead>
<tr>
<th></th>
<th>HPV16</th>
<th>HPV18</th>
<th>HPV31</th>
<th>HPV35</th>
<th>HPV45</th>
<th>HPV58</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of all infected women (median age 46 years)</td>
<td>24.3</td>
<td>7.3</td>
<td>4.2</td>
<td>2.7</td>
<td>3.5</td>
<td>2.3</td>
</tr>
</tbody>
</table>

1.6 HPV in the development of cervical lesions

The development of cervical cancer due to persistent infection with a HR HPV is not a spontaneous event, instead it is preceded by the development of dysplastic precursor lesions.

Within a few months of infection a flat condyloma lesion of the cervical epithelia can develop. In most cases the infection is cleared effectively and the lesion is resolved. However in a minority of cases the lesion can persist and progress to CIN. CIN is graded from I through to III based upon the histology of the lesion (Arends et al., 1998). In CIN I only the lower third of the epithelia is affected, whilst in CIN II the lower two thirds of the epithelial structure is altered. In CIN III the lesion has progressed such that the full thickness of the cervical epithelia contains abnormal cells. The progression from CIN I to CIN III is accompanied by notable changes in the virus-host cell interaction. In low grade CIN the HPV genome is still maintained as an episome. However, high grade lesions are associated with integration of the HPV genome, resulting in higher levels expression of E6 and E7 oncogenic proteins leading to uncontrolled cellular proliferation (Arends et al., 1998). CIN I lesions can spontaneously regress without any treatment in 60% of cases. In contrast the spontaneous regression of CIN III occurs in only 33% of cases (Ostor, 1993). The progression from CIN III to invasive cervical cancer occurs when the transformed cells breach the basement membrane of the epithelial tissue, allowing spread to other anatomical sites.
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Annually there are over 510,000 new cases of cervical cancer worldwide, making it the second most common form of cancer in women (Padma, 2005). Every year over 288,000 women die from this disease making it an important global health issue. Over 80% of all cervical cancer cases occur in developing countries, with India alone hosting one third of cases (Padma, 2005). The high rates seen in these countries are largely due to the lack of cervical screening programs, resulting in the late detection of cervical cancer and consequently high mortality rates. In the United States, the incidence of cervical cancer has been dropping steadily since the introduction of cervical screening programs (Sirovich and Welch, 2004). The routine screening of women for cervical abnormalities facilitates detection of lesions at a much earlier stage in development, increasing the chance of treatment being effective. For patients with cervical cancer, the survival rates are closely related to the stage of disease. Following treatment, the 5 year survival rate of women with invasive cervical cancer is ~20%, whilst for women with very early stage cervical cancer there is a 95% 5 year survival rate (Cannistra and Niloff, 1996).

1.7 Risk factors in the development of cervical cancer

It is not entirely understood why some HPV lesions are effectively cleared whilst others persist and progress to cervical cancer. A number of genetic and/or immunological and environmental co-factors have been implicated in increasing the risk of disease progression. Environmental co-factors include smoking tobacco and the use of oral contraceptives (Hildesheim et al., 2001; Kjellberg et al., 2000; Moreno et al., 2002). A number of epidemiological studies have described an association between the development of cervical neoplasia and several HLA genes. HLA-DRB1*13 has been strongly implicated in protecting against progression to cervical cancer (Hildesheim and Wang, 2002; Madeleine et al., 2002). Whilst no alleles have been shown to consistently correlate with an increased risk of disease progression, the HLA class I genes HLA-B*44 and HLA-B*51, and the HLA class II genes HLA-DRB1*11, HLA-DRB1*15 and HLA-DQB1*03 have all been implicated by several studies (Hildesheim and Wang, 2002; Krul et al., 1999; Madeleine et al., 2002; Zehbe et al., 2003). Infection by intratypic variants of both HPV16 and HPV18 are associated with
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differential oncogenicity. Infection with non-European variants of HPV16 correlates strongly with an increased risk of developing cervical neoplasia (Matsumoto et al., 2000). Smaller studies have also provided evidence that infection with non-European versions of HPV18 is associated with a higher risk of disease progression (Hecht et al., 1995).

1.8 The immune response to infection
The cells of the immune system are constantly patrolling the body in order to efficiently detect and eliminate pathogens. The innate immune response offers the first line of anti-microbial defence (Tosi, 2005). If this response fails to completely eradicate the pathogen, the subsequent activation of the adaptive immune response produces vast numbers of lymphocytes that specifically recognise the pathogen. A population of cells called dendritic cells (DCs) act as a bridge between the innate and adaptive immune system responses. DCs are the major antigen presenting cells (APCs) of the immune system, capable of activating naive populations of T-lymphocytes, thereby initiating a primary immune response directed at a specific antigen (Rossi and Young, 2005).

1.8.1 Innate immunity
Innate immunity functions to prevent infection by pathogens. Where infection is established, the innate immune response acts to keep it under control until the adaptive immune response develops. A major component of the innate immune system is the mechanical barrier provided by epithelia (skin, mucosal surfaces, and respiratory tract), which acts to prevent entrance of pathogens (Tosi, 2005). Where the integrity of the epithelia is compromised and infection is established, a number of other innate immune system components come into play. The major effector cells of the innate immune response are neutrophils, macrophages, DCs, and NK cells (Janeway and Medzhitov, 2002). The recruitment and activation of these cells is largely controlled by the precise cytokine environment, the composition of which varies according to the nature of the infecting pathogen.
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NK cells are particularly important in the clearance of both virally infected and transformed cells (Smyth et al., 2005). NK cell lytic activity can be induced by a number of mechanisms including the down regulation of HLA class I expression by virally infected cells (Karre, 2002). This down regulation is an immune evasion strategy practised by a number of viruses in order to avoid recognition by cytotoxic T-lymphocytes (CTL) (Bartholomew et al., 1997; Keating et al., 2002). NK cells kill through either the release of perforin and granzyme, or by the engagement of FAS (Smyth et al., 2005). Activated NK cells also secrete IFNγ which is a potent antiviral cytokine, which among other things activates macrophages and neutrophils and upregulates HLA class I and class II expression on APCs (Boehm et al., 1997).

Macrophages, neutrophils and DCs are potent phagocytes which ingest and endogenously process bacteria and viruses, presenting the resulting microbial peptide fragments to T-cells and B-cells (Ishikawa and Miyazaki, 2005; Taylor et al., 2005). APCs therefore act as a link between the innate and adaptive immune responses, which is particularly important when the innate system is breached.

In addition to specialised effector cells, the virally infected cells can themselves contribute to the control of infection through the secretion of type I IFNs (IFNα and IFNβ), which function to inhibit viral replication (Young et al., 2003). These cytokines predominantly exert their effects in a paracrine manner, effectively protecting neighbouring cells from viral infection.

1.8.2 Adaptive immunity

The adaptive immune response is a more highly evolved system than the innate, allowing responses to be generated against specific regions (epitopes) of a foreign molecule. These responses are accompanied by the generation of immunological memory allowing a more rapid and potent response upon subsequent exposure to the pathogen. The adaptive immune response will be discussed in the context of an antiviral immune response. There are two major divisions of the adaptive immune response, humoral immunity and cell-mediated immunity.
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1.8.2.1 Humoral immunity
Humoral immunity is mediated by the production of antibodies by activated B-cells. The binding of antibodies to the outer capsid of a virus particle can prevent viral entry into the host cell (Christensen et al., 1995). The production of neutralizing antibodies is important (in the early stages of an immune response) in preventing viral infection, however they are ineffective in the elimination of virally infected cells.

1.8.2.2 Cell-mediated immunity
The cellular immune response is mediated by T-cells. These cells express T-cell receptors (TCR) on their surface, enabling them to recognize short peptide fragments presented by HLA molecules expressed on the surface of APCs (Engelhard, 1994; Rudensky et al., 1991). T-cells can be divided into two distinct types based upon their expression of CD4 or CD8. CD8+ T-cells predominantly recognize peptides derived from endogenously expressed proteins (including viral proteins in an infected cell). HLA class I molecules are expressed on the surface of most cell types permitting CD8+ T-cells to examine the majority of cells in the body for viral infection or transformation. In a normal cell where the peptides presented by HLA class I are all host derived, a CD8+ T-cell response is not elicited due to host immune tolerance. However, recognition of virally derived epitopes leads to the activation of the CD8+ T-cell, culminating in the destruction of the infected cell. CD4+ T-cells recognize peptides presented by HLA class II. HLA class II molecules are mainly expressed on cells with highly specialized antigen presenting functions (Engelhard, 1994). The principal APCs include DCs, macrophages and B-cells.
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1.8.2.2.1 Antigen presenting cells in the generation of T-cell responses

APCs play a highly significant role in the generation of epitope specific T-cell responses. The most potent APC is the DC. These cells are especially crucial in the activation of naive CD4+ and CD8+ T-cells during a primary immune response.

During an immune response DCs must carry out two major functions, namely antigen processing and antigen presentation to T-cells. The phenotype of a DC changes so as to complement the specific task that it must carry out (summarised in Table 1.4). Immature DCs enter the peripheral blood system where they account for only 0.5% of the total blood leukocytes (Reinhard et al., 2002). The circulating DCs are then deposited in peripheral tissues where they are able to constantly screen the local environment for the presence of antigenic material through macropinocytosis of soluble proteins (Sallusto et al., 1995). A DC can be activated by the detection of pathogenic debris including lipopolysaccharide (LPS), dsRNA (Alexopoulou et al., 2001), or unmethylated CpG DNA (Rossi and Young, 2005). This activation initiates an intracellular signalling pathway terminating in DC maturation. DC activation can also be stimulated by the presence of pro-inflammatory cytokines such as TNFα and IL-1β secreted by infected tissue.

The immature DC can phagocytose microbes and apoptotic or necrotic bodies. The internalised material is processed within a phagolysosome, producing peptide fragments which bind to either HLA class I (through cross-priming) (Albert et al., 2001) or HLA class II molecules which are then displayed on the surface of the DC (Banchereau and Steinman, 1998). The mature phenotype of the DC is specifically directed towards efficient antigen presentation rather than phagocytosis and antigen processing. Maturation is characterised by the up-regulation of a number of adhesion molecules (ICAM-1, ICAM-3 and LFA-3) and co-stimulatory molecules (CD80, CD86) that are respectively required for the migration of the DC and the priming of T-cells (Timmerman and Levy, 1999).
Table 1.4 Phenotypic and functional differences between immature and mature DCs (adapted from O'Neill et al., 2004).

<table>
<thead>
<tr>
<th>Immature DC</th>
<th>Mature DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor stimulator of T-cells</td>
<td>Potent stimulator of T-cell responses</td>
</tr>
<tr>
<td>No dendrites</td>
<td>Prominent dendrites</td>
</tr>
<tr>
<td>Low levels of HLA class I and II</td>
<td>High levels of HLA class I and II</td>
</tr>
<tr>
<td>Limited antigen presentation</td>
<td>Enhanced antigen presentation</td>
</tr>
<tr>
<td>Active as phagocytes</td>
<td>Up-regulation of DC-LAMP</td>
</tr>
<tr>
<td>High expression of antigen-uptake receptors; DC-SIGN, DEC-205, Dectin-1,</td>
<td>Little or no expression of antigen-uptake receptors</td>
</tr>
<tr>
<td>Langerin, FcγRI and II, LOX-1, αVβ5, TLRs</td>
<td>High expression of adhesion molecules</td>
</tr>
<tr>
<td>Low expression of adhesion molecules</td>
<td>ICAM-1, ICAM-3, LFA-1, LFA-3, DC-SIGN,</td>
</tr>
<tr>
<td>Low motility</td>
<td>High motility</td>
</tr>
<tr>
<td>Express chemokines receptors; CCR1, CCR2, CCR5, CXCR1, CXCR2</td>
<td>Express chemokines receptors; CCR7</td>
</tr>
<tr>
<td>Do not secrete immunoregulatory cytokines which dictate T-cell effector</td>
<td>Secrete immunoregulatory cytokines which can dictate the phenotype of the</td>
</tr>
<tr>
<td>phenotype</td>
<td>resulting T-cell response</td>
</tr>
<tr>
<td>Do not secrete chemokines</td>
<td>Secrete chemokines which recruit key immune cells</td>
</tr>
<tr>
<td>Lower expression levels of co-stimulatory molecules CD80, CD86 and CD40</td>
<td>MIP-1α, MIP-1β, RANTES, IL-8 and DC-CK,</td>
</tr>
<tr>
<td></td>
<td>Express high levels of co-stimulatory molecules CD80, CD86 and CD40</td>
</tr>
</tbody>
</table>

Once activated, DCs secrete a number of cytokines such as MIP-1α, IL-8 and RANTES that are involved in the recruitment of T-cells, NKs, phagocytes and further DCs (O'Neill et al., 2004; Sallusto et al., 1999b). The DC-specific chemokine called DC-CK1 is secreted predominantly by DCs residing in the T-cell areas of lymph nodes (Adema et al., 1997; Lindhout et al., 2001). This cytokine plays an important role in the initiation of the primary immune response as it specifically recruits naïve T-cells, encouraging their interaction with antigen-displaying DCs (Adema et al., 1997). In the lymph nodes, mature DCs present the antigen (complexed with a HLA molecule) to naïve T-cells. A T-cell recognises a peptide through its expression of a unique TCR. The recognition of an epitope by the TCR is not sufficient to activate a naïve T-cell.
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Secondary signals provided by the ligation of co-stimulatory molecules on the DC (CD80 and CD86) with their cognate molecule (CD28) on the T-cell, are required for activation and clonal expansion (Linsley et al., 1991; Sansom et al., 2003). The expression of these co-stimulatory molecules is up-regulated in the mature DC phenotype.

The outcome of the T-cell-DC interaction is influenced by a number of peripheral factors. These include the signals delivered by the activated T-helper cells (Th) cells to the DC, the nature of the infecting pathogen, and the tissue microenvironment (including immunoregulatory cytokines and chemokines). The secretion of IL-12 by an activated DC preferentially stimulates a Th1 response (IFNγ, IL-2) (Macatonia et al., 1995), whilst the presence of IL-4 in the tissue microenvironment leads to the generation of a Th2 response (IL-4, IL-5) (Seder et al., 1992). DCs exposed to IL-10 have also been shown to induce T-cell anergy (Steinbrink et al., 1999; Steinbrink et al., 1997).

1.8.2.2 CD4+ T helper responses

CD4+ T helper cells (Th) play an important role in the activation and co-ordination of CD8+ T-cell and B-cell responses, and can themselves demonstrate anti-viral effector functions (Bickham et al., 2001). Naïve CD4+ T-cells become primed and activated through the recognition of their cognate epitope presented by HLA class II molecules expressed on mature DC.

Depending upon the nature of the microbial infection a Th1 and/or Th2 polarised T-cell response develops. The Th1 response to viral infection is characterized by the secretion of IL-2 and IFNγ. IL-2 is a T-cell growth factor and is important for the proliferation of activated CD8+ T-cells (Morgan et al., 1976). The production of IFNγ has several positive anti-viral effects. In particular it activates the anti-microbial activity of macrophages (Taylor et al., 2005) and up-regulates the expression of HLA and other molecules involved in antigen processing (Boehm et al., 1997). Moreover, when IFNγ is secreted locally it limits viral spread by making the cells refractory to
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infection (Christensen et al., 1999; Guidotti and Chisari, 2001). Th2 responses are characterized by the production of IL-4 and IL-13 which results in the activation of the B-cell antibody response and influences isotype switching (Tangye et al., 2002).

1.8.2.2.3 CD4+ Th-DC interactions are critical for activation of naïve CD8+ T-cells

The main effector cells responsible for clearance of virally infected cells are CD8+ CTL. In many instances the success of an anti-viral CTL response is reliant upon a strong proliferative Th1 response. In patients infected with Hepatitis C (HCV), the control of primary infection requires a strong HCV-specific CD4+ and CD8+ T-cell response. The absence of an effective Th1 response directly correlates with a failure to clear the virus (Gerlach et al., 1999), resulting in lifelong chronic infection.

It has been shown that during their interaction with mature DC, CD4+ Th cells provide some form of stimulatory signal, enhancing the DCs capacity to effectively activate naïve CD8+ T-cells via a cross-priming mechanism of antigen processing (Bennett et al., 1997). This stimulation or ‘licensing’ by the CD4+ Th cell is believed to be reliant on the ligation of CD40L on the activated Th cell with CD40 on the DC. This ligation has been shown to result in enhanced surface expression of co-stimulatory molecules such as CD80 and CD86, cell adhesion molecules such as ICAM-1 and also high levels of IL-12 production (Caux et al., 1994; Cella et al., 1996; Shinde et al., 1996). In the absence of CD4+ T-cell help, the interaction between mature DCs and naïve CD8+ T-cells can result in T-cell tolerisation (Albert et al., 2001). In vitro studies have revealed that this need for CD4 help can be bypassed through the addition of CD40L (Albert et al., 2001). CD40/CD40L ligation is clearly an important pathway through which CD4+ T-cells provide cognate help.

The requirement for CD4+ T-cell help in the generation of a primary CD8+ T-cell response can differ depending on the infecting virus. The clearance of non-cytopathic or poorly immunogenic viruses such as HCV appears to require CD4+ T-cell help (Godkin et al., 2002). The need for CD4+ T-cell help is circumvented for viruses such as Influenza A which directly infect DCs. DCs infected with Influenza A become
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appropriately activated and present the endogenously derived viral antigens via the HLA class I pathway (independent of cross-priming), resulting in effective priming of naïve CD8+ T-cells (Albert et al., 2001). Murine studies have revealed that although CD4+ T-cell help is not always required for a primary CD8+ T-cell response, it is important for the generation of a robust CD8+ T-cell memory population (Janssen et al., 2003; Shedlock and Shen, 2003).

1.8.2.2.4 CD8+ T-cells
Following viral infection, a primary immune response must develop to clear the infected cells. CD8+ CTL are the main effector cells in this response. Virus-specific naïve CD8+ T-cells are primed and activated by mature DCs. Subsequent to activation, the CD8+ T-cells proliferate secreting IL-2 and differentiate into effector CTL. The activated CTL migrate into inflamed tissue in search of virally infected cells, which are detected by the presentation of virus-derived epitopes complexed with HLA class I molecules on the cell surface. The recognition of HLA class I restricted epitopes by the T-cell leads to the re-activation of the CTL, resulting in the death of the infected cell. The principal mechanism through which a CTL kills is the release of pre-formed cytotoxic granules containing perforin and granzymes (Podack et al., 1985). Perforin creates pores in the plasma membrane of the infected cell that firstly encourages osmotic lysis of the cell, and secondly permits the entrance of the proteolytically active granzymes leading to apoptosis (Heusel et al., 1994). CTL can also induce apoptosis via the ligation of Fas-L with Fas expressed on the target cell (Bossi and Griffiths, 1999). This activates a signaling pathway downstream of FAS which culminates in the activation of caspases resulting in apoptosis of the target cell. Activated CD8+ T-cells also secrete the anti-viral cytokines IFNγ and TNFα (Guidotti et al., 1996; Jassoy et al., 1993). Together these cytokines can induce Fas expression in target cells, making them more susceptible to Fas-mediated apoptosis (Guidotti et al., 1996). The ligation of TNFα with the TNF receptor (part of the TNF superfamily of death receptors) expressed by a target cell can result in the induction of caspase-mediated apoptosis (Thorburn, 2004).
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1.8.2.2.5 T-cell responses to tumours
In addition to their important anti-viral function, T-cells also play a role in the recognition and clearance of malignantly transformed cells. Whilst tumour-specific CTL tend to be the main effector cells in tumour rejection, a number of murine tumour studies have revealed an important role for CD4+ T-cell help in the generation of an effector CTL response (Hung et al., 1998; Ossendorp et al., 1998). Regardless of the absence of HLA class II expression by most tumours, CD4+ T-cells have themselves been shown to reject tumours possibly through the recruitment of macrophages (Corthay et al., 2005; Mumberg et al., 1999). The proposed mechanism for tumour rejection in these studies is as follows. During the primary response, tumour-specific CD4+ T-cells become primed and activated within the lymphoid tissue. Activated T-cells upon migrating to the tumour site, interact with macrophages presenting tumour-derived epitopes (bound to HLA class II molecules). The tumour-specific T-cells become re-activated and secrete IFNγ which activates the macrophages tumoricidal functions including the generation of cytotoxic compounds such as reactive oxygen species and nitrous oxide.

In cases where tumours are not rejected, they can progress virtually unchallenged by the immune system. Initially tumour development is not accompanied by a pro-inflammatory response. In the absence of any ‘danger’ signals, the presentation of tumour antigen via APC to naïve T-cells can result in T-cell tolerance. T-cell anergy could also be induced via the interaction with HLA molecules expressed by tumour cells in the absence of co-stimulatory signals (Staveley-O'Carroll et al., 1998).

1.8.2.2.6 T-regulatory cells
In addition to the CD4+ T-cell Th1 and Th2 populations described in section 1.8.2.2.2 there is an additional population of CD4+ T-cells to which a regulatory or ‘suppressive’ function has been attributed (Bluestone and Abbas, 2003; Sakaguchi, 2000). The best characterized CD4+ T-regulatory cells (Tr) are CD4+ CD25+ and constitute on average 6% of peripherally circulating CD4+ T-cells (Dieckmann et al., 2001). To date, two major CD4+ CD25+ Tr subsets have been identified, the natural Tr and the adaptive Tr.
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Natural Tr cells develop in the thymus (Papiernik et al., 1998) and recognize self antigen via their TCR (Thornton and Shevach, 2000). The principal function of these Tr cells (subsequent to antigenic stimulation) is to suppress the activity (activation, proliferation and IL-2 production) of autoreactive CD4+ CD25- and CD8+ T-cells (Thornton and Shevach, 1998). The precise mechanism through which these Tr cells mediate suppression of an autoreactive T-cell response is not known, but is believed to involve an element of cell-cell contact (Bluestone and Abbas, 2003; Shevach, 2002). Tumours express host-derived peptides complexed with HLA molecules on their cell surface. Failure to reject a tumour can be in part attributed to immunotolerance. Several groups have utilized murine models to investigate the effect of removing CD4+ CD25+ Tr cells on breaking immunotolerance to tumours (Jones et al., 2002; Shimizu et al., 1999). Interestingly, in the absence of these Tr cells, tumour-specific CD4+ CD25- T-cell and CD8+ T-cell responses develop, resulting in tumour rejection (Casares et al., 2003). This work has obvious important clinical implications for the treatment of human cancers.

The adaptive or ‘induced’ Tr cells are produced in the periphery in response to repeated antigen-specific activation (Grundstrom et al., 2003). These Tr cells are thought to be involved in the suppression of anti-viral immune responses. In CMV seropositive donors and HIV positive patients, the removal of CD4+ CD25+ T-cells was shown to augment the T-cell immune response to these viruses (Aandahl et al., 2004). It appears likely that these Tr cells develop from persistently activated CD4+ CD25- T-cells during chronic viral infection, although this is still a matter of much debate.

1.8.2.2.7 Memory T-cells

During an anti-viral immune response, antigen-specific T-cells undergo proliferation and differentiation. Following resolution of infection the effector T-cell population contracts, leaving behind a memory T-cell population that persists at much higher levels than the initial T-cell frequency (Sallusto et al., 2004). Memory T-cells are maintained over long periods of time and function to permit an increasingly rapid and potent immune response with each subsequent exposure to a specific pathogen.
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In most cases, naïve and antigen-experienced T-cells can be differentiated by their respective expression of CD45RA and CD45RO. The memory T-cell pool consists of two functionally heterogeneous populations, which differ in both their expression of cell surface markers and in the tissues in which they reside. The two populations are designated ‘effector’ memory (EM) and ‘central’ memory (CM) (Sallusto et al., 1999a). Figure 1.4 provides a simplified overview of the generation of memory T-cell populations. Central memory T-cells constitutively express CCR7 and CD62L which are markers for lymphoid homing. Upon re-stimulation with antigen, these T-cells secrete IL-2 but do not exhibit effector functions. Instead they must undergo a period of proliferation and differentiation to acquire their effector function (Sallusto et al., 2004). When compared to naïve T-cells, the CM cells are more sensitive to antigen stimulation and exhibit a lower activation threshold (Sallusto et al., 2004). Activated CM cells also up-regulate CD40L to comparatively high levels, thus providing greater ‘help’ to DCs which facilitates the activation of further antigen-specific T-cells (Sallusto et al., 2004).

The EM T-cell subset are CCR7- and express chemokine receptors (CCR1, CCR3 and CCR5) and adhesion molecules (β1 and β2 integrins) which facilitate T-cell homing to inflamed tissues. Large numbers of EM cells are present in the liver, lungs, and gut. These cells are rapidly recruited to sites of inflammation and infection, where upon antigenic re-stimulation they exhibit immediate effector functions including the production of IFNγ (Lanzavecchia and Sallusto, 2005).

These two subsets represent a ‘division of labour’ within the memory response. EM represents a population of cells that are rapidly recruited to sites of infection and elicit an immediate effector response. The CM cells located in the T-cell areas of lymphoid tissue can provide important signals to DCs (via CD40L/CD40 ligation) and proliferate and differentiate into a second wave of effector T-cells following infection.

The proportion of antigen-specific memory T-cells within the central and effector subsets varies depending on the infecting virus and the nature of infection (acute or
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chronic). Several studies have started to characterise the memory responses to a number of different viruses (Bachmann et al., 1997; Baron et al., 2003; Godkin et al., 2002; Harari et al., 2005; Hislop et al., 2001). Most recently a study was carried out characterising the memory CD4+ T-cell responses against several viruses representing different types of exposure and antigen dose (Harari et al., 2005). Tetanus toxoid (TT) was used to test responses against a viral infection in which the viral antigen is effectively cleared. Interestingly, the vast majority of TT-specific CD4+ T-cells were CM cells (IL-2+), with almost no detectable EM cells (IFNγ+) present. In contrast the memory responses to viruses associated with chronic infection and therefore antigen persistence (albeit at low levels), namely EBV, CMV and HSV, consisted of three major cytokine secreting populations. These were IL2+ CM cells, IFNγ+ EM cells, and an IL-2+/IFNγ+ population which may be an intermediate population. Lastly, in patients with progressive HIV infection (associated with uncontrolled viraemia and high antigen dose) the HIV-1 gag-specific memory T-cell population was dominated with IFNγ+ EM cells with only a small number of CM cells present.

This data is in agreement with the theory that memory T-cell responses are heterogeneous between different viruses. It appears that CM populations are present in all memory T-cell populations, which fits with the hypothesis that these cells constitute a population of long lived stem cell-like T-cells that are responsible for the generation of the EM T-cell pool (Lanzavecchia and Sallusto, 2005). The EM population appears to be more substantial for viruses which can enter latent periods of infection. This is likely to be due to the persistence of low level antigen, which may assist in the maintenance of EM T-cell population long after primary infection.
Figure 1.4 Overview of the generation of T-cell memory.
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1.9 HLA and antigen presentation
The ability of a T-cell to specifically recognise foreign antigen on the surface of either APC or infected cells is a critical component of the adaptive immune response. This recognition is mediated by the interaction of HLA-peptide complexes with the TCR. This sophisticated system enables the immune system to screen both the intracellular and extracellular environment of the host for pathogens.

1.9.1 HLA class I structure and function

Structure
HLA class I molecules are responsible for the presentation of virally derived peptide epitopes to CD8+ T-cells. The HLA class I molecule is a membrane-bound heterodimer consisting of an α chain associated non-covalently with β2-microglobulin (β2m) (Figure 1.5). The α chain contains 3 extracellular globular domains, with the polymorphic α1 and α2 regions forming the peptide binding groove. The ends of the peptide binding groove are closed, thereby restricting the length of peptide that can be accommodated. HLA class I restricted peptides tend to be between 8 and 11 amino acids in length (Rammensee, 1995).

Antigen processing and presentation
Peptides presented by the HLA class I antigen presentation pathway are mostly derived from endogenously processed proteins. In a healthy cell the peptides presented by HLA class I molecules are host derived. However in a virally infected cell, peptides generated from endogenously expressed viral proteins can also gain access to the HLA class I pathway. This system effectively permits sampling of the cytoplasmic environment of a cell, allowing the CD8+ T-cell to discriminate between normal and virally infected cells.

In the cytosol proteins become ubiquitinated which targets them for proteasomal degradation (Ciechanover, 1994). Peptide fragments generated by the proteasome are translocated into the ER via the transporter associated with antigen processing complex (TAP), where they can bind to newly synthesised HLA class I molecules (Pamer and
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Cresswell, 1998). A number of chaperone proteins assist in the formation of the HLA class I-peptide complex, including tapasin, calnexin and calreticulin (Capps and Zuniga, 1994; Sadasivan et al., 1996). The complex then traverses the ER and Golgi apparatus, before transport to the plasma membrane (Figure 1.6A). The TCR expressed by CD8+ T-cells can then interact with the HLA class I-peptide complex.

Cross-priming

Cross-priming is the process by which APC such as DCs and macrophages can process exogenously derived antigen (most commonly protein), and present the resulting peptides complexed with HLA class I molecules to CD8+ T-cells (reviewed in (Shen and Rock, 2006). APC acquire antigen via endocytosis and phagocytosis. There are several proposed mechanism as to how the antigen gains access to the HLA class I pathway. One of the major pathways is thought to involve the release of antigen from the phagosomes into the cytosol. The antigen is then degraded by the proteasome, and the resulting peptides are transported via the TAP complex into the ER where they bind to newly synthesised HLA class I molecules (Kovacsovics-Bankowski and Rock, 1995). The second main pathway is independent of TAP and proteasome activity. Instead the antigen is processed in the phagosome by the cysteine protease cathepsin S (Shen et al., 2004). The resulting peptides bind to HLA class I molecules within the endocytic compartment, however the precise mechanism through which this occurs is not clear.
Figure 1.5 Structure of HLA class I and HLA class II molecules (adapted from Alberts et al., 2002). The HLA class I heterodimer consists of an α chain, non-covalently associated with β2-microglobulin (left). The α chain contains 3 extracellular globular domains (α1, α2 and α3), with the polymorphic α1 and α2 regions forming the peptide binding groove. The α chain also contains a transmembrane domain and a short cytoplasmic tail. The HLA class II heterodimer consists of an α and a β chain. These chains both contain two extracellular domains (α/β1 and α/β2). The α1 and β1 domains of the chains come together to form a polymorphic peptide binding groove. The chains also contain a transmembrane domain and a cytoplasmic tail.
Figure 1.6 The HLA class I and II processing pathways (adapted from Cannon and Pate, 2003). (A) The HLA class I pathway presents peptides derived from endogenously expressed host and viral proteins. The proteins become ubiquitinated which targets them for proteasomal degradation (1). The proteasome cleaves the protein, generating short peptide fragments (2), which can then be transported via the TAP complex into the ER (3). In the ER, peptide fragments associate with the HLA class I heterodimer, assisted by several chaperone molecules (4). The HLA class I-peptide complex is then transported to the cell surface (5), where it is presented to CD8+ T-cells. (B) The HLA class II pathway predominantly presents peptides derived from an exogenous source of protein. Protein is endocytosed by APC and enters the endocytic pathway (1), where it progresses through increasingly acidic endosomes, finally fusing with a lysosome (2). The majority of proteolysis takes place in the lysosomal compartment which contains the cathepsins B, D, S and L (acid proteases) (3). The resulting peptide fragments are introduced to the HLA class II molecules in the MHC class II compartment (MIIC) (4). Peptide loading requires the release of the CLIP fragment from the HLA class II binding groove. This process is facilitated by the HLA-DM heterodimer. The HLA class II-peptide complex is then transported to the cell surface where it is presented to CD4+ T-cells (5).
1.9.2 HLA class II structure and function

Structure

HLA class II molecules expressed on APC present epitopes to CD4+ T-cells. The HLA class II molecule consists of an α and β chain (Figure 1.5). These chains are integral membrane glycoproteins, each consisting of two extracellular domains, a transmembrane domain and a cytoplasmic tail. The most distal regions of the two chains come together to form a polymorphic antigen binding groove. The walls of the antigen binding groove are formed by 2 α-helices, whilst the base of the groove consists of 8 anti-parallel β-pleated sheets (Wolf and Ploegh, 1995). The extremities of the binding groove are open, allowing the bound peptide to protrude from each end. Therefore the length of peptide capable of binding is far less restricted than for HLA class I, ranging in lengths of 12-25 amino acids (Rammensee, 1995) although most commonly identified peptides are 13-17 amino acids in length (Rudensky et al., 1991).

HLA class II processing

-Endocytosis and processing of antigen

The vast majority of peptides presented by HLA class II molecules are derived from an exogenous antigen source. A protein antigen enters APC by endocytosis, which includes specific receptor-mediated endocytosis and non-specific adsorptive endocytosis or phagocytosis. Endocytosed protein is then delivered into the endocytic pathway, where it progresses through endosomes/phagosomes (which become increasingly acidic), finally fusing with lysosomes. The acidic endosome environment activates a number of acid proteases, in particular the cathepsins B, D, S and L (Watts, 2001). Limited proteolysis occurs in the endosomes, with the majority of protein degradation taking place in the lysosomal compartments. For efficient proteolysis the antigen must be unfolded. This requires the breaking of intramolecular disulphide bonds which is catalysed by GILT (IFNγ-inducible lysosomal thiol reductase) (Phan et al., 2000). This proteolytic pathway produces peptide fragments, some of which are capable of binding to the HLA class II molecules. A small amount of intracellular protein can also enter the HLA class II pathway via an endosomal route. Therefore in
an infected cell some virally derived peptides can enter the HLA class II pathway of antigen presentation.

*Synthesis and trafficking of the HLA class II molecule*

Newly synthesised HLA class II α and β chains enter the ER where they form αβ heterodimers through non-covalent interactions. The newly formed HLA class II heterodimer then interacts with a non-polymorphic protein called the invariant chain (Ii). Occupation of the HLA class II peptide binding groove with a region of the Ii protein known as the CLIP domain prevents binding of ER resident peptides (Li et al., 2005).

Within the ER, three Ii chains interact to form a trimer to which three αβ heterodimers bind, forming a nonameric structure (Roche et al., 1991) which can then interact with calnexin (Anderson and Cresswell, 1994). The αβ-Ii complex traverses the trans-Golgi network before being diverted to the endocytic pathway. Within the endocytic vesicle, acid proteases proteolytically cleave the Ii chain, leaving the CLIP domain bound to the HLA class II heterodimer (Maric et al., 1994).

The precise vesicular compartment where peptide loading of the HLA class II molecule occurs has not been clearly defined, but is believed to be late in the endosomal pathway. It is referred to as the MIIC (MHC class II compartment) (Peters et al., 1991). For peptides to bind the HLA class II molecule, the CLIP peptide must first be released. This is facilitated by the de-stabilisation of the αβ heterodimer by HLA-DM. HLA-DM is an intracellular heterodimeric protein, similar in structure to the HLA-class II molecule. This dimer also acts to supervise the binding of peptides to the HLA class II molecule, mediating the release of weakly bound peptides in preference for stronger binding peptides (Busch et al., 2005). This is important for efficient presentation to CD4+ T-cells, as the peptides must interact strongly with the HLA class II molecule and remain bound for long periods of time. The HLA class II-peptide complexes are then transported to the cell surface, however the precise pathway
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through which this occurs has not been well defined. This pathway is summarised in Figure 1.6B.

1.9.3 The HLA genes
The human HLA region (HLA complex) is located on chromosome 6 and extends over four million base pairs of DNA (Marsh, 2000). It can be divided into three major regions. The first contains the HLA class I HLA-A, -B, -C, -E, -F and -G genes. The second region contains the HLA class II -DR, -DP and -DQ genes, as well as HLA-DM and the genes encoding TAP and low molecular weight proteins (LMP) (immunoproteasome subunits). The class III region is situated between the class I and II regions and contains over 75 genes including those encoding several complement proteins. HLA genes can be highly polymorphic allowing the presentation of a huge range of potential peptide epitopes. Multiple alleles exist for most genes, with the majority of individuals being heterozygous for each locus. For some isotypes both the α and β chains can contribute to the polymorphism of the HLA class II molecule. The HLA-DR isoform is encoded by a monomorphic α chain and a highly polymorphic β chain gene. For HLA-DRB1 alone, there are 221 different alleles (Marsh, 2000). Both the α and β chains of the DP and DQ isotypes are highly polymorphic which permits a large number of different α and β chain combinations.

1.9.4 Peptide binding to HLA molecules
The majority of polymorphic amino acids found in HLA molecules are clustered in the peptide binding groove. These amino acids often contribute to the formation of peptide binding pockets. Pockets differ between allelic variants in their spatial and chemical characteristics and as a consequence preferentially accommodate specific amino acid side chains (Nielsen et al., 2004). The amino acid residues or the peptide epitope that sit in the pockets of the binding groove are known as anchor residues and constitute the HLA allotype-specific binding motif.
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*Binding motifs*

HLA class I restricted peptides are normally 8-10 amino acids in length and residues at positions 2 and 9 have been implicated as the peptide anchor residues (Rammensee *et al.*, 1999). Due to the restricted length of these peptides it has been possible to identify HLA class I binding motifs by peptide elution studies.

HLA class II restricted peptides range in length from 13-25 amino acids (Rammensee, 1995). Due to the open structure of the HLA class II molecule, the region of a peptide that interacts with the binding groove can lie anywhere within the primary amino acid sequence. Peptides that bind to a specific HLA class II molecule share a similar core region spanning 9 amino acids, with the key anchor residues located at the relative positions 1, 4, 6 and 9 (Rammensee *et al.*, 1999). To assess if a peptide may bind to a given HLA class II molecule, the primary amino acid sequence must be aligned with the binding motif to identify this core binding region (Figure 1.7).

A number of peptide prediction programmes are available for both HLA class I and II, however due to the inherent difficulties in defining motifs for class II restricted peptides (long peptide length and the open structure of the HLA class II molecule), their use is limited (Nielsen *et al.*, 2004; Rammensee *et al.*, 1999).

![Figure 1.7 Alignment of HLA class II peptides based on a common 'core' binding motif (adapted from Marsh, 2000).](image)

HLA class II restricted peptides vary in length from 13-25 amino acids. Peptides that bind to a specific HLA class II molecule can be aligned based upon the presence of a common binding motif. The black squares represent the key anchor residues in the binding motif at the relative positions of 1, 4, 6 and 9.
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1.10 The T-cell receptor

1.10.1 T-cell receptor genetics and structure

The TCR is an antigen-specific membrane-bound molecule present on the surface of T-cells. Between 90 and 99% of peripheral T-cells express TCR αβ chains, whilst the remaining population express TCR γδ chains (Lefranc and Lefranc, 2001). The α and β chains are covalently linked by disulphide bonds. These TCR chains consist of an N-terminal region (containing a variable and constant domain), a transmembrane region and a cytoplasmic tail (Figure 1.8) (Bentley and Mariuzza, 1996). The TCR chains are glycoproteins, with the α chain containing between four and five N-inked oligosaccharides and the β chain containing up to two N-linked glycans (Lefranc and Lefranc, 2001). The constant region is encoded by a constant gene (C). The variable domain however, is encoded by a variable gene (V), a joining gene (J), and in the case of β chains, a diversity gene (D). There are potentially over 40 functional Vα genes, and ~50 Jα genes. In the TCR β locus there are approximately 40 functional Vβ genes, 2 diversity genes, and 12 Jβ genes. The somatic rearrangement of the V-J (α) or V-D-J (β) genes occurs in thymus during the early stages of lymphocyte maturation (Janeway, 2001). This results in the generation of large numbers of T-cells expressing diverse TCRs and is referred to as combinatorial diversity. Further diversity is also achieved by the addition or removal of nucleotides at the V-D-J or V-J segments, a result of terminal transferase and exonuclease activity (Lefranc and Lefranc, 2001).

The TCR chains contain three hypervariable regions (CDR1-3) which are involved in the recognition and binding of the HLA-peptide complex (Lefranc and Lefranc, 2001). The CDR1 and 2 regions are encoded by the variable genes, whilst the CDR3 region occurs at the V-J (α-chain), or V-D-J (β-chain) junctions. Therefore the CDR3 regions are the most variable section of the TCR chain, and are the principal determinants of specificity (Lefranc and Lefranc, 2001). The antigen-recognition site of the TCR consists of a flattened region formed by the CDRs of the α and β chain. The CDR3 regions of the α and β chains interact with the peptide binding region of the HLA molecule.
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1.10.2 T-cell receptor nomenclature
A number of monoclonal antibodies which specifically recognise different families of TCR Vβ chain are commercially available. The TCR Vβ chain families were designated numbers in the order of their antibody-based identification. This terminology was agreed upon at the first human TCR monoclonal antibody workshop in 1996 (Posnett et al., 1996).

**International immunogenetics database (IMGT)**
The IMGT database created in 1989 by Marie-Paule Lefranc (Lefranc et al., 2005), provides an alternative nomenclature system to identify and compare T-cell receptor gene and protein sequences. The IMGT unique numbering system was created after aligning more than 5000 TCR gene sequences, taking into account the defined CDR regions, structural data provided by X-ray diffraction studies and the characterised hypervariable loops. An advantage of this system is that it permits simple comparisons between sequences encoding variable regions of different origin. Furthermore, the conserved amino acids within TCR chains are always found at the same position using this system (Lefranc and Lefranc, 2001). The classification of TCR genes is first based upon the chromosomal location of the TCR gene; TRA (α), TRB (β), TRD (δ) and TRG (γ). TCR genes can be further divided into the gene groups V, J, D, or C, as an example, the TCR β chain variable gene group would be referred to as TRBV. Variants within these gene groups (subgroups) are designated with a number, for example TRBV4. Subgroups can be further subdivided to identify a specific TCR gene e.g. TRBV4-3.
Figure 1.8 Simplified structure of the TCR (adapted from Janeway, 2001).
A schematic representation of the TCR heterodimer. The TCR is a heterodimeric membrane bound molecule consisting of an α and β chain which are covalently linked by disulphide bonds. The chains consist of an N-terminal region containing a variable and constant domain, a transmembrane region and a cytoplasmic tail. The variable domains are encoded by the variable gene (V), the joining gene (J), and in the case of the β chain the diversity gene (D). The antigen-recognition site of the TCR consists of a flattened region formed by the α and β chain.
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1.10.3 T-cell signalling
Efficient T-cell signalling is a necessary event in the activation of T-cells and their subsequent proliferation. The TCR complex is non-covalently associated with a complex of five polypeptide chains termed the CD3 complex. These proteins associate to form several dimers (CD3γ:CD3ε, CD3δ:CD3ε, CD3ζ:CD3η and CD3ζ:CD3ζ) (Lefranc and Lefranc, 2001) and play a critical role in transducing the activation signal intracellularly after peptide-recognition by the TCR (Pitcher and van Oers, 2003). This recognition event initiates a signalling cascade. The TCR-associated CD3 molecule becomes phosphorylated on the tyrosine residue within an ITAM motif (immunoreceptor tyrosine-based activation motif), resulting in the subsequent recruitment, phosphorylation and activation of ZAP-70 (Pitcher and van Oers, 2003). The phosphorylated ZAP-70 protein can then recruit LAT (linker for activation of T-cells) adapter proteins, which act as a link between TCR activation and downstream PLC-γ1 and PI3K signalling pathways (Sommers et al., 2004).

The activation of T-cells can be enhanced through additional signalling through accessory molecules such as CD28. This molecule is expressed on nearly all CD4 T-cells and approximately 50% of CD8 T-cells (Riley and June, 2005). Its ligands B7-1 and B7-2 are expressed on the surface of APCs (Cella et al., 1996). The signalling cascade (subsequent to ligand engagement) synergises with the TCR complex-mediated signalling in the production of IL-2 and in cellular proliferation (Ward, 1996).

1.11 Immune responses to HPV

1.11.1 Immune evasion
Whilst infection with HR HPVs is common, nearly all infections are cleared effectively, with less than 1% of women with clinical evidence of infection progressing to cervical cancer (Tindle, 2002). The median duration of HPV infection (as detected by PCR) is approximately 8 months (Ho et al., 1998), which clearly indicates that the virus is persisting in the host for a protracted period of time without being detected. HPV is widely regarded as a weakly immunogenic virus and avoids detection in several
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ways. Firstly, the replication of HPV in the infected keratinocytes does not result in cell lysis, instead the virions are assembled in terminally differentiated cells which are naturally shed from the epithelial surface (Stanley, 2001). The absence of cell lysis and the minimal exposure of virus particles to immune cells of the epithelia, results in little or no inflammation within the infected tissue. This results in a failure to activate and recruit key immune effector cells of both the innate and adaptive immune system.

In the cervical epithelium a unique subset of DCs called Langerhans cells (LCs) act as the principal APCs. Ordinarily these cells endocytose antigen within the infected epithelia (resulting in their activation and migration) and present the processed antigen to T-cells within the draining lymph node (Carbone et al., 2004). Recent data provided by an in vitro study revealed that unlike peripheral DCs, the LCs do not become activated following internalisation of HPV16 virus-like particles (VLPs) (Fausch et al., 2002). VLPs consist of the L1 and/or L2 capsid proteins and contain no DNA (described in section 1.12.1). This lack of activation could subsequently result in the failure of LCs to migrate and prime the HPV-specific cell-mediated immune response. In HPV-induced cervical lesions the number of LCs present in the epithelia are greatly reduced (Connor et al., 1999), which would further impede the presentation of the HPV antigen to the immune system. In transformed cervical tissue, the capacity of the remaining LCs to effectively promote a Th1 immune response may be impeded by the local cytokine environment. Cervical cancer cells have been shown to secrete IL-10, TGFβ (Sheu et al., 2001), prostaglandin E2 (Sales et al., 2001), and vascular endothelial growth factor (VEGF) (Fujimoto et al., 1999). The presence of these cytokines has been shown (in vitro) to negatively affect the capacity of (DCs) LCs to deliver co-stimulatory signals and produce IL-12 (necessary for Th1 polarisation). Depending on the cytokines present in the tissue microenvironment these LCs could induce T-cell tolerance (Steinbrink et al., 1997), or redirect the immune response by inducing Th2 polarised T-cell populations (Liu et al., 1998; Sheu et al., 2001).

Several proteins of the HR HPVs have been shown to contribute to immune evasion. The direct infection of epithelia should result in the production of type I IFNs (α and β)
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which demonstrate anti-proliferative effects and also act to stimulate the maturation of DC (Luft et al., 1998). E7 has been shown to inhibit the effects of the interferon pathway by decreasing the expression of IFNα inducible genes (Barnard and McMillan, 1999) and interfering with the activation of the IFNβ promoter (Park et al., 2000). The HPV16 E5 protein was recently shown to selectively down-regulate the cell surface expression of HLA-A and HLA-B, thereby decreasing viral peptide presentation to CD8+ T-cells (Ashrafi et al., 2005). By not affecting the expression of HLA-C and HLA-E the HPV infected cells can avoid recognition by NK cells. The absence of pro-inflammatory signals associated with HPV infection, together with the immune evasion strategies employed by the viral proteins, permits the virus to establish infection by temporarily evading the immune system.

1.11.2 Cell-mediated response to HPV

The cellular immune response is thought to play a significant role in the clearance and control of HPV infection. This is substantiated by an abundance of indirect evidence provided by both human and animal studies.

In immunocompromised transplant patients and HIV positive individuals there is a striking increase in the prevalence of progressive anogenital disease (Fruchter et al., 1996; Petry et al., 1994). Studies investigating the spontaneous regression of genital warts and cervical lesions have provided a more detailed picture of the immune response during lesion clearance. The cellular infiltrate of regressing HPV-induced genital warts is dominated by CD4+ T-cells, with significant levels of CD8+ T-cells and macrophages also present (Coleman et al., 1994). The cytokine environment was shown to be dominated by pro-inflammatory molecules such as TNFα and IFNγ. In a recent study, the spontaneous regression of high grade vulval intraepithelial neoplasia (VIN) in one individual, was accompanied by E6 and E7-specific T-cell responses in peripheral blood, which were undetectable in the non-regressing patients (Bourgault Villada et al., 2004). These studies provide interesting preliminary information regarding the cellular immune response during regression. However, due to the unpredictable nature of spontaneous lesion regression and obvious ethical restrictions
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when studying humans, it has not been possible to obtain a complete picture of the events initiating at infection and terminating in regression. In animals, a more complete picture of the chronological events culminating in wart regression has been investigated. The canine oral papillomavirus (COPV) model is particularly appropriate for study as the experimentally induced mucosal papillomas are subject to rapid and predictable regression (Nicholls et al., 2001). Immunohistochemical analysis of biopsies taken at regular intervals post infection revealed that just prior to regression, the papilloma was infiltrated by large numbers of CD4+ T-cells (the dominant population) and CD8+ T-cells, which peaked during resolution (Nicholls et al., 2001). This was followed by a protective antibody response. Taken together the evidence provided by human and animal studies highlights the importance of the cellular immune response to HPV. Numerous studies have investigated the contribution of CD8+ T-cells and CD4+ T-cells to the clearance of HPV disease.

1.11.3 CD8+ T-cell response to HPV

The vast majority of studies investigating HPV-specific immunity have focussed on type 16 as this is the most prevalent oncogenic HPV type detected in high grade CIN and cervical cancer (Cuschieri et al., 2004; Munoz et al., 2003). In particular, responses to the E6 and E7 proteins have been extensively studied due to their constitutive expression in cervical cancer. Many of the findings regarding the CD8+ T-cell response to the HPV16 E6 and E7 proteins are to some extent contradictory, and as yet the relationship between these T-cell responses and disease clearance or progression, has not been resolved.

E6 and E7-specific CTL detected in many women with persistent HPV16 infection or cervical cancer appear to be absent in healthy women (Bontkes et al., 2000). This suggests that these CTL responses do not play a role in disease clearance. In support of these findings, HPV16 E7(11-20)-specific CTL responses have been detected in the PBMC of CIN and cervical cancer patients and not in healthy women (Ressing et al., 1996). Previously our group has demonstrated that short-term in vitro culture with the HPV16 E7(11-20) peptide, expanded E7-specific CD8+ T-cells to a greater extent in the
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PBMC from CIN3 patients than from healthy volunteers (Youde et al., 2000). In contrast, Gurski et al., 2001 were able to detect HPV E7\((11, 20)\)-specific CTL responses in 3/6 healthy women but not in cervical cancer patients (Gurski et al., 2001). It should be noted however, that these may be primary responses, as the T-cells had been cultured (and re-stimulated) with DCs pulsed with peptide for at least 14 days. Another group studying HPV16 positive women (no CIN) showed that HPV16 E6-specific CTL can be detected in over 50% of those women who clear the infection, but not in women in which the infection persists (Nakagawa et al., 2000). This suggests that the lack of E6-specific CTL may be a contributing factor in persistence. This is not supported by a recent study by Steele et al., 2005, who directly detected E6-specific CD8+ T-cell responses in the majority of patients with CIN and cervical cancer, suggesting that this response does not play a critical role in clearance of HPV-mediated disease. It should be noted however that this study provided no evidence that the CD8+ T-cells were CTL, as they were identified on the basis of IFN\(\gamma\) secretion rather than cytotoxic effector function (Steele et al., 2005). Longitudinal studies using standardised immunological assays, containing larger cohorts of women with varying severity of cervical disease will be required to gain a better understanding of the role of HPV-specific CTL in protection from viral persistence and disease progression.

1.11.4 CD4+ T-cell response to HPV

Previously, attention has focussed on the role of CTL in the clearance and control of HPV infection, primarily as these cells are the major effector cells in viral clearance. More recently the contribution of CD4+ T-cells to this response has been studied in greater depth as evidence has emerged for their critical role in the clearance of virally infected (Christensen et al., 1999; Xiang et al., 2005) or transformed cells (Mumberg et al., 1999). As with the CTL studies, HPV16 E6 and E7 proteins have received the most attention. Several studies have investigated the responses to other HPV16 proteins such as L1, L2, E2 and E5 (Bontkes et al., 1999a; de Jong et al., 2002; Shepherd et al., 1996; Steele et al., 2005).
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The study of HR HPV E6 and E7-specific CD4+ T-cell responses has not been restricted to women with HPV related disease, a number of studies have also investigated the memory Th1 response present in healthy women who have cleared the virus. Presently 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.

HPV16 E6-specific memory CD4+ T-cell responses have been detected in ~50% of healthy female donors (de Jong et al., 2004; Smith et al., 2005; Welters et al., 2003). For HPV18 E6 the frequency of detection for E6-specific Th1 responses in healthy women is ~20%, reflecting a lower incidence of infection (Facchinetti et al., 2005; Welters et al., 2005). In a recent study, all 10 HPV18 positive cancer patients tested failed to demonstrate an HPV18 E6-specific Th1 response (Welters et al., 2005). de Jong et al., 2004 observed that the CD4+ T-cell response to HPV16 E6 peptides (assessed in proliferation assays) was detected less frequently in CIN patients (1/8), compared to healthy donors (13/20) and cancer patients (7/17) (de Jong et al., 2004). The E6-specific CD4+ T-cells from the majority of cancer patients did not secrete pro-inflammatory cytokines. Antigen-specific IL-10 secretion was detected from some patients and this was rarely accompanied by IFNγ. This suggests that in cancer patients the CD4+ T-cell response is either absent or inappropriately polarised (de Jong et al., 2004). This evidence further implies that the absence of effective E6-specific CD4+ T-cells early in the HPV immune response, could contribute to the risk of viral persistence and disease progression. It appears that the progression from CIN to cervical cancer is often accompanied by the development of an E6-specific CD4+ T-cell proliferative response (without cytokine production). Where invasive cervical cancer develops, the basement membrane of the epithelia is breached and HPV-transformed cells are exposed, providing the immune system with a large amount of viral antigen against which a CD4+ T-cell response can be generated. It is likely that at this stage the T-cell response is ineffective due to the large mass of transformed tissue (Steele et al., 2005). Additionally the cytokine environment surrounding the cancerous tissue may not be conducive to the generation of a Th1 response (Giannini et al., 1998;
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Sheu et al., 2001). Welters et al., 2003, also reported that relatively few (3/11) HPV16 positive cervical cancer patients generated Th1 responses to HPV16 E6 compared to healthy donors (12/20) (Welters et al., 2003).

Cross-sectional analysis of CD4+ T-cell responses to HPV16 E7 has revealed that they are rarely detected from healthy donors (1/7), but frequently occur in HPV16 positive patients with cervical disease (5/11) (van der Burg et al., 2001). The low frequency of E7 responses in healthy donors has been reported elsewhere for both HPV16 and HPV18 (Welters et al., 2003; Welters et al., 2005). Longitudinal studies have provided evidence that the development of Th reactivity to E7 can correlate both with viral clearance and lesion progression (de Gruijl et al., 1998). It was proposed that the responding Th 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 a result of the detection and clearance of the HPV infected cells.

It is clear from these studies that in healthy individuals the development of a Th1 E6 response is a relatively frequent event, whilst E7 responses in the same individuals are far rarer. The relevance of these responses in protection from disease progression is unclear and further study is required.

1.11.5 Humoral response to HPV

Following infection with HPV most people will normally seroconvert within 6 to 12 months (Carter et al., 2000). However, some individuals with persistent HPV infection will remain seronegative and therefore seropositivity cannot be used as a test for HPV infection. Antibodies specific for HPV proteins L1 and L2 can be detected from HPV experienced individuals using VLP-based ELISAs. IgG is the most abundant antibody isotype detected in serum, followed by IgA (Onda et al., 2003).

Species-specific (dogs, rabbits and cows) PV neutralising antibodies have been shown to provide resistance to further viral infection (Breitburd et al., 1995; Gaukroger et al.,
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1996; Suzich et al., 1995). Recent clinical trials showed that HPV16 and HPV18 VLP vaccines induce protection against persistent HPV infection through the generation of strong antibody responses to the L1 (Harper et al., 2004; Koutsky et al., 2002). The development of an L1 and L2-specific humoral response is effective in the prevention of HPV infection, but is not thought to contribute to the clearance of established infection. This is highlighted by the finding that seropositivity to these structural proteins is reported to rise with increasing disease severity (Bontkes et al., 1999b; de Gruijl et al., 1997). Antibodies to the E6 and E7 proteins (both expressed intracellularly) have also been detected in a proportion of cervical cancer patients (Ghosh et al., 1993; Jochmus-Kudielka et al., 1989; Kochel et al., 1991). This is presumably a result of increased exposure of the HPV proteins to the immune system, as the mass of neoplastic tissue increases.

1.12 HPV vaccines

1.12.1 Prophylactic vaccines

HPV has been shown to be a necessary agent in the development of cervical cancer (Walboomers et al., 1999). Therefore, prevention of persistent infection with HR HPVs should have a major impact on the prevalence of malignant cervical lesions. The focus of current prophylactic strategies is the induction of effective humoral responses that protect vaccinated subjects from infection with HPV. The most promising vaccines are DNA-free VLPs which consist of the L1 and /or L2 capsid proteins (Galloway, 2003). VLPs mimic the natural virus structure and are thought to function by eliciting a strong type-specific neutralising antibody response. Convincing evidence provided by animal studies has demonstrated that neutralising antibodies effectively block viral infection (Breitbart et al., 1995; Ghim et al., 2000). Data from a clinical trial using HPV16 VLP (L1) as an immunogen has shown encouraging results (Koutsky et al., 2002). HPV16 negative females (16-23 years of age) participated in this double-blind, placebo controlled trial. None of the women who received the 3 dose VLP vaccine regimen showed persistent HPV16 infection over a median 17.4 month monitoring period, although a small number of individuals were positive for HPV16 DNA at a single time point post vaccination. Persistent HPV16 infection was only
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detected in women who received the placebo. This suggests that the VLP vaccine prevents persistent HPV16 infection, but does not always induce sterilising immunity. Recently a clinical trial using a bivalent HPV16 and HPV18 L1 VLP vaccine has also demonstrated 100% efficacy against persistent infection with these HPV types (Harper et al., 2004). It is interesting to note that immunisation with VLPs leads to a higher titre of L1-specific antibodies, than would result from natural infection (Harper et al., 2004; Koutsy et al., 2002).

A bivalent vaccine which induces protection against HPV16 and HPV18 should prevent up to 70% of cervical cancer cases (Franco and Harper, 2005). A recent clinical trial was conducted using a tetravalent HPV6, 11, 16 and 18 VLP vaccine. The success of this trial indicates the feasibility of vaccinating against multiple HR HPV types (Villa et al., 2005). A tetravalent vaccine that could protect against HPV16, 18, 31 and 45 could potentially prevent 80.3% of cervical cancers (Franco and Harper, 2005).

Whilst VLP-based vaccines have shown very promising results in the prevention of persistent HPV infection, the duration of protection has not yet been established. Longitudinal studies will be required to show if the vaccines provide life-long protection or whether a booster vaccination is required. Moreover, these studies will be necessary to confirm that the vaccines ultimately protect against the development of cervical cancer. Other potential issues concerning the use of these vaccines include the difficulties and cost of delivering a 3 dose VLP immunogen requiring cold chain transport in developing countries, where the burden of cervical disease is greatest (Padma, 2005).

Should a widespread immunisation program be implemented using these VLP vaccines, there would still be a requirement for cervical screening for several decades, especially as these vaccines are very unlikely to benefit individuals with pre-existing infection. Therefore there is still a need for the development of therapeutic strategies that could control or eradicate HPV-mediated disease.
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1.12.2 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. It is thought that the development of a T-cell response specific for HPV antigens would be required to induce these effects. The E6 and E7 proteins of HR HPVs are constitutively expressed from infection through to cervical cancer, making these proteins ideal targets for immunotherapy. Therefore, the central aim of a wide range of therapeutic vaccine strategies is to induce an effective E6 and E7 specific immune response.

1.12.2.1 Peptide vaccines

Peptides are attractive candidates for vaccines as they can be produced on a large scale and allow precise targeting of the immune response. A major limitation of peptide vaccines is that the immunogenic peptides must be specifically selected to complement the HLA haplotype of the patient. Most studies have focussed on HLA-A*02 restricted CTL epitopes derived from the E7 protein (Kast et al., 1993; Muderspach et al., 2000; Ressing et al., 1995). Peptide vaccination studies performed using animal models have yielded promising results. The vaccination of mice with the HPV16 E7(49-57) peptide (emulsified in incomplete Freund’s adjuvant (IFA)), was shown to confer protection from subsequent tumour challenge. This response was mediated by E7(49-57)-specific CTL (Feltkamp et al., 1993). These CTL were later shown to be effective in the clearance of established tumours (Feltkamp et al., 1995). A concern regarding such studies is that the transplant of HPV positive tumours into mice does not closely reflect the human disease situation. Most crucially, the mouse model does not include a period of persistent HPV infection, which in humans precedes the development of cervical cancer (Hildesheim et al., 1994). Persistent infection results in protracted exposure of HPV antigen which may induce viral tolerance in the absence of pro-inflammatory signals.

Clinical trials using peptide vaccines have been carried out by several groups. It appears that the outcome of these trials may be related to the disease status of the
patient cohorts studied. In a phase I trial, fifteen HPV16 positive cervical cancer patients (all of whom where HLA-A*02+) were vaccinated with 3 peptides; the CTL epitopes HPV16 E7\textsubscript{(11-20)} and HPV16 E7\textsubscript{(86-93)}, and an un-related pan-HLA-DR CD4+ T-cell epitope (PADRE). Following vaccination, a PADRE-specific Th response was detected in some donors, however no E7-specific CTL response was detected (Ressing \textit{et al}., 2000). In a similar trial, HPV16 positive patients with either CIN II/III or VIN were vaccinated with HPV16 E7\textsubscript{(12-20)} and E7\textsubscript{(86-93)} epitopes. Enhanced E7-specific CTL responses were detected in over 60\% of patients. 3 of the 18 patients vaccinated cleared their dysplasia, but a correlation could not be made between this regression and the E7-specific immune response (Muderspach \textit{et al}., 2000). Taken together, these trials indicate that peptide vaccines may be more immunogenic in patients with pre-invasive disease who are not immunocompromised.

Murine studies suggest that the use of long peptides (~30-35mers) that contain a helper T-cell epitope linked to a CTL epitope are more efficient at eliciting a CTL response than the minimum CTL epitope alone. In one such study, mice immunised with the HPV16 E7\textsubscript{(43-77)} peptide and a DC activating agent (ODN-CpG) induced both an E7-specific CD4+ Th and a CTL response which mediated the eradication of an established tumour (Zwaveling \textit{et al}., 2002). Similarly, immunisation of rabbits (persistently infected with CRPV) with long overlapping peptides from CRPV E6 and E7 (in Montanide ISA51) resulted in the controlled growth of established papillomas (Vambutas \textit{et al}., 2005). In the future, long peptides containing multiple Th and CTL epitopes combined with an appropriate adjuvant could be used to efficiently induce broader T-cell responses.

Use of peptide-based vaccines to induce HPV-specific T-cell responses capable of clearing established cervical lesions has met with limited success and further study and optimisation will be required.
1.12.2.2 Protein vaccines

One of the major disadvantages of using peptide-based vaccines is that they are HLA restricted. The use of whole protein as a therapeutic vaccine would circumvent this problem as it provides all of a protein's potential immunogenic epitopes for all HLA haplotypes. Protein-based vaccines can generate both CD4+ and CD8+ T-cell responses. This is reliant upon protein uptake, processing and presentation (cross-presentation for HLA class I peptides) by APC (most likely DCs). One of the major drawbacks of vaccinating with protein is that they are poorly immunogenic, requiring administration with an adjuvant for the development of a potent immune response. Another potential issue with immunising using whole protein is that there is no control over which epitopes will generate the dominant T-cell response. It is feasible that the immune response could be targeted at dominant epitopes that are not optimal for viral clearance. In this respect, the use of well-characterised peptide epitopes in vaccination studies could 'encourage' the immune response to focus on sub-dominant epitopes that are more effective in the resolution of infection.

In animal models, vaccination with HPV proteins has been shown to elicit protein-specific CTL responses (reviewed in Steller, 2002). One such study utilised the HPV16 E7 protein (in the PROVAX adjuvant) to immunise mice which resulted in protection from subsequent tumour challenge and prevented further growth of established tumours (Hariharan et al., 1998).

Several clinical trials have been carried out using HPV protein vaccination strategies, although with little success. In a recent double-blind, placebo controlled Phase I clinical trial, patients with CIN were immunized with a bacterially derived HPV16 E6/E7 fusion protein (in ISCOMATRIX adjuvant) (Frazer et al., 2004). Overall, patients immunised with the fusion protein tended to demonstrate enhanced HPV16 E6 and E7-specific CTL and antibody responses when compared to the placebo controls. Whilst the vaccine was well tolerated and did generate protein-specific immune responses, there was no correlation with disease clearance. As the cohort of patients
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used in the trial was relatively small, a larger cohort of women would need to be studied to truly evaluate the vaccine efficacy.

1.12.2.3 Dendritic cell-based vaccines
DCs are highly potent APCs that are capable of priming both CD4+ and CD8+ T-cells in vivo. By utilising the ability of these cells to efficiently present tumour antigen to immune effector cells, DCs may serve an important role in immunotherapeutic approaches to cancer. Vaccine strategies based on DCs as adjuvants involve either the pulsing of DCs with peptides/proteins or the transfection of DCs with genes encoding tumour antigens (Fong and Engleman, 2000).

To date, the majority of studies utilising DC vaccines to generate HPV-specific immunity have been performed in murine models (Kim et al., 2004; Mayordomo et al., 1995). Vaccination of mice with DCs pulsed with HPV16 E7(49-57) peptide has been shown to confer protection against challenge with tumour cells. This protection was mediated by CTL (Ossevoort et al., 1995). Administration of DCs pulsed with full length E7 protein conferred 100% protection in another murine model and also elicited an E7-specific CTL response (De Bruijn et al., 1998). A mouse DC line constitutively expressing the E7 gene from HPV16 was used to immunise mice intramuscularly. This vaccine induced both CD4+ and CD8+ T-cell immune responses specific for E7, which was associated with a greater capacity to reject tumour challenge (Wang et al., 2000).

Limited human studies have investigated the ability of DC-based therapies to elicit HPV-specific immune responses. In a Phase I clinical trial, women with advanced cervical cancer were vaccinated with immature DCs loaded with sonicated HPV positive tumour cell lysate. Of the 7 patients treated, only 1 demonstrated a small increase in the frequency of HPV-specific CTL (Adams et al., 2001). In a more recent trial, patients with advanced cervical cancer were vaccinated with DCs pulsed with either HPV16 or HPV18 E7 protein (Ferrara et al., 2003). A proportion of these patients did generate E7-specific humoral and cellular immune responses,
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demonstrating that even patients with advanced disease can develop HPV immunity using DC-based therapy. In this study, no clinical responses were observed.

The use of DC-based vaccines in several human disease conditions has demonstrated some degree of clinical success (Kugler et al., 2000; Nestle et al., 1998). In the treatment of HPV-related disease however, this is not the case (Adams et al., 2001; Ferrara et al., 2003; Santin et al., 2005). Nonetheless, the ability to generate 'new' T-cell responses in patients with late stage cancer (who are often immunocompromised) is promising. One issue concerning the use of autologous DCs in immunotherapy is the expensive and labour intensive process of generating high numbers of these cells ex vivo under highly sterile conditions. Moreover, the DCs generated must possess the correct activated phenotype before administration for an appropriate and effective immune response to be induced.

1.12.2.4 Recombinant virus vaccines

The advantage of using a viral vector to introduce genes expressing tumour-specific antigen is that it provides additional stimulatory signals to the host immune cells. Vaccinia virus-based vaccines have the advantage that they can carry large inserts, permitting the expression of whole protein/s. The lytic nature of the vaccinia virus ensures that the infected cell will not persist, which is an important consideration when immunising with an oncogenic protein (Galloway, 2003). There is also a large safety profile for vaccinating with vaccinia as a result of its widespread use in the eradication of smallpox (Henderson, 2002).

In murine models, vaccinia viruses expressing HPV16 E6 and E7 fusion proteins have been shown to induce strong HPV-specific CTL responses (Gao et al., 1994; Zhu et al., 1995). HPV16 E7-specific CD4+ T-cell responses have been generated in murine models by retargeting the E7 (via fusion with LAMP-1) to the endosomal pathway, resulting in class II presentation (Wu et al., 1995).
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A vaccinia virus termed TA-HPV expressing modified HPV16 and HPV18 E6 and E7 proteins was shown to induce a HPV16 E7-specific CTL response in vaccinated mice (Boursnell et al., 1996). TA-HPV was also tested for immunogenicity and safety in a Phase I/II clinical trial in which 8 patients with late stage cervical cancer were recruited (Borysiwicz et al., 1996). A HPV-specific humoral response was generated in 3/8 patients as a result of the vaccine. HPV18-specific CTL were detected in 1/3 of the patients evaluated 9 weeks post vaccination, however this response disappeared after 14-20 weeks post-vaccination.

More impressive results have been obtained from a Phase II study in which 12 women with high grade HPV positive vaginal or vulval intraepithelial neoplasia were vaccinated with TA-HPV (Baldwin et al., 2003). A decrease in lesion size (average 40% reduction) was detected in 83% of the women and enhanced HPV16 E6 and E7-specific T-cell responses were detected in 6/10 patients evaluated. Whilst this result is promising, the absence of a placebo control group complicates interpretation, as the naturally occurring oscillations in lesion size have not been accounted for.

1.12.2.5 DNA vaccines

DNA has become an attractive vaccine candidate for several reasons, most saliently it is versatile, safe and cheap to produce (Prud'homme, 2005).

In a recent murine study it was shown that co-vaccination with calreticulin-E6 linked and calreticulin-E7 linked DNA constructs led to the generation of E6 and E7-specific T-cell responses. Moreover, co-vaccination with these two constructs resulted in a superior anti-tumour effect when compared to vaccination with either construct in isolation (Peng et al., 2005). A number of earlier studies have also demonstrated HPV-specific T-cell responses and anti-tumour responses using DNA vaccine constructs (Cheng et al., 2002; Osen et al., 2001; Velders et al., 2001).

A double-blind, placebo-controlled trial was carried out using a plasmid-DNA vaccine (ZYC101a) encoding fragments from HPV16 and HPV18 E6 and E7 proteins in
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women with CINII/III disease (Garcia et al., 2004). The proportion of patients in which disease was resolved was higher in women vaccinated with the DNA construct than with the placebo (43% versus 27%), however this was not shown to be statistically significant.

To date, no HPV-related DNA-based vaccine trial has demonstrated a correlation between the induced immune response and a positive clinical outcome. However this technology is still developing and it is possible that in the future the use of combinational DNA therapy (such as genes encoding pro-inflammatory cytokines as well as HPV peptides) may provide more success. A reason that could potentially limit the transfer of success seen in rodent models, to the clinical setting is that the expression of Toll-like receptor 9 (TLR9), which is necessary for CpG recognition, varies considerably between mice and humans. TLR9 is expressed on a broad range of murine cells including myeloid DCs, monocytes and macrophages, however expression of this receptor in humans has only been described on plasmacytoid DCs and B-cells (Krieg, 2003). Therefore in humans, DNA vaccines may fail to adequately activate the immune system.

At present the main issue with testing vaccine efficacy in humans is that in the majority of cases, the patients recruited in clinical trials have advanced cervical cancer. Such individuals are likely to be immunocompromised as a result of treatment received (chemotherapy and radiotherapy), which obviously limits their capacity to respond to immunogens. Furthermore advanced lesions are more genetically unstable, often harbouring defects in antigen processing and presentation (Brady et al., 2000; Fowler and Frazer, 2004), which could effect the presentation of viral epitopes. It is feasible that many of these vaccine strategies may provide greater clinical success in patients with less severe disease. It should also be noted that cervical cancer is a localised disease, thus the use of systemic vaccine strategies may not be optimal and mucosal delivery may provide more success.
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1.13 Project Aims

Infection with human papillomavirus (in particular types 16 and 18) is the causative factor in the development of cervical cancer. The E6 and E7 proteins are constitutively expressed in cervical cancer and are therefore attractive targets for T-cell based immunotherapy.

To date, the majority of studies have investigated T-cell immune responses to HPV16 and HPV18 E6 and E7 proteins in women with high grade CIN and cervical cancer. Only recently have the memory responses to these proteins been studied in healthy women. As these women routinely clear the HPV infection, the epitopes against which the immune response was generated are more likely to correlate with protection from persistent infection. However, relatively few E6 or E7 derived epitopes have been defined from healthy donors’ responses.

Therefore the principal aims of this project were

- To assess T-cell immunity against HPV16 and HPV18 E6 and E7 proteins in healthy female donors.
- To define novel HPV16 and HPV18 E6 and E7 specific T-cell epitopes.
- To generate T-cell lines which specifically recognise the defined HPV epitopes and to identify the CD4/CD8 restriction of responses.
- To use these lines to further characterise these epitope specific responses in terms of their HLA-restriction, effector function and TCR chain usage.
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2.1 Tissue culture

2.1.1 Tissue culture media

FCS-RPMI media
RPMI 1640 (Invitrogen) was supplemented with 100 units/ml penicillin, 100µg/ml streptomycin (Invitrogen), 25mM HEPES (Sigma), 2mM L-glutamine (Invitrogen), and 10% foetal calf serum (Invitrogen).

AB-RPMI media
RPMI 1640 (PAA), was supplemented with 100 units/ml penicillin, 100µg/ml streptomycin (Invitrogen), 25mM HEPES (Sigma), 2mM L-glutamine (Invitrogen), and 10% heat inactivated human AB serum (Welsh Blood Transfusion Service).

MACs culture media
RPMI 1640 (PAA), was supplemented with 100 units/ml penicillin, 100µg/ml streptomycin (Invitrogen), 25mM HEPES (Sigma), 2mM L-glutamine (Invitrogen), and 5% heat inactivated AB serum (WBTS).

Serum-free RPMI media
RPMI 1640 (Invitrogen) supplemented as above, but with no serum added.

Freezing mix
10% Dimethyl Sulphoxide (DMSO) (Sigma), 40% RPMI 1640 (Invitrogen), and 50% Foetal calf serum (Invitrogen).
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2.1.2 Tissue culture vessels
All standard plasticware was obtained from either Nunc, Greiner, or Fisher, with the exception of 48 well plates from Falcon. The MultiScreen –HA plates used in ELISpot assays were purchased from Millipore.

2.1.3 Maintenance of established cell lines used in this study
Table 2.1 displays the established cell lines used in this project. The BLCL (suspension cells) were cultured in T25/T75 flasks in FCS-RPMI media. Adherent cell lines were cultured in 25cm$^3$ (T25) or 75cm$^3$ (T75) flasks in FCS-RPMI media. Cells were routinely split 1:4 when confluent (every 3-4 days). After washing with PBS (Invitrogen), cells were incubated at 37°C with either 0.5mls (T25) or 1.5mls (T75) EDTA-trypsin (Invitrogen). Following detachment, the cells were split between new flasks. FCS-RPMI media was then added to the cells (which inactivates trypsin) and flasks were incubated at 37°C.

Thawing cryopreserved cells: Cells were thawed 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 10% AB serum).

After thawing a T-cell line or clone, the cells are plated out in AB-RPMI media supplemented with 10ng/ml human recombinant IL-7 (Genzyme). Half the media was removed on day 2 and replaced with AB-RPMI + 40 IU/ml IL-2 (Chiron) (final concentration of 20U/ml IL-2). Cells were fed with AB-RPMI + IL-2 (20 IU/ml) every 2-4 days.
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Table 2.1 Established cell lines used

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLCL</td>
<td>B-cell lymphoblastoid cell lines generated by EBV transformation (Rowe et al., 1992)</td>
<td>Generated from HLA-typed donors</td>
</tr>
<tr>
<td>Mouse L-cells</td>
<td>Murine fibroblast line. Either: untransfected (HLA-DR negative), Transfected with HLA-DRB1<em>01 (DR1) Transfected with HLA-DRB1</em>04 (DR4) Transfected with HLA-DRB1*15 (DR15)</td>
<td>(Lechler et al., 1988; Wilkinson et al., 1988)</td>
</tr>
<tr>
<td>Clone 25</td>
<td>CTL line recognizing the HLA-A2 restricted Influenza A matrix epitope M1(_{(58-66)})</td>
<td>(Lawson et al., 2001)</td>
</tr>
<tr>
<td>7E7</td>
<td>CTL line recognizing the HLA-A2 restricted HPV16 E6(_{(29-38)}) epitope</td>
<td>(Evans et al., 2001)</td>
</tr>
</tbody>
</table>

2.1.4 Generating EBV-transformed B-cell Lines (BLCL)

PBMC were isolated from whole blood as described in section 2.2.2. 1ml of the EBV viral supernatant B95.8 was used to resuspend 5x10\(^6\) PBMC (Rowe et al., 1992). Cells were transferred to a T25 tissue culture flask and 4mls of FCS-RPMI, supplemented with 100ng/ml Cyclosporin A (CsA) (Novartis) was added. After 7 days of culture in a tilted position, half of the media was replaced with fresh FCS-RPMI media supplemented with CsA (100ng/ml). Half the media was replaced every week (no further CsA added) until cells started to form clumps. The flask was set upright and the BLCL were fed more regularly (every 2-4 days) in accordance with increased growth.

2.1.5 Estimation of cell viability

Cells were analysed for viability by combining a 15μl of the well mixed cell suspension with an equal volume of Trypan blue (Sigma), before examining cells using a microscope. Viable cells remain colourless, whilst non-viable cells appear blue. The percentage of total cells counted that remained white equates to the viability of the culture.
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2.2 Origin of blood samples and their preparation

2.2.1 Blood donors
The donors whose blood was used in the screen for HPV responses were all female members of laboratory staff aged between 21 and 31 years. These women had no known history of cervical dysplasia or neoplasia. As these donors were colleagues, it would have been inappropriate to test for current HPV infection by PCR or serotyping. Those donors whose blood was used as either APC or for feeders were also laboratory staff, but of any age or gender. Blood was taken with informed consent. Since donors were healthy volunteers, no ethical approval was required.

2.2.2 Isolation of peripheral blood mononuclear cells (PBMC) from whole blood
Human blood was collected into 50ml Falcon tubes and heparin was added at 10U/ml. PBMC were enriched from whole blood by density gradient centrifugation. This is a rapid and effective method, yielding 1-1.5x10^6 PBMC/ml of whole blood. 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 interface between Histopaque and plasma. This monolayer was harvested into a 50ml Falcon tube, to which serum-free RPMI media was added at a ratio of 1:5. Enriched PBMC were centrifuged at 612g for 10 minutes. If the pellet was contaminated with a large proportion of red blood cells, it was resuspended in 1ml of red blood cell lysis buffer and incubated for 10 minutes at 4°C. Serum-free RPMI media was then used to wash the PBMC, with the first 2 washes centrifuged at 425g (for 5 minutes) and the final spin at 272 g (for 5 minutes) so as to reduce the platelet content of the pellet.
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2.2.3 HLA typing of blood donors
The HLA class I and class II haplotypes of the blood donors were typed by the Welsh Transplantation and Immunogenetics Laboratory, Pontyclun, UK. This was carried out using PCR-SSP (sequence specific primers).

2.3 Primary PBMC cultures

2.3.1 Cryopreservation of PBMC
Following isolation of PBMC from fresh blood, a proportion of cells were cryopreserved at -70°C in freezing mix, before long term storage in liquid nitrogen. Aliquots ranged from 5-15x10^6 cells depending on the intended use. These PBMC were later used to re-stimulate effector T-cells in either the IFNγ ELISpot assay or in tissue culture wells.

2.3.2 Short term in vitro culture of PBMC with peptide antigens
PBMC isolated from fresh blood were plated out (in 24 well plates) at 2x10^6 cells/ml in AB-RPMI (1ml/well). Peptides were then added to these cultures; HPV pools or individual peptides at 5µg/ml of each peptide; positive peptide pool (PPP) at 10µg/ml of each peptide. On day 2, 0.5ml of AB-RPMI media containing IL-2 was added to each well to give a final concentration of 20U/ml IL-2. On day 6, 0.5ml of media was removed and replaced with 1ml of fresh AB-RPMI media without IL-2 added. On day 7, either some or all of the PBMC cultures were tested for the presence of peptide-specific T-cells in the IFNγ ELISpot assay (section 2.5.1).

2.3.3 Re-stimulation of PBMC cultures
PBMC/T-cell cultures were re-stimulated with the appropriate peptide or peptide pool. The cultured PBMC were harvested, counted, and re-plated at 1-2x10^6 cells/well in 0.5ml of AB-RPMI media supplemented with 40 U/ml IL-2. Autologous cryopreserved PBMC were used as a source of antigen presenting cells. These cells were irradiated in FCS-RPMI at 4,000 rads. After 1 wash in serum-free RPMI, they were added to wells in 0.5ml AB-RPMI at a ratio of 2 irradiated-APC: 1 cultured
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PBMC/T-cells. HPV peptide/s were then added to the wells at 5μg/ml. After 3 days, 0.5ml of AB-RPMI supplemented with 20 U/ml IL-2 was added to each well. One day prior to testing cells in ELISpot, 0.5ml of media was removed from each well and replaced with 1ml AB-RPMI without IL-2.

2.4 Antigens

2.4.1 Non-HPV peptides

Positive Peptide Pool (PPP); A pool of peptides containing common recall antigens for both CD8+ T-cells and CD4+ T-cells, was added to cultures at 10μg (of each peptide) per ml of culture medium (Smith et al., 2005). These peptides are shown in Table 2.2. Several different antigen sources (Tetanus, Influenza, CMV, and EBV) were included so as to increase the chance that a donor will have encountered at least one of the viruses. Each peptide synthesized (Severn Biotech), is >90% pure. Stocks of individual peptides were made by dissolving each peptide in DMSO. The PPP mixture was made by combining aliquots taken from each peptide stock, with each peptide being present at 10μg/μl. Peptide stocks and PPP aliquots were stored at -20°C.

In experiments where clone 25 (CTL) was tested, the Influenza A M1\(^{(58-66)}\) peptide was used. This peptide (synthesised by Immune systems) was >80% pure (as confirmed by mass spectrometry). This peptide was dissolved in DMSO to give a working stock of 10mg/ml.

2.4.2 HPV protein and peptide pools

The HPV peptide pools consist of 15mer peptides that overlap by 9 amino acids. The peptides span the entire length of the E6 and E7 proteins of HPV16 and HPV18. The peptides were synthesized with purity not guaranteed to be greater than 64% (Mimotopes). Individual peptides were dissolved in DMSO to give stock solutions of 100mg/ml. Peptide aliquots were combined to give 4 pools; HPV16 E6 (25 peptides), HPV16 E7 (15 peptides), HPV18 E6 (25 peptides), HPV18 E7 (16 peptides). In each pool, an individual peptide was present at 4mg/ml. For peptide mapping, 6 smaller
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Split pools from HPV16 E6 and HPV18 E6 were made as shown in Table 2.3. As whole E6 pools consist of 25 peptides, there were 5 split pools of 4 peptides, and 1 split pool containing 5 peptides, with each peptide present at 5μg/μl. The peptides were allocated numbers to allow simple identification. The HPV16 E7 peptides are numbered p1-p15, HPV16 E6 p16-p40, HPV18 E7 p41-p56, and HPV18 E6 p57-p81. HPV16 E6 and HPV18 E6 split pools were both numbered as pools 1-6. Peptides were allocated to split pools as shown in Table 2.3. All individual peptide stocks and HPV pools were stored at -20°C.

Due to low purity of the HPV pepset, a new batch of peptide 37 was synthesized at a purity of >95% (Severn Biotech). This peptide was dissolved in DMSO to give a stock of 100 mg/ml. From this, a working stock solution of 10mg/ml was created through 1:10 dilution with DMSO.

The HLA-A*02-restricted HPV16 E7(29-38) peptide was used in experiments involving the CTL clone 7E7. The peptide was synthesised by Immune systems, to a purity of >80% (as confirmed by mass spectrometry). This peptide was dissolved in DMSO to give a working stock of 10mg/ml.

The recombinant HPV16 E6 protein was diluted in 1:1 mixture of PBS and DMSO to give a working stock of 10mg/ml. The protein was produced in recombinant E.Coli transformed with Pet-19b-HPV16 E6, and was a kind gift from Dr. Marij Welters, Leiden University (Welters et al., 2003).

The HPV-11 L1 VLP was a generous gift from Merck Sharp & Dohme (West Point, PA, USA). The HPV-11 L1 protein was generated using a Saccharomyces cerevisiae expression system as previously described (Cook et al., 1999; Neeper et al., 1996).
### Table 2.2 Peptides included in the PPP

<table>
<thead>
<tr>
<th>Sequence (aa)</th>
<th>Source Antigen</th>
<th>Residue</th>
<th>HLA Restriction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKYVKQNTLKLAT</td>
<td>Influenza A HA</td>
<td>306–324</td>
<td>DR4/DR7/DR11</td>
<td>(Gelder et al., 1995)</td>
</tr>
<tr>
<td>QYIKANSKFIGITEL</td>
<td>Tetanus toxoid</td>
<td>830–844</td>
<td>Multiple DR</td>
<td>(Panina-Bordignon et al., 1989)</td>
</tr>
<tr>
<td>FNNFTVSFWLRVPSASHLE</td>
<td>Tetanus toxoid</td>
<td>947–967</td>
<td>Multiple DR</td>
<td>(Panina-Bordignon et al., 1989)</td>
</tr>
<tr>
<td>TSLYNLRRGTALA</td>
<td>EBV EBNA1</td>
<td>515–527</td>
<td>DR1*0701</td>
<td>(Khanna et al., 1997)</td>
</tr>
<tr>
<td>AGLTLLSLVCYLYFISRG</td>
<td>EBV BHRF1</td>
<td>171–181</td>
<td>DR15(2)</td>
<td>(Rickinson and Moss, 1997)</td>
</tr>
<tr>
<td>IVTDFSVIKAIEEE</td>
<td>EBV EBNA3c</td>
<td>416–429</td>
<td>A11</td>
<td>(Rickinson and Moss, 1997)</td>
</tr>
<tr>
<td>LTKGILGFVFTLTVPSERG</td>
<td>Influenza A M1</td>
<td>55–73</td>
<td>A2</td>
<td>(Gotch et al., 1987)</td>
</tr>
<tr>
<td>IQNAGLCTLVAMLEE</td>
<td>EBV BMLF1</td>
<td>276–290</td>
<td>A2</td>
<td>(Rickinson and Moss, 1997)</td>
</tr>
<tr>
<td>RPFFHPVGEADYFEY</td>
<td>EBV EBNA1</td>
<td>403–417</td>
<td>B35</td>
<td>(Rickinson and Moss, 1997)</td>
</tr>
<tr>
<td>QEFFWDANDIYRIFA</td>
<td>CMV pp65</td>
<td>511–525</td>
<td>B44</td>
<td>(Wills et al., 1996)</td>
</tr>
<tr>
<td>EENLDFVRF</td>
<td>EBV EBNA3c</td>
<td>281–290</td>
<td>B44</td>
<td>(Rickinson and Moss, 1997)</td>
</tr>
<tr>
<td>RKPRVTGGGAMAGA</td>
<td>CMV pp65</td>
<td>415–429</td>
<td>B7</td>
<td>(Wills et al., 1996)</td>
</tr>
<tr>
<td>RPQKRPSGCGCKGT</td>
<td>EBV EBNA1</td>
<td>71–85</td>
<td>B7</td>
<td>(Rickinson and Moss, 1997)</td>
</tr>
<tr>
<td>RKCRAKFQLLQHYR</td>
<td>EBV BZLF1</td>
<td>187–201</td>
<td>B8</td>
<td>(Rickinson and Moss, 1997)</td>
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<td>CTELKLSDY</td>
<td>Influenza A NP</td>
<td>44–52</td>
<td>A1</td>
<td>(DiBrino et al., 1993)</td>
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<tr>
<td>ILRGSVAHK</td>
<td>Influenza A NP</td>
<td>265–273</td>
<td>A3</td>
<td>(DiBrino et al., 1993)</td>
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</table>
### Table 2.3 HPV peptide pools

<table>
<thead>
<tr>
<th>Split pool</th>
<th>HPV16 E6 peptides (+ designated number)</th>
<th>HPV16 E7 peptides (+ designated number)</th>
<th>Split pool</th>
<th>HPV18 E6 peptides (+ designated number)</th>
<th>HPV18 E7 peptides (+ designated number)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>MHQKRTAMFQDPQER 16</td>
<td>MHGDTPTLHEYMLDL 1</td>
<td>1</td>
<td>MARFEDPTRRPYKLP 57</td>
<td>MHGPATLQDIVHL 41</td>
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<tr>
<td>2</td>
<td>AMFQDPQERPRKLPQ 17</td>
<td>TLHEYMLDLQPETTD 2</td>
<td>2</td>
<td>PTRRPYKLPDLCTEL 58</td>
<td>TLQDIVLHLPQNEI 42</td>
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<tr>
<td>3</td>
<td>QERPRKLPQLCTELQ 18</td>
<td>LDLQPTETTDLICYEQ 3</td>
<td>3</td>
<td>KLPDLCTELNTSLQD 59</td>
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</tr>
<tr>
<td>4</td>
<td>LPQLCTELQ11HTHIDI 19</td>
<td>TTDLYEQIDQNLDSSE 4</td>
<td>4</td>
<td>TELNTSLQIDETCV 60</td>
<td>NEIPVDLLLCEQILSD 44</td>
</tr>
<tr>
<td>5</td>
<td>ELQTTIDHIILECVY 20</td>
<td>YEQLNSDESEIDEID 5</td>
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<td>LLCHEQLESSEEND 45</td>
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<td>6</td>
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<td>SSEEEDIDGPGQIA 6</td>
<td>6</td>
<td>TCYVCKVQELTEVF 62</td>
<td>LSDSEEENIDGVN 46</td>
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<td>1</td>
<td>CVYCKQQLRREVYD 22</td>
<td>EIDGPAGQAEFPDRA 7</td>
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<td>QLLREVYDVFAFRDL 23</td>
<td>QYPFQLKDIQTPOQ 8</td>
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<td>RYSDDVSYGTDEKL 70</td>
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<td>4</td>
<td>RDLCIVYRDNQTV 25</td>
<td>KLPCLTEKQHRLDKK 35</td>
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<td>VYRDSIPHAACHCKI 66</td>
<td>EPQRHTLMCCCKCE 50</td>
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<td>YRDNQTVAYDCKCLK 26</td>
<td>EKTLNTGLNILLRC 4</td>
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<td>PHAACHKCIDFYISRI 67</td>
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<td>6</td>
<td>YAVCDKCLKFYSKIS 27</td>
<td>QYKINCPGLCCLCPQ 32</td>
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<td>IRCRQKPLPNAE 74</td>
<td>HSCCNARQERLQNRR 80</td>
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<td>CLKFYSKISEYRHYC 28</td>
<td>LCDLLIRCINCQKPL 33</td>
<td>1</td>
<td>SRIRELHYSVDSVG 69</td>
<td>ARQERLQRRRQRETQ 81</td>
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<td>KISEREHYCISLYGTYT 29</td>
<td>RCINQKPLCEEEQK 34</td>
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- TLHEYMLDLQPETTD 2
- LDLQPTETTDLICYEQ 3
- TTDLYEQIDQNLDSSE 4
- YEQLNSDESEIDEID 5
- SSEEEDIDGPGQIA 6
- EIDGPAGQAEFPDRA 7
- QCAEPDRAHYNIVTF 8
- RAHYNIVTFCCCKDS 9
- VFCCCKCDSTLRLCV 10
- CDSLRLCVQSTHVD 11
- LCQSTHVDIRTED 12
- HVDIRELTDLMGMITL 13
- LEDLMLMTLGVICPI 14
- GTLGIVCPICSQKP 15

**HPV18 E7 peptides (+ designated number):**
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- TLQDIVLHLPQNEI 42
- LHLQPNIEIPVDDL 43
- NEIPVDLLLCEQILSD 44
- LLCHEQLESSEEND 45
- LSDSEEENIDGVN 46
- ENDEIDGVHMNHQHLA 47
- GVNHQLHPPRAEAPQ 48
- LPAAPFRQHRMLC 49
- EPQRHTLMCCCKCE 50
- MLCMCKCEARIEL 51
- KCEARIELVVESSAD 52
- ELVVESSADDLRAFQ 53
- SADDLRAFQGLFNT 54
- AFQQLFLNTLSFVC 55
- LNTLSFVCPCASQQ 56
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2.5 Detection of peptide-specific T-cells by ELISpot

2.5.1 IFNϒ ELISpot Assay

ELISpot wells were set up either in triplicate or quadruplicate. All experiments included wells containing T-cells + APC, and T-cells + APC + peptide. In the initial screening and mapping experiments (of Chapter 3) wells containing cultured T-cells alone were included.

For a response to be deemed positive, the mean number of spots from the test wells (T-cells + APC + peptide) must be greater than 20 spots/1x10^5 cells, after subtraction of the background response from control wells (T-cells + APC). This criteria is more stringent than used by a number of other studies (Bourgault Villada et al., 2004; Godard et al., 2004; van der Burg et al., 2001; van Poelgeest et al., 2005), and should reduce the possibility of detecting false positives. The protocol used is as follows:

- The mouse anti-human IFNϒ antibody 1-DIK (Mabtech) was diluted to 10μg/ml with PBS, and 50μl was added to each well of an ELISpot plate.
  Plates were either incubated over night at 4°C, or for 3 hours at 37°C.
- Plates were washed thoroughly 5 times with 150μl PBS/well, before blocking with 100μl of FCS-RPMI media. Plates were incubated for 1 hour at room temperature, after which media was discarded.
- The responder cells (either cultured PBMC or T-cell lines) were added to the wells in 50μl of AB-RPMI media. The number of cells/well ranged from 5x10^2-1x10^5.
- The antigen presenting cells (PBMC/L-cells/BLCL) were then added in 50μl of AB-RPMI media to the appropriate wells at varying ratios that will be made clear in the individual experiments.
- The peptide was added to appropriate wells in 50μl of AB-RPMI media. In some experiments the APC were pre-pulsed with peptide for 90 minutes at 37°C. These cells were washed twice in PBS to remove unbound peptide,
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before adding to the ELISpot wells. Control wells that did not require peptide received 50µl of the media alone.

- The plate was then wrapped in silver foil and incubated at 37°C for 16-18 hours.
- Plates were washed 4 times with 150µl PBS and then incubated with 100µl sterile water for 10 minutes at room temperature, thus lysing the remaining bound cells. An additional incubation step was included where adherent cells were used, so as to avoid cell debris remaining bound to the membrane. This involved the incubation of 100µl PBS + 2mM EDTA for 5 minutes. The plate was then washed a further 5 times with 100µl/well PBS.
- The secondary biotinylated antibody 7-B6-1-Biotin (Mabtech) was diluted 1:1000 with PBS. This was added at 50µl/well and incubated in the dark at room temperature for 2 hours.
- The plate was washed 5 times with PBS, followed by the addition of 50µl of Streptavadin-Alkaline phosphatase (diluted 1:1000 with PBS) to each well. The plates were incubated for 2 hours in the dark at room temperature, before washing 5 times with PBS.
- Developing solution was made freshly using 25xAP colour development buffer (Bio-Rad), AP conjugate substrate (Bio-Rad), and sterile water. The solution was added at 50µl/well and left to develop in the dark for a minimum of 15 minutes, until the spots were clearly visible. To stop the developing reaction, tap water was used to wash the plates 6 times. Plates where then air dried in the dark before counting. Spots were initially all counted by eye using a stereomicroscope (magnification 3.2x) (Leica MZ6). However, near the end of this study, an automated ELISpot counting system became available; AID ELISpot reader (Cadama Medical).

2.5.2 HLA-DR blocking in ELISpot assays
In some experiments where the aim was to determine if HLA-DR molecules were capable of presenting a peptide, blocking antibodies were added directly to the ELISpot wells. L243 is a mouse IgG2a anti-human HLA-DR monoclonal blocking antibody, (Godkin et al., 2001). W6/32 is a mouse IgG2a anti-human HLA class I monoclonal
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blocking antibody (Peoples et al., 1993). The antibodies were added to the ELISpot wells at a concentration of 25μg of protein/ml.

2.5.3 Mapping Epitopes from HPV pool responses

When a HPV response was detected, the next step was to try and map the epitope/s that were being recognized. To do this, a new PBMC culture was set up with the HPV pool and cultured as before for 7 days (described in section 2.3.2). In the ‘mapping’ ELISpot, the whole HPV E6 pool was tested, along with the appropriate 6 split pools. This allowed the response to be narrowed down significantly. The remaining cells of the PBMC culture were then re-stimulated with the whole HPV pool as described in section 2.3.3, and cultured for 7 further days. This was in an effort to increase the number of peptide specific T-cells, to facilitate mapping of the individual epitopes. In a final ELISpot the whole pool, the positive split pool/s, and the individual peptides from the split pool/s were tested. For the majority of peptides identified as immunogenic, a new PBMC culture was set up with the peptide and cultured as before for 7 days, before testing in an ELISpot, in order to confirm the finding.

2.5.4 Enrichment of cell populations from PBMC using specific microbeads

Magnetic microbeads specific for either CD8 or CD14 were used in enrichment protocols. The CD8 enrichment was carried out using PBMC cultured with peptide for 7 days (8-12x10^6 input cells), whilst the CD14+ cells were enriched from freshly isolated PBMC (2x10^7 input cells). Cells were washed in PBS and spun twice at 272g in a 15ml Falcon tube. Supernatant was removed with a pipette and pellets were resuspended in 80μl cold MACs buffer and 20μl anti-CD8 or anti-CD14 microbeads (MACs, Miltenyi) per 10^7 cells. Cells were incubated for 15 minutes at 4°C and resuspended by flicking every 2-3 minutes. Pellets were then washed (in 20x the incubation volume) with cold MACs buffer and spun at 272 g for 10 minutes. Supernatant was removed and cells resuspended in 500μl cold MACs buffer. This was then loaded onto a magnetic MS column (Miltenyi). Unlabelled cells passed through the column, which was washed 3 times with 500μl MACs buffer. These cells were collected as the CD8 or CD14 depleted fraction. To collect the CD8 or CD14 enriched
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fraction, the column was removed from the magnetic field and 1ml MACs buffer was applied to the column. A plunger was then used to force the buffer and bead-labelled cells through.

2.6 Enrichment of IFNγ-secretting T-cells using magnetic beads

In order to enrich T-cells based upon their secretion of IFNγ, the ‘Cell enrichment and detection kit’ (MACs, Miltenyi) was adapted for use with cultured T-cells. All volumes of reagents added on day 7 were on a per 10⁷ cell basis, and all centrifugation was carried out for 10 minutes at 272 g at 4°C unless otherwise stated.

PBMC were isolated from 25 or 50mls of blood as described in section 2.2.2. Cultures were set up (as described in section 2.3.2) with either PPP (10µg/ml) or p37 (5µg/ml). On day 6 of culture, blood was taken from the same donor and the PBMC isolated were irradiated at 4,000 rads in FCS-RPMI. The cells were then washed and plated out at 10⁷/ml in 2 wells of a 48 well plate. Cells were incubated at 37°C for 2-3 hours. The non-adherent fraction was then removed by pipetting, and wells were washed with PBS twice. This step removed the majority of T-cells and enriched for APC, namely DC, B-cells and monocytes/macrophages. The adherent cells were incubated over night in 1ml of AB-RPMI.

On day 7, media was removed from the adherent PBMC wells and the cultured PBMC were added on top at 10⁷ cells/well in 1ml MACs culture media. To one well, either p37 or the PPP was added at 10µg/ml, and 2µl DMSO was added to the second well. A positive control was not included due to a limiting number of T-cells. Cells were incubated for 6 hours at 37°C, after which they were harvested into a 15ml Falcon tube. Cells were washed with 10mls of cold MACs buffer, and centrifuged. Supernatant was removed and the pellet was incubated on ice for 5 minutes with 80µl cold serum-free RPMI and 20µl of IFNγ catch reagent. The latter is a mouse anti-human IFNγ antibody conjugated to a mouse anti-human CD45 antibody. Therefore, all leukocytes are labelled with an antibody capable of binding secreted IFNγ.
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Cells were then diluted with 10mls of warm MACs culture media, thus restarting the IFNγ secretion process. Cells were incubated at 37°C for 45 minutes whilst being gently rotated using a MACsmix machine (Miltenyi). During this period, the secreted IFNγ was captured onto the surface of the cell that produced it. The IFNγ secretion was terminated by topping up the tube with cold MACs buffer and placing the falcon on ice for 2 minutes. Cells were centrifuged, supernatant removed, and the pellet was resuspended in 80μl of cold MACs buffer, 20μl of IFNγ detection antibody and 10μl of mouse anti-human CD4-FITC antibody. After 10 minutes incubation on ice, 10mls of cold MACs buffer was added to the tube and the cells were centrifuged. Supernatant was removed and the pellet was resuspended in 80μl cold MACs buffer and 20μl of anti-PE magnetic beads, mixed thoroughly and incubated at 4°C for 15 minutes. Cells were washed in 10mls of cold MACs buffer and the pellet was resuspended in 500μl cold MACs buffer. A small aliquot of 5μl (~1x10^5) was taken at this stage for later analysis by flow cytometry. The remaining cells were loaded onto a prepared MS magnetic column (Miltenyi). The unlabelled and labelled fractions were collected as described in section 2.5.4, with one additional step in which the labelled fraction was expelled and then reloaded onto a second MS column to ensure a high purity of IFNγ-secreting cells in the enriched population.

Aliquots of the pre and post enrichment fractions were analysed by flow cytometry. 7-AAD was added to the appropriate samples to allow exclusion of dead cells by negative gating during flow cytometric analysis (section 2.9.3).

2.7 Generating p37 specific T-cell lines

2.7.1 Culture of IFNγ enriched p37-specific T-cells
The 4x10^4 T-cells enriched in section 2.6, were seeded into 1 well of a 48 well plate in 0.5ml AB-RPMI. The media contained 20 U/ml IL-2, and 5x10^5 irradiated PBMC isolated from 3 allogenic donors. After 6 days, 0.5ml of fresh AB-RPMI + 20 U/ml IL-2 was added and the cells were cultured for a further 3 weeks. During this time half the culture media was replaced with fresh AB-RPMI + IL-2 when the media turned yellow.
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every 2-5 days). On day 14, the cell culture was split 1:2 and the two wells were re-stimulated with 1x10^6 irradiated allogenic PBMC. At 4 weeks post-enrichment, the cells were harvested and three different strategies were employed for the generation of T-cell lines. In the first, 1x10^6 T-cells were put into an allogenic feeder expansion (section 2.8), so as to generate large numbers of the enriched T-cells. Enriched cells were also used in the 96 well cloning protocol (section 2.7.2), so as to generate highly specific lines or clones. The remaining 1x10^6 T-cells were re-stimulated with 2x10^6 irradiated autologous PBMC and 5μg/ml p37 (see section 2.3.3).

2.7.2 Generating T-cell lines from IFNγ-enriched T-cells in 96 well cloning plates
A bulk cloning mix was added to the 60 innermost wells of ten 96-well, round-bottomed culture plates. The 50μl of cloning mix in each well consisted of AB-RPMI, 40U/ml IL-2, 2μg/ml PHA (Sigma), 5x10^4 irradiated allogenic PBMC (from 3 donors). The IFNγ enriched cells were diluted such that 1, 10, 100, or 1000 cells/well, were added in 50μl AB-RPMI to the appropriate wells. Seven plates were seeded with 1 cell/well and 1 plate each of the other cell densities was also set up. On day 7, cells were fed with 50μl of fresh AB-RPMI + 30U/ml IL-2. On day 14, 100μl was removed from each well and replaced with 100μl cloning mix with a final concentration of 20U/ml IL-2, 1μg/ml PHA, and 5x10^4 irradiated autologous PBMC (from 3 donors). Feeding and re-stimulating were alternated for 2 further weeks. Lines that had grown to a large pellet size, were plated out into new plates, split 1:2 or 1:3 and fed with AB-RPMI + IL-2 (20 IU/ml). By day 34 there were 189 different lines that had grown sufficiently to have a minimum of 2 well replicates. These cultures were all tested for recognition of the p37 by ELISpot (section 2.7.3).

2.7.3 Selection of peptide-specific T-cell lines from cloning plates
The 189 lines isolated from the cloning protocol in section 2.7.2 were tested for recognition of the p37 in an IFNγ ELISpot assay. This method is described in section 2.5.1, however this experiment differed in aspects of the ELISpot set up. Firstly, the precise number of T-cells in each well was not known, instead 7 different culture wells
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were harvested and counted and an average of 1.9x10^5 cells/well was calculated. An average of 3.5x10^4 cells were taken from each line and added to each of two wells of an ELISpot plate and AB-RPMI added to make the volume up to 50μl. The APC were autologous BLCL and these were added at 1x10^5 cells/well in 50μl AB-RPMI. As the number of responder cells was limiting, only 2 wells were set up, one of which was a control for spontaneous IFNγ secretion (T-cell + BLCL) and the other contained p37 (T-cell + BLCL + p37). p37 was added (at a final concentration of 5μg/ml) in 50μl AB-RPMI media to one well of each pair, whilst 50μl of AB-RPMI media alone was added to the control well.

It was decided that the 7 most specific lines would be expanded using the protocol described in section 2.8. Due to the large number of wells (~380), that needed to be examined in a short space of time, it was not possible to accurately count each well by eye using a microscope. Instead, a basic comparison was used; the (estimated) relative number of spots in the peptide wells (lines that generated high background in control wells were excluded); the size of the cell pellet in each lines replicate well. The latter is important as obviously if less cells generate more spots, the frequency of peptide-specific T-cells is higher. The plates have since been counted using an AID ELISpot reader (Cadama).

2.7.4 Cell sorting
2x10^7 line 46 T-cells were incubated with 15μl of anti-TCR Vβ16-PE antibody for 20 minutes at 4°C. Cells were then washed once with warm PBS before being resuspended in 0.5ml AB-RPMI. Cells were sorted using a MoFlo cell sorter (Dako), into two populations based upon the presence and absence of TCR Vβ16 staining.

2.8 Expansion of T-cell lines using an allogenic feeder system
This non-specific expansion protocol was used to generate large numbers of T-cell lines. In a T75 flask, 50mls of AB-RPMI was added, supplemented with 20U/ml IL-2, 0.5μg/ml PHA, and 2x10^7 irradiated autologous PBMC feeders (from 3 donors). 1x10^6 T-cells were added to the flask which was placed into a 37°C incubator tilted at 45°, so
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as to enhance cell-cell contact. When the number of T-cells available for expansion was less than $1 \times 10^5$, cells were seeded into a T25 tissue culture flask with half the amount of the above expansion mix. On day 5 of the expansion, half the media was removed and replaced with the same volume of AB-RPMI media supplemented with 20 U/ml IL-2. Cells were resuspended and cultured for a further 2 days upright. Cells were then harvested and plated out in fresh AB-RPMI supplemented with 20 U/ml IL-2, at $2 \times 10^6$ cells/well in 24 well plates. Every 2-3 days when media turned yellow, half the media was replaced with AB-RPMI + 20 U/ml IL-2.

2.9 Flow cytometric analysis and associated techniques

Cells were analysed using a BD FACs calibur and analysis was performed using CellQuest Pro analysis software (BD). Analysis of T-cell populations by flow cytometry involved the construction of a live lymphocyte gate which was based upon the forward and side scatter profiles of these cells.

2.9.1 Phenotyping human PBMC/T-cell populations

Expression of a number of different cell surface markers was tested for using the reagents in Table 2.4. Ordinarily, 1-2x10^5 cells were pelleted in wells of a 96 well, round-bottomed plate. The pellets were washed twice with 100μl cold FACs buffer and centrifuged at 755g at 4°C for 2 minutes. Supernatant was removed and the appropriate cells were resuspended in 2-3μl of commercially available antibody, or 20μl of the L243 antibody. For two-colour analysis, both antibodies (FITC and PE conjugated) were added in the same incubation. Cells were incubated for 15-30 minutes at 4°C in the dark. Cells were washed with 100μl cold FACs buffer and centrifuged at 755 g for 2 minutes at 4°C. Supernatant was removed and the wash process repeated twice. Where directly conjugated antibodies were used, the cells were resuspended in FACs buffer and were either fixed with PAF (described in section 2.9.2.1) or analysed immediately. Where un-conjugated antibodies were used, cells were subsequently incubated with the secondary rabbit anti-mouse-FITC antibody (Dako). Cells were incubated for 15-30 minutes at 4°C in the dark, before being washed 3 times with 100μl FACs buffer. Cells were then either fixed or analysed
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immediately. Negative control wells were included in all experiments. For directly conjugated antibodies a mouse IgG-FITC/PE (Dako) negative control was used. The control for un-conjugated antibodies was incubation with the rabbit α-mouse-FITC alone. A well to which nothing was added was also included as an additional control.

2.9.2 Fixation of cells using paraformaldehyde (PAF)

2.9.2.1 Fixation of FACs samples using PAF
After the staining and washing procedure of cells described in section 2.9.1, samples were sometimes fixed if analysis was not possible immediately. Cells were resuspended in 100μl FACs buffer + 100μl 4% PAF, giving a final concentration of 2% PAF. Cells were incubated at 4°C in the dark until analysis could be performed.

2.9.2.2 Fixation of BLCL for use in ICS
BLCL were harvested, counted, and washed in PBS (centrifuged at 425g for 5 minutes). The BLCL were resuspended in 500μl of PBS containing 2% PAF and incubated at 4°C for 20 minutes. The cells were then washed 2 times with PBS before resuspending the BLCL in AB-RPMI.

2.9.3 Staining with cell dyes
To exclude dead cells from the IFNγ-pullout FACs analysis (section 2.6) 7-AAD (Table 2.4), was used. Cells had been resuspended in 200μl FACs buffer, ready for analysis. 5μl of 7-AAD was added to the appropriate samples and incubated at room temperature in the dark for 10 minutes before analyzing by flow cytometry.

In some ICS assays the T-cells were pre-stained before combining with the APC population, allowing a clearer distinction between the two populations in the FACs analysis. Cells were harvested, washed in PBS, and then centrifuged at 425g. The pellet was resuspended in Cell Trace Far red DDAO-SE tracker dye (Molecular Probes, Invitrogen) (0.05-1μg dye/ml PBS), and incubated at 37°C for 15 minutes. AB-RPMI
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was added and cells were centrifuged at 425 g for 5 minutes. Cells were then used in
the ICS assay.

2.9.4 IFNγ-Intracellular Cytokine Staining (ICS) analysis of T-cell lines
T-cells were plated out in 0.5ml AB-RPMI at 5x10⁵ cells/well in a 48 well culture
plate. APC were either autologous PBMC, CD14+ enriched cells, or BLCL. In some
experiments a proportion of BLCL were fixed with 2% PAF prior to the ICS assay
(section 2.9.2.2). APC were added to the wells in 0.5ml AB-RPMI at 1x10⁶/well, and
the cells were incubated at 37°C in the presence of either p37 (0.05-5µg/ml),
recombinant E6 (1-40µg/ml), or DMSO. In the initial experiments, PHA 2µg/ml,
phorbol myristate acetate (PMA) (Sigma) 100ng/ml, ionomycin (Sigma) 1.5µg/ml and
Concavalin A (Sigma) 20µg/ml were added to an extra well to facilitate the set-up
procedure during FACs analysis. 1µl of golgi plug (BD) was added to each well after
90 minutes incubation and cells were further cultured for 11-13 hours. The following
day, cells were harvested into 15ml Falcon tubes and washed twice with PBS. Cell
pellets were then labelled with 5µl antibody (usually anti-TCR Vβ16, but occasionally
anti-CD3 or -CD4) and incubated for 25 minutes at 4°C. Cells were washed twice with
cold ICS sample buffer and centrifuged at 4°C for 4 minutes at 612g. The supernatant
was carefully removed and each cell pellet was resuspended in 250µl of
Cytoperm/Cytofix reagent (BD) and incubated at 4°C in the dark for 20 minutes. Cells
were washed twice with 1x perm/wash solution (BD), with the supernatant being
carefully removed by pipette to avoid cell loss. The cell pellets were resuspended in
50µl perm/wash + 2µl of mouse anti-human IFNγ antibody (see Table 2.4) and
incubated in the dark for 30 minutes at 4°C. During this period, cells were resuspended
by flicking the tube every 10 minutes. Cells were then washed twice with perm/wash.
The cells were resuspended in 300µl of ICS sample buffer and analysed by flow
cytometry.
Table 2.4 Antibodies and cell dye reagents used

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2.9.5 Analysis of the Th1/Th2 cytokine secretion profiles of lines Belx1 and Belx2 using cytometric bead array (CBA)

Cytokine production from lines Belx1 and Belx2 during peptide stimulation was analysed by using a human Th1/Th2 Cytokine Cytometric Bead Array assay (BD). The lines were harvested and plated out 5x10^4 cells/well in a 48 well plate. 1x10^5 autologous BLCL were added to each well in a final volume of 1ml AB-RPMI.

Duplicate wells were set up for each line as follows; T-cell + BLCL + DMSO, T-cell + BLCL + p37, and T-cell + BLCL + p64 (irrelevant peptide). The p37 and p64 were added at 5μg/ml. The cells were incubated at 37°C, and 60μl aliquots of supernatant were taken at 1, 6 and 10 hours. The aliquots were stored immediately at -20°C.

A CBA assay was carried out according to manufacturers instructions. Briefly, the assay allows the quantity of IFNy, IL-2, TNFα, IL-4, IL-5 and IL-10 within a supernatant sample to be assessed using flow cytometry. The CBA kit includes 6 different cytokine-capture bead populations (one specific for each cytokine). Each bead type can be discriminated in flow cytometry based upon their distinct fluorescent intensity. Aliquots of each capture bead (5μl/bead/test) were combined and 25μl of the bead mix was added to assay tubes. Th1/Th2 PE detection reagent (25μl/test) was then
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added to each assay tube, followed by 50μl of cytokine standards or test samples and the tubes were incubated for 3 hours at room temperature protected from light. The human recombinant Th1/Th2 cytokine standard was serially diluted with a concentration range from 5000pg/ml to 4pg/ml, with the diluent buffer used as a negative control (0pg/ml). Following incubation with the samples, the beads were washed and centrifuged at 425g for 5 minutes. Supernatant was carefully removed by pipette and each bead pellet was resuspended in 300μl of CBA wash buffer before analysis by flow cytometry. BD CBA analysis software was used to analyse the sample data. The limits of detection in this assay range from 6 to ~5000pg/ml.

Due to the high cost of the CBA kits, duplicate supernatants were analysed for 1 and 10 hour time points, whilst only one aliquot (for each condition) was analysed for the 6 hour time point.

2.9.6 CD107 in assessing the cytotoxic effector function of Belx2

A CD107 assay was carried out to investigate the cytotoxic behaviour of a T-cell line and clone following peptide stimulation (Betts et al., 2003). This assay allows the direct detection of degranulation by activated T-cells. CD107 is present in the membrane of cytotoxic granules. During T-cell degranulation the granule fuses with the T-cell plasma membrane and CD107 can be detected on the cell surface. Briefly, 5x10⁵ T-cells were incubated in 1ml AB-RPMI with 1x10⁶ BLCL in the presence and absence of either p37 (5μg/ml) or HPV16 E7 (29-38) (5μg/ml). BLCL were HLA-DRB1*01+ and HLA-A*02+. The CD107-FITC antibody was added to each well at 2μl/ml and golgi stop (BD) was added at 1μl/well. Cells were incubated at 37°C for 4 hours, after which they were harvested and washed (in FACs buffer) before analysing by flow cytometry.
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2.10 Viral Stocks
All viral stocks were stored at -70°C.

Wyeth
A Vaccinia virus strain used in the construction of TA-HPV. Viral titre =\(2 \times 10^8\) pfu/ml. The virus was kindly donated by Cantab Pharmaceuticals.

TA-HPV
A recombinant vaccinia virus based on the Wyeth vaccine strain, encoding modified forms of the HPV16 and HPV18 E6 and E7 proteins (Boursnell et al., 1996). Viral titre =\(2 \times 10^9\) pfu/ml. TA-HPV was kindly donated by Cantab Pharmaceuticals.

2.11 Infection of BLCL with TA-HPV or Wyeth vaccinia viruses
BLCL were infected with either TA-HPV or Wyeth at a multiplicity of infection of 15 pfu/cell and incubated at 37°C. After 1 hour, 3mls of FCS-RPMI was added and cells were incubated for 12 hours at 37°C. The BLCL were used the next day in an ICS assay (section 2.9.4) as the APC. As the BLCL were infected with a lytic virus, the incubation time of BLCL with T-cells in the ICS assay was decreased to 6 hours.

2.12 IFNγ-ELISA

2.12.1 Analysis of supernatant taken from p37-stimulated line B by IFNγ ELISA
T-cells were plated out at 1\(\times\)10\(^5\) cells/well in a 48 well plate. BLCL were added at a ratio of 2:1 in a final volume of 0.5 ml AB-RPMI. Cells were cultured in the presence of either p37 (5μg/ml) or DMSO (1μl/ml). Triplicate wells were set up for each of the time points measured. Cells were incubated at 37°C and 250μl aliquots of supernatant were taken at 2, 4, and 6, hours. Supernatants were stored at -20°C until they could be analysed by ELISA (section 2.12.2).

2.12.2 Analysis of IFNγ content of supernatants
A 96 well flat-bottomed Maxisorp plate (Nunc) was coated with 50μl of anti-human IFNγ antibody 1-D1K (diluted with coating buffer to 2μg/ml). Plates were incubated
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overnight at 4°C. The plates were washed twice with 200µl/well PBS. Blocking buffer was added at 100µl/well and incubated at room temperature for 1 hour. Wells were then washed 5 times with ELISA wash buffer. The IFNγ standard (Mabtech) was reconstituted according to manufacturers instructions to give a 10µg/ml stock which was then aliquoted and stored at -20°C. For each experiment, 1µl of thawed IFNγ stock was diluted 1 in 10,000 with dilution buffer to give a 1000pg/ml concentration. 200µl of the standard 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 supernatant samples were added in duplicate or triplicate at 100µl/well or were diluted appropriately with dilution buffer. The plates were incubated at room temperature for 2 hours and then washed 5 times with 200µl/well ELISA wash buffer. The antibody 7-B6-1-biotin was diluted in incubation buffer to 1µg/ml and added at 50µl/well. After 1 hour incubation at room temperature, the plate was washed 5 times and streptavidin-HRP reagent (diluted 1:1000 with incubation buffer) was added at 50µl/well. The plate was incubated for 1 hour at room temperature and washed 5 times. Substrate was made up by adding equal volumes of Substrate reagent A and reagent B (BD). 50µl of the substrate mix was added to each well and the plates were incubated in the dark for 15-30 minutes until the standards had clearly developed. The reaction was stopped by adding 50µl of ELISA stop reagent (1M phosphoric acid). The optical density was then analysed using a Benchmark Microplate reader (Bio-Rad) at 450nm. A standard curve was created using the serially diluted IFNγ standard, allowing the concentration of IFNγ in the test supernatants to be determined. The upper limit of the standard curve used was 500pg/ml.

2.13 Analysis of the TCR chains used by Belx1 and Belx2 T-cell lines using molecular techniques

2.13.1 Isolation of RNA from Belx1 and Belx2 T-cell lines

The lines Belx1 and Belx2 were harvested and 5x10^6 of each line was washed in PBS and centrifuged at 252 g for 5 minutes. Supernatant was removed and the pellets were
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resuspended and lysed in 1ml Trizol reagent (Invitrogen). The lysate was transferred to an eppendorf and 200µl of chloroform was added. The lysate was incubated at room temperature for 3 minutes and then centrifuged at 12,000 g for 15 minutes at 4°C. The upper aqueous phase was transferred into a new eppendorf and 500µl of isopropanol added. This was incubated for 10 minutes at room temperature, and then centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was removed, the pellet washed with 1ml 75% ethanol before centrifugation at 7,500 g for 5 minutes at 4°C. The supernatant was removed, and the pellets air-dried for -2 minutes. The pellet was resuspended in 50µl of RNAse-free water, 5µl was taken for determining optical density, and the remaining RNA was stored at -80°C.

The optical density of the samples was determined by diluting the RNA 1 in 100 with double distilled water. The absorbance was then measured at 260nm, from which the RNA concentration was calculated.

2.13.2 Cloning and sequencing of TCR α and β chains

The amplification, cloning and sequencing of the TCR α and β chains from Belx1 and Belx2 was performed by Dr. Jonathan Boulter (Cardiff University), essentially as described in Moysey et al., 2004. Briefly, 1-2µg of RNA (from each T-cell line) was subjected to a reverse transcription reaction using SuperScript III CellsDirect cDNA Synthesis System (Invitrogen). The cDNA produced was then used as template in nested PCR reactions using primer sets previously described (Moysey et al., 2004). The primer sets (see appendix 1) are capable of amplifying all possible α and β TCR chain variable regions. The amplified products were then cloned into a TA-cloning vector (pCR3.1) (Invitrogen) and sequenced using a vector-specific M13 primer. A comparison of the sequences detected with the IMGT database allowed the identification of the TCR α and β chains used by Belx1 and Belx2 T-cell lines.

2.14 Molecular modelling of peptide-HLA-DRB1*01 interactions

The modelling was carried out by Dr. Istvan Bartok, Imperial College, London and is as described in Quaratino et al., 2004. Briefly, the p37 model was constructed on a
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Silicon Graphics Octane 2 workstation using Insight II modelling suite (MSI/Accelrys), on the basis of previously described crystallised HLA-DRB1*01-peptide complexes (Stern et al., 1994; Sundberg et al., 2002; Zavala-Ruiz et al., 2004).

2.15 Buffers

Red blood cell lysis buffer
0.155M ammonium chloride (Sigma)
0.01M potassium bicarbonate (Sigma)
0.1mM potassium EDTA (Sigma)

MACs buffer
PBS
0.5% BSA (Sigma)
2mM EDTA (Sigma)

ELISPOT buffer for removing adherent cells
PBS
2mM EDTA

ICS sample buffer
PBS
1% FCS

1x ICS Perm/wash
10x perm/wash solution (BD) diluted 1:10 with sterile water → 1x perm/wash

FACS buffer
PBS
0.1% FCS
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**ELISA Coating buffer**
0.04M Na$_2$CO$_3$ (Sigma)
0.06M NaHCO$_3$ (Sigma)
pH should be 9.6 (+/- 0.1); adjust with HLC or NaOH.

**ELISA Blocking buffer**
PBS
1% FCS

**ELISA Wash buffer**
PBS
0.05% Tween (Sigma)

**ELISA dilution buffer**
PBS
0.1% BSA

**ELISA incubation buffer**
PBS
0.05% Tween
0.1% BSA
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Chapter 3

Defining CD4+ T-cell responses against HPV16 and HPV18 E6 and E7 proteins in healthy females

3.1 Introduction

Until recently the study of HPV16 and HPV18-specific T-cell responses has been restricted to women with current HPV infection, high grade cervical dysplasia, or cervical cancer (Bontkes et al., 2000; Kadish et al., 1997; Nakagawa et al., 1999; Todd et al., 2004). These studies have revealed important information regarding both the nature of the T-cell responses in the disease state and the viral proteins against which these responses are directed. The application of these findings in a therapeutic context is less clear. It could be argued that as the T-cell response in patients with cervical disease has failed to control the virus, the epitopes that have been defined may be ineffective in immunotherapy.

Several recent studies have investigated the memory CD4+ T-cell response against HPV16 and HPV18 E6 proteins in healthy women who have previously encountered and cleared the virus (de Jong et al., 2004; Facchinetti et al., 2005; Smith et al., 2005; Welters et al., 2003; Welters et al., 2005). In healthy women these memory T-cell responses are likely to have been a component of an effective anti-HPV immune response. Therefore, CD4+ T-cell epitopes identified from a memory response that correlated with viral clearance could be useful in the development of effective immunotherapeutic approaches. To date, the majority of studies has focussed on studying the overall immune response to HPV viral proteins without a specific view to mapping the epitopes from these responses (de Jong et al., 2002; de Jong et al., 2004; van Poelgeest et al., 2005; Welters et al., 2003). As few HPV specific CD4+ T-cell epitopes have been defined, the central aim of this project was to define novel HPV E6 and E7-derived T-cell epitopes from healthy women (presumed to have previously
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encountered and cleared the virus), in an attempt to contribute to this neglected area of the field.

To define HPV-specific T-cell epitopes it was first necessary to identify donors that respond to the HPV16 and HPV18 E6 and E7 peptide pools. Ten healthy female donors aged between 21 and 31 years, were recruited to the study. For ethical reasons we could not ask the donors if they had a history of abnormal smear results, and in most cases they did not offer this information. In this age group it would be expected that a number of donors would have encountered a HR HPV (Cuschieri et al., 2004).

The strategy employed to detect HPV responses is summarised in Figure 3.1. PBMC were isolated from donors blood and cultured with HPV peptide pools. These pools contained overlapping 15mer peptides spanning the entire length of the HPV16 and HPV18 E6 and E7 proteins. After 7 days in culture, PBMC were tested for the presence of peptide specific T-cells in an IFNγ ELISpot assay. This assay involves the re-stimulation of the cultured cells with the HPV pools. Each cell that secreted IFNγ was represented by a purple spot in the assay (Figure 3.2). The spots were enumerated using an automated ELISpot counter. A response was considered positive if the average number of spots in the test wells was ≥20 per 1x10^5 cells, after the subtraction of background. All spot values reported in this section (unless otherwise stated) are per 1x10^5 cells, after subtraction of background. This protocol is a well established method in this laboratory, in the detection of T-cell responses (Smith et al., 2005), and in this project it was used to define novel CD4+ T-cell epitopes. A number of other studies have used similar protocol for the detection of HPV-specific CD4+ T-cell responses from PBMC (van der Burg et al., 2001; Welters et al., 2003).

Additional cultures were set up in parallel with a positive peptide pool (PPP) containing common recall antigens as detailed in Table 2.2. This allowed the overall viability of the T-cells and their ability to respond to antigen to be assessed, as a PPP response would be expected from the vast majority of donors. This was especially important for donors that did not demonstrate an HPV specific response.
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Once HPV responders were identified, new PBMC cultures were set up with the positive HPV pool/s. In a second ELISpot assay, the response was narrowed down by using smaller split pools of peptide. If a split pool generated a response, the culture was re-stimulated for 7 days using irradiated autologous PBMC and the whole HPV peptide pool. This was done to further increase the number of peptide-specific T-cells, as it was possible that several sub-dominant epitopes could be contributing to an overall response. A final ELISpot assay in which individual peptides from the appropriate split pools were tested, allowed identification of the epitopes.
Figure 3.1 Overview of the protocol used to define epitopes from HPV16 E6 and HPV18 E6 responses in healthy female donors. A summary of the individual steps taken, leading from the screen for HPV responses, to the mapping of individual peptide epitopes. No E7 responses were detected and therefore this element of the screening process has been excluded for clarity.
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**Figure 3.2 Use of the IFNγ ELISpot assay in detecting peptide-specific T-cells.**
ELISpot wells were set up in (at least) triplicate with the cultured T-cells + APC, in the presence and absence of the test peptide. Each spot that develops in the assay represents an IFNγ-secreting T-cell. In the wells without peptide, a certain degree of non-specific IFNγ secretion occurs and this must be subtracted from the spot counts of the peptide test wells in order to determine the peptide specific response. The ELISpot well images shown above were captured using an AID ELISpot reader.

Positive ELISpot response =
Peptide response - Background response = ≥ 20 spots/1x10⁶ cells
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3.2 Screening female donors for HPV16 and HPV18 responses
Ten healthy donors were tested for responses to HPV16 and HPV18 E6 and E7 in the IFNγ ELISpot assay. The positive control pool (PPP) was included for all donors tested. This pool contains a mixture of CD4+ and CD8+ T-cell epitopes from common recall antigens.

3.2.1 Donor responses
Five of the ten donors tested (donors 1, 5, 6, 7, and 9), responded to HPV E6 peptides. PPP responses ranging from 107 to 390 spots were detected from all of these five donors. The HPV and PPP specific responses detected from the HPV responding donors are illustrated in Figure 3.3. No donors demonstrated immunity to the HPV E7 peptides. The five remaining donors (2, 3, 4, 8 and 10) did not elicit a HPV-specific T-cell response. PPP responses were however detected from all of these donors, with the average number of spots ranging from 102-188. The data for all donors who failed to demonstrate a HPV specific response is shown in Figure 3.4.

HPV responding donors

Donor 1
A positive response was only detected against the HPV16 E6 peptide pool, with an average of 41.0 spots (+/- 22.4) after subtraction of background.

Donor 5
Positive responses were detected against both HPV16 E6 and HPV18 E6 peptide pools. For the HPV16 E6 response, an average of 97.8 spots (+/- 24.3) was counted, whilst for the HPV18 E6, 199.3 spots (+/- 58.1) were detected.

Donor 6
In this experiment a response was detected against HPV16 E6 with 140.0 spots (+/- 90.1) detected. Against HPV18 E6 and 89.0 spots (+/- 82.2) were detected. Due to the high standard deviation it was deemed necessary to repeat these experiments, so as to
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confirm the results as positive. No positive response was detected against HPV18 E7 peptides, however, a weak positive response was detected against HPV16 E7 with 22.0 spots (+/- 53.7) counted. Due to the borderline nature of this response coupled with the high standard deviation, the experiment was repeated for all 3 dubious 'positive' responses. In the repeat experiment the HPV16 E7 response was negative with no spots detected. The HPV16 E6 and HPV18 E6 responses were confirmed as positive, with the detection of 96.8 spots (+/-39.0) and 74.0 spots (+/-17.4), respectively. The PPP response in the second experiment was >3 fold higher than detected in the previous experiment.

Donor 7
Positive responses were detected against the HPV16 E6 and HPV18 E6 peptide pools with an average count of 83.5 spots (+/-22.2), and 36.5 spots (+/-29.5), respectively.

Donor 9
A positive response was generated against the HPV18 E6 peptide pool with an average spot count of 97.8 (+/- 64.2).

To summarise, HPV specific T-cell responses were detected from five of the ten donors tested, of which one donor responded to HPV16 E6 alone, one donor responded to HPV18 E6 alone, and three donors responded to both HPV16 and HPV18 E6 peptides. The spot counts detected in these experiments range from 36.5 to 199 per 1x10^5 cells. For the remainder of this section, spot count values will only be stated where they fall well outside this range e.g. <20-25 and >350 spots.
Figure 3.3 HPV16 E6 and HPV18 E6 responses were detected by IFNγ ELISpot from 5 healthy female donors. PBMC from 10 donors were cultured with HPV16 E6, HPV16 E7, HPV18 E6 and HPV18 E7 peptides pools, and the control PPP. Cells were tested by IFNγ ELISpot assay against the 4 HPV pools (5μg/ml) and the PPP (10μg/ml). The results from donors 1, 5, 6, 7, and 9 who demonstrated an HPV specific response are shown. Background responses have been subtracted from data. The spot counts for PPP responses are not shown above 300. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown.
Figure 3.4 HPV specific responses were not detected from 5 healthy female donors by IFNγ ELISpot. PBMC from 10 donors were cultured with HPV16 E6, HPV16 E7, HPV18 E6 and HPV18 E7 peptides pools, and the control PPP. Cells were tested by IFNγ ELISpot assay. The results from donors 2, 3, 4, 8 and 10 who failed to make an HPV specific response are shown here. Background responses have been subtracted. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown.
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3.3 Mapping epitopes from HPV16 and HPV18 E6 peptide responses
Four donors demonstrated responses to HPV16 E6 and four responded to HPV18 E6, with three responding to both. To define the epitopes from these responses PBMC were cultured with the positive HPV peptide pools. Cultures were tested by ELISpot, first using split peptide pools, followed by the individual peptides (Figure 3.1).

Donor 1
PBMC were cultured with the HPV16 E6 pool and the PPP control. These cells were tested by ELISpot with the whole HPV16 E6 pool and 6 small split pools representing HPV16 E6. A strong positive response against the HPV16 E6 whole pool was detected. This response was narrowed down further to two split pools (Figure 3.5A). The pool 4 response was half that of the HPV16 E6 whole pool, whilst the split pool 5 response was relatively weak.

To map individual peptides from the pool 4 and pool 5 responses, PBMC cultures were re-stimulated with the whole HPV16 E6 pool and cultured for a further 7 days. Cells were tested by ELISpot against the whole HPV16 E6 pool, the split pools 4 and 5 and the individual peptides from these split pools (Figure 3.5B). The positive response to split pool 4 was mapped to p37. The p37 response was approximately equal to that of the HPV16 E6 whole pool response, suggesting it to be a dominant epitope in the HPV16 E6 pool. The response to p37 was confirmed by setting up a new PBMC culture with this peptide alone (Figure 3.14).

The split pool 5 response was shown to have doubled since the previous ELISpot, however none of the individual peptides tested elicited a positive response. A further attempt to map an epitope from split pool 5 failed, but did confirm the positive response (data not shown).

Overall from donor 1 an HPV16 E6 response was mapped to split pools 4 and 5. From split pool 4 response the novel epitope p37 was defined.
Figure 3.5 Mapping the HPV16 E6 response from donor 1 to split pools and individual peptides. PBMC cultured with the HPV16 E6 pool were tested by IFNγ ELISpot against the HPV16 E6 whole pool, and split pools 1-6 (A). PBMC cultures re-stimulated with the HPV16 E6 pool were tested by ELISpot against the whole HPV16 E6 pool, split pools 4 and 5, and individual peptides 19, 20, 25, 26, 31, 32, 37, and 38 (B). Background responses have been subtracted from the data shown here. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown.
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Donor 5

Donor 5 responses were previously detected against both the HPV16 E6 and HPV18 E6 peptide pools. New PBMC cultured with these HPV pools and the PPP control were tested by ELISpot assay against the HPV16 and HPV18 E6 pools and the corresponding split pools (Figures 3.6A and 3.7A respectively).

The response to HPV16 E6 was mapped to split pools 4 and 5. The split pool 4 response was half that of the HPV16 E6 whole pool response, whilst the split pool 5 response was far lower with only 24.1 spots (+/- 6.3) detected. The HPV18 E6 response was mapped to split pool 3. The split pool 3 response was approximately half that detected against the HPV18 E6 whole pool.

To define the epitopes from HPV16 E6 split pool 4 (Figure 3.6B) and from HPV18 E6 split pool 3 (Figure 3.7B), the PBMC cultures were re-stimulated with the appropriate HPV E6 peptide pools and cultured for a further 7 days. Cells were tested by ELISpot against the whole HPV pools, the positive split pools, and their respective individual peptides.

The HPV16 E6 response of 545.8 spots (+/- 14.0) was 3.5 fold higher than previously detected. The response to HPV16 E6 split pool 4 was also greatly increased to 474.8 spots (+/- 98.7). Of the individual HPV16 E6 peptides tested, a strong response of 502.0 spots (+/- 24.0) was detected against p37 alone. Individual split pool 5 peptides were not tested due to limiting cell numbers. The response against the HPV18 E6 split pool 3 was mapped to p65 alone. This response was approximately 60% of that detected against the whole HPV18 E6 pool. Responses to p37 (Figure 4.2B) and p65 (Figure 3.15) have both been confirmed as positive in separate experiments in which PBMC were cultured with the individual peptides alone and tested by ELISpot assay.

As mentioned above, the individual epitope/s from the HPV16 E6 split pool 5 response could not be mapped in this experiment. A second attempt was made to define this response from a day 7 PBMC culture. Yet again the pool 5 response was positive but
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no individual peptide could be identified. This raised the possibility that several peptides could be weakly contributing to the overall response.

To increase the magnitude of the split pool 5 response, PBMC were cultured with split pool 5 peptides alone for 14 days, with a re-stimulation step included on day 7. In the subsequent ELISpot assay (Figure 3.6C), pool 5 and the individual peptides were tested. Pool 5 gave a strong positive response with over 9 fold more spots than had previously been detected. The peptides 20, 26, and 32 also elicited positive responses in the range of 26-80 spots. Unfortunately due to limiting donor samples it was not possible perform a repeat experiment to confirm these responses, and as such they must be regarded as putative epitopes.

Overall from donor 5, a HPV16 E6 response was mapped to p37 from split pool 4, and an HPV18 E6 response was mapped to split pool 3 from which p65 was defined.
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Figure 3.6 Mapping the HPV16 E6 response from donor 5 to split pools and individual peptides. PBMC cultured with the HPV16 E6 pool were tested by IFNγ ELISpot against the HPV16 E6 whole pool, and split pools 1-6 (A). PBMC cultures were re-stimulated with the HPV16 E6 pool and tested by ELISpot against the whole HPV16 E6 pool, split pool 4 and individual peptides 19, 25, 31 and 37 (B). A separate PBMC culture was set up with split pool 5 peptides (5μg/ml) for 14 days and tested by ELISpot against the split pool 5, and the peptides 20, 26, 32 and 38 (C). Background responses were subtracted from this data. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown.
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HPV16 E6 +ve response

Test HPV16 E6 split pools 1-6

Split pools 4 and 5 are +ve

Test peptides from HPV16 E6 split pool 4

p37 from split pool 4 is +ve

Test peptides from HPV16 E6 split pool 5

Peptides 20, 26 and 32 appear +ve but have not been confirmed
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Figure 3.7 Mapping the HPV18 E6 response from donor 5 to split pools and individual peptides. PBMC cultured with the HPV18 E6 pool were tested by IFNγ ELISpot against the HPV18 E6 whole pool, and split pools 1-6 (A). PBMC cultures were re-stimulated with the HPV18 E6 pool and tested by ELISpot against the whole HPV18 E6 pool, split pool 3 and individual peptides 59, 65, 71 and 77 (B). Background responses have been subtracted from the data shown. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown.
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Donor 6
Fresh PBMC cultures were set up with the HPV16 E6 and HPV18 E6 peptide pools to allow further study of these responses. Cells were tested by ELISpot against the whole HPV E6 pools, and the appropriate split pools. The response against the HPV16 E6 whole pool was mapped to split pools 2, 3, 4 and 5 (Figure 3.8A). The split pool 3 response was the strongest, accounting for approximately 75% of the response to the HPV16 E6 whole pool. The pool 4 and pool 5 responses were fairly equivalent, however split pool 2 elicited a very low positive response with spot counts of only 23.4 (+/- 15.5). The HPV18 E6 peptide pool response was mapped to split pools 1, 4 and 6 (Figure 3.9A). Responses against split pools 1 and 4 were fairly equivalent, whilst the response to split pool 6 was approximately one third of that detected against the other two pools.

PBMC cultures re-stimulated with the appropriate HPV pools were tested by ELISpot against the whole HPV pools, the split pools 3, 4 and 5 from HPV16 E6, and split pools 1, 4, and 6 from HPV18 E6. Due to limiting cell numbers it was only possible to test the individual peptides from the HPV16 E6 split pool 3 (Figure 3.8B). Responses to split pools 3, 4, and 5 responses were confirmed as positive. The split pool 3 response of 425.5 spots (+/- 38.7), was mapped to p30 and p36. The p30 (Figure 4.5) and p36 (data not shown) responses were confirmed as positive (similar frequency as detected here) in later experiments using PBMC cultured with the individual peptides alone. The responses against HPV18 E6 (526.8 spots (+/- 30.7)) and the split pools 1, 4 and 6 were confirmed as positive, but no epitope mapping was possible due to limiting cell numbers (Figure 3.9B). Two subsequent attempts to map epitopes from the responses against HPV16 E6 split pools 4 and 5, and HPV18 E6 split pools 1, 4 and 6, were unsuccessful due to technical issues.

Overall, donor 6 responded to both HPV16 and HPV18 E6 peptide pools. The 16 E6 response was mapped to split pools 2, 3, 4 and 5 demonstrating the breadth of the response. Peptides 30 and 36 were both mapped from split pool 3. The HPV18 E6 response was mapped to pools 1, 4 and 6, but no individual peptides were defined.
Figure 3.8 Mapping the HPV16 E6 response from donor 6 to split pools and individual peptides. PBMC cultured with the HPV16 E6 pool were tested by IFNγ ELISpot against the HPV16 E6 whole pool, and split pools 1-6 (A). PBMC cultures re-stimulated with the HPV16 E6 pool were tested by ELISpot against the whole HPV16 E6 pool, split pool 3 and individual peptides 18, 24, 30 and 36, and split pools 4 and 5 (B). Background responses were subtracted from the data shown. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown.
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Figure 3.9 Mapping the HPV18 E6 response from donor 6 to split pools and individual peptides. PBMC cultured with the HPV18 E6 pool were tested by IFNγ ELISpot against the HPV18 E6 whole pool, and split pools 1-6 (A). PBMC cultures were re-stimulated with the HPV18 E6 pool and tested by ELISpot against the whole HPV18 E6 pool and split pools 1, 4, and 6 (B). Background responses were subtracted from the data shown. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown.
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Donor 7
This donor previously demonstrated responses to both HPV16 E6 and HPV18 E6 peptide pools. PBMC were cultured with the two HPV E6 pools and the PPP control. After 7 days in culture, cells were tested in the ELISpot assay against the whole HPV E6 pools and their respective split pools. The HPV16 E6 response was mapped solely to split pool 4 (24.3 spots (+/- 10.4)) (Figure 3.10A). The HPV18 E6 response was mapped exclusively to split pool 2 (Figure 3.11A).

PBMC cultures re-stimulated with the appropriate HPV pools were tested by ELISpot against the HPV16 E6 split pool 4 and corresponding individual peptides, or against the HPV18 E6 whole pool, split pool 2 and the corresponding individual peptides. Due to limiting cell numbers it was not possible to test against the HPV16 E6 whole pool. The HPV16 E6 split pool 4 response was mapped to p19 (Figure 3.10B). The HPV18 E6 split pool 2 response was mapped to p64 (Figure 3.11B). The p64 response was later confirmed to be positive (Figure 3.16). The p19 response was also confirmed as positive in a later experiment in which PBMC were cultured with p19 alone before testing in an ELISpot assay (data not shown).

Overall, donor 7 responded to both HPV16 E6 and HPV18 E6 peptide pools. The HPV16 E6 response was mapped to peptide 19 in split pool 4, and the HPV18 E6 response was mapped to p64 from split pool 2.
Figure 3.10 Mapping the HPV16 E6 response from donor 7 to split pools and individual peptides. PBMC cultured with the HPV16 E6 pool were tested by IFNγ ELISPOT against the HPV16 E6 whole pool, and split pools 1-6 (A). PBMC cultures were re-stimulated with the HPV16 E6 pool and tested by ELISPOT against the HPV16 E6 split pool 4 and individual peptides 19, 25, 31 and 37 (B). Background responses were subtracted from the data shown. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown.
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**Figure 3.11 Mapping the HPV18 E6 response from donor 7 to split pools and individual peptides.** PBMC cultured with the HPV18 E6 pool were tested by IFNγ ELISpot against the HPV18 E6 whole pool, and split pools 1-6 (A). PBMC cultures were re-stimulated with the HPV18 E6 pool and tested by ELISpot against the whole HPV18 E6 pool, split pool 2 and individual peptides 58, 64, 70 and 76 (B). Background responses were subtracted from the data shown. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown.
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Donor 9

PBMC cultured with the HPV18 E6 peptide pool were tested by ELISPOT for recognition of the HPV18 E6 whole pool and the 6 split pools. The HPV18 E6 pool response was mapped to split pools 2, 3 and 5 (Figure 3.12A).

PBMC re-stimulated with the HPV18 E6 peptide pool were cultured for a further 7 days. Cells were tested in the ELISPOT against the whole HPV18 E6 pool, split pools 2 and 5, and the respective individual peptides (Figure 3.12B). Due to limiting cell numbers pool 3 peptides could not be tested. The split pool 5 response was weak and no individual epitope was mapped. The response to split pool 2 however, was mapped to peptide 64. The response against p64 was later confirmed as positive in an ELISPOT assay using PBMC cultured with p64 alone (data not shown).

Overall, the HPV18 E6 response detected from donor 9 was mapped to split pools 2, 3 and 5. p64 from split pool 2 was the only epitope identified.

Summary of Epitopes Mapping

In this section four immunogenic epitopes were defined from the HPV16 E6 peptide pool and two from the HPV18 E6 peptide pool. Table 3.1 summarises the epitopes defined from each donor and allows the magnitude of response to each epitope to be compared.
Figure 3.12 Mapping the HPV18 E6 response from donor 9 to split pools and individual peptides. PBMC cultured with the HPV18 E6 pool were tested by IFNγ ELISpot against the HPV18 E6 whole pool and split pools 1-6 (A). PBMC cultures were re-stimulated with the HPV18 E6 pool and tested by ELISpot against the whole HPV18 E6 pool, split pools 2 and 5 and the individual peptides 58, 61, 64, 67, 70, 73, 76 and 79 (B). Background responses were subtracted from the data shown. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown.
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Table 3.1 Summary of all epitopes mapped from the HPV16 E6 and HPV18 E6 responding donors. Data is from the ELISPot epitope mapping experiments performed at day 14 of culture for all HPV responding donors. Specific spot counts are shown with the standard deviation (background subtracted). The non-specific responses (background) are shown in brackets below the specific spot counts.

<table>
<thead>
<tr>
<th>Donors</th>
<th>HPV16 E6 peptides</th>
<th>HPV18 E6 peptides</th>
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<tbody>
<tr>
<td></td>
<td>Epitopes</td>
<td>Spots/1x10^6 cells</td>
</tr>
<tr>
<td>1</td>
<td>Peptide 37 (127-141)</td>
<td>293 +/- 73.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(background 0.8)</td>
</tr>
<tr>
<td>5</td>
<td>Peptide 37 (127-141)</td>
<td>502 +/- 24.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(background 36.0)</td>
</tr>
<tr>
<td>6</td>
<td>Peptide 30 (85-99)</td>
<td>219.5 +/- 25.8</td>
</tr>
<tr>
<td></td>
<td>Peptide 36 (121-135)</td>
<td>382.8 +/- 38.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(background 48.5)</td>
</tr>
<tr>
<td>7</td>
<td>Peptide 19 (19-33)</td>
<td>35.1 +/- 13.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(background 18.6)</td>
</tr>
<tr>
<td>9</td>
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3.4 Demonstration of epitope recognition by CD4+ T-cells

The ELISpot assay detects IFNγ-secreting CD4+ or CD8+ T-cells. The peptides used in this study are 15mers which can potentially be recognised by both CD4+ and CD8+ T-cells. It was therefore important to establish the phenotype of those T-cells responding in the ELISpot assay.

CD8+ enriched and CD4+ enriched T-cell fractions were obtained from cultured PBMC using MACs anti-CD8 microbeads (section 2.5.4). Briefly, after 7 days in culture, PBMC were incubated with anti-CD8+ beads, allowing labelling of the CD8+ T-cells. Cells were then passed through a magnetic column, permitting bead-labelled CD8+ T-cells to be retained in the column (CD8+ enriched), whilst the remaining PBMC flowed through the column (CD4+ enriched). These fractions were then tested by ELISpot to determine which contained the responding cells.

The enrichment protocol was first tested with PBMC cultured for 7 days with PPP. The CD4+ and CD8+ enriched fractions were analysed by flow cytometry to determine the purity of the fractions (Figure 3.13). The cells were stained for expression of CD3, CD4 or CD8 and analysed by flow cytometry. In the initial PBMC culture, the CD3+ cells (within the lymphocyte gate) were constituted of 58% CD4+ T-cells and 31% CD8+ T-cells. After separation, the CD8+ enriched fraction contained 92% CD8+ CD3+ T-cells and 5% CD4+ CD3+ T-cells. The CD4+ enriched fraction contained 23% CD8+ CD3+ T-cells, whilst the CD4+ T-cell population was increased to 66%.

This data indicates that this system does not provide a high degree of purity in the negatively selected population (CD4+ enriched). In each subsequent experiment the purity of the CD8+ T-cell enriched fraction was always greater than 90% (data not shown). Having established that this enrichment protocol was appropriate, the HPV peptide studies were initiated.
Figure 3.13 Flow cytometric analysis of CD8+ and CD4+ enriched PBMC fractions generated by magnetic-based cell sorting. PBMC were sorted into the 2 fractions using magnetic anti-CD8 microbeads. The whole PBMC, CD4+ enriched and CD8+ enriched cells were co-stained using anti-CD3 PE, anti-CD4 FITC, and anti-CD8 FITC antibodies, before analysis by flow cytometry. T-cells were gated based upon their forward and side scatter profile.
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Donor 1
PBMC were cultured with p37 alone for 7 days. Cells were sorted into CD8+ and CD4+ enriched fractions which were tested for recognition of p37 in the ELISpot assay, as well as whole cultured PBMC (Figure 3.14). The responses detected from the CD4+ enriched population was >8 fold higher than detected with the CD8+ enriched fraction. The weak positive p37 response detected from the CD8+ enriched fraction was mostly likely due to the presence of contaminating CD4+ cells. The recognition of p37 by CD4+ T-cells was later confirmed (Figure 4.10).

Donor 5
PBMC cultures were set up with p65 alone. On day 7 cells were sorted into CD4+ and CD8+ T-cell enriched fractions and tested for recognition of p65 by ELISpot (Figure 3.15). The CD8+ enriched fraction did not generate a positive response, whilst for the CD4+ enriched fraction 209.3 spots (+/- 42.8) were detected. This is good evidence that CD4+ T-cells and not CD8+ T-cells recognise p65.

Donor 7
PBMC cultures were set up with p64 alone for 7 days. Cells were sorted into two enriched fractions and tested by ELISpot for recognition of p64. Due to technical issues this first experiment failed, however the remaining cells from the PBMC culture were used on day 8 to generate the enriched fractions which were tested by ELISpot (Figure 3.16). Due to limiting cell numbers it was not possible to include whole cultured PBMC in the experiment. The CD8+ enriched fraction did not generate a positive response. In contrast the CD4+ enriched fraction generated spot counts of 69.5 (+/- 20.9). This suggests that the epitope is recognised by CD4+ T-cells.
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**Figure 3.14 Donor 1: The CD4+ enriched fraction recognises HPV16 E6 p37 in an ELISpot assay.** PBMC were cultured with p37 for 7 days. Cells were then sorted into CD4+ and CD8+ enriched fractions. The whole PBMC, CD4+ enriched and CD8+ enriched cells were tested by ELISpot for recognition of p37. Background responses were subtracted from the data shown.

**Figure 3.15 Donor 5: The CD4+ enriched fraction recognises HPV18 E6 p65 in an ELISpot assay.** PBMC were cultured with p65 for 7 days. Cells were then sorted into CD4+ and CD8+ enriched fractions. The whole PBMC, CD4+ enriched and CD8+ enriched cells were tested by ELISpot for recognition of p65. Background responses were subtracted from the data shown.
Figure 3.16 Donor 7: The CD4+ enriched fraction recognises HPV18 E6 p64 in an IFNγ ELISpot assay. PBMC were cultured with p64 for 7 days. Cells were then sorted into CD4+ and CD8+ enriched fractions. The CD4+ and CD8+ enriched fractions were tested by ELISpot for recognition of p64. Background responses were subtracted from the data shown.
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3.5 Discussion
In the first stage of this study ten healthy female donors were tested for memory T-cell responses against the HPV16 and HPV18 E6 and E7 peptide pools. In total, five donors responded to either HPV16 E6 or HPV18 E6 peptides. Interestingly, HPV specific responses from donors 1 and 7 had previously been detected two years before the initiation of this study (Smith et al., 2005). No responses were detected against HPV16 or HPV18 E7 peptide pools. This is unsurprising as several studies have already shown that memory T-cell responses against the E7 protein in healthy women are very rare (van der Burg et al., 2001; Welters et al., 2005).

An HPV-specific response was not detected from five of the ten donors tested. However, each of these donors generated positive responses to the PPP control, demonstrating their T-cells were capable of responding to antigen. All ten donors demonstrated a strong response against the PPP whose magnitude was higher than any HPV specific response. This is unsurprising as the PPP contains CD4+ and CD8+ T-cell epitopes from common recall antigens such as EBV, CMV and Influenza A (Currier et al., 2002; Gelder et al., 1995; Rickinson and Moss, 1997), and responses can be detected directly ex vivo.

40% of donors tested displayed immunity against the HPV16 E6 peptide pool. Although the cohort used was relatively small, the frequency of HPV16 E6 immunity detected is similar to that found by other studies (Welters et al., 2003). HPV18 E6 responses were also detected in 40% of donors. The frequency of this response was higher than previously reported (Facchinetti et al., 2005; Smith et al., 2005; Welters et al., 2005), but more accurate measures both HPV16 and HPV18 immunity could be obtained studying larger cohorts.

Immunity to both HPV16 and HPV18 E6 was detected in three of the ten donors. Infection with multiple high risk HPVs is not uncommon in young women. In a recent report by Cuschieri et al., a large number of cervical smear samples from primary care clinics were tested for the presence of HPV DNA (Cuschieri et al., 2004). In women
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aged between 16 and 25 years, 57% of those who tested positive for a HR-HPV, were also positive for at least one other HR-HPV type. The two most prevalent HR HPV types detected were HPV16 and HPV18 (Cuschieri et al., 2004). It is unlikely that the peptide responses detected in this study are due to cross-reactivity between the two viruses. Firstly the homology between HPV16 E6 and HPV18 E6 is relatively low (55%) (Welters et al., 2005). Also by mapping epitopes from the majority of responses, it is clear that HPV16 E6 and HPV18 E6 responses from the same donor are being directed against peptides from non-homologous regions (Figure 3.17).

![Figure 3.17: Position of peptides defined from the HPV16 and HPV18 E6 protein.](image)

The positions of the HPV16 E6 peptides 19, 30, 36 and 37 and the HPV18 E6 peptides 64 and 65 defined in this study are depicted. The full length E6 protein is shown. The conserved cysteine residues (C) involved in the formation of zinc binding regions are shown.
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From the HPV16 and HPV18 E6 responses, six T-cell epitopes have been mapped using the ELISpot assay from PBMC cultures. To date, very few HPV-specific CD4+ T-cell epitopes have been mapped from healthy women and the protocol used here represents a powerful and efficient approach to define a relatively large number of HPV-specific epitopes without extended culture periods.

The ELISpot assay was selected for use in the detection of low frequency peptide-specific T-cells in cultured PBMC. The ELISpot is a very sensitive technique, detecting antigen-specific T-cells at frequencies lower than 1 in 10,000 (Helms et al., 2000). It is also a simple, cost effective and robust assay, making it ideally suited for screening large numbers of donors for immune responses. The assay is particularly useful where low cell numbers are available for analysis (Shacklett et al., 2003). Techniques such as intracellular cytokine staining (ICS) and chromium release cytotoxicity assays require far higher cell numbers, and are more useful in the analysis of T-cell lines and clones, rather than detecting low frequency responses.

This assay has been used successfully by a number of groups studying T-cell responses against a wide range of viruses (Draenert et al., 2003; Godard et al., 2004; Goon et al., 2002; Li Pira et al., 2004; Tan et al., 1999). Welters et al., 2003 have previously shown that HPV16 E6-specific memory CD4+ T-cell responses can be detected by ELISpot from 60% of healthy donors. They were able to detect these responses after 4 days of in vitro culture with HPV16 E6 32mer peptides overlapping by 14 residues (Welters et al., 2003). In this study, PBMC were cultured for 7 days before testing by ELISpot and the magnitude of the responses detected were often several fold higher than those detected by Welters et al., 2003. Several other studies have also detected similar magnitudes of T-cell responses to HPV16 and HPV18 E6 peptides, once the data is adjusted for differences in methodology (de Jong et al., 2002; Facchinetti et al., 2005; van der Burg et al., 2001; Welters et al., 2005). The criteria used to define a positive response of ≥20 spots per 1x10^5 cells after subtraction of background is either similar or more stringent than that used by several other groups (Bourgault Villada et al., 2004; Godard et al., 2004; van der Burg et al., 2001; van Poelgeest et al., 2005). In
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cases where a response was not a clear cut positive e.g. high standard deviation, a repeat experiment was performed.

The requirement for a period of *in vitro* culture in order to detect the HPV-specific memory T-cells has been shown by a number of groups (de Jong *et al.*, 2002; Facchinetti *et al.*, 2005; van der Burg *et al.*, 2001; van Poelgeest *et al.*, 2005). One possible explanation is that the cells detected in the ELISpot assay are central memory CD4+ T-cells, rather than effector memory T-cells. Central memory T-cells require re-stimulation with antigen followed by a period of expansion and differentiation in order to acquire their effector function (Lanzavecchia and Sallusto, 2005; Sallusto *et al.*, 1999). In contrast, effector memory T-cells can secrete IFNγ within hours of re-stimulation with antigen (discussed in section 1.8.2.2.7) and would therefore be detectable *ex vivo* by ELISpot. In this laboratory it has not been possible to reliably detect HPV specific memory T-cells *ex vivo* (personal communication, Dr. S. Man). In healthy donors who have cleared HPV infection, this may be because the HPV-specific effector CD4+ T-cells are no longer present, or the precursor frequency has dropped below the levels of detection.

In patients with either recent or current HPV16 infection or related disease, it has recently been shown that E6-specific CD4+ and CD8+ T-cell responses can be detected *ex vivo* by ELISpot using 30-35mer overlapping peptides (Steele *et al.*, 2005). The criteria used in that study to assign positivity to a response, is very different to the criteria used in this study. A specific frequency of responding cells was not used, instead a patients response against a particular epitope was only considered positive if the spot count was greater than that obtained from the virgin control group. This meant that for some epitopes a positive response could be as low as 5 spots/1x10^3 T-cells, whilst in others a response would need to be greater than ~50 spots/1x10^5 T-cells in order to be deemed positive. For this reason it is likely that some false positives will have been included in the data, highlighting the usefulness of generating peptide-specific T-cell lines in the confirmation of responses. In addition to this, the cells used in the ELISpot assay were either CD4 or CD8 enriched, and the positive cut-off point
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for an epitope was often different for the CD4 versus the CD8 fraction. Due to these methodological and analytical differences it is not simple to compare the magnitude of responses detected by the ex vivo study with this investigation. In terms of CD4+ T-cell responses against E6 peptides, no ex vivo response detected was greater than 80 spots/1x10⁵ CD4+ T-cells. It is most likely that the T-cells detected in this ex vivo study are either effector or effector memory T-cells due to the recent or current HPV infection.

The frequency of HPV16 and HPV18 E6 responses detected in this study after 7 days of in vitro culture rarely exceeds 0.2% of the PBMC. This suggests that the precursor frequency of the HPV-specific T-cells in vivo is much lower. When considered next to viruses such as CMV and EBV these frequencies appear comparatively low. Dunn et al., 2002 reports that up to 10% of CD4+ T-cells tested directly ex vivo from a healthy CMV seropositive individual can be specific for CMV (Dunn et al., 2002). CD4+ T-cell responses to EBV latent-cycle proteins can also be detected ex vivo from healthy virus carriers by IFNγ ELISpot, with a frequency of up to 28 spots per 1x10⁵ PBMC (Leen et al., 2001).

In this study a re-stimulation step was included in the in vitro culture protocol on day 7, so as to further increase the frequency of peptide-specific T-cells. This was deemed necessary as it was likely that some responses to weakly immunogenic peptides would not be detected by day 7. The inclusion of the re-stimulation step routinely correlated with an increased number of peptide-specific T-cells. It is possible that this increase could in part be a result of non-specific T-cells dying and the expansion of T-cells directed against sub-dominant epitopes. Figure 3.18 shows examples of these boosted responses in donors 1, 5 and 6. In most cases the responses increased at least 2 fold. Larger increases were also detected, the most impressive being the donor 5 response to HPV16 E6 split pool 4, which saw a 6 fold rise.

The HPV peptides used in this investigation were all 15mers overlapping by 9 amino acids. As the majority of naturally presented HLA class II epitopes have been shown to
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range from 13 and 17 amino acids (Rudensky et al., 1991), it is possible that some responses have been missed using the 15mers. It has previously been shown that both CD4+ and CD8+ T-cell responses can be detected against 15mer peptides derived from viral proteins using the IFNγ ELISpot assay (Draenert et al., 2003; Li Pira et al., 2004). However in this study all three epitopes tested in the CD4/CD8 enrichment experiments were confirmed to be recognised by CD4+ T-cells. In chapter 4 it was further demonstrated that two additional peptides are also likely to be CD4+ T-cell epitopes through HLA-DR blocking studies. Thus it appears that this protocol preferentially detects CD4+ T-cell epitopes.

In the second part of this chapter, four HPV16 E6 epitopes were mapped: p19 (E6(19-33)), p30 (E6(85-99)), p36 (E6(121-135)), and p37 (E6(127-141)). All peptide responses have been confirmed as positive from the analysis of PBMC cultured with the individual peptide alone. Based on the current literature (2005) these CD4+ T-cell epitopes have not previously been defined and are therefore novel.

The p37 response was detected in both donor 1 and donor 5 suggesting that it could be a dominant epitope in HLA-DRB1*01+ individuals. Interestingly, donors 1 and 5 also demonstrated responses to the HPV16 E6 split pool 5. Initial attempts to map epitopes from this response in both donors failed. This suggested that more than one epitope was weakly contributing to the pool 5 response. To investigate this, donor 5 PBMC were cultured with pool 5 peptides alone instead of the HPV16 E6 whole pool, thus preventing the generation of the dominant p37 epitope response. The frequency of pool 5 specific T-cells generated was higher than previously detected and peptides 20, 26 and 32 were all mapped from this response. Unfortunately due to limiting donor blood samples, it has not been possible to repeat these results. These peptides are however putative epitopes worthy of further investigation. This illustrates some of the potential problems that can be encountered with epitope mapping as it tends to favour the identification of immunodominant epitopes.
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The two epitopes p64 and p65 were mapped from HPV18 E6 responses. p65 (E6\(_{69-83}\)) was mapped from the response detected in donor 5. This year Facchinetti et al., 2005, published an almost identical CD4+ T-cell epitope HPV18 E6\(_{52-66}\). This is significant for several reasons. Firstly, it demonstrates that this epitope can be defined using two different protocols, which helps to validate both the method and the peptide response. Further to this, the physiological relevance of the E6\(_{52-66}\) epitope was demonstrated as it was shown to be naturally processed (using PBMC-APC) from a full length E6 protein. The second epitope p64 (HPV18 E6\(_{63-77}\)) was detected in donors 7 and 9, suggesting it may be a common response in a particular HLA class II context. This is a novel epitope having not previously been published. In donors 6 and 9 it was not possible to map epitopes from every positive split pool, however both donors did demonstrate broad responses against the HPV18 E6 pool.

The testing of CD4 or CD8 enriched populations of cultured PBMC strongly suggested that peptides 37, 64 and 65 were all recognised by CD4+ T-cells. As mentioned previously an E6 epitope almost identical to p65 has been shown to be CD4+ T-cell-restricted which supports this data (Facchinetti et al., 2005). Based on these findings it appears that this method preferentially detects epitopes recognised by CD4+ T-cells.

In summary, 50% of the healthy women tested responded to HPV16 E6 and/or HPV18 E6 peptides. From these responses six epitopes have been defined, five of which are novel. Of the three epitopes examined in further detail, all were shown to be recognised by CD4+ T-cells. Overall the protocol used here has proved to be a powerful and efficient method for the rapid definition of CD4+ T-cell epitopes.
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Figure 3.18 Re-stimulation of PBMC cultures correlates with an increase in the number of IFNγ-secreting peptide-specific T-cells. PBMC were cultured for 7 days with HPV peptide pools before being tested by ELISpot against HPV split pools. Remaining cultured PBMC were re-stimulated with the whole HPV pool using irradiated autologous PBMC as antigen presenting cells. Cultures were tested on day 14 against the split pools and individual peptides. Shown here is a comparison of the average spot counts on days 7 and 14, from donors 1 (A), 5 (B), and 6 (C), generated against HPV16 E6 whole pool and split pools.
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Chapter 4

Characterisation of polyclonal T-cell lines generated against HPV E6 peptides

4.1 Introduction
In chapter 3, six T-cell epitopes were defined from HPV E6 peptide pools using short term PBMC cultures. To confirm the immunogenicity of these epitopes attempts were made to generate long term, stable T-cell lines. In this chapter the successful generation of several T-cell lines is described together with partial functional and phenotypic characterisation.

4.2 Investigating the potential role of HLA-DR in the presentation of HPV16 and HPV18 E6 epitopes

4.2.1 Validating the use of L243 and W6/32 in antibody blocking studies
The majority of defined peptide epitopes recognised by CD4+ T-cells are presented by either HLA-DR or HLA-DQ molecules. To determine if the peptides defined in this study were presented by HLA-DR, the monoclonal antibody L243 was used in an HLA-DR blocking ELISpot experiment. This binding prevents T-cell recognition of DR-restricted epitopes. Other studies have used this antibody in HLA-DR blocking experiments (Elferink et al., 1985; Gehring et al., 2003; Godkin et al., 2001). The isotype control antibody selected for use is W6/32, a monoclonal antibody that has been used previously as an HLA class I blocking studies (Peoples et al., 1993).

The blocking experiments were performed as described in section 2.5.2. L243 or W6/32 was added to peptide-containing ELISpot wells at 25µg/ml. This concentration has been shown to be appropriate for use in DR blocking studies (Godkin et al., 2001). Before proceeding with the HPV E6 peptide investigation it was first necessary to demonstrate that L243 (at the specified concentration) did not non-specifically decrease T-cell responses to peptide. Clone 25, a CD8+ T-cell clone was selected for use in this
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preliminary antibody blocking ELISpot assay (Figure 4.1). The clone recognises an HLA-A2 restricted Influenza A Matrix peptide M1_{58-66} (Lawson \textit{et al.}, 2001). Clone 25 was plated out with BLCL and the M1 peptide either in the presence or absence of L243 or W6/32. The addition of W6/32 resulted in a marked reduction in the number of spots detected. L243 addition however, had no effect on the T-cell response. In view of this data it was concluded that at 25\mu g/ml L243 does not demonstrate a non-specific blocking effect and was therefore appropriate for use in the next stage of this study.
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Figure 4.1 Recognition of M1_{58-60} by Clone 25 is reduced by the addition of W6/32, but not L243. Clone 25 was plated out at 1x10^3 cells/well of an ELISpot plate and HLA-A*02+ BLCL were added at a ratio of 1:1. Cells were incubated either with media alone or with M1 peptide (10μg/ml) in the presence and absence of either L243 (25μg/ml) or W6/32 (25μg/ml). Background responses have been subtracted from the data shown.
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4.2.2 Peptides 30, 37, 64 and 65 are presented by HLA-DR molecules
In this section the potential role of HLA-DR molecules in the presentation of peptides 30, 37, 64 and 65 was investigated. Due to a limiting number of blood samples from donors 6 and 7, it was not possible to include p36 and p19 in this stage of the investigation. The HLA haplotypes of the blood donors were determined as described in section 2.2.3.

PBMC were cultured with the individual HPV peptides for 7 or 14 days (section 2.3.2), before being tested in the HLA-DR blocking ELISpot assay. It was anticipated that if a peptide required HLA-DR for its presentation no response would be detected in a well containing L243. The data stated in this section is per $1\times10^5$ cells after the subtraction of background. In all experiments the addition of the isotype control antibody W6/32 did not exhibit any blocking effects.

Peptide 37
The p37 response was detected in PBMC cultures from donors 1 and 5. The HLA-DR types of these donors are shown in Table 4.1. To determine if p37 was presented by HLA-DR, PBMC were cultured with p37 for 14 days before being tested using the L243 DR blocking ELISpot protocol (Figure 4.2A). The addition of L243 to p37 containing wells resulted in a complete abrogation of the response.

An identical experiment was also carried out using PBMC cultures from donor 5 (Figure 4.2B). Again the number of spots in the L243 wells was greatly reduced from that detected in the p37 alone wells (96% decrease). Collectively the data from donors 1 and 5 clearly indicates that p37 is an HLA-DR restricted epitope.

Peptide 64
The p64 response was detected in donors 7 and 9. The HLA-DR types of these donors are shown in Table 4.1. Donor 7 PBMC cultured with p64 for 14 days were tested in the HLA-DR blocking ELISpot (Figure 4.3). The presence of L243 in p64 containing wells was shown to reduce the number of spots detected by 97%.
<table>
<thead>
<tr>
<th>Donor</th>
<th>HLA-DR</th>
<th>Peptide Defined</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B1*01</td>
<td>p37&lt;sub&gt;(127-141)&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>B1*03</td>
<td>DKKQRFFHNIRGRWTG</td>
</tr>
<tr>
<td>5</td>
<td>B1*01</td>
<td>p37&lt;sub&gt;(127-141)&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>B1*14</td>
<td>DKKQRFFHNIRGRWTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p65&lt;sub&gt;(49-63)&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FKDLFVYRDSIPHA</td>
</tr>
<tr>
<td>6</td>
<td>B1*07</td>
<td>p30&lt;sub&gt;(85-99)&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>B1*13</td>
<td>HYCYSLYGTTLEQQY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p36&lt;sub&gt;(121-135)&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EKQRHLDKKQRFHNI</td>
</tr>
<tr>
<td>7</td>
<td>B1*04</td>
<td>p64&lt;sub&gt;(43-57)&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>B1*15</td>
<td>EVFEFAFKDLFVYYR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p19&lt;sub&gt;(19-33)&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPQILCTELQTTIHDI</td>
</tr>
<tr>
<td>9</td>
<td>B1*11</td>
<td>p64&lt;sub&gt;(43-57)&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>B1*15</td>
<td>EVFEFAFKDLFVYYR</td>
</tr>
</tbody>
</table>

Table 4.1 HLA-DR types of donors from which HPV E6 epitopes were defined.
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Figure 4.2 Demonstration that peptide 37 is presented by HLA-DR through the use of the HLA-DR blocking antibody L243 in an ELISpot assay. PBMC from donors 1 and 5 were cultured with p37 for 14 days. Cultured cells were plated out at 1x10^5 cells/well in an ELISpot plate, to which autologous PBMC were added at a ratio of 1:2. Donor 1 PBMC (A) and donor 5 PBMC (B) were tested with p37 (5μg/ml) in the presence and absence of L243 (25μg/ml) or W6/32 (25μg/ml). Background responses have been subtracted from the data.
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Figure 4.3 Demonstration that peptide 64 is presented by HLA-DR through the use of the HLA-DR blocking antibody L243 in an ELISpot assay. PBMC from donor 7 were cultured with p64 for 14 days before testing in an ELISpot assay. Cultured cells were plated out at 1x10^5 cells/well in an ELISpot plate to which autologous PBMC were added at a ratio of 1:2. Cells were incubated with either media alone or p64 (5μg/ml) in the presence and absence of L243 (25μg/ml) or W6/32 (25μg/ml). Background responses have been subtracted from the data.
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**Peptide 65**
The response to p65 was only detected in PBMC from donor 5. The HLA-DR type of donor 5 is shown in Table 4.1. Donor 5 PBMC cultured with p65 for 7 days were tested in the HLA-DR blocking ELISpot assay (Figure 4.4). The response to p65 was shown to be completely inhibited by the addition of L243, clearly suggesting this peptide is presented by HLA-DR.

**Peptide 30**
The p30 response was detected in PBMC from donor 6. The HLA-DR types for this donor are shown in Table 4.1. PBMC were cultured for 7 days with p30 before testing in the HLA-DR blocking ELISpot protocol (Figure 4.5). In the presence of L243 no spots were detected after the subtraction of background, indicating the involvement of HLA-DR in p30 presentation.

All four peptides tested in the antibody blocking experiments were shown to be presented by HLA-DR. The donors from which these peptides were defined have all been HLA typed making it possible to nominate specific HLA-DR allotypes that could be involved in presentation. Table 4.1 shows the HLA-DR types of each donor along with the corresponding peptide epitope.

A subsequent aim in this study was to identify the HLA-DR allotypes involved in the presentation of newly defined epitopes. The peptides 37 and 64 were selected for study as responses to these epitopes had been defined from two donors and for each peptide there was one strong candidate HLA-DR allotype identified.
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Figure 4.4 Demonstration that peptide 65 is presented by the HLA-DR molecule through the use of the HLA-DR blocking antibody L243 in an ELISpot assay.
Donor 5 PBMC were cultured with p65 for 7 days before testing in an ELISpot assay. Cultured cells were plated out at 1x10⁵ cells/well in an ELISpot plate, to which autologous PBMC were added at a ratio of 1:2. Cells were incubated with either media alone or p65 (5µg/ml) in the presence and absence of L243 (25µg/ml) or W6/32 (25µg/ml). Background responses have been subtracted from the data.

Figure 4.5 Demonstration that peptide 30 is presented by the HLA-DR molecule through the use of the HLA-DR blocking antibody L243 in an ELISpot assay.
PBM were cultured with p30 for 7 days before testing in an ELISpot assay. Cultured cells were plated out at 1x10⁵ cells/well in an ELISpot plate, to which autologous PBMC were added at a ratio of 1:2. Cells were incubated with either media alone or p30 (5µg/ml) in the presence and absence of L243 (25µg/ml) or W6/32 (25µg/ml). Background responses have been subtracted from the data.
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4.3 Characterisation of the peptide 64 T-cell response

4.3.1 Generation of a peptide 64-specific T-cell line
In order to precisely define the HLA-DR allotype involved in the presentation of p64, large numbers of a line with a maintainable specificity was required for use in ELISpot assays. To generate the p64 specific T-cell line, PBMC were cultured with the peptide for 7 days (culture conditions described in section 2.3.2) after which 5x10^5 cells were expanded using non-specific stimulation (section 2.8). An ELISpot assay carried out prior to expansion, demonstrated that approximately 0.27% of the cultured PBMC recognised p64 (data not shown).

The expansion yielded 2.6x10^7 cells (52 fold increase), which rose to 6x10^7 cells after one week in culture with media containing IL-2. The line was tested by ELISpot to determine if the frequency of responding T-cells had changed post expansion. The number of spots detected halved, revealing only 0.13% of the line now recognised p64. However this was still an appropriate level of response for use in the HLA class II restriction studies.

4.3.2 Identification of the HLA-DR allotype responsible for peptide 64 presentation through the use of allogenic PBMC-APC
To determine the HLA-DR allotype responsible for p64 presentation, a panel of eleven HLA typed PBMC was employed as the antigen presenting cells (PBMC-APC) in ELISpot assays. In theory the specific HLA class II molecule/s responsible for presentation could be pinpointed based upon which of the HLA-typed PBMC-APC demonstrated the ability to present p64 to the T-cell line.

PBMC-APC were selected based specifically on their similarities or differences with the HLA class II haplotype of donors 7 and 9 (from whom the p64 response was originally defined). The only HLA-DR allotype common to these responding donors was HLA-DRB1*15, making it a strong candidate for p64 presentation. Whilst this allotype appeared the most likely molecule involved in p64 presentation, it was still
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necessary to exclude the other potential candidates. HLA-DRB1*04 and HLA-
DRB1*11 expressed by donor 7 and donor 9 respectively could potentially be capable
of p64 presentation. In section 4.2.2, p64 was shown to be presented by HLA-DR,
however this did not rule out the possibility that HLA-DQ molecules could also be
capable of presenting the peptide. As both donors 7 and 9 express HLA-DQB1*03 and
-DQB1*06, the potential involvement of these HLA-DQ allotypes in peptide
presentation was also assessed in this experiment. Table 4.2 shows the HLA-DR and
HLA-DQ types of the PBMC-APC used in these experiments.

The p64 T-cell line was tested in an ELISpot assay on day 10 post expansion. The
ability of different PBMC-APC to present p64 to the T-cell line was assessed (Figure
4.6). Of the eleven PBMC-APCs tested, six were able to present p64 to the T-cell line.
These PBMC-APCs were all HLA-DRB1*15+ and no other HLA-DR allele was
common to all. These positive responses ranged from 60.0 to 121.2 spots, with a mean
response of 90.5. No positive response to p64 was detected using the 5 PBMC-APC
that were negative for HLA-DRB1*15. Based upon the HLA class II haplotypes of
these five PBMC-APC (Table 4.2), the other candidate allotypes (HLA-DRB1*04 and
-DRB1*11, and -DQB1*03 and -DQB1*06), were shown to be uninvolved in the
presentation p64. This data clearly indicates that p64 is presented by HLA-DRB1*15.

It was possible to confirm these results through the use of APCs expressing only one
HLA-DR allotype. The APCs selected were mouse L-cells (murine fibroblast lines
described in Table 2.1), kindly donated by Dr. Altmann (Imperial College, UK) and Dr.
Lombardi (Guy's Kings and St. Thomas' School of Medicine, University of London,
UK). Other studies have used HLA-DR transfected mouse L-cells in antigen
presentation experiments (Altmann et al., 1989; Lombardi et al., 1989; Wilkinson et
al., 1988). In this experiment both wild type L-cells (DR-) and L-cells transfected with
either HLA-DRB1*01, -DRB1*04, or DRB1*15 were used as APC in an ELISpot
assay (Figure 4.7). The p64 T-cell line was tested on day 12 post expansion. The
ELISpot revealed a weak yet positive response with the p64-pulsed HLA-DRB1*15 L-
cells, with a spot count of 21.8 (+/- 8.4). No positive response was detected using the
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other L-cells. Although this provides further evidence that HLA-DRB1*15 can present p64, the response detected was >4 fold lower than the average response detected using the HLA-DRB1*15 PBMC-APC (90.5 spots).

p64 was studied in parallel with the p37 study. It was decided that p37 would become the focus for the remainder of this project due to the interesting results obtained.
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<table>
<thead>
<tr>
<th></th>
<th>HLA-DR</th>
<th>HLA-DQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive p64 response</td>
<td>DR4, DR15 *1</td>
<td>DQ3, DQ6</td>
</tr>
<tr>
<td>detected</td>
<td>DR11, DR15 *2</td>
<td>DQ3, DQ6</td>
</tr>
<tr>
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<td>DR1, DR15</td>
<td>DQ5, DQ6</td>
</tr>
<tr>
<td></td>
<td>DR3, DR15</td>
<td>DQ2, DQ6</td>
</tr>
<tr>
<td></td>
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<td>DQ6, DQ8</td>
</tr>
<tr>
<td></td>
<td>DR12, DR13</td>
<td>DQ1, DQ3</td>
</tr>
</tbody>
</table>

*1 Donor 7 PBMC
*2 Donor 9 PBMC

Table 4.2 HLA-DR and HLA-DQ types of the PBMC-APC used to determine the HLA-DR allotype involved in the presentation of p64.

Figure 4.6 Demonstration that peptide 64 is presented by HLA-DRB1*15 using HLA typed PBMC-APC. The p64 T-cell line was tested on day 10 post expansion in an ELISpot assay. 1x10^5 T-cells were incubated in wells of an ELISpot plate with 11 HLA-typed PBMC-APC at a ratio of 1:1. The HLA-DR haplotype for each PBMC-APC is shown. The cells were incubated in the presence and absence p64 (5μg/ml). Background responses have been subtracted from the data.
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![Graph showing spot forming cells per 1x10^5 cells for different conditions: DR-, DR1, DR4, DR15.]

**Figure 4.7** Mouse L-cells transfected with HLA-DRB1*15 can present peptide 64 in an ELISpot assay to the peptide 64-specific T-cell line. The p64-specific T-cell line was tested in an ELISpot assay on day 12 post expansion. 1x10^5 T-cells were plated out in quadruplicate with the L-cells at a ratio of 1:2. The L-cells (either DR-, DR1, DR4, or DR15), had been pulsed prior to the assay for 90 minutes in the presence of either 5µg/ml p64 or an equivalent volume of DMSO. L-cells were washed to remove unbound peptide before adding to the assay. Background responses have been subtracted from the data shown.
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4.4 Generation and characterisation of peptide 37-specific polyclonal T-cell lines

4.4.1 Generation of peptide 37-specific T-cell lines
To characterise the p37 response in detail it was necessary to generate highly specific T-cell lines. An approach was employed involving the magnetic enrichment of peptide specific T-cells based upon their secretion of IFNγ (section 2.6). These enriched T-cells were then used to generate several T-cell lines using several different strategies (summarised in Figure 4.8).

4.4.1.1 Enrichment of IFNγ-secreting T-cells using magnetic-based cell sorting
A pilot experiment was carried out to ensure that the cultured peptide specific T-cells could be enriched using this protocol. Fresh PBMC were cultured for 7 days with the PPP (Table 2.2). The cultured cells were then seeded into 2 wells containing irradiated autologous (adherent) PBMC. PPP was added to the first well at 10μg/ml and an equivalent volume of DMSO was added to the second, to serve as a negative control for non-specific IFNγ secretion. IFNγ secreting T-cells were labelled with anti-PE magnetic beads, allowing them to be detected by flow cytometry and enriched using magnetic columns. Cells were taken for analysis pre and post magnetic enrichment and stained with an anti-CD4-FITC antibody before analysis by flow cytometry to assess the enrichment of the IFNγ+ T-cells (Figure 4.9).

Analysis of the pre-sort PPP re-stimulated sample showed that 1.5% of cells within the lymphocyte gate were CD4+ and IFNγ+, whilst 0.6% were CD4- and IFNγ+. The latter population presumably represents the PPP-specific CD8+ T-cells. Both of these populations were greatly enriched in the post-sort fraction. The enrichment of IFNγ+ cells was greater for the CD4- population than the CD4+ population. The CD4+ IFNγ+ population was enriched by 19 fold whilst the CD4- IFNγ+ cells were increased by 86 fold. It was unexpected to see such a marked difference in the enrichment of two IFNγ+ populations from the same culture. One feasible explanation is that the
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Donor 1 PBMC + peptide 37

7 days

Enrichment of p37-specific T-cells based on IFNγ-secretion and magnetic-based sorting

6x10⁴ IFNγ-enriched T-cells

4 weeks in culture to increase cell numbers

3x10⁶ IFNγ-enriched T-cells

1x10⁶ cells expanded

1x10⁶ cells re-stimulated with p37 for 14 days

96 well cloning plates

Expansion

Expansion of 7 p37-specific T-cell lines

Lines 12, 46, 62, 68, 85, 87, 178

Line 1

Line B

Figure 4.8 Overview of the process by which peptide 37-specific T-cell lines were generated from donor 1 PBMC.
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Figure 4.9 Enrichment of IFNγ-secreting PPP-specific T-cells from cultured PBMC using a magnetic bead cell sorting system. PBMC were cultured with PPP for 7 days. After re-stimulation with PPP the IFNγ-secreting cells were stained with anti-IFNγ-PE antibody and were enriched using anti-PE magnetic beads. Cells were also stained with an anti-CD4 FITC antibody. Data shown here represents the percentage of IFNγ-secreting cells within the lymphocyte gate of the PPP re-stimulated culture, pre- and post-sorting. The enrichment data for both IFNγ-secreting CD4+ and CD4- cells are displayed. T-cells were gated based upon their forward and side scatter profile, and dead cells were excluded from analysis using negative gating, based on 7-AAD staining.
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efficiency of enrichment is related to the quantity of IFNγ produced by the T-cells. In essence a cell that secretes more IFNγ would become more highly labelled with the anti-IFNγ-PE antibody. This in turn may increase the cells association with the anti-PE magnetic beads, improving its chance of magnetic enrichment. In support of this idea, the MFI of IFNγ staining for the CD4- cells in the pre-sort fraction was over 2 fold higher than observed for the CD4+ cells, a result indicative of superior IFNγ production by the CD4- cells. Further to this, the MFI of IFNγ+ cells in the post-sort fraction is several fold higher for both the CD4+ and CD4- populations (compared to the pre-sort fraction), suggesting that this method preferentially selects for cells that secrete high levels of IFNγ.

As expected, the yield obtained from the cells incubated with DMSO was very low compared to that of the PPP re-stimulated cells (data not shown). With the enrichment protocol shown to be effective, it was then possible to proceed to generate a p37-specific T-cell line.

PBMC from donor 1 were cultured with p37 for 7 days, after which the p37-specific T-cells were enriched using the protocol described above. Analysis by flow cytometry revealed that all IFNγ-secreting cells were CD4+ T-cells (Figure 4.10). In the p37 re-stimulated cultures IFNγ+ cells were enriched from 0.7% to 61.7% of cells within the lymphocyte gate. As previously described with the enrichment of PPP cultured cells, the MFI of the IFNγ+ cells in the post-sort fraction was over 2 times higher than in the pre-sort fraction. The yield from the enrichment of DMSO treated cells (data not shown) was 30 fold lower than obtained from the p37 re-stimulated cultures, from which 6x10⁴ cells were collected.

The implementation of a magnetic-based cell sorting technique permitted the isolation of an enriched p37-specific Th1 CD4+ T-cell population. These enriched cells were then used in the generation of T-cell lines containing high frequencies of p37-specific T-cells.
Figure 4.10 Enrichment of IFNγ-secreting p37-specific T-cells from a PBMC culture using a magnetic bead cell sorting system. PBMC were cultured with p37 for 7 days. After re-stimulation with p37 the IFNγ-secreting cells were stained with anti-IFNγ-PE antibody and were enriched using anti-PE magnetic beads. Cells were also stained with an anti-CD4-FITC antibody. The pre-sort (A) and post-sort (B) fractions were analysed by flow cytometry. Values shown in the dot plots represent the percentage of gated cells in each quadrant. T-cells were gated based upon their forward and side scatter profile, and dead cells were excluded from analysis using negative gating, based on 7-AAD staining.
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4.4.1.2 Generation of line 1 through the expansion of IFNγ-enriched T-cells

The enriched p37-specific T-cells were cultured for several weeks (section 2.7.1) to increase cell numbers, before using them to generate T-cell lines. To increase the likelihood of generating a highly specific T-cell line from the enriched cells, three different methods were utilised; expansion (section 2.8); peptide re-stimulation (section 2.3.3) followed by expansion (section 2.8); cloning in 96 well plates (section 2.7.2).

The first method involved the non-specific expansion of $1\times10^6$ enriched cells to secure a large stock of these cells. From this expansion over $1\times10^8$ cells were recovered and these are referred to as line 1. Analysis by flow cytometry revealed that $>99\%$ of line 1 are CD4+ T-cells. The frequency of p37-specific T-cells in line 1 was estimated using the ELISpot assay, in which the T-cells were incubated with autologous BLCL in the presence and absence of p37. After the subtraction of background approximately 1.8% of T-cells were estimated to have responded to p37 (Figure 4.11). As an additional control in this experiment the HPV18 E7 peptide pool was included as an irrelevant peptide control. A positive response was not detected in these wells. Wells containing T-cells with p37 alone were also set up, and no spots were detected from these wells.
Figure 4.11 The frequency of peptide 37-specific T-cells in line 1 as assessed by ELISpot. The line 1 T-cells were plated out at 2\times10^4 cells/well and incubated either alone with p37 (5\mu g/ml), or with autologous BLCL at a ratio of 1:1 in the presence and absence of peptide 37(5\mu g/ml). The HPV18 E7 peptide pool (5\mu g/ml) was included as an irrelevant control. Background responses have been subtracted from the data.
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4.4.1.3 Generation of p37-specific T-cell lines using a 96 well plate cloning protocol
To generate highly specific T-cell lines, the IFNγ-enriched cells were seeded at varying concentrations into ten 96 well cloning plates as described in section 2.7.2. After five weeks 189 cultures had grown sufficiently to have at least two replicate wells. These were tested by ELISpot with p37 to indicate the specificity of each culture (section 2.7.3). To help estimate the average cell number in the cultures, replicate wells from seven different cultures were counted. The cell counts ranged from $1 \times 10^5$ to $3 \times 10^5$, with a mean average of $1.9 \times 10^5$ cells/well. Based upon this an estimated $3.5 \times 10^4$ cells from each culture were plated out into two ELISpot wells. Autologous BLCL were added to all wells and p37 was added to one of each well pair.

The purpose of the ELISpot was to assist selection of the seven most specific cultures for further study. At the time when this experiment was undertaken an automated ELISpot counter was unavailable and therefore the wells had to be assessed by eye. Cultures 12, 46, 62, 68, 85, 87 and 178 were identified to be among the most specific. Cells from each culture were expanded using non-specific stimulus (section 2.8). More recently it was possible to count the ELISpot plates using an automated ELISpot counter. From these results (Figure 4.12) it can be seen that the vast majority of cultures recognised p37, although with greatly varying magnitudes of response. The spot counts for the aforementioned cultures selected for expansion ranged from 107 to 313, with a mean average of 216.3 spots detected. The actual frequency of p37-specific T-cells in each line could not be determined in this experiment as the exact number of cells added per well was unknown.

The seven lines selected, were tested for p37 recognition in an ELISpot assay 7 days post expansion (Figure 4.13). The number of spots detected (per $2 \times 10^4$ cells) from each line ranged from 23 to 113 after background subtraction. Lines 46, 68 and 87 demonstrated the greatest number of spots with respective counts of 113.0, 99.3, and 98. These three lines were all shown to consist of >99% CD4+ T-cells using flow cytometry (data not shown).
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Figure 4.12 189 lines generated from 96 well cloning plates were screened for recognition of p37 by ELISpot assay. On average 3.5x10^4 cells/well from each culture were plated out with 1x10^5 autologous BLCL in the presence and absence of p37 (5 μg/ml). For each condition there was only one well. The results from the 189 lines tested with p37 are spread over the 4 graphs. Those lines selected for expansion are highlighted (*). Background responses have been subtracted.
Figure 4.13 The specificity of seven T-cell lines derived from the 96 well cloning plate strategy was assessed by ELISpot. Lines 12, 46, 62, 68, 85, 87 and 178 were tested for recognition of p37 in an ELISpot assay. Each line was plated out at $2 \times 10^3$ cells/well to which autologous BLCL were added at a ratio of 1:1, in the presence and absence of p37 (5μg/ml). Background responses were subtracted from this data.
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4.4.1.4 Generation of line B from IFNγ-enriched cells through p37 re-stimulation
The remaining cells that were not expanded or used in the cloning protocol, were re-
stimulated with p37 using irradiated autologous PBMC at a ratio of 1 T-cell:2 PBMC.
The cells were cultured for 14 days before putting them into an expansion (section 2.8).
This additional period of re-stimulation (described in section 2.3.3) was included in an
attempt to further increase the specificity of the line, which is hereon referred to as line
B. This was seen as a necessary precaution in case the specificity of line 1 was not
maintained during expansion. In total over 2x10⁸ line B cells were harvested from two
T75 expansions.

Analysis by flow cytometry revealed that line B consisted of > 99% CD4 T-cells (data
not shown). The specificity of this line was estimated 7 days post expansion using the
ELISpot assay (data not shown). Line B was plated out at 2x10⁴ cells/well with
autologous PBMC at a ratio of 1:1. After the subtraction of background approximately
1.6% of the line was estimated to have responded to p37. The magnitude of this p37
response is very similar to that obtained with line 1.

4.5 Characterisation of polyclonal T-cell lines and the p37 response
From the enriched p37-specific T-cells (section 4.4), line 1, line B and lines 12, 46, 62,
68, 85, 87 and 178 were all generated. In this section a number of these lines were
characterised in more detail in terms of the specificity of the p37-specific T-cells, the
IFNγ secretion response and the TCR Vβ usage. The lines also proved to be useful
tools in investigating the HLA-DR allotype responsible for p37 presentation.

4.5.1 Investigating the HLA-DR allotype responsible for the presentation of
peptide 37 to T-cell lines
In section 4.2.2, HLA-DR was shown to present p37. As both donors 1 and 5 share
expression HLA-DRB1*01, it seemed likely that this specific allotype was responsible
for p37 presentation. In a first simple experiment the T-cell line 1 (from donor 1) was
used in an ELISpot assay to determine if the PBMC from donor 5 could effectively
present p37 without a strong alloreactive response being elicited in parallel. It should
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be noted that due to technical failure, spot counts could only be included from two wells containing p37. The results of this experiment revealed that T-cell line 1 efficiently recognises p37 presented by donor 5 PBMC (Figure 4.14). Moreover no alloreactive response was detected (i.e. no strong background response in control wells), making the use of allogenic HLA-typed PBMC-APC a viable approach in confirming the proposed involvement of HLA-DRB1*01.

In the subsequent experiment, line B was tested in an ELISpot assay with a panel of eleven HLA-typed APC (Table 4.3). The APC included 10 different PBMC-APC (including PBMC from donors 1 and 5) and two BLCL-APC of which one was donor 1 derived. The inclusion of donor 1 BLCL was a necessary control for the second BLCL-APC used. Positive responses against p37 were detected using five PBMC-APC, all of which were HLA-DRB1*01 positive (Figure 4.15). From wells containing the six HLA-DRB1*01 negative PBMC/BLCL-APC, a weak response was detected (on average 7.6 fold lower than with HLA-DRB1*01+ PBMC-APC). However, the level of response detected with these HLA-DRB1*01 negative PBMC-APC was equivalent to that detected from T-cells incubated with p37 in the absence of APC (after background subtraction). This suggests that these T-cells are capable of low levels of p37 presentation and therefore the weak response seen with the HLA-DRB1*01 negative PBMC-APC could be the result of T-cell mediated peptide presentation. This is a feasible explanation as the line B T-cell line expresses high levels of HLA-DR (Figure 4.16).

Interestingly the magnitude of response generated in the presence of the donor 1 BLCL-APC was over 3 fold higher than detected with the corresponding donor 1 PBMC-APC. This suggested that the specificity of line B had been underestimated due to the nature of the APC used in the ELISpot assay. This was further investigated in section 4.5.3.

The data obtained using the HLA-typed APC strongly suggests the involvement of HLA-DRB1*01 in p37 presentation. This result was confirmed using mouse L-cells

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that expressed only one HLA-DR allotype (HLA-DRB1*01, -DRB1*04, or -DRB1*15).

Line B T-cells were tested in an ELISpot assay using transfected L-cells as APC (Figure 4.17). A strong response to p37 was only detected in wells containing the HLA-DRB1*01 transfected L-cells. A weak response was detected using HLA-DR negative and HLA-DRB1*15 L-cells as APC (over 12 fold lower than HLA-DRB1*01+ L-cells). This clearly indicates that HLA-DRB1*01 is responsible p37 presentation. The low-level responses detected with the HLA-DR negative and HLA-DRB1*15 L-cells are again presumably due to low levels of self-presentation by the T-cell line.

It has been demonstrated using both allogenic PBMC and transfected mouse L-cells that p37 is presented to T-cells by HLA-DRB1*01. Having identified the allotype responsible for p37 presentation it was possible to investigate this response in additional HLA-DRB1*01 donors.
Figure 4.14 Donor 5 PBMC can present peptide 37 to T-cell line 1 generated from donor 1. Line 1 T-cells were plated out at 1x10⁴ cells/well with PBMC from donor 5 at a ratio of 1:1 in an ELISpot assay. Cells were incubated either in the presence or absence of p37 (5μg/ml). Background responses were subtracted from this data.
Figure 4.15 HLA-DRB1*01+ APC present peptide 37 to T-cell line B.
A panel of HLA typed PBMC and BLCL were used as APSC in an ELISpot assay with line B. T-cells were plated out at 5x10^3 cells/well to which APC were added at a ratio of 1:1 in the presence and absence of 5μg/ml p37. Background responses were subtracted from this data.

<table>
<thead>
<tr>
<th>HLA-DR</th>
<th>HLA-DQ</th>
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<tbody>
<tr>
<td>Positive p37 response detected</td>
<td>DR1, DR3</td>
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<tr>
<td></td>
<td>DR1, DR14</td>
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<td></td>
<td>DR1, DR13</td>
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<td>DR1, DR11</td>
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<td></td>
<td>DR1, DR15</td>
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<tr>
<td>No p37 response detected</td>
<td>DR3, DR13</td>
</tr>
<tr>
<td>(Low responses detected but not greater than control)</td>
<td>DR14, DR15</td>
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<td>DR11, DR15</td>
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<td>DR7, DR13</td>
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<tr>
<td></td>
<td>DR11, DR15</td>
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<td></td>
<td>DR4, DR13</td>
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</tbody>
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*1 Donor 1 PBMC
*2 Donor 5 PBMC
*3 BLCL

Table 4.3 HLA-DR and HLA-DQ types of the PBMC- and BLCL-APC used to determine the HLA-DR allotype involved in the presentation of p37.
Figure 4.16 HLA-DR expression by T-cell line B
Line B T-cells were stained using a HLA-DR specific antibody L243 and FITC conjugated anti-mouse secondary antibody (shown in black fill). Cells were also incubated alone with the FITC conjugated anti-mouse secondary as a negative control (shown unfilled).
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Figure 4.17 HLA-DRB1*01+ L-cells can present peptide 37 to T-cell line B.
Line B was plated out at 2x10^4 cells/well with HLA-DR negative, HLA-DRB1*01, and HLA-DRB1*15 transfected L-cells at a ratio of 1:3. The cells were incubated in the presence and absence of p37 (5μg/ml). Background responses were subtracted from the data.
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4.5.2 Testing additional HLA-DRB1*01 donors for a peptide 37 T-cell response
In the previous section p37 was shown to be presented by HLA-DRB1*01. p37 specific CD4+ T-cells were originally detected in two HLA-DRB1*01 donors suggesting that this may be a common response. To investigate this further, two additional HLA-DRB1*01+ female donors (referred to as donors R and L) were tested for recognition of both the HPV16 E6 peptide pool and p37. The HLA-DR types for donors R and L are -DRB1*01 -DRB1*15, and -DRB1*01 -DRB1*13 respectively.

PBMC from these donors were cultured with both the HPV16 E6 peptide pool and peptide 37 alone for 7 days before testing by ELISpot. The cultures stimulated with p37 alone were tested in the ELISpot assay against only this peptide. Cells cultured with the HPV 16 E6 peptide pool were tested against the whole pool and p37. In the case of donor L, additional wells were tested against the HPV16 E6 peptides 30 and 36. This additional analysis was performed as donor 6 from whom these peptides were originally identified, shared expression of HLA-DRB1*13 (and HLA-DQB1*06) with donor L. Responses from donors R and L are shown in Figures 4.18 and 4.19 respectively.

Both donors R and L generated strong positive responses against the HPV16 E6 peptide pool. Donor R also demonstrated a positive response against p37 from PBMC cultured with the HPV16 E6 peptide pool and p37 alone. In contrast no p37 response was detected in either peptide culture from donor L. Also no positive response was detected against p36. However a positive p30 response was detected from the HPV16 E6 peptide pool culture. As p30 has been shown to be HLA-DR restricted (section 4.2.2), this implicates HLA-DRB1*13 as the allotype responsible for presentation. Overall, of the four HLA-DRB1*01 donors that generated responses against HPV16 E6 three have demonstrated a p37 specific response.
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Figure 4.18 Detection of a peptide 37 T-cell response from the HLA-DRB1*01+ donor R. PBMC were cultured with either the HPV16 E6 peptide pool or p37 alone for 7 days before testing in an ELISpot assay. Cultured cells were plated out at 1x10^5 cells/well with autologous PBMC at a ratio of 1:1. Cells cultured with the HPV16 E6 pool were incubated in the presence and absence of the whole pool (5μg/ml) and peptide 37 (5μg/ml). Cells cultured with peptide 37 were incubated in the presence and absence of peptide 37 (5μg/ml) alone. Background responses were subtracted from the data.

Figure 4.19 No peptide 37 T-cell response was detectable from the HPV16 E6 response of HLA-DRB1*01+ donor L. PBMC were cultured for 7 days with the HPV16 E6 peptide pool before testing in an ELISpot assay. Cultured cells were plated out at 1x10^5 cells/well with autologous PBMC at a ratio of 1:1. Cells were incubated in the presence and absence of the HPV16 E6 pool, peptide 37, p30 and p36 (all at 5μg/ml). Background responses were subtracted from the data.
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4.5.3 Specificity of T-cell lines determined by ELISpot is dependent upon the ratio of antigen presenting cells to the responding T-cells

In section 4.5.1, data was shown suggesting that the frequency of p37-specific T-cells in a line can be underestimated using PBMC as the APC, as higher spot counts were obtained using BLCL. This result prompted further investigations regarding the determination of T-cell specificity by ELISpot. Line 46 T-cells were tested in an ELISpot assay with varying ratios of BLCL:T-cells (8:1, 4:1, 2:1, 1:1, 1:2, and 1:4) (Figure 4.20). Increasing the ratio of BLCL:T-cells resulted in an increase in the number of spots detected.

In previous experiments the APC:T-cell ratio of 1:1 was commonly used. In this experimental system using the BLCL at a 1:1 ratio, only 4% of line 46 T-cells responded to p37. However 22% of T-cells responded at a BLCL:T-cell ratio of 8:1. This clearly demonstrates that at lower BLCL:T-cell ratios, the actual frequency of epitope specific T-cells within a line can be greatly underestimated.

4.5.4 Temporal analysis of IFNγ secretion by line B

Whilst the ELISpot assay is a useful tool to investigate the specificity of a T-cell line, it does not provide any information about the quantity of IFNγ secreted. An IFNγ ELISA was used to characterise line B in terms of the quantity of IFNγ produced and the kinetics of the IFNγ response (described in section 2.12.1). Supernatants harvested 2, 4 and 6 hours after p37 stimulation were analyzed in an ELISA for IFNγ (section 2.12.2) (Figure 4.21). No IFNγ was detected from any of the supernatants collected at 2 hours. By 4 hours IFNγ was detectable in the p37 test well supernatants, with an average concentration of 331.0 (+/- 24.5) pg/ml after the subtraction of background secretion (BLCL + T-cells + DMSO). In the 6 hour supernatants this had increased over 3.5 fold to 1205.1 (+/- 82.8) pg/ml (after background subtraction). From this data it can be seen that within 4 hours of re-stimulation with p37, line B can mount an IFNγ response, which increases further by 6 hours.
Figure 4.20 The peptide 37 response detected from line 46 rises with increasing ratios of BLCL to T-cells. An ELISpot assay was set up in which 5x10^2 line 46 T-cells were incubated in the presence and absence of peptide 37 (5μg/ml) with various ratios of autologous BLCL (8:1, 4:1, 2:1, 1:1, 1:2, and 1:4). Background responses were subtracted from the data.
Figure 4.21 Quantitative analysis of IFNγ secretion by line B over 6 hours post restimulation with peptide 37. Autologous BLCL were plated out in 0.5mls media at 2x10^5 cells/well with 1x10^5 line B T-cells in the presence of either p37 (5μg/ml) or DMSO. Supernatants were taken from triplicate wells at 2, 4 and 6 hours and analysed in an IFNγ ELISA. The concentration of IFNγ (pg/ml) in each supernatant was calculated using an IFNγ-standard curve. The average IFNγ concentration detected from triplicate wells is stated.
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4.5.5 Analysis of the TCR Vβ chains used by peptide 37-specific polyclonal T-cell lines

There are over 20 different groups of the TCR Vβ chain family that can be detected serologically. In this section we aimed to identify the TCR Vβ chains used by the p37 T-cell lines. This was done using a panel of 19 PE-conjugated antibodies recognising different TCR Vβ chains to stain the lines, which were subsequently analysed by flow cytometry. The purpose of this experiment was not only to determine which Vβ chain/s were involved in p37 recognition, but also to indicate the number of different T-cell populations each line contained.

Donor 1 PBMC were initially tested in order to determine what proportion of the TCR Vβ chains could be identified using these antibodies. In Figure 4.22 the percentage of CD4+ cells expressing each TCR Vβ chain is shown. This panel of TCR Vβ antibodies was able to identify the Vβ chain usage of 62% of CD4+ cells. Thus over one third of the TCR Vβ chains are not being detected.

In subsequent experiments the Vβ usage of the p37 specific T-cell lines was examined. Of the lines generated in section 4.4.1.3, lines 46, 68 and 87 were the three most specific. These three lines were analysed using the full panel of anti-TCR Vβ antibodies (data not shown). Analysis of line 46 revealed that 88.7% of T-cells expressed TCR Vβ16, with no other Vβ chain being detected. Similarly with line 68 and line 87, TCR Vβ16 was the only chain detected with 98.7% and 12.9% of cells expressing this chain respectively. This data indicates that lines 46 and 68 contain at least two T-cell populations, a Vβ16+ and a Vβ16- population/s. The TCR Vβ16 staining of all cell lines tested is shown in Figure 4.23.

Based on these results, line 1 and line B were tested solely for TCR Vβ16 expression. Both of these lines consist of over 99% CD4+ T-cells. In line 1, 9.0% of gated cells were TCR Vβ16+. In line B the proportion of cells expressing TCR Vβ16 was 3 fold
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Figure 4.22 Staining PBMC for TCR Vβ expression on CD4+ T-cells. PBMC from donor 1 were co-stained with a CD4-FITC antibody and 19 different TCR Vβ chain-PE antibodies. Data shown represents the percentage of CD4+ cells expressing each Vβ chain. T-cells were gated, based on their forward and side scatter profile.

Figure 4.23 Expression of TCR Vβ16 is variable between different peptide 37-specific T-cell lines. Donor 1 PBMC and lines 1, B, 46, 68, and 87 were co-stained with an anti-CD4-FITC antibody and an anti-TCR Vβ16-PE antibody. Data shown represents the percentage of CD4+ cells expressing Vβ16 in each line.
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higher at 26.3%. This suggests that the extra period of peptide re-stimulation undergone by line B but not by line 1, did lead to further expansion of p37-specific T-cells. In light of these results it seemed likely that TCR Vβ16 was involved in p37 recognition. However it was also possible that another as yet unidentified TCR Vβ chain could also recognise p37.

4.5.6 Analysis of polyclonal T-cell lines using an IFNγ-intracellular cytokine staining assay (ICS)

To determine which T-cell population/s in line 46 recognised p37, an ICS assay (flow cytometry-based technique) was employed as it permitted phenotypic analysis of the IFNγ-secreting cell (section 2.9.4). By co-staining line 46 T-cells for TCR Vβ16 expression and IFNγ production it was possible to examine which cellular populations were involved in p37 recognition. Line 46 (~88% TCR Vβ16+) was tested by ICS on 14 days post expansion. T-cells were incubated with BLCL for 12 hours with either DMSO or p37 (5μg/ml, 1μg/ml, or 0.2μg/ml) before ICS analysis.

Duplicates were carried out for each condition and representative results are shown in Figure 4.24. In the DMSO control wells background the levels of IFNγ+ cells did not exceed 0.5% of cells gated (Figure 4.24A). At 5μg/ml of p37, up to 53.2% of gated cells were IFNγ+ (Figure 4.24B). Interestingly, the IFNγ+ cells consisted of both Vβ16- and Vβ16+ T-cells. Of the total Vβ16+ T-cell population, 47.5% were IFNγ+, with a MFI of 27.0. In contrast, 85.8% of Vβ16- cells were positive for IFNγ, with an MFI of 233.9. Therefore it appears that as a proportion of their individual populations, a greater number of Vβ16- cells were IFNγ+ and produced higher levels of the cytokine as compared to the Vβ16+ cells.

Decreasing the peptide dose resulted in a clear reduction in the number of IFNγ+ cells. Dissection of this effect revealed that the proportion of Vβ16+ cells positive for IFNγ was dramatically reduced at lower peptide doses with the response almost disappearing at 1μg/ml p37. The proportion of Vβ16- cells producing IFNγ was not observed to
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decrease over this peptide dose range, however the quantity of IFNγ produced by these cells (assessed by MFI of IFNγ staining) did decrease as the peptide dose was lowered.

Line B was also tested in an ICS assay to determine if a similar trend in IFNγ responses could be detected from this less specific T-cell line. This line had previously been shown to contain >99% CD4+ T-cells, of which ~ 26% expressed TCR Vβ16 (section 4.5.5). Up to 4% of line B T-cells were estimated (by ELISpot) to recognise p37 (Figure 4.15). In the presence of DMSO, 0.6% of gated cells non-specifically secreted IFNγ (Figure 4.25A). In the presence of p37, over 7% of gated cells were IFNγ+ (Figure 4.25B). Of these IFNγ+ cells >80% were negative for Vβ16 expression. As observed with line 46, the TCR Vβ16- cells were shown to produce IFNγ at levels several fold higher than the Vβ16+ T-cells. This result was interesting not only because the frequency of p37-specific T-cells in line B were shown to be higher than previously thought, but also because it confirms the differential IFNγ secretion profiles of the TCR Vβ16 positive and negative cells.

Collectively, this data reports of at least two T-cell populations that recognise p37 which can be discriminated by their positive or negative staining for TCR Vβ16 and their differential IFNγ secretion profiles. Vβ16+ T-cells were the dominant population in several lines tested. However, these T-cells were demonstrated to be functionally inferior in terms of their IFNγ response, compared to the Vβ16- T-cells. Further characterisation of these two populations would require their purification in order for more direct comparisons to be made.
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Figure 4.24 ICS analysis of line 46 incubated with decreasing concentrations of peptide 37 reveals IFNγ-secreting T-cells are constituted of TCR Vβ16+ and Vβ16- cells. Line 46 T-cells were plated out at 5x10^5 cells/well with autologous BLCL at a ratio of 1:2 in an ICS assay in the presence of either DMSO (A), 5µg/ml p37 (B), 1µg/ml p37 (C), or 0.2 µg/ml (D) for 12 hours with golgi plug. Cells were stained with an anti-TCR Vβ16-PE antibody and anti-IFNγ-FITC antibody. The dot plots show the percentage of gated cells in each quadrant. The proportion of Vβ16+ and Vβ16- cells that were positive for IFNγ for each peptide test condition are depicted in histograms (black fill), with the DMSO control results shown in red. Representative data is shown for each duplicate sample. The corresponding mean fluorescent intensities of the IFNγ positive TCR Vβ16+ and TCR Vβ16- cells for each peptide test are also shown (E).
Figure 4.25 ICS analysis of line B reveals that the majority of IFNγ+ cells are negative for TCR Vβ16 expression. Line B T-cells were plated out at 5x10^4 cells/well with autologous BLCL at a ratio of 1:2 and incubated in an ICS assay in the presence of either DMSO (A) or 5μg/ml peptide 37 (B) for 12 hours with golgi plug. Cells were stained with an anti-TCR Vβ16-PE antibody and anti-IFNγ-FITC antibody. The dot plots show the percentage of gated cells in the quadrants. T-cells were gated based on their forward and side scatter profile. Representative data is shown from duplicate samples.
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4.6 Discussion

Data presented in this section revealed that the HPV E6 peptides 30, 37, 64 and 65 were all presented by HLA-DR molecules. T-cell lines were generated that recognised peptides 37 (HPV16 E6) and 64 (HPV18 E6). Using these lines the specific HLA-DR allotypes responsible for presentation of the epitopes were identified. Characterisation of CD4+ T-cell lines recognising p37 revealed that TCR Vβ16 was involved in p37 recognition (at least in donor 1). A second, more potent IFNγ-secreting T-cell population (negative for TCR Vβ16 expression) was identified.

In chapter 3, peptides 37, 64 and 65 were shown to be recognised by CD4+ T-cells. Peptides are presented to CD4+ T-cells as part of an HLA class II-peptide complex. Three HLA class II isotypes are capable of presenting peptides; HLA-DR, HLA-DQ and HLA-DP. HLA-DR is expressed at higher levels on primary APC than HLA-DQ and -DP. To determine if epitopes identified in this study were HLA-DR restricted, a HLA-DR blocking antibody (L243) was employed in ELISpot assays. Peptides 30, 37, 64, and 65 were all shown to be presented by HLA-DR. The L243 antibody has also been used successfully for HLA-DR blocking in other studies (Elferink et al., 1985; Godkin et al., 2001). It was not possible to investigate the potential involvement of HLA-DQ and -DP molecules in the presentation of these peptides due to the lack of appropriate antibodies and therefore the possibility remains that some of these epitopes could also be presented by other HLA class II isotypes.

Strategies using two different types of APC (allogenic PBMC/BLCL and transfected L-cells) were employed to determine the HLA-DR allotypes that present both p37 and p64. The application of allogenic APC (with defined HLA haplotypes) in determining the allotype-restriction of a peptide has previously been demonstrated (Cao et al., 2002). The disadvantage of this strategy is that for each APC, numerous HLA class I and class II allotypes are expressed, making data interpretation more complicated. It is therefore critical to carefully select the APC panel to contain the appropriate combinations of HLA alleles, both to allow confirmation of a particular allotype and to
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exclude other potential candidates. The use of mouse L-cells (homozygous for one HLA-DR allotype only) as APC was an ideal alternative strategy. The advantage of this system is that where a positive peptide-specific T-cell response is detected, only one allotype can be implicated in presentation. Several other studies have used HLA-DR+ L-cells to present antigens to human T-cells (Altmann et al., 1989; Lombardi et al., 1989; Wilkinson et al., 1988).

Interestingly, the magnitude of the p37 response detected from T-cell line B in the presence of HLA-DRB1*01+ L-cells was similar to that previously detected using PBMC-APC (Figure 4.15 and Figure 4.17). In marked contrast the positive response generated by the p64-specific T-cell line in the presence of the HLA-DRB1*15+ L-cells was -4 fold lower than that detected using PBMC-APC (Figures 4.6 and 4.7). The difference between these two peptide responses cannot be attributed to lower HLA-DR expression on the DRB1*15+ L-cells, as these cells were confirmed by flow cytometry to express higher levels of HLA-DR than the DRB1*01+ L-cells (data not shown). It has previously been shown that some T-cell lines can be adequately stimulated using HLA-DR+ L-cells (Wilkinson et al., 1988), however other T-cell lines appear to require the expression of additional co-stimulatory molecules such as ICAM-1 for effective activation (Altmann et al., 1989). The L-cells used in this study do not express human co-stimulatory molecules. It is therefore feasible that the p37 and p64 specific T-cell lines require different levels of co-stimulatory signals for efficient activation.

The precise allotype involved in the presentation of p37 was identified to be HLA-DRB1*01. The DRB1*01 allele occurs at a relatively high frequency within caucasian (9.4%) and black populations (5.4%) (Marsh, 2000). The allotype responsible for p64 presentation was identified to be HLA-DRB1*15 which occurs at a frequency of 10.7% in caucasian and 9.9% in black populations (Marsh, 2000). As no one HLA-DRB1 allele occurs at a frequency >16% in either caucasian or black populations, DRB1*01 and DRB1*15 represent two of the more common alleles (Marsh, 2000).
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Successful allotype characterization in this study was restricted to p37 and p64 only. However other attempts were made to investigate the HLA-DR allotypes involved in the presentation of p65 and p30.

The p65 response was detected in donor 5 who is positive for HLA-DRB1*01 and HLA-DRB1*14. To determine which allotype was responsible for p65 presentation, a p65-specific T-cell line was generated. ELISpot assays utilising either a panel of HLA typed PBMC or HLA-DRB1*01 L-cells as APC failed to demonstrate p65 presentation by HLA-DRB1*01 (data not shown). By default, this suggested HLA-DRB1*14 could be involved in p65 presentation. However, the appropriate APC were not available to test this hypothesis. This could be attributed to the low frequency of this allele in caucasian populations (~3%) (Marsh, 2000). As no clear binding motif for HLA-DRB1*14 has been defined, it is not possible to comment further on this epitope.

A peptide epitope that contained the p65 sequence (HPV18 E6(49-63)) was defined recently (Facchinetti et al., 2005). The E6(52-60) peptide in this paper was shown to be a promiscuous epitope binding to both HLA-DRB1*03 and HLA-DRB1*16. Collectively, these two alleles occur in 14.8% of Caucasians and ~15% of the black population. The results from this chapter, although not definitive, suggest that this epitope may be able to bind to an additional HLA-DR allotype, possibly HLA-DRB1*14. The promiscuous nature of this epitope could be beneficial therapeutically, for example it could be used to monitor the CD4+ T-cell immune responses to a HPV16 E6 vaccine in a large proportion of the population e.g. between 18% (including HLA-DRB1*14) and 24.2% (including HLA-DRB1*01) of the caucasian population.

The candidate allotypes for p30 presentation were HLA-DRB1*07 and HLA-DRB1*13. In addition to donor 6, a p30 response was also identified from donor L. The detection of a p30 response from a second donor suggests it is a genuine immunogenic epitope. Based upon the HLA haplotype of Donor L (HLA-DRB1*01 and -DRB1*13), HLA-DRB1*13 is the strongest candidate allotype for p30 presentation, and warrants further investigation, especially as the expression of HLA-
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DRB1*13 strongly correlates with protection from the development of cervical cancer (Madeleine et al., 2002).

Following the identification of the allotype responsible for p37 presentation, it was possible to select two further HLA-DRB1*01 positive female donors to investigate the frequency of the p37 immune response. Both donors responded to HPV16 E6 peptide pools but were excluded from the initial screen for HPV-specific immunity as they were both over the age of 50. Of the four HLA-DRB1*01+ donors tested that demonstrated HPV16 E6 T-cell immune responses, three responded to p37. This suggests that the response to p37 is common in HLA-DRB1*01+ women, although larger numbers would need to be tested to confirm the frequency of this response.

In this chapter, several T-cell lines recognising p37 were generated from donor 1 PBMC. Initially the aim was to generate a p37-specific T-cell line as rapidly as possible. The use of a commercially made HLA-DRB1*01-p37 tetramer to enrich p37 specific T-cells was not an option due to the high cost of production. Instead, the IFNγ-enrichment protocol (section 2.6) was selected for use as it was a rapid, cost-effective, and relatively simple technique. Several other groups have used this method for the enrichment of peptide-specific T-cells (Koehne et al., 2002; Oelke et al., 2000; Welters et al., 2003). This technique could potentially be used to isolate virus-specific T-cells from the PBMC of healthy donors for use in adoptive transfer immunotherapy. Adoptive transfer of virus-specific T-cells isolated using tetramers has proven successful in reducing viral load in infected patients (Cobbold et al., 2005). The advantage of the IFNγ based enrichment protocol is that it would select T-cells based on their effector function, whereas not all tetramer positive cells secrete IFNγ. In the case of HPV, a period of in vitro culture would be required before using either enrichment technique, in order to isolate an appropriate number of HPV-specific T-cells for immunotherapy.

In this study, the CD4+ IFNγ+ T-cell population was enriched from 0.7% to 61.7%. As the number of cells isolated using this method was relatively low (6x10^4), several
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weeks of culture was required to obtain sufficient cells for generating lines. Following expansion of these cells to generate line 1 the frequency of p37-specific T-cells was determined by ELISpot to be only ~1.5%. This was far lower than anticipated and it is possible that the long periods of culture followed by expansion resulted in a loss of specificity. This could have occurred for a number of reasons. The addition of IL-2 to the cultures in the absence of p37 stimulation over a long time in culture could have allowed the non-specific T-cells to out-grow the p37-specific population. Moreover, the expansion protocol uses non-specific stimuli (IL-2 and PHA) which encourages multiple rounds of T-cell proliferation. During this process it is again possible that the non-specific T-cell population ‘expanded’ to a greater degree compared to the p37-specific T-cells. Alternatively a proportion of the highly activated p37-specific T-cells could have undergone apoptosis subsequent to the IFNγ-pullout method, as a result of the conditions used during this protocol to stimulate the cells (extremely high density of T-cells activated with p37 in one well).

Isolation of highly specific T-cell lines revealed a limitation of the IFNγ ELISpot assay in its capacity to estimate the frequency of peptide specific T-cells. In such lines, the ratio of APC:T-cells plays a critical role in determining the number of cells that respond. Increasing this ratio from 1:1 to 8:1 lead to a 5.5 fold increase in the number of IFNγ-secreting T-cells detected from line 46 (Figure 4.20). This suggests that at lower ratios, the number of peptide-loaded APC available to activate the T-cells becomes limiting. Even at a ratio of 8:1 the ELISpot underestimated the frequency of cells in line 46 capable of responding, as ICS analysis detected over twice as many p37-specific T-cells at a ratio of 2:1 (Figure 4.24). For highly specific T-cell lines, ICS is a more suitable technique for assessing the frequency of peptide specific T-cells. A comparative study using ICS and ELISpot has also shown that ICS is able to detect a higher frequency of antigen specific T-cells, however the ELISpot was shown to be more effective at detecting low-level immune responses (Karlsson et al., 2003). Therefore, when screening for a clear cut positive or negative T-cell response to peptide, the ELISpot is the more appropriate technique. However, where more advance analysis is required, ICS is greatly superior to the ELISpot assay as it permits the
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phenotype of the IFNγ+ cells to be identified and also indicates the magnitude of IFNγ secretion.

Lines containing high frequencies of p37-specific T-cells were required for a more in depth study of the p37 immune response. These lines were generated through the use of a 96 well plate cloning protocol. Only polyclonal T-cell populations were isolated using this method. This is most likely due to the minimum seeding density used, as no less than 1 cell was plated out per well and also a degree of inaccuracy in counting is not uncommon. To reliably generate clonal lines, cells would have to be plated out using a more accurate technique such as a MoFlo cell sorter. Analysis of several lines revealed that they all contained a TCR Vβ16+ population, with no other Vβ chain being detected (using the available monoclonal antibodies). As line 68 (estimated specificity 5% by ELISpot, Figure 4.13) consisted of over 98% TCR Vβ16+ T-cells this was clearly indicated that the TCR Vβ16+ cells were capable of recognising p37. However, this did not exclude the possibility of a second p37 responsive T-cell population.

ICS analysis of line 46 (estimated by ELISpot to contain the highest frequency of p37-specific T-cells), demonstrated that both TCR Vβ16+ and TCR Vβ16- T-cell populations recognised p37. Interestingly, the TCR Vβ16+ and TCR Vβ16- T-cells demonstrated major differences in their IFNγ effector response. At all p37 concentrations tested, the TCR Vβ16- T-cells were superior both in the proportion of cells that were IFNγ+ and also in the amount of cytokine produced. This is an interesting finding as it suggests that the dominant T-cell population within this line is functionally inferior to the sub-dominant population of T-cells.

In this section, peptides 30, 37, 64 and 65 have all been shown to be presented by HLA-DR. Furthermore, peptides 37 and 64 were shown to be presented by HLA-DRB1*01 and HLA-DRB1*15 respectively. Several p37-specific T-cell lines have been generated from a single donor. Characterisation of these lines revealed that the TCR Vβ16 chain is involved in the recognition of p37. An additional T-cell population
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expressing an unidentified TCR Vβ chain was demonstrated to be functionally superior in its IFNγ response to p37. To study these Vβ16+ and Vβ16- T-cell populations in further detail it was deemed necessary to generate two sub-lines from line 46, which is discussed in chapter 5.
Chapter 5

Characterisation of TCR Vβ16+ and Vβ16- T-cell lines generated against HPV16 E6(127-141)

5.1 Introduction
In the previous chapter a number of T-cell lines were generated recognising p37. Line 46 was shown to contain the highest frequency of p37-specific T-cells. Characterisation of this line revealed the presence of two T-cell populations, one of which expressed TCR Vβ16, whilst the other was negative for Vβ16 staining. In ICS assays these two populations demonstrated different IFNγ responses upon peptide re-stimulation, with the TCR Vβ16- cells being superior in both the proportion of IFNγ+ cells and in the amount of cytokine produced. In order to effectively investigate these differences it was necessary to study each T-cell population in isolation.

This chapter describes the generation of two sub-lines from line 46: a TCR Vβ16+ line (Belx1) and a TCR Vβ16- line (Belx2). Lines were characterised in terms of cell surface marker expression, Th1/Th2 cytokine production, and the TCRαβ chains they expressed. These lines were also used to investigate the endogenous processing of the p37 epitope (HPV16 E6(127-141)).

5.2 Generation of TCR Vβ16+ and TCR Vβ16- T-cell lines from line 46
To generate a TCR Vβ16+ and Vβ16- T-cell line, line 46 T-cells were stained with a TCR Vβ16-PE antibody and sorted based upon positive and negative PE staining using a MoFlo cell sorter (section 2.7.4). 3.6x10⁶ TCR Vβ16+ cells and 1x10⁶ TCR Vβ16- T-cells were recovered. Cells were cultured overnight in media containing IL-2 before expanding (section 2.8). Expansions yielded >7.5x10⁷ cells for both T-cell lines. The TCR Vβ16+ T-cell line is hereon referred to as Belx1 and the TCR Vβ16- line as Belx2. Flow cytometric analysis revealed the Belx1 T-cell line was ~94% positive for Vβ16 expression and the Belx2 T-cell line was 99.6% negative for Vβ16 (Figure 5.1).
Figure 5.1 Generation of the TCR Vβ16+ and TCR Vβ16- T-cell lines from line 46. Line 46 was shown to consist of 87.6% TCR Vβ16+ CD4+ T-cells and 12.4% TCR Vβ16- CD4+ T-cells (left panel). To generate a TCR Vβ16+ line and a TCR Vβ16- line, 2x10⁷ line 46 T-cells were incubated with an anti-TCR Vβ16-PE antibody. Cells were sorted based upon positive and negative PE staining using a MoFlo cell sorter. The cells obtained were expanded for 7 days. The purity of the Belx1 and Belx2 lines was assessed by flow cytometry using an anti-TCR Vβ16-PE antibody (right panel) and histograms depict the percentage of the lines expressing TCR Vβ16.
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5.3 Characterisation of Belx1 and Belx2 T-cell lines

5.3.1 ICS analysis of IFNγ secretion by Belx1 and Belx2
ICS analysis of line 46 had previously shown a difference between the TCR Vβ16 positive and negative T-cell populations, both in the proportion of IFNγ+ cells and in the amount of cytokine produced following p37 re-stimulation (section 4.24). In this section the IFNγ secretion profiles of the Vβ16+ and Vβ16- T-cell lines were investigated to determine if the aforementioned differences had been maintained after cell sorting.

14 days post expansion the lines were tested by ICS with either p37 or DMSO (Figure 5.2A) (described in section 2.9.4). In the presence of p37, 31.3 % of Belx1 cells were positive for IFNγ, with a MFI of 29.5 (non-specific production of IFNγ represented 0.3% of gated cells). In the presence of p37, 96.4% of Belx2 cells were positive for IFNγ, with a MFI of 145.0 (non-specific IFNγ production represented 0.7% of cells). When this experiment was performed 21 days post expansion (Figure 5.2B), almost identical results were obtained for the Belx2 T-cell line. In the case of Belx1 however, the proportion of IFNγ+ cells had increased to 83.1% and the MFI had at least doubled to 64.7.

This data reveals that the Belx2 T-cell line elicits a superior IFNγ response compared to that demonstrated by Belx1. The vast majority of Belx2 T-cells were p37-responsive (IFNγ+) on days 14 and 21 post expansion. In contrast the proportion of IFNγ+ Belx1 T-cells was shown to increase by approximately 2.5 fold between days 14 and 21.
Figure 5.2 ICS analysis of Belx1 and Belx2 incubated with peptide 37 on days 14 and 21 post expansion. The T-cell lines Belx1 and Belx2 were plated out at 5x10^5 cells/well with autologous BLCL at a ratio of 1:2 in the presence of either DMSO or p37 (5μg/ml) and tested by ICS. T-cell lines were tested on day 14 (A) and 21 (B) post expansion. Cells were stained with an anti-TCR Vβ16-PE antibody (to identify the contaminating T-cells in each population) and an anti-IFNγ-FITC antibody. Histograms depict the percentage of cells that were IFNγ+, with the DMSO control shown in grey and the p37 tests shown in black fill. The percentage of IFNγ+ cells stated, refers only to the Vβ16+ cells in Belx1 and Vβ16- cells in Belx2. Duplicate samples were tested and representative data is shown.
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5.3.2 Differential expression of cell surface markers by Belx1 and Belx2 lines

There are a number of potential reasons that could underlie the functional differences between Belx1 and Belx2. One possibility was that the lines could differentially express cell surface molecules relevant to T-cell activation (TCR recognition and signalling). Belx1 and Belx2 lines were tested for expression of key cell surface markers (CD3, CD4, CD25 and CD28) by flow cytometry (section 2.9.1). CD3 is a component of the TCR complex and functions to couple TCR recognition of antigen with intracellular signalling, CD4 is a co-receptor which interacts with invariant regions of HLA class II molecules and facilitates antigen recognition, CD25 (IL-2 receptor) is a marker of T-cell activation and CD28 is an important accessory molecule which provides co-stimulatory signals to augment TCR signalling (Ward, 1996).

Additional molecules involved in T-cell activation were not investigated as antibodies were not available in the laboratory. Lines were tested on days 14 and 21 post expansion (in parallel with ICS experiments in section 5.3.1).

For CD3 stains, the MFI associated with Belx1 cells was 2.8 fold lower than for Belx2 T-cells (149.1 verses 414.4) on day 14. On day 21 however, the CD3 MFI of the Belx1 cells had increased to 297.3, whilst the MFI for Belx2 had remained stable at 426.2 (Figure 5.3A). This data indicates two interesting points. Firstly, levels of CD3 expression appear consistently higher on Belx2 T-cells, and secondly CD3 expression on Belx1 T-cells seems to increase with time in culture. This increase in CD3 expression correlates with the observed rise in the number of IFNγ+ Belx1 T-cells detected by ICS between days 14 and 21 post expansion (Figure 5.2).

CD25 was detected on >97.3% of Belx2 T-cells at both time points. In contrast the equivalent stains for Belx1 revealed only ≤59.6% of T-cells expressed CD25 (Figure 5.3B for day 21 data). The intensity of CD25 staining was very similar for both cell lines at both time points (day 21 is shown in Figure 5.3B). CD28 expression by the two lines differed in both the number of cells positive for CD28 and in the intensity of the staining (Figure 5.3C). The MFI of CD28 staining on Belx2 T-cells was 2.4 fold higher than detected on Belx1 at both time points. The proportion of CD28+ Belx2 T-
Chapter 5 - Results

cells was 1.8 and 1.5 fold higher than observed for the Belx1 cells on days 14 and 21 respectively. Figure 5.3C shows both the percentage CD28+ gated cells and the MFI for both lines on day 21. Both lines demonstrated ~100% CD4 expression and the MFI of staining was very similar at both time points tested (day 21 data shown in Figure 5.3D).

In summary this data shows that the expression of CD3, CD25, and CD28 (either percentage expressing cells or MFI of staining) is greater for the Belx2 T-cell line compared to Belx1. These differences were observed at both the time points analysed.

At this stage a clear difference had been identified between Belx1 and Belx2 T-cell lines in terms of the cell surface markers expressed and in the IFNγ response to p37. In addition to IFNγ, it was also feasible that the lines could also differ in their secretion of other immunoregulatory cytokines in response to p37 recognition.
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A

![Bar chart showing MFI for CD3 at Day 14 and Day 21 for Belx1 and Belx2.]

B

![Bar chart showing % Gated for CD25 and MFI for Belx1 and Belx2.]

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Figure 5.3 Differential expression of cell surface markers by Belx1 and Belx2.
The T-cell lines Belx1 and Belx2 were assessed for the expression of cell surface markers; CD3, CD4, CD25 and CD28. Cells were stained with FITC or PE conjugated antibodies recognising these CD molecules and analysed by flow cytometry. T-cells were gated based on their forward and side scatter profile. Data shown depicts the MFI for CD3 on days 14 and 21 post expansion (A), the percentage of gated cells and MFI for CD25 (B), CD28 (C), and CD4 (D) on day 21. The standard deviation for the day 21 sample duplicates never exceeded +/- 5.1 for both the MFI and percentage gated values, and therefore representative data is shown.
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5.3.3 Analysis of Th1/Th2 cytokine production by Belx1 and Belx2 T-cells
The ICS analysis of Belx1 and Belx2 revealed that these lines differentially produce IFNγ in response to p37. In this section the production of IFNγ and other immunoregulatory cytokines by the two lines was directly compared on a per cell basis using a cytokine bead array (CBA). This method (described in section 2.9.5) was used to quantify the amount of IFNγ produced by each line, along with five other cytokines; IL-2, IL-4, IL-5, IL-10 and TNFα. The detection limits range from 6pg/ml to ~5000pg/ml.

No cytokines were detectable in the 1 hour supernatants from either cell line. The levels of IFNγ detected in 6 and 10 hour supernatants are shown for both cell lines in Figure 5.4A, and the concentrations of IL-2, IL-4 and IL-10 produced by the two lines at 6 hours are shown in Figure 5.4B, and at 10 hours in Figure 5.4C. No cytokine was detected above 13.6pg/ml from the DMSO or p64 (irrelevant control) supernatants of both lines, with nearly all cytokine levels being below the detectable limit. IL-5 was the only cytokine not detected in any supernatant. At 6 hours, the p37 Belx1 supernatant contained only IFNγ and IL-2 (after background subtraction). The equivalent Belx2 supernatant contained >31 fold more IFNγ (5378.0pg/ml), and almost 10 fold more IL-2 (81.4pg/ml). IL-4 and IL-10 were also detected in the Belx2 p37 6 hour supernatant (41.6 and 53.3pg/ml respectively).

At 10 hours the Belx2 p37 supernatant contained 5.5 fold more IL-2 (108.3pg/ml (+/- 2.6)) than Belx1. IL-4 and IL-10 were now detectable in the Belx1 supernatant, however, the concentration of these cytokines was at least 12 fold lower than detected in the Belx2 supernatants (88.7pg/ml (+/- 9.1) IL-4, and 197.7 pg/ml (+/- 8.4) IL-10). The concentration of IFNγ in the 10 hour p37 Belx1 supernatant was increased over 6 fold compared to the 6 hour supernatant. However, Belx1 still produced less IFNγ (at least 5 fold) than Belx2 T-cells at 10 hours (>>5,000pg/ml). TNFα was detected in the 10 hour supernatants from both cell lines. However the amounts produced from duplicate samples for this cytokine were so variable that no clear conclusions could be reliably drawn and as a consequence this data has been excluded from the analysis.
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In the Belx2 p37 10 hour sample, the IFNγ concentration was higher than the upper limit of detection of the CBA. Therefore an IFNγ ELISA was used to establish the concentration of IFNγ in the replicate samples (described in section 2.12.2). To verify that all IFNγ responses followed the same pattern as detected by CBA, supernatants from both T-cell lines at 6 and 10 hours were tested in the ELISA (Figure 5.4D). No IFNγ was detected in the supernatants taken from either T-cell line incubated with DMSO. In the Belx2 p37 10 hour supernatant, the mean average concentration of IFNγ detected was 11,020pg/ml. For the equivalent Belx1 supernatant 880pg/ml of IFNγ was detected, which is of a similar magnitude to levels detected by CBA (1,040pg/ml). The concentration of IFNγ detected in the p37 Belx1 and Belx2 6 hour supernatants were similar to those detected by CBA (217.5 and 3,485 pg/ml respectively).

To summarise, at 6 hours Belx2 secreted 10 fold more IL-2 and up to 31 fold more IFNγ (by CBA) than Belx1. In the 10 hour supernatant the quantity of IL-2 produced by Belx2 was 5.5 fold higher than that secreted by Belx1. Analysis of the 10 hour supernatants by ELISA revealed that Belx2 T-cells secreted 12.5 fold more IFNγ compared to Belx1. A comparison of IL-4 and IL-10 levels was only possible at the 10 hour time point, with Belx1 supernatant containing far lower levels (13 and 12 fold less respectively) than detected from the Belx2 supernatant.
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A

\[ [\text{FNt}] \text{ pg/ml} \]

- 6 hrs
- 10 hrs

B

6 hours

\[ \text{[cytokine]} \text{ pg/ml} \]

- IL-2
- IL-4
- IL-10

- Belx1
- Belx2

C

10 hours

\[ \text{[cytokine]} \text{ pg/ml} \]

- IL-2
- IL-4
- IL-10

182
Figure 5.4 Analysis of the cytokine production by Belx1 and Belx2.
5x10^4 Belx1 or Belx2 T-cells were incubated with autologous BLCL in the presence of p37 (5μg), DMSO, or the irrelevant peptide control p64 (5μg/ml). Supernatants taken from duplicate wells at 6 and 10 hours, were analysed for cytokine content by CBA. Duplicate supernatants were only analysed for the 10 hour time point. Data shown depicts the concentration of IFNγ in the p37 supernatants from both cell lines at 6 and 10 hours (A), the concentration of IL-2, IL-4, and IL-10 in the p37 Belx1 and Belx2 supernatants at 6 hours (B) and 10 hours (C). The IFNγ concentration of the 6 and 10 hour supernatants of p37 re-stimulated Belx1 and Belx2, were additionally analyzed by IFNγ ELISA (D). Background cytokine secretion detected in the negative controls has been subtracted for all data shown here.
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5.3.4 Investigating the relationship between peptide dose and the Belx2 response

Previously Belx2 had been demonstrated to secrete high levels of IFNγ in response to p37 (5μg/ml), with up to 96% of T-cells capable of responding in an ICS assay. To determine the ability of the line to be activated by lower concentrations of peptide, Belx2 T-cells were incubated with autologous BLCL in the presence of either DMSO or p37 at 5, 0.5, 0.25 and 0.05μg/ml (Figure 5.5). These peptide doses were selected as a similar range of peptide concentrations had been tested with line 46 in the previous chapter (Figure 4.24).

At the highest concentration tested, 96.4% of T-cells were positive for IFNγ. When the peptide concentration was reduced from 5 to 0.25μg/ml, the percentage of IFNγ+ cells only decreased by 15.6, however the MFI of IFNγ staining decreased by 61%. The major decrease in the number of IFNγ+ T-cells from 80.8% to 45.5% occurred as the peptide concentration was reduced from 0.25 to 0.05μg/ml. This was accompanied by a 50% decrease in the MFI of IFNγ staining. It thus appears that even at a peptide concentration 0.05μg/ml almost 50% of Belx2 T-cells demonstrate a p37-specific IFNγ response.
Figure 5.5 ICS analysis of the Belx2 IFNγ response to decreasing doses of peptide 37. The T-cell line Belx2 (day 14 post expansion) was incubated in an ICS assay with autologous BLCL at a ratio of 1:2 in the presence of either DMSO or p37 at 5.0, 0.5, 0.25 and 0.05 μg/ml. Cells were stained with an anti-TCR Vβ16-PE antibody and an anti-IFNγ-FITC antibody. Histograms depict the percentage of gated cells (based on forward and side scatter profiles) that were IFNγ+, with the DMSO control shown in red and the p37 tests shown in black fill. The values shown in bold type refer to the percentage of IFNγ+ cells within the lymphocyte gate, whilst the values shown in italic refer to the MFI of the IFNγ stain.
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\[ \text{IFN}_\gamma \]

\[ \text{Counts} \]

\[ \text{p37 (\mu g/ml)} \]

96.4% 145.0

86.9% 93.4

80.8% 56.4

45.5% 27.2

5

0.5

0.25

0.05
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5.3.5 Belx2 does not exhibit a granule-mediated cytotoxic effector function
Although cytotoxic effector function is usually associated with CD8+ T-cells, CD4+ T-cells demonstrating cytotoxic behaviour have been described (Bickham et al., 2001; Facchinetti et al., 2005; Sun et al., 2002). Belx2 was examined in terms of its ability to secrete cytotoxic granules in response to peptide induced activation using a CD107 assay (described in section 2.9.6). This assay allows the direct detection of degranulation by activated T-cells (Betts et al., 2003). CD107 is present in the membrane of cytotoxic granules. During T-cell degranulation the granule fuses with the T-cell plasma membrane and CD107 can be detected on the cell surface. The CTL line 7E7 was included as a positive control.

No CD107 surface expression was detected on the Belx2 line, however 25% of 7E7 T-cells were positive for CD107 in the presence of the cognate peptide (Figure 5.6). This indicates that Belx2 does not exhibit granule-mediated cytotoxic effector function.

5.3.6 Analysis of the TCRαβ chains expressed by Belx1 and Belx2 T-cell lines
Belx1 expresses TCR Vβ16 on ~94% of its T-cells (Figure 5.1). The TCR Vβ chain expressed by Belx2 could not be identified using the panel of 19 different monoclonal anti-TCR Vβ antibodies. In this section the central aim was to identify the Vβ chain/s used by Belx2, and also to investigate the TCR Vα chains used by both T-cell lines. As part of this process the precise CDR3 regions of the TCR chains were defined.

RNA was extracted from Belx1 and Belx2 T-cell lines (described in section 2.13.1). A reverse transcription reaction was carried out to generate cDNA. This cDNA was then used as a template for the PCR reaction using a set of TCR Vα and Vβ specific primers as described in section 2.13.2. The PCR products were purified, cloned, and sequenced. This part of the project was performed in collaboration with Dr John Boulter (Wales College of Medicine, Cardiff University), who was responsible for the cloning and sequencing of the TCR chains.
Figure 5.6 Belx2 does not exhibit a granule-mediated cytotoxic effector function in a CD107 assay. Belx2 T-cells or 7E7 were incubated with HLA-DRB1*01+ HLA-A*02+ BLCL with an anti-CD107 FITC antibody and golgi stop, in the presence of DMSO or either p37 (5μg/ml) (Belx2), or HPV16 E7(29-38) peptide (5μg/ml) (7E7) for 4 hours before analysing for CD107 expression by flow cytometry. T-cells were gated based upon their forward and side scatter profiles. Histograms depict the percentage of gated cells that were CD107+ with the DMSO control shown in grey and the 7E7 + T1H (A) and the Belx2 + p37 (B) shown in black fill. The values shown in bold type refer to the percentage of CD107+ cells within the lymphocyte gate.
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Both the IMGT unique numbering system for TCR sequences and the old serological-based system (described in section 1.10.2) are used in this analysis. The TCR Vβ chain used by Belx1 was confirmed to be TCR Vβ16, which is referred to as TRBV14 using the IMGT system. The CDR3 region of the TCR β chain was shown to consist of TRBV14/ TRBJ1-2. The TCR β chain used by Belx2 was identified as TRBV4-3 (TCR Vβ7). The CDR3 region of this β chain was shown to consist of TRBV4-3/TRBJ1-2. The amino acid sequence of the CDR3 regions for both β chains is shown in Table 5.1. From both Belx1 and Belx2 derived cDNA, the α chain TRAV17 (TCR Vα 3.1) was identified. For both cell lines the CDR3 region was shown to be composed of TRAV17/TRAJ49, the sequence of which is also shown in Table 5.1. It should be noted that the Vα chain detected from both lines was identical at the nucleotide level. It is possible but unlikely that the same chain could be used by both T-cell populations. It is more probable that the expression of this chain is restricted to either the Vβ16+ or Vβ16- population. PCR is a highly sensitive technique and may be simply detecting the TRAV17 α chain from the contaminating population in one of the lines. If this is the case, the failure to detect any other Vα chain, implies that there could have been a problem with one of the PCR primers. There is no commercially available antibody to specifically detect the TRAV 17 α chain, therefore it was not possible to identify on which T-cell population it was expressed. Isolation of clonal T-cell populations using a MoFlo cell sorter could help to resolve this uncertainty by removing contaminating populations.

In summary, TRBV4-3 (TCR Vβ7) has been identified as the TCR Vβ chain expressed by Belx2. It was also possible to confirm by molecular techniques that Belx1 expresses TRBV14 (TCR Vβ16). Only one TCR Vα chain (TRAV17) was identified, and it is not yet known with which TCR Vβ chain (if not both) it is expressed. All three chains have subsequently been cloned into vectors containing the TCR α and β constant domain genes, so as to enable the future production of soluble TCR.
### Table 5.1 Amino acid sequence of the CDR3 region of TCR Vα and Vβ chains expressed by Belx1 and Belx2 T-cell lines.

RNA extracted from Belx1 and Belx2 T-cell lines was used to make cDNA. TCR Vα and Vβ specific primers were used to amplify the specific TCR chain gene from each line by PCR. PCR products were cloned and sequenced. The amino acid sequence of the CDR3 regions for each chain identified is shown.

<table>
<thead>
<tr>
<th>CDR3 Region</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRBV4-3/TRBJ1-2</td>
<td>CASSQDPGGVFGYTFGSGLTVVE</td>
</tr>
<tr>
<td>TRBV14/TRBJ1-2</td>
<td>CASSQQGIGYTFGSGLTVVV</td>
</tr>
<tr>
<td>TRAV17/TRAJ49</td>
<td>CATDAGTGNQFYFGTGLTVIP</td>
</tr>
</tbody>
</table>
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5.4 Endogenous processing and presentation of the HPV16 E6\textsubscript{(127-144)} epitope

In this study a CD4+ T-cell response against p37 (HPV16 E6\textsubscript{(127-144)}) has been detected in 3 out of 4 HLA-DRB1*01+ women tested. This frequency of detection strongly suggested that this was a genuine epitope response. At this stage however, all responses had been generated against the synthetic 15mer peptide p37. It was important to demonstrate that the E6\textsubscript{(127-144)} epitope could be presented from an endogenously processed full length HPV16 E6 protein. Due to the lack of HPV16+ cervical carcinoma cell lines expressing HLA-DRB1*01 it was necessary to employ alternative methods to investigate the processing and presentation of the epitope.

5.4.1 E6\textsubscript{(127-144)} is not presented by HLA-DRB1*01+ BLCL infected with TA-HPV

This first approach involved the infection of HLA-DRB1*01+ and HLA-A*02+ BLCL with TA-HPV, a recombinant vaccinia virus expressing an HPV16 E6-E7 fusion protein (section 2.11). These BLCL were used as APC in an ICS assay with the Belx2 T-cell line and 7E7 CTL (Evans et al., 2001). Infection of BLCL with TA-HPV resulted in endogenous expression of HPV16 E6-E7 fusion protein that was recognised by 37% of HPV16 E6\textsubscript{(29-38)}-specific CTL (7E7) (Figure 5.7B). However when the same target was tested with the Belx2 T-cell line there was no recognition (Figure 5.7A). TA-HPV infected BLCL pulsed with p37 (as a positive control) were recognised by 85% of Belx2 (data not shown). Neither Belx2 or 7E7 recognised the BLCL infected with the control vaccinia strain Wyeth (≤0.2% IFNγ+ cells detected) (Figure 5.7).

The data shows that whilst Belx2 demonstrated recognition of p37-pulsed infected BLCL (exogenous source of peptide) no recognition was detected when APC were expressing an endogenous source of the E6-E7 protein.
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Figure 5.7 The HPV16 E6_{127-141} epitope is not presented by BLCL infected with TA-HPV. HLA-DRB1*01+ and HLA-A*02+ BLCL infected with either TA-HPV or Wyeth, were incubated with Belx2 (A) or 7E7 (B) at a ratio of 2:1 for 6 hours. The cells were stained with an anti-CD3-PE antibody and an anti-IFNγ-FITC antibody. T-cells were gated based upon their forward and side scatter profiles. The dot plots show the percentage of gated cells within the upper quadrants.
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The failure here to demonstrate endogenous processing and presentation of the HPV16 E6\textsubscript{(127-141)} epitope was most likely related to the fact that the majority HLA class II restricted peptides are processed from exogenously derived proteins, although some can be presented from endogenously expressed proteins. As TA-HPV infection results in the intracellular expression of the E6 (fusion) protein, it was not wholly unexpected that the HLA class II epitope was not presented. For this reason all subsequent approaches involved exogenously sourced E6 antigen.

5.4.2 The HPV16 E6\textsubscript{(127-141)} epitope is presented by APC incubated with soluble full-length E6 protein

In order to demonstrate endogenous processing and presentation of the HPV16 E6\textsubscript{(127-141)} epitope, a purified exogenous source of E6 was required. The laboratory was fortunate to receive a stock of recombinant, soluble full-length E6 protein (section 2.4.2), kindly donated by Dr. Marij Welters, Leiden University.

The first experiment carried out was an ELISpot assay. The HPV16 E6 protein was tested at 40μg/ml so as to achieve a similar molarity to the p37 used at 5μg/ml (2.08μM and 2.63μM respectively). Strong positive responses were detected against both the E6 protein and p37 containing wells (Figure 5.8). No response was detected from T-cells incubated with the HPV11 L1 protein (described in section 2.4.2), which shares no homology with HPV16 E6. From this data it appears that the HPV16 E6\textsubscript{(127-141)} epitope was presented by the BLCL to the Belx2 T-cell line.
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![Bar Graph]

**Figure 5.8 Belx2 T-cells demonstrate responses against the HPV16 E6 full-length protein in an ELISpot assay.** Belx2 T-cells were plated out at 5x10^3 cells/well with HLA-DRB1*01+ BLCL at a ratio of 1:1 in the presence and absence of either 40µg/ml E6 protein, 40µg/ml HPV11 L1 protein (negative control), or 5µg/ml p37. Samples were plated out in triplicate or quadruplicate. Background responses have been subtracted from the data shown.
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In the next stage of this investigation the concentration of E6 protein required for activation of Belx2 was determined by ICS. In the previous experiment it was shown that 40μg/ml E6 protein could activate similar numbers of Belx2 T-cells as p37 at 5μg/ml. As there was limited full length E6 protein available it was necessary to investigate Belx2 T-cell responses at lower concentrations of the E6 protein.

Belx2 T-cells were tested in an ICS assay in the presence of either DMSO, p37, or E6 protein (25, 10 and 1 μg/ml) (Figure 5.9). As predicted from the ELISpot response, there was a clear IFNγ T-cell response to soluble E6 protein. The IFNγ response detected at 25μg/ml and 10μg/ml of E6 protein was similar both in the number of IFNγ+ cells and in the MFI of staining. As the dose was decreased from 10μg/ml to 1μg/ml, both the number of cells positive for IFNγ and the level of cytokine production (MFI) reduced substantially. No background response was detected in the DMSO control.

Collectively this data suggests there is no advantage in using concentrations of E6 protein >10μg/ml in an ICS assay, and that 1μg/ml was an adequate concentration of E6 protein for use in future experiments.

Belx1 was tested simultaneously with the experiment just described. The experimental setup was identical with the exception that cells were only tested with either DMSO, p37 (5μg/ml), or E6 protein (25μg/ml) (Figure 5.10). In the presence of p37, 35.3% of gated cells (within the TCR Vβ16+ quadrant) were IFNγ+. However, in the presence of E6 protein no IFNγ response was detected. This experiment was repeated using 50μg/ml E6 protein and again no response was detected (data not shown). Due to limiting amounts of E6 protein, it was not possible to further investigate the Belx1 response with higher concentrations.
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Figure 5.9 Belx2 T-cells demonstrate responses against the HPV16 E6 full-length protein in an ICS assay. Belx2 T-cells were plated out at 5x10^5 cells/well in the presence of HLA-DRB1*01+ BLCL at a ratio of 1:2. Cells were incubated in the presence of either DMSO, p37 (5μg/ml), or E6 protein (25, 10 and 1 μg/ml) for 12 hours and an ICS assay was performed. Cells were co-stained with an anti-TCR Vβ16-PE antibody and an anti-IFNγ-FITC antibody. T-cells were gated based upon their forward and side scatter profile. Histograms depict the percentage of gated cells that were IFNγ⁺, with the DMSO control shown in grey and the E6 protein tests (A) or p37 test (B) shown in black fill. The values shown in bold type refer to the percentage of IFNγ⁺ cells, whilst the values shown in italics refer to the MFI of the IFNγ stain. Duplicate tests were carried out, and representative data is stated.
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Figure 5.10 Belx1 T-cells do not recognise the HPV16 E6 full-length protein in an ICS assay. Belx1 T-cells were plated out at 5x10^5 cells/well in the presence of HLA-DRB1*01+ BLCL at a ratio of 1:2. Cells were incubated in the presence of either DMSO, p37 (5µg/ml), or E6 protein (25µg/ml) for 12 hours and an ICS assay was performed. Cells were co-stained with an anti-TCR Vβ16-PE antibody and an anti-IFNγ-FITC antibody. T-cells were gated based upon their forward and side scatter profile. Histograms depict the percentage of TCR Vβ16+ cells that were IFNγ+, with the DMSO control shown in grey and the p37 test (A) or the E6 protein test (B) shown in black fill. The values shown in bold type refer to the percentage of IFNγ+ cells.
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The data generated from these experiments clearly indicated that the full length E6 protein could be processed and the epitope presented to the Belx2 T-cells. In order to verify that uptake of the E6 protein was required for Belx2 activation, the BLCL used in this experiment were either added directly to the T-cell containing wells as normal, or were first fixed using 2% paraformaldehyde (section 2.9.2.2). This fixation process inhibits the uptake of exogenous protein by the BLCL, yet should not abrogate their capacity to present exogenously pulsed peptide.

Belx2 T-cells were incubated with either unfixed BLCL or fixed BLCL, in the presence of either DMSO, p37, or E6 protein. Fixation of BLCL abolished recognition of E6 protein by Belx2 T-cells (Figure 5.11). This was not due to loss of antigen presenting function because Belx2 were able to recognize both fixed and unfixed p37-pulsed BLCL.

Collectively this data confirms that the HPV16 E6_{127-141} epitope can be processed endogenously from an exogenous source of full length E6 protein and presented via the HLA class II pathway. It was also demonstrated that the 15mer peptide (p37) can be recognised by Belx2 without a requirement for endogenous processing. These experiments were all carried out using BLCL as the APC. These cells are not entirely representative of the B-cells that would be found in vivo as they have been immortalized through EBV infection and express consistently high levels of HLA class II. It was therefore important to verify that the E6_{127-141} epitope could also be endogenously processed and presented by primary APC from the E6 protein, making the test system more representative of the in vivo situation.
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Figure 5.11 Effect of fixation on presentation of E6(127-141) epitope processed from full length E6 protein, to Belx2 T-cells. Belx2 T-cells were plated out at 5x10^5 cells/well in the presence of either unfixed (A) or fixed (B) BLCL at a ratio of 1:2. BLCL were fixed using 2% paraformaldehyde. Cells were tested with either DMSO, p37 (5μg/ml), or E6 protein (1μg/ml) for 12 hours and an ICS assay was performed. Cells were co-stained with an anti-TCR Vβ16-PE antibody and an anti-IFNγ-FITC antibody. T-cells were gated based upon their forward and side scatter profile. Histograms depict the percentage of cells that were IFNγ+, with the DMSO control shown in grey and the E6 protein or p37 tests shown in black fill. The values shown in bold type refer to the percentage of IFNγ+ cells, whilst the values shown in italic refer to the MFI of the IFNγ stain. Duplicate tests were carried out, and representative data is stated.
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To address this, an ICS assay was performed using either freshly isolated HLA-DRB1*01+ PBMC or CD14+ cells as the APC. CD14 is expressed on both monocytes and macrophages, which are competent antigen presenting cells which express HLA class II (Taylor et al., 2005). CD14+ cells were enriched to a purity of 97% (data not shown) from PBMC using anti-CD14-microbeads as described in section 2.5.4.

T-cells can be identified based upon their forward and side scatter profiles, which is used to create a ‘lymphocyte gate’. An issue with using PBMC as the APC is that a significant proportion of the PBMC are T-cells. As a result, cells present within the lymphocyte gate will be both Belx2 T-cells and PBMC-derived T-cells. It was therefore necessary to label the Belx2 T-cells prior to the assay to allow these cells to be specifically selected for analysis. Belx2 T-cells were labelled using a cell tracker dye (Cell Trace Far red DDOA-SE tracker dye) as described in section 2.9.3. This dye was selected as it did not effect cell viability (assessed by trypan blue staining as described in section 2.1.5) over 24 hours (data not shown), and it can be detected in the FL-4 channel by flow cytometry, therefore not interfering with the IFNγ or TCR Vβ16 stains (detected in FL-1 and FL-2 channels). T-cells were incubated with several concentrations of the dye to determine a concentration at which an appropriate level of fluorescence was detected (Figure 5.12). 0.05μg/ml DDAO (MFI 1,473) was selected for use in this assay.

Having identified an appropriate concentration of cell tracker reagent it was possible to proceed to the ICS assay. Fluorescently labelled Belx2 cells were incubated with either PBMC or CD14+ enriched cells in the presence of either DMSO or E6 protein (Figure 5.13). This assay demonstrated that PBMC were capable of processing the full-length E6 protein and presenting the cognate epitope, as 98.0% of T-cells were IFNγ+, with background IFNγ production accounting for only 0.1%. Similar results were obtained using CD14+ cells as the APC, with 94.4% of Belx2 T-cells being IFNγ+.
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This indicates that the ability to present the E6_{127-141} epitope from full length E6 protein is not restricted to BLCL, but can also be performed by PBMC. It was also established that the CD14+ population of PBMC are competent APC in this system.

5.5 Molecular modelling of the p37-HLA-DRB1*01 complex

In the previous chapter, p37 was shown to be presented by HLA-DRB1*01 and could be aligned to complement the binding motif for this HLA-DR allotype. X-ray crystallisation studies have determined the 3-D structure of several peptide-HLA-DRB1*01 complexes (Stern et al., 1994). Preliminary molecular modelling of the p37 epitope complexed with HLA-DRB1*01 has allowed the predicted binding of this epitope to be compared with that of a number of other HLA-DRB1*01-restricted peptides (section 2.14).

The modelling was carried out by Dr. Istvan Bartok, Imperial College, London. The p37 epitope was superimposed onto the crystallised structures of HLA-DRB1*01 bound to 1) Influenza A HA_{306-318} (Stern et al., 1994), 2) HIV-1 gag_{35-47} (Zavala-Ruiz et al., 2004), and 3) Triose phosphate isomerase_{23-37} (Sundberg et al., 2002) (Figure 5.14A). From these models it appears that p37 'sits' in the binding groove of the HLA-DR molecule in a similar way to the known, well-characterised epitopes.

The epitope (DKKQRFHNJRGRWTG) was aligned based upon the HLA-DRB1*01 binding motif with P1=F, P4=I, and P6=G. In Figure 5.14B the epitope is shown with the HLA-DR molecule excluded 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 (positively charged) at positions P-1, P5 and P7, and the glutamine (P-2), tryptophan (P8) and glycine (P10) residues all protrude on the water accessible surface of the bound peptide. It is possible that one or more of these residues could form contacts with the CDR3 loops of the TCR structure, and therefore be relevant to the recognition of the epitope by the T-cell. The CDR3 region of the α
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Figure 5.12 Titration of DDAO-SE cell tracker dye with T-cells
Belx2 T-cells were incubated with 0.5 (blue), 0.25 (red) and 0.05µg/ml (black fill) of DDAO-SE cell tracker dye for 15 minutes at 37°C. Unstained control cells are shown in grey. Cells were washed and cultured for 12 hours before analysis of stain intensity by flow cytometry.

Figure 5.13 PBMC and CD14+ enriched cells process and present E6(127-141) from full-length E6 protein to Belx2 T-cells in an ICS assay. Belx2 T-cells pre-stained with DDAO cell tracker dye were plated out at 5x10^5 cells/well in the presence of PBMC or CD14+ enriched cells at a ratio of 1:2. Cells were incubated in the presence of either DMSO or E6 protein (10µg/ml) for 12 hours and an ICS assay was performed. Cells were co-stained with an anti-TCR Vβ16-PE antibody and an anti-IFNγ-FITC antibody. T-cells were gated, based upon their forward and side scatter profiles and Belx2 T-cells were selected based upon DDAO staining. Histograms depict the percentage of Belx2 T-cells that were IFNγ+, with the DMSO control shown in red and the E6 test shown in black fill.
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chain (TRAV17/TRAJ49) (CATDAGTGNQFYFGTGTSLTVIP) identified from the Belx1 and Belx2 T-cell lines, contains a negatively charged aspartic acid residue (D), which could in theory form electrostatic interactions with a positively charged arginine in p37.

Molecular modelling of the p37-HLA-DRB1*01 complex suggests that this epitope binds to the HLA-DR molecule in a similar fashion to other well characterised HLA-DRB1*01-restricted peptides. The anchor residues predicted by the 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 future studies investigating the effect amino acid substitutions (in p37) on T-cell recognition.
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A

Influenza A HA 306-318
PKYVKONLKLAT

HIV-1 gag 35-47
PEVPMFSALEN

Triose phosphate isomerase 23-37
GELIGTLNAAKVPAD


Figure 5.14 Molecular modelling of p37 complexed with HLA-DRB1*01.
The p37 epitope was superimposed onto the models of Influenza A HA (306-318), HIV-1 gag (15-47), and Triose phosphate isomerase (23-37) peptides complexed with HLA-DRB1*01 (defined from X-ray crystallisation studies) (A). The E6 peptide is shown in red ribbon whilst the reference peptides are shown in grey ribbon. The p37 epitope is also depicted in ‘ball and stick’ with the HLA-DR molecule excluded for clarity (B). The anchor residues proposed to sit in the specificity 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 workstation using Insight II modelling suite (MSI/Accelrys). The first three amino acid residues DKK were excluded from analysis to simplify the modelling.
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5.6 Discussion

The T-cell lines Belx1 and Belx2 were successfully generated by cell sorting the TCR Vβ16 positive and negative populations constituting line 46. It was previously shown that these two populations whilst both recognising p37 exhibited highly differential IFNγ response profiles when assessed by ICS. These functional differences were interesting, considering that the two populations originated from the same PBMC culture. Whilst the Vβ16+ T-cells were the most abundant population in several lines, they were shown to be inferior to the Vβ16- T-cells in their IFNγ effector response. The Belx1 and Belx2 T-cell lines were generated in order to investigate some of the potential reasons that could explain these differences and also to determine if the two lines differed in any other significant functional or phenotypic way.

The purity of the lines obtained by cell sorting was reasonably high. The Belx2 T-cell line contained <1% Vβ16+ cells. The Belx1 line however, was contaminated with ~6% Vβ16- cells. As it is well established that the Vβ16- cells are superior in both the proportion of responding cells as well as the quantity of IFNγ they produce, this contamination (of Belx1) must be considered in experiments where it was not possible to discriminate between the dominant and contaminating cell populations.

The use of ICS to examine the IFNγ responses to p37 avoided this problem, as it permits the exclusion of the contaminating population from the overall IFNγ response. ICS analysis revealed that the Belx2 T-cells were as expected, superior to Belx1 T-cells in their IFNγ response, with >96% of cells responding to p37 (5µg/ml). Interestingly the proportion of Belx1 (Vβ16+) T-cells that produced IFNγ in response to p37 was shown to increase over two fold between days 14 and 21 post expansion, with 83% of cells responding at the latter time point. In the previous chapter, line 46 (the parental line of Belx1 and Belx2) was not assessed by ICS beyond day 14 post expansion. This analysis of Belx1 IFNγ production beyond day 14, revealed that the proportion of Vβ16+ T-cells capable of recognising p37 was previously underestimated. The analysis of cell surface markers expressed by the T-cell lines on days 14 and 21 post
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expansion allowed the differences in the IFNγ responses between a) the two T-cell lines and b) Belx1 on day 14 and 21, to be investigated.

Analysis of cell surface markers on the Belx1 and Belx2 T-cell lines revealed differences in the expression of CD3, CD25 and CD28 that correlated with a superior IFNγ response from Belx2. The results of this experiment are summarised in Table 5.2.

Perhaps the most significant difference noted between the two cell lines was that CD3 expression (at both time points) was greater for Belx2 T-cells. It is possible that this difference in CD3 expression could contribute to the superior IFNγ response demonstrated by Belx2. CD3 expression indicates directly the level of TCR complex expressed, as TCR αβchains are non-covalently associated with CD3 proteins on the cell surface. The primary function of the CD3 protein is to couple the recognition of antigen with intracellular signalling, resulting in functional activation of the T-cells. It is therefore unsurprising that the high levels of TCR expression (MFI of CD3 stain) by Belx2 would correlate with high levels of T-cell activation. Furthermore, the increase in the number of Belx1 T-cells producing IFNγ between days 14 and 21 could in part be attributed to the increase in TCR expression over time.

In addition to CD3, another molecule involved in T-cell signalling was also shown to be differentially expressed by the two T-cell lines. CD28 interacts with B7 co-stimulatory molecules expressed on APC and serves to transduce signals that augment T-cell activation. This molecule is expressed by nearly all CD4+ T-cells (in vivo) (Riley and June, 2005). The loss of CD28 expression has been shown to correlate with immune senescence, and its re-introduction (through IL-12 signalling) can restore T-cell effector function (Warrington et al., 2003). Compared to Belx1, the Belx2 line contained a greater proportion of CD28+ T-cells (up to 1.8 fold more), and the MFI of CD28 staining for this line was also higher (2.4 fold). It would be interesting to investigate by ICS what proportion of IFNγ+ Belx1 T-cells express CD28, in order to evaluate the importance of this molecule in Belx1 T-cell activation. To investigate the
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The relative contribution of differential CD28 expression to the Belx1 and Belx2 T-cell lines IFNγ response, CD28 blocking experiments could also be performed.

In summary, it is possible that differences in IFNγ production (in response to p37) between the two cell lines could be partly related to differential expression levels of the TCR and accessory molecules involved in co-stimulation. The fact that Belx1 and Belx2 use two different TCR β chains should also be considered as a potential explanation, however the relative avidity of these chains for the p37-HLA class II complex is not known.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Day14</th>
<th>Day21</th>
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<tbody>
<tr>
<td></td>
<td>Belx1</td>
<td>Belx2</td>
</tr>
<tr>
<td>CD3</td>
<td>100</td>
<td>149.1</td>
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<tr>
<td>CD4</td>
<td>100</td>
<td>644.0</td>
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<tr>
<td>CD25</td>
<td>56.5</td>
<td>25.8</td>
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<tr>
<td>CD28</td>
<td>56.0</td>
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Table 5.2 Expression of cell surface markers by Belx1 and Belx2.
The T-cell lines Belx1 and Belx2 were assessed on days 14 and 21 post expansion for the expression of cell surface markers; CD3, CD4, CD25 and CD28. Cells were stained with FITC or PE conjugated antibodies recognising these CD molecules and analysed by flow cytometry. Live T-cells were gated based on their forward and side scatter profile. The standard deviation for the day 21 sample duplicates never exceeded +/- 5.1 for both the MFI and percentage gated values and therefore representative data is shown.
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It was previously shown using line 46 that decreasing the p37 concentration from 5μg/ml to 0.2μg/ml did not effect the percentage of IFNγ+ Vβ16- cells, but dramatically reduced the proportion of IFNγ+ Vβ16+ cells (Figure 4.24). Therefore the effect of lower p37 concentration on only Belx2 (Vβ16-) activation was investigated in this chapter. A marginal decrease in the proportion of Belx2 T-cells recognising p37 was noted as the concentration of peptide was reduced from 5μg to 0.25μg/ml, whilst the MFI of IFNγ was shown to decrease by >50% (Figure 5.5). This suggests that whilst the T-cells were still being activated at the lower peptide concentration, the strength of the activation signal was decreased, resulting in the reduced production of IFNγ. It is feasible that at lower doses of peptide fewer TCRs engage the p37-HLA class II complexes, leading to a decrease in the number of TCRs triggered. This is supported by a previous study which correlated TCR occupancy with the strength of the effector response (IFNγ production) (Valitutti et al., 1995).

Whilst the ICS assay provides information regarding the percentage of cells producing IFNγ, it is not possible to ascertain the concentration of IFNγ secreted by the T-cells. To allow a direct comparison between the quantity of IFNγ produced by Belx1 and Belx2 lines, a CBA assay and an IFNγ ELISA were performed. The major advantage of the CBA is that in addition to IFNγ, it allows the detection of several other immunoregulatory cytokines from the same sample. CBA is a sensitive technique which is both simple and quick to perform. The very high cost of this assay does however limit the ability to perform multiple experiments.

The results of this experiment suggest that both Belx1 and Belx2 display a Th1 phenotype, as the dominant cytokine expressed was IFNγ. Overall IL-2, IL-4 and IL-10 were detectable in the 10 hour supernatant of both cell lines (incubated with p37). Whilst the concentration of each cytokine was higher in Belx2 supernatant, all were detected below 200pg/ml. It is not unusual to detect several cytokines, as other groups have described similar findings for Th1 cells (Davignon et al., 1996; Sun et al., 2002). In the case of IFNγ, CBA analysis of the supernatants from both time points
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demonstrated that Belx2 produced several fold more IFNγ than Belx1. Unfortunately at the 10 hour time point, the concentration of IFNγ produced by Belx2 T-cells exceeded the upper limits of detection by the assay. As it was not possible to repeat the CBA experiment due to high costs, the IFNγ ELISA was used to analyse the equivalent replicate samples. This revealed that in the 10 hour supernatant, Belx2 produced 11,020 pg/ml IFNγ, which is over 12 fold more than produced by Belx1. On a per cell basis the amount of IFNγ secreted by Belx2 was either equivalent or greater when compared with other HPV, CMV and EBV specific CD4+ T-cell lines analysed using similar systems (Davignon et al., 1996; Facchinetti et al., 2005; Long et al., 2005). For the Belx1 line it should be taken into account that a large proportion of the cytokines detected in the supernatant could have been secreted by the 6% contaminating Vβ16-T-cells. A 100% pure TCR Vβ16+ clone would be required in order to determine a more accurate picture of the cytokine secretion profile of these cells.

It was not possible to identify the Vβ chain used by the Belx2 T-cell line by antibody staining. Therefore the specific Vβ chain from this line was identified using a PCR-based approach. Using this technique, previously used to identify a number of TCR chains from T-cell clones (Moysey et al., 2004), it was also possible to investigate the Vα chain usage of the two lines. The TRBV14 (Vβ16) TCR chain was cloned and sequenced from Belx1, confirming the results previously obtained using antibody staining. Analysis of the TCR Vβ chain sequence from Belx2 indicated usage of a TRBV4-3 (Vβ7) chain. An antibody specific for the TCR Vβ7 chain was used in the original antibody panel and failed to detect the Belx2 TCR Vβ chain. The precise epitope recognised by this antibody has not been defined and it is therefore likely that the antibody does not recognise all Vβ7 chains. This identification of TCR chains from T-cell lines using a molecular-based technique thus has an obvious advantage over antibody-based identification. The detection of only two TCR Vβ chains by PCR suggests that the parental line (line 46) was oligoclonal, containing only two T-cell populations.
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The question still remains as to which (if not both) TCRβ chain the TCRα chain (TRAV17) is expressed with. In the absence of an antibody for this α chain it may necessary to make the two TCRαβ chain combinations (TRBV14 and TRBV4-3) and test them in a functional assay. Potentially, the cloned TCR chain genes could be used to express soluble TCR molecules for use in TCR-peptide-HLA-DR affinity studies. The introduction (by transfection or infection) of the cloned TCR chains into other T-cells could potentially be used to retarget these cells to recognise the HPV16 E6(127-141) epitope. This is a viable prospect as a number of groups have successfully redirected the specificity of recipient T-cells using TCR gene transfer (Cooper et al., 2000; Fujio et al., 2000; Orentas et al., 2001).

In this study the TCRαβ chains have only been defined from two T-cell lines from one donor. Future work should investigate the TCR usage by other p37-specific T-cell lines from several other donors in order to get a clear picture of how frequently the α chain (TRAV17) and β chains (TRBV14 and TRBV4-3) identified in this study are used. A HLA-DRB1*01-p37 tetramer would greatly facilitate this process, as it would enable the selection of p37-specific T-cells from short term PBMC cultures, without bias towards their capacity to secrete IFNγ.

The HPV16 E6(127-141) epitope was shown to be processed from an exogenous source of recombinant full-length E6 protein and presented to the Belx2 T-cell line. This was demonstrated not only with BLCL but also using freshly isolated autologous PBMC and CD14+ enriched cells. The predicted pathway for epitope generation is that the exogenous E6 protein is endocytosed by the APC. The E6 protein then undergoes proteolysis in the endosomal/lysosomal pathway and the resulting peptide fragments include the epitope recognised by Belx2 T-cells. Within the MIIC compartment the epitope binds to HLA-DRB1*01 and the HLA class II-peptide complex is expressed on the cell surface.

The major APCs in PBMC are DCs, B-cells and monocytes. It is not possible to precisely identify which of these APC populations (if not all) are capable of E6-epitope
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presentation, although enriched CD14+ cells are clearly capable of processing the E6 protein and presenting the epitope.

The use of BLCL fixation in an ICS assay confirmed that uptake of the E6 protein by BLCL was a necessary event in order for the E6(127-141) epitope to be presented. As protein endocytosis was required, this experiment also ruled out the possibility that a contaminating peptide fragment in the E6 sample could be causing the T-cell response by binding directly to the HLA class II molecules. This method of APC fixation has been used previously (Elferink et al., 1985), and is a simple and elegant way to investigate the requirement for endogenous processing of a protein or peptide for T-cell recognition.

The failure to detect a T-cell response using TA-HPV infected BLCL as APC was not altogether surprising. TA-HPV infection results in an endogenously expressed HPV16 E6 antigen source (as part of an E6-E7 fusion protein). The antigen-presentation pathway primarily used by endogenously derived protein is the HLA class I pathway. It is clear from the 7E7 CTL response that an E6 derived epitope was presented by the class I pathway. Most peptides presented by the HLA class II pathway are derived from an exogenous source of protein antigen, however reports of CD4+ T-cells recognising epitopes derived from endogenously expressed proteins are becoming increasingly common (Facchini et al., 2005; Munz et al., 2000). Attempts have been made to specifically target intracellularly expressed proteins into the HLA class II pathway, so as to generate an effective CD4+ T-cell response (Bonini et al., 2001; Su et al., 2002). Mice immunised with vaccinia expressing the chimeric E7/LAMP-1 protein demonstrated enhanced levels of E7-specific lymphoproliferation compared to immunization with the wild type E7 protein alone (Wu et al., 1995). The LAMP-1 molecule (lysosomal-associated membrane protein) was proposed to have targeted the E7 protein into the endosomal/lysosomal compartments, resulting in HLA class II presentation of E7-derived epitopes to CD4+ T-cells. It is therefore possible that the infection of BLCL by vaccinia expressing a HPV16 E6/LAMP-1 chimeric protein could result in epitope recognition by the Belx2 T-cell line.
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The demonstration that the E6\textsubscript{(127-141)} epitope is processed and presented from the full length protein, validates the original responses detected against p37. The p37 used in these experiments was synthesised to a purity of >95% which was confirmed by mass spectrometry. The BLCL fixation studies demonstrated that p37 does not require endogenous processing in order for it to be recognised by Belx2. However, this does not show that the 15mer is the minimum epitope that can be recognised. To determine the minimum epitope for recognition a number of truncated peptides could be synthesised and tested with Belx2 in an ICS assay. Based upon the p37-HLA-DRB1*01 model, the peptide could still bind and be recognised by the TCR without the first three amino acids (DKK), as these were not predicted to be involved. This hypothesis could only be verified experimentally.

Following the identification of HLA-DRB1*01 and -DRB1*15 as the allotypes responsible for p37 and p64 presentation respectively, the peptide sequences were compared to the corresponding binding motifs (www.syfpeithi.de). Peptide binding motifs for HLA-DRB1*01 and -DRB1*15 allotypes (Rammensee \textit{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-DRB1*01 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 \textit{et al.}, 1999).

\begin{align*}
P1 &= YVLFIAMW \\
P4 &= LAIMMNQ \\
P6 &= AGSTCP \\
P9 &= AIVNFY\
\end{align*}
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The sequence of p37 can be aligned to compliment 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 p37 is a conventional HLA-DRB1*01 restricted peptide. Using the SYFPEITHI predictive database (Rammensee et al., 1999) p37 is predicted to bind to HLA-DRB1*01 molecules. The database provides a score which relates to how strongly a peptide may bind, based upon the amino acids it contains. p37 was given high a score of 25. As a comparison, the Influenza A HA\(_{306-318}\) peptide which is known to strongly bind HLA-DRB1*01 (Stern et al., 1994), was given a score of 34.

This same approach was taken when assessing how well p64 conforms to the HLA-DRB1*15 binding motif. The binding motif for HLA-DRB1*15 consists of 3 anchor positions; P1, P4 and P7. The potential amino acids for each of the anchor residues are listed below (Rammensee et al., 1999).

\[
P1 = \text{L V I} \\
P4 = \text{F Y I} \\
P7 = \text{I L V M F}
\]

The sequence of p64 can be successfully aligned either to compliment P1 and P4 (shown in bold underline), or P4 and P7 (shown in bold italic) as highlighted in the following: E V F E F A F K D L F V V Y R. The SYFPEITHI database predicts that this peptide would bind to HLA-DRB1*15. This peptide was given a score of 24. As a comparison the myelin basic protein \(_{84-102}\), which is known to bind strongly to HLA-DRB1*15 (Wucherpfennig et al., 1994) was given a score of 36.

Whilst the binding motifs for HLA-DRB1*01 and HLA-DRB1*15 have been defined, there are a number of other DR allotypes for which there is either a very limited motif or no motif available e.g. HLA-DRB1*11, -DRB1*14, and -DRB1*16. In this study, overlapping 15mer peptides that covered the entire sequence of the E6 proteins were used. This approach to define epitopes is not biased towards predicted binding motifs and is therefore less likely to 'miss' epitopes that do not match such criteria.
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The molecular models produced in this chapter indicate that p37 binds to the HLA-DRB1*01 molecule in a similar fashion to peptides previously crystallised with HLA-DRB1*01. The model suggests that the anchor residues at relative positions 1, 4, 6 and 9 fit well in the specificity pockets of the HLA class II peptide binding groove. To confirm this model, peptides containing amino acid substitutions at the key positions 1, 4, 6 and 9, could be tested to determine if the substitutions prevented binding to the HLA-DR molecule (thereby abrogating recognition by the Belx2 T-cell line). It may also be possible to alter the sequence of the p37 epitope so as to enhance its capacity to bind to the HLA-DR molecule, increasing its immunogenic potential. One obvious target for substitution is the threonine (T) anchor residue at P9, as this does not ‘fit’ with the HLA-DRB1*01 binding motif (discussed in section 4.6). Potentially, by substituting the T residue with one which is predicted to bind by the motif, namely A, F, V, N, F, or Y, the peptide may bind more strongly to the HLA-DR molecule. Such an approach has been used successfully in several studies (Huart et al., 2002; Sarobe et al., 1998). The model also highlighted six residues which could potentially interact with the TCR structure (Figure 5.14) and which therefore may contribute to TCR recognition. By sequentially implementing non-conservative substitutions of these residues in the E6(127-141) epitope, it may also be possible to get a clearer indication of which residues are important in T-cell recognition.

Alignment of the p37 sequence (HPV16 E6(127-141)) with the E6 protein sequences from eight different HR HPVs revealed that residues in the core region involved in HLA-DR binding are relatively well conserved. In particular, the p37 anchor residues (at positions 1, 4, 6 and 9) are very well conserved in the E6 proteins from all the HR HPVs assessed (summarised in Table 5.3). Furthermore, the putative TCR contact residues at P-1, P7 and P8 are also well conserved (Table 5.3). It would be interesting to test the E6 peptides from these HPV types with the Belx2 T-cell line to determine if they could be recognised. It also may be possible to engineer the peptide sequence to allow cross-reactive recognition between HPV16 E6-specific T-cells and other HR HPV E6-specific T-cells. It would also be possible to test for antagonism effects, as peptides which are similar in sequence to a cognate peptide, can in some cases result in
Chapter 5 - Results

only partial activation of the T-cell. This effectively turns off the T-cells ability to respond to its original cognate epitope (Jameson, 1998). Therefore, it is possible that the exposure of Belx2 to the HPV33 E6 peptide for example, could prevent T-cell activation upon subsequent p37 re-stimulation.

In this chapter the p37-specific T-cell lines Belx1 and Belx2 were isolated. Characterisation of these lines revealed a correlation between a superior IFNγ response by Belx2 with higher expression levels of molecules involved in T-cell activation. The Belx2 line was used to demonstrate the endogenous processing of the E6_(127-141) epitope from full length E6 protein. Identification of the TCR α and β chains used by these lines will prove useful in the further investigation of p37 responses both in vitro and ex vivo.

Table 5.3 Alignment of amino acid sequences from HR HPV E6 proteins with p37 demonstrates conservation of anchor residues used in HLA-DRB1*01 binding.
The region of p37 predicted to bind to HLA-DRB1*01 is shown in bold. The relative positions (P) of the amino acids are shown, with anchor residues located at P1, P4, P6 and P9. The amino acid sequences from HPV18, 31, 33, 51, 52, 58 and 82 E6 were aligned with the HPV16 E6_(131-140) sequence (predicted binding region in p37), and anchor residues conserved with the HPV16 E6 sequence are highlighted in red. All non-conserved residues are shown in underlined italics. Putative TCR contact residues are found at positions P-1, P5, P7 and P8.

<table>
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<th>P-1</th>
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<th>P3</th>
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Chapter 6 – Discussion

Chapter 6

Final Discussion

The causative agent in the development of cervical cancer is infection with 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 et al., 1994; Petry et al., 1994), and also animal studies (Nicholls 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 et al., 1999; Bontkes et al., 2000; de Gruijl et al., 1998; de Jong et al., 2004; Steele et al., 2005;Welers et al., 2005) and more recently in healthy donors (de Jong et al., 2002; Facchinetti et al., 2005; Welers et al., 2003; Welers et al., 2005). 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 et al., 2001; Welers et al., 2003). As these women have successfully cleared viral infection, it is feasible that the epitopes against which the antiviral 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. A central aim in this project was to define novel immunogenic HPV16 and HPV18 E6 and E7-derived epitopes from healthy donors. This is important as very few such epitopes have been identified to date, with most studies focussed on the identification of immunogenic regions of the protein.

Ten healthy women were screened for T-cell responses to pools of overlapping 15mer peptides representing the HPV16 and HPV18 E6 and E7 proteins. Responses to the E6 peptide pool was detected in 4/10 donors for HPV16 and 4/10 for HPV18. This is the
Chapter 6 – Discussion

first study to define both HPV16 and HPV18 E6-derived CD4+ T-cell epitopes from the same individuals.

Overall, four novel epitopes (p19, p30, p36, and p37) were identified from HPV16 E6 and two further epitopes (p64 and p65) were defined from HPV18 E6. This is an important contribution to the field as very few HPV16 E6 and HPV18 E6 (Facchinetti et al., 2005; Welters et al., 2003) CD4+ T-cell epitopes have been defined from healthy donors. Of the four peptides that were further investigated, all were shown to be CD4+ T-cell-restricted and were presented by HLA-DR molecules. The precise HLA-DR allotypes involved in the presentation of p37 and p64 were identified to be HLA-DRB1*01 and HLA-DRB1*15 respectively. These are the first HR HPV epitopes demonstrated to bind HLA-DRB1*01 and HLA-DRB1*15. The potential HLA-DR allotypes responsible for presentation of the other peptide epitopes can be narrowed down by determining the HLA class II haplotypes of the donors from which the responses were detected (Table 6.1). To fully exploit the potential usefulness of these epitopes it will be necessary to precisely define the specific HLA-DR allotypes responsible for their presentation. This could be achieved by using HLA-typed APC as described in this study, or alternatively by screening the peptides in HLA class II binding assays (Sidney et al., 2002). An advantage of using the latter technique is that it could provide further information regarding the binding promiscuity of each epitope, as multiple HLA-DR allotypes could be screened.

One benefit of identifying CD4+ T-cell epitopes rather than purely defining immunogenic regions of the E6 protein is that, armed with the knowledge of the HLA-DR restriction, it will be possible to directly monitor the appropriate donors/patients to identify the frequency and nature of immune responses in an epitope-specific context. This knowledge also allows the design and generation HLA class II-peptide tetramers. Tetramers are an invaluable immunological tool, which can be used to rapidly enrich peptide-specific T-cells and also facilitate precise phenotyping of such T-cells by flow cytometry. Another advantage of tetramer technology is the ability to detect low frequency T-cells directly \textit{ex vivo} (from PBMC or tumour infiltrating lymphocytes).
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Whilst HLA-DRB1*01 tetramers are commercially available, they were not used in this study due to their high cost.

It will be important to fully investigate the frequency with which these epitopes can be detected in healthy women, so as to evaluate their potential utility in an immunotherapeutic setting. This could be achieved by recruiting a large cohort of women (selected based upon their HLA-DR haplotypes) and testing for responses against the individual peptides. Responses to three of the epitopes have been detected in at least two donors, indicating that this method of epitope mapping may favour the identification of dominant responses.

Following on from this, it would be interesting to screen for T-cell responses against these epitopes in HLA-matched women with varying severity of cervical disease. A study including women with incident HPV infection, low grade CIN, high grade CIN or cervical cancer would give an indication as to how frequently responses can be detected against these epitopes in the different disease states. By studying this cohort over an extended period of time it would also be possible to track changes in the immune responses to these epitopes that may correlate with disease clearance or progression.

The epitopes defined in this study could potentially be used as a ‘read out’ for immunological responses to therapeutic vaccination strategies. The lack of defined T-cell epitopes (for which the HLA restriction is known), has limited the thorough evaluation of the immunogenicity of vaccines tested in clinical trials (Adams et al., 2001).

In this study, all of the epitopes defined were recognised by CD4+ T-cells. CD4+ T-cells are known to play a vital role in the activation and co-ordination of CD8+ cytotoxic T-cells which are the major effectors in the clearance of virally infected or transformed cells. CD4+ T-cells can themselves exhibit potent effector functions relevant to the control of infected or transformed tissue (Bickham et al., 2001). As the CD4+ T-cell response is likely to be an important component of the overall anti-HPV immune response, it seems likely that an optimal therapeutic strategy should direct responses
against both CD4+ and CD8+ T-cell epitopes (Zwaveling et al., 2002). Therefore, the
generation of a vaccine construct incorporating the epitopes defined in this study in
combination with previously defined CTL epitopes could be could be tested for its ability
to elicit appropriate and effective immune responses. Vaccination of mice with multiple
CTL epitope linked together in a ‘string of beads’ formation has been successful in
generating effective anti-tumour responses (Toes et al., 1997). Immune responses have
also been detected against a polyepitope vaccine containing several CD4+ T-cell epitopes
linked together in a similar fashion (Falugi et al., 2001).

<table>
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<th>Epitope Defined</th>
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<tr>
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<td>HLA-DRB1*04</td>
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<td>HPV16 E6(19-33)</td>
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<td>p30</td>
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<td>HPV18 E6(49-63)</td>
<td>HLA-DRB1*16</td>
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Table 6.1 HLA-DR types potentially responsible for presentation of peptide epitopes
defined in this study. The potential HLA-DR allotypes involved in the presentation of
the HPV16 E6 and HPV18 E6 epitopes defined are shown. Where a particular allotype
has been confirmed (either in this study or published elsewhere) it is underlined.
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For the remainder of this study the immune response to p37 (HPV16 E6_{127-141}) was the primary focus. In the course of this work, two p37-specific Th1 T-cell lines were generated which could be phenotypically differentiated, based upon their expression of either TRBV14 (TCR Vβ16), or TRBV 4-3 (TCR Vβ7). Despite the fact that both these lines were generated from the same PBMC culture from donor 1, there were major functional differences between these lines when re-stimulated with p37. Most strikingly, the TRBV14+ T-cells (Belx1) demonstrated an inferior IFNγ response to p37 compared to the TRBV4-3+ T-cells (Belx2), both in the quantity of cytokine produced and in the proportion of cells which responded. The difference in the IFNγ secretion profiles of the two T-cell lines correlated with the differential expression of TCR complex and CD28 (accessory molecule involved in T-cell signalling). It would be possible to further investigate the importance of CD28 in the functional differences noted, through the use of HLA-DRB1*01+ L-cells transfected with the CD28 ligands (CD80 and CD86) as APC in an ICS assay.

It is likely that the different TCR Vβ chains used by the two populations could also be an important contributing factor to the differential cytokine secretion profiles. As the TCR α and β chains from the T-cell lines have been cloned it will be possible to generate soluble TCR molecules and test their avidity for soluble HLA-DR-p37 complex using surface plasmon resonance technology (Biacore) (Boulter et al., 2003).

It is also interesting to note that in line 46 (the parental line of Belx1 and Belx2), the TRBV14+ T-cell population was dominant (88.7%), despite the fact that these cells demonstrated an inferior IFNγ response compared to the sub-dominant T-cell population, (later revealed to be TRBV4-3+). This finding could have important implications for T-cell immunotherapeutic strategies, indicating that the dominant T-cell populations generated in vitro may not be the most potent effector cells. For this reason it would be important when generating T-cells for adoptive transfer, to specifically select the T-cell populations based not only upon their epitope specificity but also upon their capacity to elicit a potent functional response.
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Responses to p37 have been detected in 3 out of 4 HLA-DRB1*01+ donors tested to date, however examination of a larger cohort of HLA-DRB1*01+ women will be required to determine more accurately the relative frequency of this response. Such a study would also permit a broader investigation of TCR Vβ chain usage by p37-specific T-cells, which would allow us to determine if the TCR chains identified in this study are frequently utilised in the p37 response, or if they are specific to the donor studied.

Adoptive T-cell immunotherapy has proven efficacy for treatment of several virus-mediated diseases, including those induced by CMV (Cobbold et al., 2005) and EBV (Haque et al., 2002; Khanna et al., 1999; Rooney et al., 1995). One of the issues that limit the use of such strategies in the treatment of HPV-mediated disease, is that it is difficult to obtain sufficient numbers of appropriate T-cells without extended periods of in vitro culture, in part, due to the low frequency of HPV-specific memory T-cells in healthy donors. In this study it has been possible to generate large numbers (>10⁶) of peptide-specific T-cells using an in vitro expansion protocol. Although the p37-specific T-cells were not tested for recognition of cervical cancer cells, it is feasible that these T-cells could contribute to an anti-tumour response, irrespective of whether the tumour expresses the cognate peptide (HPV16 E6{127,141}). CD4+ T-cells have been shown to play an important role in the anti-tumour response to HLA class II negative cancers through collaboration with macrophages (Corthay et al., 2005).

The HPV16 E6{127,141}–specific TCR chains isolated in this study could potentially be used to circumvent the problems surrounding the need for in vitro culture and clonal selection, through their introduction into bulk T-cell populations (both CD4+ and CD8+ T-cells), effectively redirecting the T-cells specificity and effector function to HPV+ cells. Such an approach has been successfully used in other studies (Schumacher, 2002; Xue et al., 2005).

Based on the known crystallised structures of HLA-DRB1*01-peptide complexes it was possible to generate a model describing the interaction between p37 and the HLA class II molecule. This model provides a basis for the rational design of p37 variants that could
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potentially be more immunogenic through modification of the interaction with both the HLA-DRB1*01 molecule and the TCR molecule. A representation of an HLA-DRB1-peptide complex interacting with its cognate TCR is represented in Figure 6.1. By enhancing the immunogenicity of the p37 epitope it could potentially be more efficacious in a therapeutic vaccine.

Figure 6.1 Interactions of TCR with an HLA-DRB1*01-peptide complex. The model depicts the interaction of a TCR with HL-DRB1*01-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.
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It was also demonstrated that the epitope recognised by the Belx2 T-cells could be endogenously processed and presented from a full length E6 protein. This validates the original responses detected against the synthetic 15mer peptide (p37) and strongly suggests that the immune response is directed against the HPV16 E6 protein. Due to the absence of a HPV16 positive cervical cancer cell line expressing HLA-DRB1*01, it has not yet been possible to determine if the epitope (p37) can be presented by HPV transformed cells.

However, even if the peptide cannot be directly presented by HPV infected cells, it is possible to envisage a scenario in which HPV-specific CD4+ T-cells could be primed and play an important role in an effective anti-HPV immune response. HPV infects the keratinocytes within the cervical epithelium. Infected keratinocytes express HLA class II molecules (Coleman and Stanley, 1994), however they are likely to be ineffective in T-cell priming due to the absence of cell surface expression of co-stimulatory molecules such as CD86 (Mota et al., 1999). 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
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collaboration with macrophages) in the control of a primary immune response against a
HLA class II negative tumour in mice (Corthay et al., 2005).

A number of clinical trials have been carried out using a range of peptide, protein, DC, or
DNA based vaccines in an attempt to elicit a potent anti-HPV immune response in
patients with high grade CIN or cervical cancer (Galloway, 2003). To date, several
clinical trials have reported varying degrees of tumour regression in vaccinated patients
with high grade cervical disease, however none of these results were statistically
significant (Baldwin et al., 2003; Borysiewicz et al., 1996; Muderspach et al., 2000).
The lack of success in these trials may in part be related to the fact that the women
included in nearly all of these trials have high grade cervical disease and many will have
been treated with chemotherapy or radiotherapy, prior to the trial. By testing vaccines in
such women who are, to a degree immunocompromised, it may be that the efficacy of
some vaccines in the treatment of less severe cervical disease is underestimated. When
considering the design of a therapeutic vaccine strategy it is important to have an
understanding of the problems that will be encountered regarding the immunological
environment in transformed cervical tissue.

LCs, which are the major APC population within healthy squamous cervical epithelia,
have been demonstrated to decrease in number with increasing severity of cervical
disease, thereby diminishing the ability of the immune system to present HPV antigens to
T-cells (Connor et al., 1999). The cytokine environment in CIN or cervical cancer differs
significantly from that of healthy cervical epithelium. In healthy cervical tissue there is
constitutive expression of TNFα (pro-inflammatory cytokine which activates resident
LCs) by basal and parabasal keratinocytes, whilst the production of IL-10 (anti-
inflammatory cytokine) is barely detectable (Giannini et al., 1998; Mota et al., 1999).
This production of TNFα is gradually lost in cervical disease, decreasing with increased
disease severity (Mota et al., 1999). IL-10 on the other hand, is detected at increasing
levels as cervical disease progresses from low to high grade (Giannini et al., 1998; Mota
et al., 1999). IL-10 may exert several effects which negatively affect the immune
systems capacity to generate an effective anti-viral response. IL-10 has been shown to
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down-regulate the expression of HLA class II and CD80 molecules on APC, which obviously could negatively influence the effective priming of naïve T-cells (Chang et al., 1995). In vitro studies have revealed that IL-10 treated DCs can induce anergy in peptide-specific CD4+ and CD8+ T-cells (Steinbrink et al., 1999; Steinbrink et al., 1997). Analysis of the cytokine secretion profiles of tumour infiltrating lymphocytes from patients with cervical cancer demonstrated these cells were inappropriately polarised from a Th1 to a Th2 response. Subsequent in vitro experiments indicated that IL-10 secretion by cervical cancer cells was responsible for this phenomenon (Sheu et al., 2001). A number of studies have also described the presence of high numbers of T-regulatory cells in the tumour environment, which could limit the effectiveness of an anti-tumour response (Liyanage et al., 2002; Somasundaram et al., 2002; Woo et al., 2002).

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 IFN or TNFα, along with the HPV vaccine construct could facilitate the generation of an appropriately polarised and effective T-cell response. Recent advances in soluble TCR technology (Laugel 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 et al., 2002), and it is possible that such an approach may be effective in the treatment of HPV-mediated disease.

Overall this study has illustrated two key issues that should be considered in the design of immunotherapy for cervical cancer. Firstly, a number of novel CD4+ T-cell epitopes have been identified which could either be included in a therapeutic vaccine construct, or be used in the precise monitoring of vaccine induced responses. Secondly, it has been shown that the dominant T-cell population generated in vitro against an epitope is not
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necessarily the most potent effector population. Therefore it is important to specifically select the appropriate effector population for immunotherapy. It may be possible to select and enrich such cells based on their expression of a specific TCR, and co-stimulatory molecules. A precise role for HPV-specific CD4+ T-cells in disease has not been defined, but it is our hope that the epitopes defined in this study may provide the basis for further investigations.
Bibliography


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release gamma interferon, tumor necrosis factor alpha (TNF-alpha), and TNF-beta when they encounter their target antigens. *J Virol*, 67, 2844-2852.


Appendix I

Primers from publication;

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α Chain primer sequences. Original sequences were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/Entrez) using accession numbers obtained from the T cell receptor factsbook [1]. Round 2 primers were designed to contain a ClaI restriction site and an ATG start codon and the CoR2 primer was designed to contain an EagI restriction site.
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**β Chain primer sequences. Original sequences were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/Entrez/) using accession numbers obtained from the T cell receptor factbook [1]. Round 2 primers were designed to contain a AseI restriction site and an ATG start codon and the CβR2 primer was designed to contain an Agel restriction site.**
Appendix II

Epitope specificity and longevity of a vaccine-induced human T cell response against HPV18

Kelly L. Smith¹, Amanda Tristram², Kathleen M. Gallagher¹, Alison N. Fiander² and Stephen Man¹

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Keywords: ELISPOT, papillomavirus, vaccinia virus

Abstract
Persistent human papillomavirus (HPV) type 16 and 18 infection can lead to pre-malignant and malignant diseases of the lower genital tract. Several lines of evidence suggest that T cell responses can control HPV infection. However, relative to other human viruses, strong effector memory T cell responses against HPV have been difficult to detect. We used an in vitro stimulation step prior to enzyme-linked immunospot assays to identify IFN-γ-secreting T cells specific for HPV16 and 18 E6/E7 peptides. This allowed the detection of HPV-specific CD4⁺ T cells that were not evident in direct ex vivo assays. T cell responses against HPV16 or 18 peptides were detected in healthy volunteers (7/9) and patients with lower genital tract neoplasia (10/20). Importantly, this assay allowed tracking of vaccine-induced T cell responses in nine patients, following inoculation with a live recombinant vaccinia virus (HPV16 and 18 E6/E7, TA-HPV). Novel vaccine-induced T cell responses were demonstrated in five patients, but no clinical responses (lesion regressions) were seen. For one vaccinated patient, the T cell response was mapped to a single dominant HPV18 E7 epitope and this response was sustained for >3 years. Our data suggest that systemic memory T cells against HPV16 and 18, induced naturally or by TA-HPV vaccination, are relatively rare. Nevertheless, the assay system developed allowed estimation of magnitude, epitope specificity, and longevity of vaccine-induced CD4⁺ T cell responses. This will be useful for vaccine design and measurement of immunological endpoints in clinical trials.

Introduction
A subset of human papillomaviruses (HPVs) can persistently infect the lower genital tract, giving rise to pre-malignant (intraepithelial neoplasia) and malignant lesions. The strong association between HPV and these diseases provides viral targets for both prophylaxis and therapy (1). Recently, proof of concept has been obtained for prophylactic vaccines for HPV (2), which ultimately aim to reduce the global burden of cancer. However, even if such vaccines were implemented immediately, there would be a substantial reservoir of infected individuals who would require treatment for pre-malignant and malignant disease for decades.

Research on HPV immunotherapy has largely focused on cervical cancer because of the poor prognosis for patients with advanced disease (3). However, current treatments for recurrent pre-malignant disease can also be problematic. For example, conventional treatment for vulval intraepithelial neoplasia (VIN) usually involves repeated local excision or laser ablation. Such treatments are painful and mutilating, and particularly distressing for young women (4). Thus, there is interest in exploring therapeutic vaccines for HPV-associated pre-malignant diseases. Such an approach may be more effective in pre-malignant disease because these patients would not have the immunosuppression observed in advanced cancer patients (5). Furthermore, lower grade lesions may be more genetically stable and thus less prone to down-regulation of immunologically important molecules such as MHC (6).

HPVs encode several potential target antigens for T cells; however, in epithelial cells transformed by ‘high risk’ HPV types, such as 16 and 18, the most attractive targets are the E6 and E7 oncoproteins. Previously, we used HLA-peptide
multimers to detect low-frequency HPV16 E6 or E7 specific CTL in the peripheral blood of patients with pre-malignant cervical intraepithelial neoplasia (CIN3) (7) and cancer (8). However, there are a paucity of confirmed human CTL epitopes for HPV, and the majority of these are based on HLA-A*0201 (9). We wished to measure systemic CD8\(^+\) and CD4\(^+\) T cell responses in a cohort of patients with HPV-associated pre-malignant disease who had been vaccinated with a live recombinant vaccinia virus vaccine (HPV16 and 18, TA-HPV). Therefore, an assay was developed that involved a short in vitro stimulation of PBMC with pools of HPV16- and HPV18-derived peptides, before measurement of IFN-\(\gamma\)-secreting T cells in enzyme-linked immunospot (ELISPOT) assays. This method allowed reliable measurement of T cell responses against HPV peptides in both healthy volunteers and patients with pre-malignant HPV-associated disease [lower genital tract neoplasia (LGTN)]. Furthermore, we demonstrate that this method allowed tracking of HPV peptide-specific T cell responses induced by a live recombinant vaccinia virus vaccine (TA-HPV).

**Methods**

**Blood**

PBMC were isolated from heparinised blood samples by centrifugation on Histopaque (Sigma, Poole, UK) density gradients and cryopreserved in aliquots of 5 \(\times\) 10\(^5\) to 10 \(\times\) 10\(^5\) cells as previously described (7).

**Healthy volunteers and patients**

Healthy male \((n = 6,\) age 29–60 years) and female \((n = 4,\) age 26–40 years) volunteers with no history of HPV-associated disease were recruited locally. Blood samples were taken with informed consent. Although HPV testing was not carried out for ethical reasons, it was assumed that several of the volunteers had previously encountered and cleared 'high risk' HPVs (10,11). After receiving approval from the local ethical committee and Gene Therapy Advisory Committee, patients with histologically confirmed LGTN were recruited from clinics at University and Llandough Hospitals (Cardiff and Vale NHS Trusts, Wales, UK). These patients had histologically confirmed high-grade CIN3, VIN3 and vaginal intraepithelial neoplasia. All patients gave informed consent for obtaining blood and where appropriate, cervical brush or biopsy samples. HPV typing was carried out on DNA extracted from patient tissue samples as previously described (12). Serological typing of HLA class I and molecular typing of HLA class II alleles were carried out on all the vaccinated patients (Welsh Blood Service, Pontyclun, Wales, UK).

**Vaccination**

A subset of the LGTN patients recruited (VAC001-011) consented to being immunized with a single dose of TA-HPV, a recombinant vaccinia virus containing HPV16 and 18 E6/E7 (13). Eleven patients were vaccinated (dermal scarification with 2.5 \(\times\) 10\(^5\) plaque-forming units) and followed up for between 3 and 20 months. Blood samples were taken on at least three separate time points post-vaccination, usually on day 28 (4 weeks), day 56 (8 weeks) and day 84 (12 weeks). All patients demonstrated vaccine 'take', with no adverse effects. Two of the patients had evidence of prior exposure to vaccinia virus from pre-existing antibody responses (VAC003, VAC004) that were boosted following vaccination. One of these patients confirmed receiving smallpox vaccine (VAC003). For the other patients, high levels of anti-vaccinia virus antibodies were induced from baseline levels following vaccination. Clinical assessment of the neoplastic lesion(s) was carried out by computer-aided measurement of digital images. The visible area of disease was measured and recorded for each patient. All patients were given standard clinical treatment within 2 months (approved by local ethics committee) with appropriate clinical follow-up.

**Cultured ELISPOT**

Cryopreserved PBMC were thawed and washed twice with RPMI1640. They were then resuspended at 2 \(\times\) 10\(^5\) to 2.5 \(\times\) 10\(^5\) cells ml\(^{-1}\) in RPMI1640 (PAAS or GIBCO, Paisley, UK) supplemented with 10% human AB serum, 2 mM glutamine, 100 \(\mu\)M \(\beta\)-estradiol, 100 U ml\(^{-1}\) penicillin and 25 mM HEPES (RPMIAB). One millilitre of PBMC suspension was added to 24-well plates (Falcon, BD Biosciences, Cowley, UK) before the addition of appropriate HPV peptide pools (each 15mer peptide was present at a concentration of 10 \(\mu\)M ml\(^{-1}\)). As a positive control, PBMC were cultured with the positive peptide pool (PPP) containing peptides from influenza, human cytomegalovirus (HCMV), Epstein Barr Virus (EBV) and tetanus at 10 \(\mu\)M ml\(^{-1}\) each. Following 3 days of incubation at 37°C, 1 ml of RPMIAB medium containing 20 IU ml\(^{-1}\) recombinant IL-2 (Proleukin, Chiron Corporation, UK) was added. On day 6, 1 ml of culture medium was removed and replaced with 1 ml of RPMIAB without IL-2. On day 7, PBMC were harvested and introduced to the ELISPOT protocol.

PBMC that had been cultured for 7 days were harvested, washed and re-suspended in RPMIAB at 10\(^6\) cells ml\(^{-1}\), before seeding in triplicate or quadruplicate at 10\(^5\) cells per well in a Multiscreen 96-well plate (Millipore, Watford, UK). The wells had been coated the day before with an IFN-\(\gamma\) capture antibody (Mabtech, Stockholm, Sweden) and left overnight at 4°C. Cells were incubated alone (as a negative control), with autologous PBMC (to deduce background response), with autologous PBMC plus specific peptide pools (test wells) and with mitogens (PHA 2 \(\mu\)g ml\(^{-1}\), phorbol myristate acetate 100 ng ml\(^{-1}\), ionomycin 1500 ng ml\(^{-1}\) and Con A 20 \(\mu\)g ml\(^{-1}\), Sigma) as a positive control. The ELISPOT plates were then incubated for 18–20 h at 37°C. The ELISPOT assay was performed the following day in accordance with the manufacturer's instructions (Mabtech). After allowing the plates to air dry, the number of spots per well was counted using an inverted stereomicroscope. The specific response was calculated by subtracting the background response (T cells + autologous PBMC) from the peptide test wells. To assign significant T cell responses, similar criteria to other studies were used (14), i.e. the mean number of spots in the peptide test wells (with background subtracted) was greater than the mean + 2SD spots of the negative control wells (T cells only) and was >20 spots (>1/5 \(\times\) 10\(^5\) cells). For the vaccinated patients, the spot counts for the ELISPOT plates were verified using an AID ELISPOT plate.
reader (AID-Diagnostika, Staßberg, Germany). Although the numbers of spots detected were different, this did not result in changes in either positive or negative responses; therefore, for consistency, the results depicted are from the manual counts for patients and healthy volunteers.

**Immunomagnetic enrichment of CD4+ and CD8+ T cells**

PBMC that had been cultured with HPV peptides for 7 days were separated into CD8+ and CD4+-enriched fractions using MACS CD8 microbeads (Miltenyi Biotec, Bisley, UK), according to manufacturer's instructions. Purity of fractions was assessed by flow cytometry. Pre-sort, CD8+ and CD4+-enriched populations were then tested in ELISPot assays as discussed above.

**Peptides**

**PPP.** To control for viability of cryopreserved samples, a PPP was constructed based on CD4+ and CD8+ T cell epitopes from common recall antigens. The source of these antigens included EBV (15–17), HCMV (18), influenza A (17, 19, 20) and tetanus (21). This was based on a previously described set of class I epitopes (22). The class I epitopes were adapted where possible to make the peptide length more comparable to the 15mer peptides used for HPV peptide stimulation. All peptides (sequences in Table 1) were synthesized to >90% purity, and amino acid sequences were confirmed by mass spectrometry (Severn Biotech, Kidderminster, UK). Individual peptides were dissolved in dimethyl sulphoxide to provide stock solutions of 40–100 mg ml⁻¹. Aliquots from these stock solutions were pooled, and the PPP was then frozen in small aliquots and stored at −20°C.

**HPV16 and 18 peptide pools.** These consisted of 81 overlapping (by nine) 15mer peptides that spanned the full-length E6 or E7 proteins of HPV16 and 18. Peptides were synthesized as part of a pepset (Mimotope, Wirral, UK). As for the PPP, individual peptides were dissolved in dimethyl sulphoxide to provide stock solutions before making pools of HPV16 E7 (15 peptides), HPV16 E6 (25 peptides), HPV18 E7 (16 peptides) and HPV18 E6 (25 peptides). Smaller pools of three to five peptides were also made to allow epitope mapping. Individual and pooled peptides were frozen in small aliquots and stored at −20°C.

**Results**

Detection of HPV peptide-specific T cell responses from healthy volunteers

Previously, we had demonstrated systemic HPV-specific CTL responses in patients who had been inoculated with a live recombinant vaccinia virus vaccine (TA-HPV) (13, 23). However, the detection systems used were relatively insensitive, not quantitative and could not map the responses to a single protein or peptide. Therefore, a major aim was to develop a more sensitive assay to measure both CD4+ and CD8+ HPV-specific T cell responses for vaccine studies. This was done initially using cryopreserved blood samples from healthy volunteers, as HPV-specific T cell responses have been frequently found in such subjects (24). Pools of 15mer peptides from HPV16 and 18 E6 and E7 were used to stimulate T cells for direct measurement of IFN-γ release. Such peptide pools have been used successfully to map anti-viral CD8+ (25) and CD4+ T cell epitopes (26, 27). However, we were unable to detect any HPV-specific T cell responses ex vivo using either intracellular cytokine staining or ELISpot assays (data not shown).

It was possible that the frequency of HPV-specific T cells in blood was too low for direct detection ex vivo, and might require in vitro amplification as previously described for melanoma (28, 29) and HPV-specific CTL (7). Initial experiments used an ELISpot protocol based on culturing PBMC with HPV peptides for 4 days to detect HPV-specific CD4* T cells (24). However, this was not successful (data not shown), so we cultured PBMC for 7 days with HPV16 or 18 peptide pools prior to testing in ELISpot assays. To control for the functional viability of cryopreserved samples, a PPP containing multiple CD4* and CD8* T cell epitopes was also designed for use alongside the HPV pool (Table 1). Using this modified protocol, a cohort of 10 healthy volunteers (HV001–0010, Table 2) was tested for T cell immunity against HPV and recall antigen peptides. One donor (HV009) failed to respond to PPP stimulus and so was excluded from the analysis, however, as

<table>
<thead>
<tr>
<th>Amino acid sequence Source protein</th>
<th>Residues</th>
<th>HLA restriction</th>
<th>Reference</th>
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<td>PKYVKQNKLKL1</td>
<td>Influenza A haemagglutinin</td>
<td>306–324</td>
<td>DR4/DR7/DR11</td>
</tr>
<tr>
<td>QYIKANSKFQITEL2</td>
<td>Tetanus toxoid</td>
<td>830–844</td>
<td>Multiple DR types</td>
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<td>FNNFNTSVFLWVPKVSHLE3</td>
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<td>947–967</td>
<td>Multiple DR types</td>
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<tr>
<td>TSLYNLRGTLAL4</td>
<td>EBV EBNA1</td>
<td>515–527</td>
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<td>AGLTLSLIVCSLYLISRG5</td>
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<tr>
<td>ILRGCSVAHK16</td>
<td>Influenza A NP</td>
<td>265–273</td>
<td>A3</td>
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exemplified by HVO03 (Fig. 1A) and HVO07 (Fig. 1B). IFN-γ-producing T cells recognizing either HPV16 or 18 peptides could often be detected (Table 2). Overall, significant T cell responses could be seen from 7/9 volunteers. T cell responses could be demonstrated against HPV16 (5/9), HPV18 (3/9) and both HPV16 and HPV18 (3/9) (Table 2). The dominant response appeared to be against HPV16 E6 peptides (5/9), confirming previous reports (24).

Table 2. T cell responses against HPV16 and 18 peptides in healthy volunteers measured by IFN-γ ELISPot

<table>
<thead>
<tr>
<th>Donor</th>
<th>Spots per cultured 10^5 cells*</th>
<th>16 E6</th>
<th>16 E7</th>
<th>18 E6</th>
<th>18 E7</th>
<th>PPP</th>
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<td></td>
<td>24</td>
<td>44</td>
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<tr>
<td>HVO02</td>
<td></td>
<td>41</td>
<td>21</td>
<td>23</td>
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<td>&gt;200</td>
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<td>HVO03</td>
<td></td>
<td>97</td>
<td></td>
<td></td>
<td></td>
<td>162</td>
</tr>
<tr>
<td>HVO04</td>
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<tr>
<td>HVO10</td>
<td></td>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td>71</td>
</tr>
</tbody>
</table>

*PBMC from cryopreserved stocks were cultured for 1 week with peptide pools of either HPV16 E6, HPV16 E7, HPV18 E6 or HPV18 E7. The cultured cells were tested in ELISpot assays against the stimulating peptide pools using autologous PBMC as antigen-presenting cells. Mean spot counts from triplicate wells are shown. Only counts that were significantly greater (>2SD) than background counts and >20 spots are shown.

Epitope specificity and phenotype of responding T cells

One attractive feature of using a peptide pool for stimulating T cells is that it provides a rapid means for mapping individual T cell epitopes. To test this, PBMC were obtained from donor HVO03 who responded against the HPV16 E6 peptide pool (Fig. 1A, Table 2). These freshly isolated PBMC were cultured with the HPV16 E6 pool before testing in ELISpot assays using six smaller HPV16 E6 pools or individual HPV16 E6 peptides. A dominant response was seen against pool 4 (data not shown), then subsequently mapped to a 15mer peptide epitope from HPV16 E6_127-141 (DKKQRFHNNIRGRWTG) (Fig. 2A). Since no

Fig. 1. T cell responses against HPV16 and 18 peptides in healthy volunteers. Representative responses are shown from (A) HVO03 (HPV16 E6 response) and (B) HVO07 (HPV18 E6 response). PBMC were cultured for 7 days with peptide pools from HPV18 E7, HPV18 E6, HPV16 E7, HPV16 E6 or the PPP. Cells were harvested and tested in ELISpot assays against autologous PBMC plus the appropriate HPV peptide pool. The counts shown are for nothing (T cells only) or plus peptide ([T cells + PBMC + peptide] - [T cells + PBMC]).

Fig. 2. Dissection of HPV16 peptide-specific T cell responses measured by ELISpot. (A) Mapping an HPV16 E6 epitope in a healthy volunteer (HVO03). PBMC were cultured with HPV16 E6 peptides before testing in ELISpot assays against the whole HPV16 E6 pool (25 peptides), pool of four peptides (19, LPOLCTELDTTHID; 25, RDLCIVYRGMPYAV 31, YGTLEOQYNKPLCD and 37, DKKQRFHNNIRGRWTG) or individual peptides. (B) Immunomagnetically enriched populations of either CD8+ (98.5% purity) or CD4+ T cells (96% purity) from HVO03 were cultured with peptide 37, before testing in an ELISpot assay.
other overlapping peptide was recognized, it is likely that the 15mer peptide constituted a CD4+ T cell epitope. To confirm this, PBMC were stimulated with the HPV16 E6_{22-31} peptide, before using MACS to enrich for either CD8+ or CD4+ T cells, prior to ELISPOT assays. The T cell responses against HPV16 E6_{22-31} correlated with a CD4+ phenotype, as depletion of CD4+ T cells greatly reduced the response compared with PBMC (Fig. 2B). Depletion of CD8+ T cells slightly augmented the response compared with PBMC (Fig. 2A). No HLA typing data were available for the healthy donors precluding further analysis. Nevertheless, these results demonstrated that the protocol used could reliably detect IFN-γ-secreting T cells specific for HPV peptides and could define dominant CD4+ T cell epitopes.

HPV16 peptide- and 18 peptide-specific T cells in patients with HPV-associated LGTN

A cohort of 22 patients with HPV-associated LGTN was recruited and their cryopreserved PBMC tested for T cell responses recognizing HPV16 and 18 peptides (Table 3). Overall, significant T cell responses against HPV16 or 18 peptides could be detected in 10/20 evaluable patients (Table 3, VAC004 and VAC007 were removed from the analysis because of lack of samples or high background responses, respectively). As with the healthy volunteers, responses against HPV16 (9/20) and HPV18 (5/20) were demonstrated (Table 3). HPV16 E6 was immunodominant, with 7/20 patients making significant ELISpot responses against the pooled HPV16 E6 peptides. Interestingly, HPV18 E6 responses were also frequently detected (3/20 patients). These responses did not strictly correlate with the HPV types present in each patient's biopsy as HPV16-specific T cell responses could be detected in patients with HPV18* biopsies (CTRL010) and vice versa (CTRL006). However, it cannot be ruled out that these patients had previously been infected with multiple HPV types. Taking into account the small sample sizes, there was no noticeable increase in the magnitude or the frequency of T cell responses in the CIN3 patients compared with the healthy donors.

### Table 3. T cell responses against HPV16 and 18 peptides in patients with HPV-associated LGTN

<table>
<thead>
<tr>
<th>Donor</th>
<th>Disease</th>
<th>HPV type</th>
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<tr>
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<td>CIN3</td>
<td>16 18</td>
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<td>CTRL005</td>
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<td>CIN3</td>
<td>16 49</td>
<td>&gt;200</td>
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<tr>
<td>CTRL009</td>
<td>CIN3</td>
<td>16 39</td>
<td>&gt;200</td>
</tr>
<tr>
<td>CTRL010</td>
<td>CIN3</td>
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<td>&gt;200</td>
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<tr>
<td>CTRL011</td>
<td>CIN3</td>
<td>16 39</td>
<td>&gt;200</td>
</tr>
<tr>
<td>CTRL014</td>
<td>VAIN3</td>
<td>HR</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

(not 16/18)

| CTRL015 | VAIN3   | NT       | 139                  |
| CTRL016 | VAIN3   | NT       | 30                   |
| CTRL020 | VAIN3   | 16 26    | >200                 |
| VAC001  | VAIN3   | 16 26    | >200                 |
| VAC002  | VAIN3   | 16 26    | >200                 |
| VAC003  | VAIN3   | 16 79    | >200                 |
| VAC004  | VAIN3   | 16 IS IS  | >200                 |
| VAC005  | VAIN3   | 16 IS IS  | >200                 |
| VAC006  | VAIN3   | 16 57    | >200                 |
| VAC007  | VAIN3   | 16 57    | >200                 |
| VAC008  | VAIN3   | 16 57    | >200                 |
| VAC009  | VAIN3   | 16 57    | >200                 |
| VAC010  | VAIN3   | 16 57    | >200                 |
| VAC011  | VAIN3   | 16 57    | >200                 |

NT denotes not tested, VAIN3 denotes vaginal intraepithelial neoplasia, — denotes no significant response above background, IS denotes insufficient sample and HB denotes non-interpretable results due to high background in several assays.

### Induction and boosting of T cell responses in CIN3 and VAIN3 patients after vaccination

A cohort of 11 patients with HPV-associated LGTN (VAC001-011, Table 3) were inoculated with TA-HPV, a recombinant vaccinia virus containing HPV16 and 18 E6 and E7 proteins. This phase I/II trial aimed to study immunogenicity of TA-HPV and to assess clinical efficacy within an ethically acceptable period. In this study, 5/9 evaluable patients made significant T cell responses against HPV16 or 18 peptides prior to vaccination (Fig. 3, Table 3). Novel HPV-specific T cell responses could be detected after vaccination in five patients (Fig. 3), with two patients (VAC001, VAC008) making responses against multiple HPV peptide pools (Fig. 3, Table 4). The timing of the T cell responses, together with the magnitude, strongly suggested that they have been induced as a direct result of vaccination. There was no concomitant increase in T cell responses against the PPI (Fig. 3), confirming that this was an HPV-specific effect.

Interestingly, four out of five of the vaccine responders made responses against HPV18 E6/E7 peptides, but only one of these patients had HPV18-associated disease (VAC010, Table 4). The strongest vaccine-induced response was in VAC002 against HPV18 E7 peptides (340 spots per 10^5 cultured cells on day 91); however, testing PBMC samples from day 68 (303 spots per 10^5 cultured cells) and day 91 in direct ex vivo ELISPOT assays (overnight incubation with peptides) did not produce significant responses (data not shown).

### Definition of a novel vaccine-induced T cell epitope and longevity of vaccine response

The magnitude of the HPV18 E7 T cell response in patient VAC002 warranted further investigation. Therefore, an additional blood sample was taken nearly 3 years (day 901) after vaccination. This provided additional PBMC to use as antigen-presenting cells to investigate epitope specificity at defined time points post-vaccination and allowed investigation of the longevity of the T cell response. Therefore, after primary in vitro re-stimulation with the whole HPV18 E7 pool (16 peptides), PBMC cultures were further tested in ELISPOT assays against pools of four peptides and then individual peptides (Fig. 4). This demonstrated that the response against HPV18 E7 was dominated by T cells recognizing a novel HPV18 E7 42-56 epitope (Fig. 4). Since no other overlapping peptide was recognized, it was likely that this was a CD4 T cell epitope. This was supported by preliminary experiments that demonstrated
immunomagnetically depleting CD4⁺ but not CD8⁺ T cells effectively abrogates the response (data not shown).

In parallel with the epitope mapping experiments, PBMC from day 901 post-vaccination were also stimulated in vitro with the whole HPV18 E7 pool. Although the magnitude of the T cell response had diminished at day 901 (Fig. 5) compared with day 91 (Fig. 4B), it was clear that the same pattern of recognition had been sustained over 3 years, with a dominant response against HPV18 E7,2-56. This suggests that TA-HPV vaccination had induced long-lasting memory T cells against this epitope.

Lack of correlation between measurable systemic T cell responses and clinical response

A secondary aim of the clinical trial was to assess clinical response to vaccination on the basis of regression of HPV-associated pre-malignant lesions. However, using digital imaging of lesions and several other clinical criteria, no complete or partial clinical response was seen within the assessment period (2 months). There was no resolution of visible disease, and no histological changes were observed when comparing pre- and post-vaccination samples.

Cervical
Fig. 4. Vaccine-induced T cell response against an immunodominant epitope of HPV18 E7. (A) Day 91 PBMC from patient VAC002 were cultured for 7 days with HPV18 E7 pool (25 peptides) before testing in ELISPOT assays against either the whole HPV18 E7 pool or smaller HPV18 E7 pools containing four individual peptides (C1-C4 pools) or mitogens. (B) Day 91 PBMC were cultured as above, before testing against entire HPV18 E7 pool, HPV18 E7 C2 pool (peptides 45, LLX5259, SE2855, 46, L355SE2855, 47, ENDEICQH-HLP and 48, G1NQHLPARG) or individual HPV18 E7 peptides (45, 46, 47 or 48). The response against mitogens in experiment B exceeded 500 spots.

Fig. 5. Longevity of vaccine-induced HPV18-specific T cell responses. Day 901 PBMC from patient VAC002 were cultured with the whole HPV18 E7 peptide pool before testing in ELISPOT assays against entire HPV18 E7 pool, HPV18 E7 C2 pool (peptides 45-48) or individual HPV18 E7 peptides 45 and 48. The response against mitogens exceeded 500 spots.

Intraepithelial disease is asymptomatic, however, none of the four patients (VAC001, 002, 004 and 005) with non-cervical disease experienced symptomatic improvement. All these patients requested surgical removal of their disease, and standard treatment (loop excision) was given to the patients with CIN3 (VAC003, VAC006–011). It should also be noted that there was no worsening of disease symptoms or increased disease progression in patients receiving vaccine.

Discussion

The study of cell-mediated immunity against HPV's has been technically challenging. This is because live virus cannot be propagated in amounts sufficient for in vitro studies, and it is difficult to grow HPV-infected or -transformed cells (30). Additionally, the localized nature of HPV infection and resultant lesions, combined with immune evasion mechanisms, probably contribute to weak systemic T cell responses (31). In this paper, we have used a short in vitro culture step followed by peptide re-challenge in the ELISPOT assay to allow quantification of IFN-γ-secreting T cells, both in healthy donors and patients receiving a candidate therapeutic HPV vaccine. This sensitive approach allowed mapping of novel CD4 T cell epitopes and demonstrated the longevity of a vaccine-induced T cell response against a dominant HPV18 E7 epitope.

The protocol used in this study was developed because we were unable to detect HPV-specific T cell responses directly ex vivo (data not shown). ELISPOT is a sensitive technique, but several studies have demonstrated that ready detection of HPV-specific CD4+ T cell requires a 4-day in vitro culture step (14,24,32). However, this protocol was not successful for the cryopreserved patient samples in this study (S. van der Burg, personal communication), and was modified by increasing the culture period to 7 days. Such an approach has also been used to detect low-frequency human T cell responses against melanoma vaccines (29) or malaria antigens (33). There is the risk that in vitro culture may create variability and skew the T cell responses detected, such that the true repertoire of responding T cells is not represented. But these risks must be balanced against the practical advantages of the protocol, which include increased recovery time from the effects of cryopreservation and amplification of low-frequency responses. We found the assay to be reproducible; the strongest T cell response in VAC002 was repeated in four separate experiments. Furthermore, the same HPV peptide-specific responses were detected in healthy volunteers (HV3 and HV7) in experiments carried out by different investigators, 2 years apart (K.L. Smith and K.M. Gallagher, unpublished observations). These arguments support the usefulness of the cultured ELISPOT assay in demonstrating vaccine responses. However, this assay should not be used for direct numerical comparison of HPV-specific T cells between individual patients.

It is likely that central memory rather than effector memory T cells (33) are being detected using our protocol. This point is
important because we did not measure T cell responses at sites of disease. The localized effects of HPV infection and transformation are unlikely to lead to induction of strong systemic effector memory T cell responses. Additionally, the vaccine used in this study was based on vaccinia virus, which by virtue of its lytic nature, and rapid clearance by the immune system, is also unlikely to sustain a population of effector memory T cells in the periphery. Nevertheless, we suggest that the assay system described is capable of detecting central memory T cells, arising from natural infection and 3 years after vaccination.

HPV16 peptide- or HPV18 peptide-specific T cell responses were demonstrated in several healthy volunteers, establishing the sensitivity of the assay. Given the small sample sizes, mixed sex of the volunteers and the constraints of the assay system, it was not valid to compare responses of the healthy volunteers against patients. Nevertheless, we confirmed previous studies of CD4+ T cell responses against HPV16 in healthy donors (24,34–36) and supported the dominance of CD4+ T cell responses against HPV16 E6 (24). More recent experiments of exclusively female volunteers (n = 9, ages 21–30 years) have produced similar results (4/9 HPV16 responders, K.M. Gallagher, unpublished observations). It is likely that these responses represent memory T cells against HPV rather than naive T cells activated in vitro. First, because HPV infection is common (11), it is likely that most adults have encountered these viruses during their lifetime. Second, the in vitro culture period is short, whereas generation of naive T cell responses requires either extended in vitro culture and/or the use of dendritic cells (37). Third, in other studies, the T cells responding against HPV in short-term in vitro culture have a memory cell (CD45RO+) phenotype (32,38). Since the majority of individuals are able to clear HPV infection, study of T cell responses in healthy subjects rather than those who have high-grade disease may provide a clearer picture of the T cell responses involved in protection against disease (24).

We are currently attempting to map further HPV epitopes using HLA-typed donors, both for use as possible vaccine candidates and to simplify measurement of immunological endpoints. Such epitopes may allow more precise quantitation in the future using HLA class II mutimers.

The use of pooled i-HPV 15mer peptides allowed rapid definition of two novel CD4+ T cell epitopes. In each case, the recognition of a single 15mer peptide suggested recognition by CD4+ T cells. Since the 15mer peptides used overlapped by 9 amino acids, it was possible that single peptides of 10 amino acid or longer could be processed and recognized by CD8+ T cells. However, this was considered unlikely, as 15mer peptides were less efficient at induction of IFN-γ secretion by CD8+ CTL clones compared with 10mer or 9mer peptides (K.L. Smith, unpublished observations). This suggests that there may be sub-optimal stimulation and detection of memory CD8+ T cells. A non-mutually exclusive explanation is that the frequency of HPV-specific CD8+ CTL is extremely low, as we have previously suggested (7).

HPV18 is the second most common virus associated with cervical cancer with an overall prevalence of 15%, compared with 58.9% for HPV16 (39). HPV45, which is closely related to HPV18, is the third most prevalent type, i.e. 5.9% (39). Thus, targeting immune responses against both HPV18 and HPV45 would have a major impact on cervical cancer. However, there have been relatively few immunological studies of HPV18 relative to HPV16. In this study, 3/9 healthy donors made a response against HPV18 versus 7/9 responses against HPV16. This ratio of responses parallels the relative prevalence of HPV18 (6%) and HPV16 (10%) detected in a recent longitudinal UK study (40). But the frequency of responders is higher than would be expected from the total incidence of HPV16 and 18 infection, raising questions about the specificity of the T cell responses. One possible explanation for these results is that T cell responses against either HPV16 or HPV18 peptides are in part composed of T cells cross-reactive against many other related HPV types including those that cause benign skin lesions. Such HPV cross-reactivity has recently been demonstrated for HPV11 L1-specific T cells (38). Another possibility is that other human pathogens may induce T cell cross-reactive on HPV peptides (41).

A novel T cell epitope HPV18 E742-56 (GVNHQHLPAR-RAEQ) was mapped in this study. Constraints on patient samples meant that we were unable to map the HLA class II restriction elements for this HPV epitope or examine cross-reactivity against a similar HPV45 epitope E7 (GVHIAQLPAR-RAEQ). Interestingly, T helper responses against a truncated form of the E743-53 (VNHQHLPARA) have been demonstrated in CBA/CaH mice (42). Larger cohorts of HLA-typed donors (healthy volunteers and donors) should be tested to assess the frequency of T cell responses against these epitopes at the population level and whether they have any role in disease.

A clear finding in our study was the lack of correlation between the T cell responses measured and any clinical response. There may be multiple reasons for the lack of clinical response after TA-HPV vaccination. We were unable to demonstrate T cell responses directly ex vivo. This suggests that the systemic T cell responses against HPV induced by TA-HPV were weak, both by comparison with other therapeutic vaccines (43) and relative to other human viruses (44). This may result from two related features of TA-HPV. First, HPV E6 and E7 are small proteins and may be poorly immunogenic to human T cells. This has been suggested by a study demonstrating the paucity of T cell epitopes in several mouse strains (45). Second, the vaccine delivery system (vaccinia) does not allow for persistent expression of these proteins. While this is a desirable safety feature when using HPV oncogenes as antigens, it could lead to the disappearance of effector memory T cells from the periphery upon viral vaccine clearance. For future vaccines for HPV, the use of peptide- or protein-based vaccines that allow safe persistence of antigen may be more effective (46).

A second confounding factor is the HPV-type specificity of the T cells induced by a vaccine that contained both HPV16 and HPV18. Vaccine-induced T cell responses against HPV18 have been observed in previous studies (13,47); however, this is the first study to demonstrate an immunodominant effect for HPV18 in certain vaccinated patients. Whether this reflects individual selection for T cell responsiveness at the level of HLA haplotype or peripheral tolerance against the HPV type in the diseased tissue is not known. However, these results do suggest that while incorporation of multiple HPV types into vaccines may be advantageous for prophylaxis (39), this may not be the case for therapy.
There may be additional explanations for lack of clinical response after TA-HPV vaccination; however, we believe that the two most important factors are the weakness of the systemic T cell response and the immunodominance of T cell responses against the ‘wrong’ HPV type in certain patients. Our clinical results do contrast somewhat with similar clinical trials using the same vaccine, both of which reported one complete response and several partial responses (48,49). While the partial and complete responses are encouraging, the overall patient response rate suggests that vaccines capable of inducing stronger systemic T cell responses are required. The natural history of HPV disease suggests that strong systemic T cell responses may not be effective in isolation. One promising approach is to use topical treatments that encourage homing of vaccine-induced T cells into disease sites (50).

Despite the absence of a clinical response in this vaccine trial, the ELISPot protocol developed did allow sensitive tracking of HPV peptide-specific T cell responses. This was particularly evident for the HPV18 peptide-specific T cell responses that were more frequently detected and of higher magnitude than previously reported (47). Importantly, dominant T cell epitopes could be mapped for HPV16 and HPV18, and for the first time the longevity of a vaccine-induced cell response against HPV could be measured. These studies provide a firm foundation for future measurement of natural and vaccine-induced immunity against HPV.

Acknowledgements
We would like to thank the many laboratory staff and patients who participated in our study. We thank Xenova plc for providing TA-HPV and Chris Boswell for performing anti-vaccinia antibody ELISAs. We are grateful to Sjoerd van der Burg, Julian Hickling and Jo Cox for helpful discussions. David Harris of Cadama Medical Ltd kindly helped with the verification of manual ELISPot counts, using the AID ELISPot plate reader. This study was funded by a project grant from Cancer Research UK to S.M. and A.N.F. S.M. was a Royal Society University Research Fellow for part of these studies. K.M.G. is funded by a Medical Research Council (UK) studentship.

Abbreviations
CIN  cervical intraepithelial neoplasia
ELISPot  enzyme-linked immunospot
HCMV  human cytomegalovirus
HPV  human papillomavirus
LGTN  lower genital tract neoplasia
VIN  vulval intraepithelial neoplasia

References

Specificity of HPV vaccine-induced T Cells 175


Appendix III

HLA haplotypes of healthy volunteers.
## HLA haplotypes of healthy volunteers

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<th>Donor</th>
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* No HLA types were available for these donors