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The Regulation of HPV Late Gene Expression and the Potential Role of iNKT Cells in Cervical Cancer

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The Regulation of HPV Late Gene Expression and the Potential Role of iNKT Cells in Cervical Cancer

A Thesis Submitted for the Award of Master of Philosophy

by

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2015

Abstract

Human papillomaviruses (HPVs) are ubiquitous, sexually transmitted viruses present in 99.7% of all cervical cancers, the second most common cancer in females worldwide. Expression of HPV L1 and L2 late genes is found only in terminally differentiated epithelial cells. As L1 and L2 proteins are highly immunogenic, it is suggested that their suppression may prevent detection of the virus by the immune system, thus acting as a prerequisite for persistence of infection. Therefore, if expression of these proteins in the lower cervical layers was induced, it could lead to clearance of the virus. One aim of this thesis was to investigate potential inducers of late gene expression in HPV-16. Functional stable cell lines containing plasmids, in which L1 is replaced by a CAT reporter gene, were treated with an array of small molecule drugs. TPA and retinoic acid were found to be inducers of late gene expression, with potential as treatments for persistent HPV infection. An additional aim of the study was to investigate the role and distribution of invariant Natural Killer T (iNKT) cells in cervical epithelium. iNKT cells are potent activators of the immune system with a predominately protective function. However, their presence may be downregulated in HPV positive epithelium, possibly helping infected cells evade protective immunological surveillance. As there is currently no available means to determine iNKT cell existence in human tissue, the objective was to develop a novel method for their detection, useful for subsequent enumeration of iNKT cells in HPV-infected cervical cancer samples. Cell blocks containing a pure population of iNKTs were initially created for use as positive controls. The staining potential of the 6B11 antibody, which targets the iNKT T cell receptor (V α 24-J α 18), was then examined for use in both iNKT cell block sections and a range of human tissue. Investigations through automated staining returned positive results in gastric and tonsil tissue, showing potential use for 6B11 as an innovative technique of iNKT cell detection in human tissue.

Declaration

I certify that this thesis which I now submit for examination for the award of Master of Philosophy, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for award in any other Institute.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

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Abbreviations

Abbreviation	Full Term
ABC	avidin-biotin complex
Ad E4orf4	adenovirus E4orf4
APC	antigen presenting cell
ASF/SF2	alternative splicing factor/splicing factor 2
CAT	chloramphenicol acetyltransferase
CIN	cervical intraepithelial neoplasia
CMV	cytomegalovirus
DAB	diaminobenzidine
DAPI	4', 6-diamidino-2-phenylindole
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EBAO	ethidium bromide acridine orange
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
eUTR	early untranslated region
FITC	fluorescein isothiocyanate
FSC	forward scatter
g	g-force
GAG	glycosaminoglycan
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIER	heat induced epitope retrieval
HIV	human immunodeficiency virus
hnRNA	heterogeneous nuclear RNA
hnRNP	heterogeneous nuclear ribonucleoprotein

HPV	human papillomavirus
HSIL	high-grade squamous intraepithelial lesion
HSPG	heparan sulfate proteoglycan
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
LC	langerhans cell
LCR	long control region
LEEP	loop electrosurgical excision procedure
LLETZ	large loop excision of the transformation zone
LSIL	low-grade squamous intraepithelial lesion
IUTR	late untranslated region
MHC	major histocompatibility complex
mRNA	messenger RNA
MTP	microsomal triglyceride transfer protein
NK	natural killer cell
NKT	natural killer T cell
ORF	open reading frame
ori	origin of replication
pAE	early polyadenylation signal
pAL	late polyadenylation signal
Pap	papanicolaou
PBA	phosphate-buffered saline containing albumin
PDGFR	platelet-derived growth factor receptor
PE	phycoerythrin
PI	propidium iodide
PIER	protease induced epitope retrieval
PKC	protein kinase C
PPT	polypyrimidine tract

pRb	retinoblastoma protein
PTB	polypyrimidine tract binding protein
RAR	retinoic acid receptor
RARE	retinoic acid response elements
RNA	ribonucleic acid
RRM	RNA recognition motif
RRP	respiratory papillomatosis
RxR	retinoid X receptor
SA	splice acceptor
SAP	sphingolipid activator protein
SC35	serine/arginine-rich splicing factor 2
SCC	squamous cell carcinoma
SCJ	squamocolumnar junction
SD	splice donor
SIL	squamous intraepithelial lesion
snRNP	small nuclear ribonucleoprotein
SR	serine-arginine rich protein
SRSF1	serine/arginine-rich splicing factor 1
SSC	side scatter
TA	tannic acid
RA	retinoic acid
TCR	T cell receptor
TGF- β	transforming growth factor- β
TMA	tissue microarray
TNF- α	tumour necrosis factor- α
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAIL	tumour necrosis factor-related apoptosis inducing ligand
T-zone	transformation zone
URR	upstream regulatory region

UTR	untranslated region
VLP	virus-like particles
VPA	valproic Acid
α -GalCer	α -Galactosyl Ceramide
β 2m	beta-2-microglobulin

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1. Chapter 1 - Introduction

1.1 The Cervix

The cervix is the narrow opening into the uterus from the vagina (Mescher 2005) and is part of the female reproductive system, which consists of internal organs (including the vagina, uterus, ovaries and fallopian tubes) together with the external genital organs (the vulva and its components). At the upper portion of the cervix, there is an opening into the uterus called the internal os, while the lower portion of the cervix contains an opening into the vagina referred to as the external os (Lowe & Anderson 2005). The cervical canal links the two, connecting the uterine cavity and the lumen of the vagina. This cervical canal varies greatly in length and width between females and over the course of a woman's life, depending on age, parity and menstrual status. The cervix is divided into the endocervix, the area proximal to the uterus and the ectocervix, proximal to the vagina (Anderson 1991) (Figure 1.1). The endocervix is lined with a single layer of mucus secreting simple columnar epithelium while the ectocervix is covered by nonkeratinizing stratified squamous epithelium. These layers of stratified squamous epithelium are divided into basal, parabasal, intermediate and superficial regions (Figure 1.2). The abrupt transition from the columnar epithelium of the endocervix to the stratified squamous epithelium of the ectocervix produces a squamocolumnar junction (SCJ) (Wadhwa 2012). Squamous metaplasia in the cervix refers to the physiological replacement of the columnar epithelium by a newly formed squamous epithelium. The region where this metaplasia occurs is known as the "transformation zone" (T-zone) and is the most likely location for abnormal or precancerous cells to develop (Pich et al. 1992). This type of cancer originating in the ectocervix is called squamous cell carcinoma (SCC) and is by far the most common tumour of the cervix. The glandular cells of the endocervix can also become cancerous, a condition referred to as adenocarcinoma of the cervix. While SCCs accounted for more than 90% of primary cervical malignancies in the past, the overall frequency has dropped

to 60–80%, while the incidence of endocervical adenocarcinoma is rising (Wei 2009). Less commonly, some cervical cancers have features of both squamous cell carcinomas and adenocarcinomas. These are referred to as adenosquamous carcinomas or mixed carcinomas.

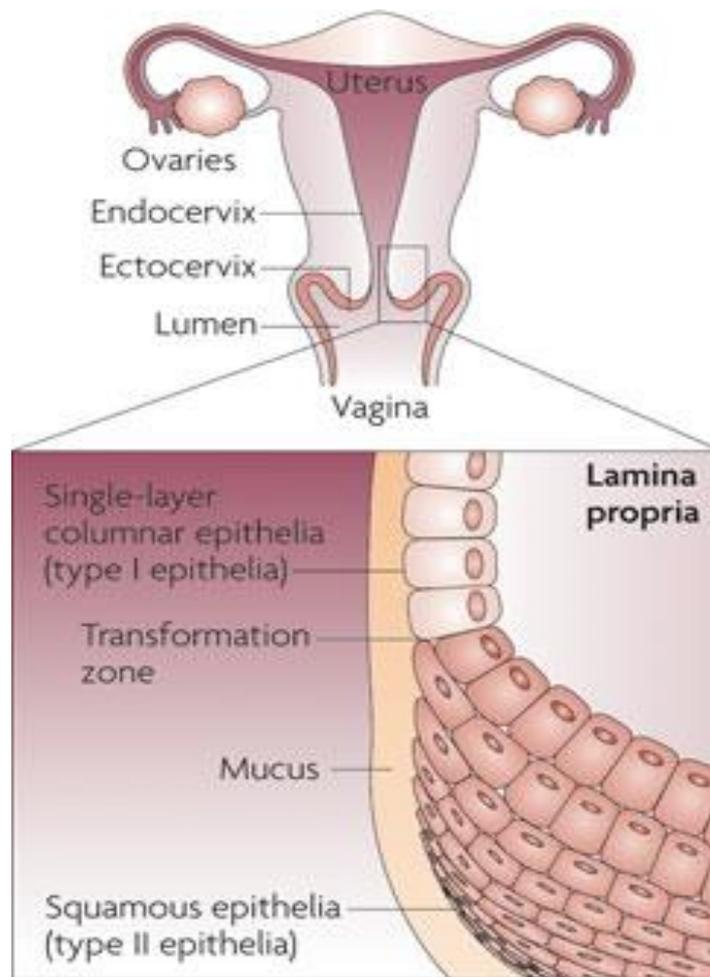


Figure 1.1 Structure of the Cervix and Transformation Zone

The diagram illustrates the female reproductive system indicating the location of the endocervix, ectocervix and the transformation zone. The transformation zone indicates the region where the columnar epithelial cells of the endocervix meet the stratified squamous epithelial cells of the ectocervix and is the area where metaplasia most commonly occurs (Iwasaki 2010).

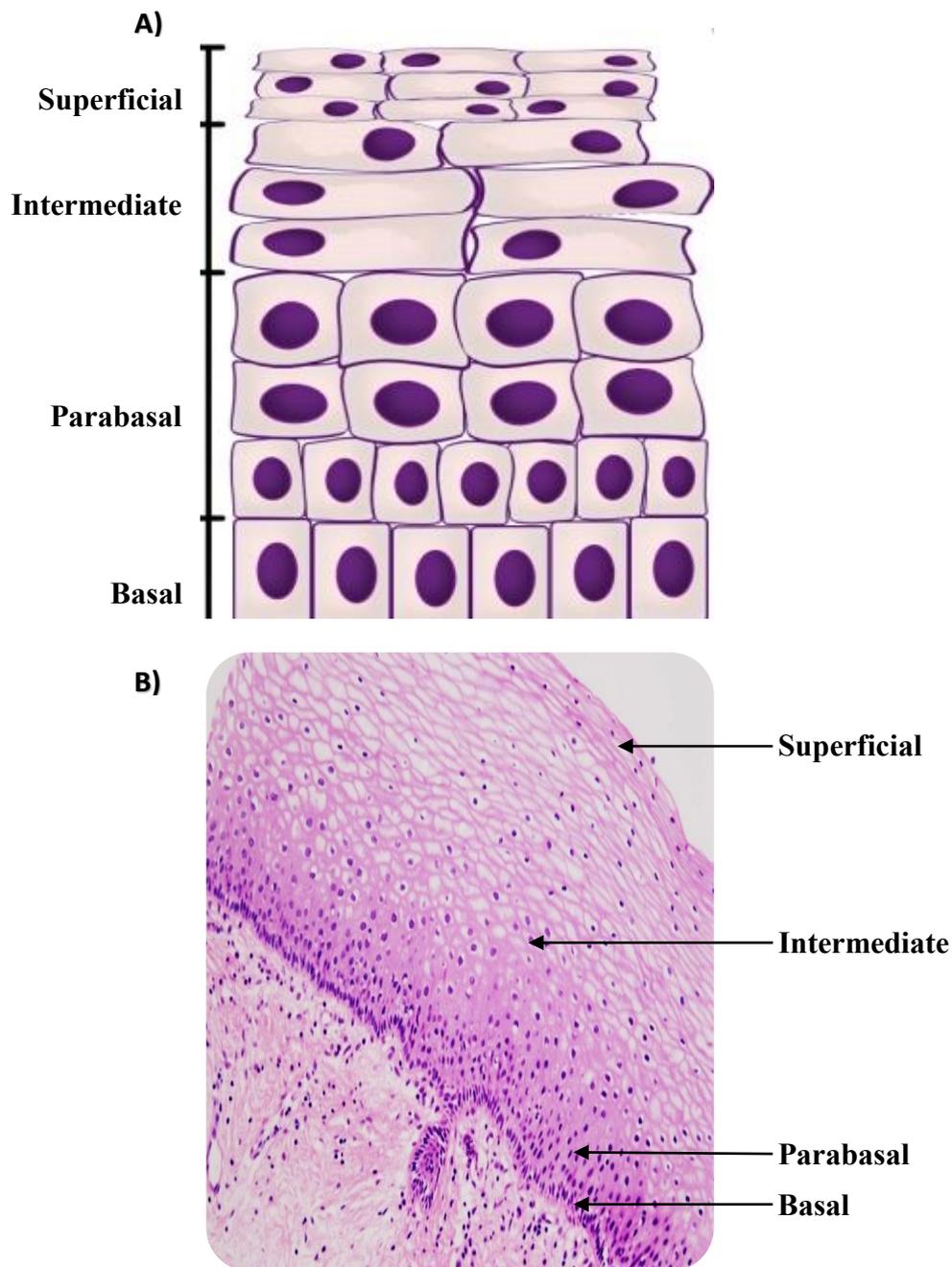


Figure 1.2 Structure of the Ectocervix

A) The diagram illustrates the layers of normal stratified epithelium (Adapted from Moody & Laimins 2010). **B)** High power H&E stain demonstrating the different layers of squamous epithelium in the ectocervix. Starting from the basement membrane, the youngest cells of the basal layer are shown, maturing as they move through the parabasal, intermediate and superficial layers (Cunniffe 2014).

1.2 Cervical Cancer Epidemiology

According to the Irish Cancer Society, approximately 200 women are diagnosed with cervical cancer each year in this country (Irish Cancer Society 2015). It is the ninth most common female cancer in Ireland and the second most common female cancer worldwide (World Health Organisation 2014b). In comparison to the majority of other cancers, cervical carcinoma primarily affects younger women, with 60% of cases occurring in women aged 50 or younger (Irish Health 2010). However, because it develops over time, it is also one of the most preventable types of cancer. The Papanicolaou (Pap) test has been established as one of the most successful cancer screening tests to date, with invasive cervical cancer incidence and mortality rates dropping dramatically in countries where this programme is implemented (Isidean & Franco 2014). A Pap smear is a simple and effective test where cells collected from the cervix are spread on a microscope slide for examination. The Papanicolaou stain, developed by Dr. George N. Papanicolaou in the late 1940s, is a polychrome staining method comprising of a nuclear stain (haematoxylin) together with two counterstains (Orange G and Eosin Azure dyes) (Papanicolaou & Traut 1997). A correctly stained Pap smear should show well distinguished purple nuclei while cytoplasmic staining should display a spectrum of colours such as orange in highly keratinised cells to ranges of orange/pink in superficial cells and turquoise green/blue in intermediate and parabasal cells (DeMay 1996) (Figure 1.3). Following Papanicolaou staining the cells are highly transparent, a feature which means diagnosis is possible even in areas of overlapping cells and when mucus and inflammatory cells are present. The cells are subsequently evaluated for irregularities, specifically for precancerous and cancerous abnormalities.

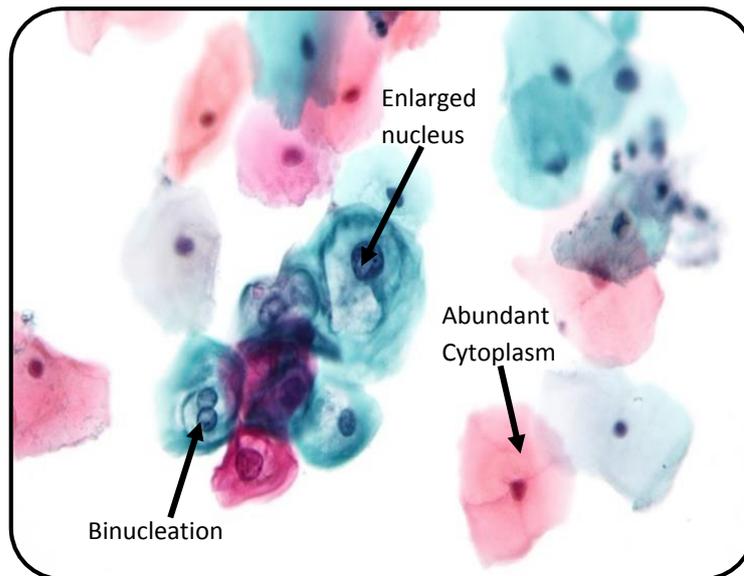


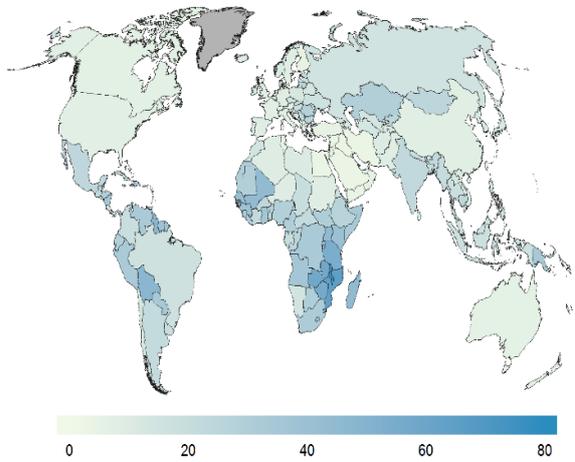
Figure 1.3 Papanicolaou Stain

Papanicolaou stain showing a low-grade squamous intraepithelial lesion (LSIL).

Superficial cells are orange to pink with intermediate and parabasal cells turquoise green to blue. Abnormal cells have an enlarged nucleus, irregular chromatin and relatively abundant cytoplasm, with binucleation also observed (Adapted from Carson & Hladik 2009).

In Ireland, CervicalCheck is the National Cervical Screening Programme and provides free Pap tests to women aged 25 to 60. The importance of access to such programmes is reinforced by World Health Organisation statistics, which state that more than 85% of cervical cancer deaths occur in developing countries, where it accounts for 13% of all female cancers (World Health Organisation 2014). Cervical cancer remains the most common cancer in women in Eastern and Middle Africa. High risk regions, with estimated age-standardised rates over 30 per 100,000, include Eastern Africa (42.7), Melanesia (33.3), Southern (31.5) and Middle (30.6) Africa, while rates are lowest in Australia/New Zealand (5.5) and Western Asia (4.4) (World Health Organisation, 2012). The estimated age-standardised incidence and mortality rates worldwide per 100,000 per year is demonstrated in Figure 1.4.

A) Estimated cervical cancer incidence worldwide in 2012



B) Estimated cervical cancer mortality worldwide in 2012

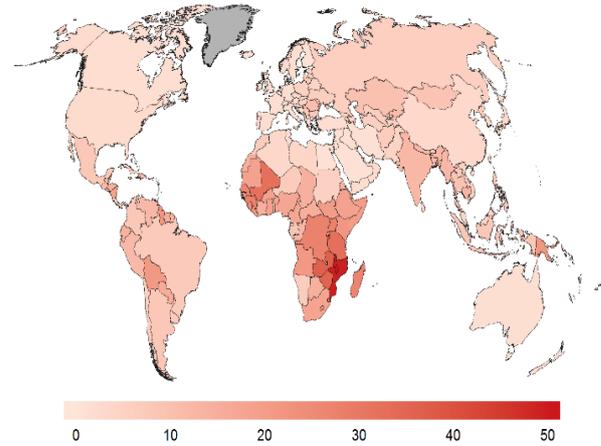


Figure 1.4 Cervical Cancer Estimated Incidence and Mortality Rates 2012

A) Estimated cervical cancer incidence worldwide in 2012. **B)** Estimated cervical cancer mortality worldwide in 2012. Figures show estimated age-standardised rates (World) per 100,000 (World Health Organisation 2012).

1.3 Human Papillomavirus and Cervical Cancer

Human Papillomavirus (HPV), one of the most common causes of sexually transmitted disease in both men and women worldwide, is found in 99.7% of women with cervical cancer (Walboomers et al. 1999). HPV was first implicated as a causative agent of cervical cancer in the early 1980s by Harold zur Hausen, who was awarded the Nobel Prize in Physiology or Medicine in 2008 for his ground-breaking discovery (zur Hausen 1989).

1.4 HPV Taxonomy

Papillomaviruses are a highly diverse family of viruses which contain 29 genera formed by 189 papillomavirus (PV) types. Numerous types have been detected in various mammals and birds, however the most extensive research has been performed in humans. Currently, the complete genomic sequence of over 240 distinct viral types has been characterised at the nucleotide level (Van Doorslaer 2013), with 120 types isolated and termed as human papillomaviruses (HPVs) (Bernard et al. 2010; de Villiers et al. 2004). HPVs infect epithelial cells and have the ability to induce an array of conditions ranging from warts to cancer (Doorbar 2006). More than 40 different strains of HPV affect the genital area and on the basis of molecular epidemiologic evidence, specific strains of HPV have been clearly indicated as principal inducers of invasive cervical cancer and intraepithelial neoplasia (Walboomers et al. 1999; Schiffman et al. 1993). All Papillomaviruses are members of the *Papillomaviridae* family. The major branches of this phylogenetic tree are divided into 5 genera, which are identified by letters of the Greek alphabet, namely alpha-papillomavirus, beta-papillomavirus, gamma-papillomavirus, mu-papillomavirus and nu-papillomavirus. The minor branches are referred to as species, which are further subdivided into strains as demonstrated in Figure 1.5 (de Villiers et al. 2004). The two major HPV genera are the alpha and beta-papillomaviruses, with approximately 90% of currently characterized HPVs belonging to one of these groups (Doorbar 2006). The largest group of HPVs comprise the Alpha-papillomaviruses, with this group containing the genital/mucosal HPV types. The remaining three genera (Gamma, Mu and Nu) infect cutaneous sites which do not normally progress to cancer (Doorbar 2006).

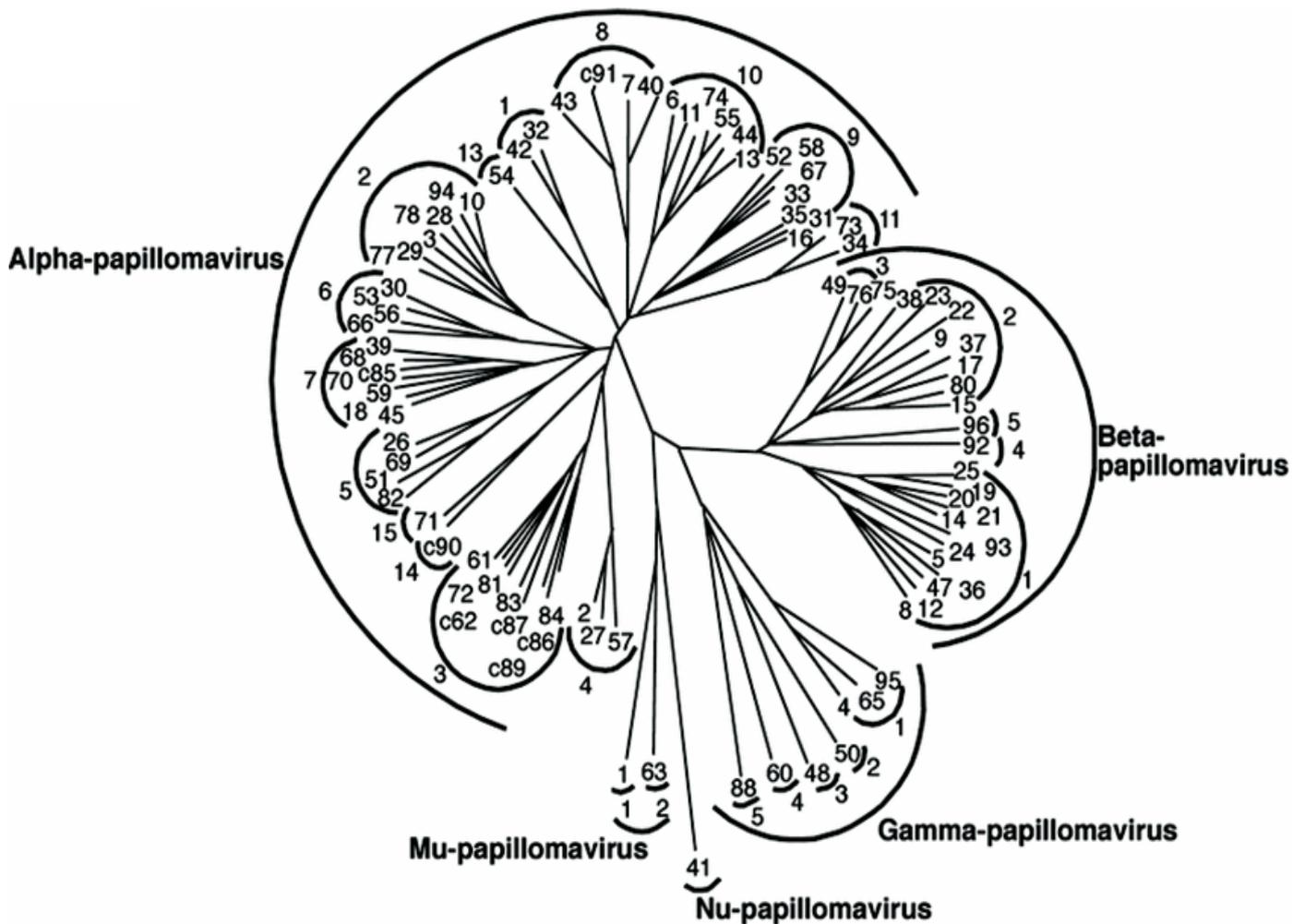


Figure 1.5 HPV Family Tree

Human Papillomaviruses are divided into 5 evolutionary groups with the Alpha genera infecting cervical epithelium (Doorbar 2006).

More than 30 different HPV types are known to infect cervical epithelium and are classified into genotypes based on their association with cervical cancer and precursor lesions (Muñoz et al. 2003; Doorbar 2006) (Table 1.1). Many of these HPV types are shown to be ubiquitous and globally distributed (de Villiers et al. 2004). Low-risk types are non-oncogenic and have the potential to induce benign or low-grade abnormalities of cervical cells. Certain low-risk strains, such as HPV-6 and HPV-11, also have the ability to cause conditions such as anogenital warts as well as recurrent respiratory papillomatosis (RRP) (Lacey et al. 2006).

Oncogenic, or high-risk HPV types can cause intraepithelial neoplasia of the anogenital region in addition to some oropharyngeal cancer.

Group	HPV Types
<i>Established High-Risk</i>	16, 18 , 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73,82
<i>Probable High-Risk</i>	26, 53, 66
<i>Established Low-Risk</i>	6, 11, 13, 40, 42, 43, 44, 54, 61, 70,72,81 and CPD108 (HPV89 Subtype)

Table 1.1 α HPV Types

Risk groups of α HPV genotypes with risk relating to the possibility of developing cervical cancer (Adapted from Muñoz et al. 2003).

It is now recognised that persistent cervical infection with high-risk HPV is a necessary prerequisite for the development of cervical cancer and its immediate precursor cervical intraepithelial neoplasia grade 3 (CIN3) (Saslow et al. 2012). Investigation into whether high-risk HPV is a true aetiological agent of cervical cancer has been examined by considering Koch's postulates, as modified by Rivers for viral diseases. River's postulates are as follows;

1. Isolate virus from diseased hosts
2. Cultivation of virus in host cells
3. Proof of filterability
4. Production of a comparable disease when the cultivated virus is used to infect experimental animals
5. Reisolation of the same virus from the infected experimental animal
6. Detection of a specific immune response to the virus (Rivers 1937).

As several of the six postulates cannot be tested ethically in patients, epidemiological data and laboratory studies have been utilised. Understandably, revolutionary advances in the scientific world have influenced the relevance of Koch's and River's postulates, with the ability to detect and evaluate nucleic acid sequences crucial for identifying infectious agents and their relationship with host cells. Fredricks and Relman have published their version of Koch's postulates for the 21st century, which may also be applied when investigating the ability of HPV to cause cervical cancer (Fredricks & Relman 1996).

Current findings offer overwhelming support for a necessary, but not sufficient, role for persistent high-risk HPV-type infection in cervical carcinogenesis (Bosch et al. 2002). A drop in cervical cancer rates following a reduction in HPV infections would provide definitive proof (Roden & Wu 2006). Infection with high-risk oncogenic HPV types is detected in 99.7% of patients with cervical carcinoma (Walboomers et al. 1999) with HPV types 16 and 18 regarded as the genotypes most closely connected with progression to cervical cancer. These types are found consistently in approximately 70% of cervical cancer biopsies worldwide with type 16 alone responsible for approximately 50% of cases (Bosch et al. 2008).

1.5 HPV Pathogenesis

Almost all sexually active men and women are exposed to the human papillomavirus in their lifetime. Eight out of 10 women will become infected at some point, with the peak prevalence of HPV infection occurring in women in their early 20s (Naucler et al. 2007). Although the incidence of infection is considerably high, most cases of HPV infection are transient and asymptomatic. The majority of instances resolve spontaneously, with up to 90% of the infected population clearing the infection within two years (Ho et al. 1998). However, a minor proportion of the infected population will become persistently infected, with the interval between persistent high-risk HPV infection and diagnosis of cervical cancer approximately 12-15 years (Snijders et al. 2006). The most common clinically significant manifestation of

persistent genital HPV infection is cervical intraepithelial neoplasia (CIN). CIN is histologically classified into 3 grades i.e. mild dysplasia (CIN1), moderate dysplasia (CIN2), or severe dysplasia (CIN3) (Massa & Cejtin 2004). Each grade depicts the extent of which the cervix is affected by abnormal cells. As mentioned, cervical lesions do not necessarily progress to invasive cancer, with CIN1 often spontaneously resolving and reverting to a normal state without treatment. However, high-risk HPV serotypes that establish persistent infections can result in high-grade dysplasia (Barroso 2013). These high-grade abnormalities are considered cancer precursors as they are at a significant risk of progression to cancer. The Bethesda classification system was also developed for reporting cervical cytologic diagnoses and recording Pap smear results. This system aims to ensure a uniform analysis with clear terminology when making a clinical diagnosis (Solomon et al. 2002). The terminology of the Bethesda system involves squamous intraepithelial lesion (SIL) classification and is divided into:

- (i) Low-grade SIL (LSIL) which includes HPV-linked cellular alterations, mild dysplasia and CIN1.
- (ii) High-grade SIL (HSIL) which includes both moderate and severe dysplasia, carcinoma *in situ* as well as CIN2 and 3.
- (iii) Squamous cell carcinoma (SCC) (Kurman 1991).

As displayed in Figure 1.6, HPV gains initial entry to the basal cell layer through micro-abrasions in the cervical epithelium. Once infection has occurred, early HPV genes are expressed and the viral DNA replicates from episomal DNA. As the viral genome migrates towards the midzone and superficial zone of the upper epithelium, it is further replicated and the late genes are expressed. When dysplastic cells constitute up to one third of the depth of the epithelium, the disease is classified as LSIL while HSIL represents dysplasia involving more

than one third and up to the entire depth of the epithelium (Mukhopadhyay et al. 2013). Over time, with persistent expression of viral genes, dysplastic cells may penetrate the basement membrane, leading to the development of an invasive cancer.

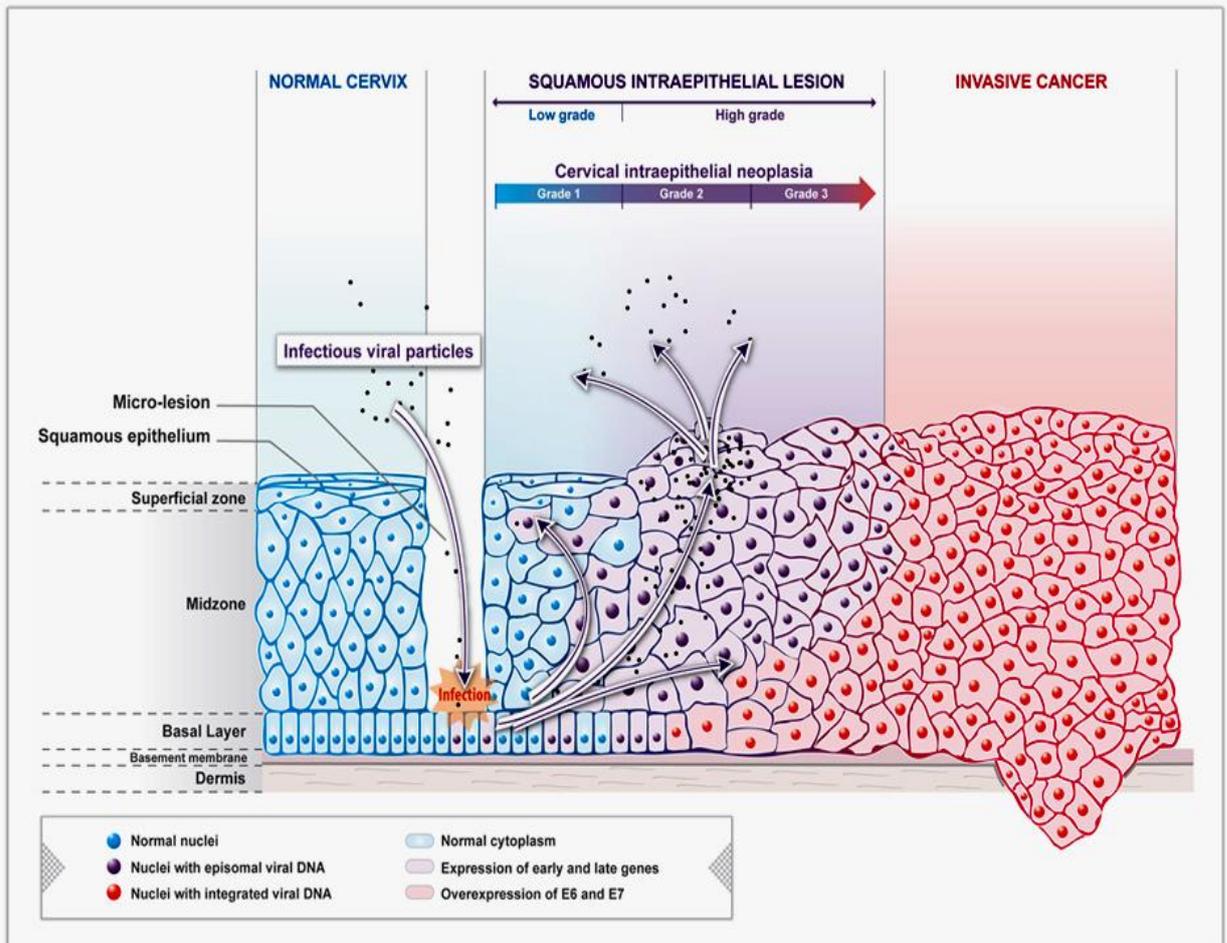


Figure 1.6 HPV-Mediated Progression to Cervical Cancer

Once initial infection has been established in the basal cell layer, viral genes are expressed which may lead to the proliferation of dysplastic cells within the epithelium. The relationship between the terminologies of the Bethesda system and CIN classification is displayed above. LSIL represents dysplasia involving the lower one-third of the squamous epithelium and comprises CIN1 while HSIL includes all dysplastic lesions beyond this and signifies moderate dysplasia (CIN2), severe dysplasia (CIN3) and carcinoma *in situ*. A small percentage of cases progress to cervical cancer with the potential to invade surrounding tissues if left untreated. (Woodman et al. 2007).

Representation of the squamous intraepithelial lesions in cervical tissue is shown in Figure 1.7.

A haematoxylin and eosin stain was performed on cervical epithelium showing no evidence of malignancy together with LSIL, HSIL and SCC tissue sections.

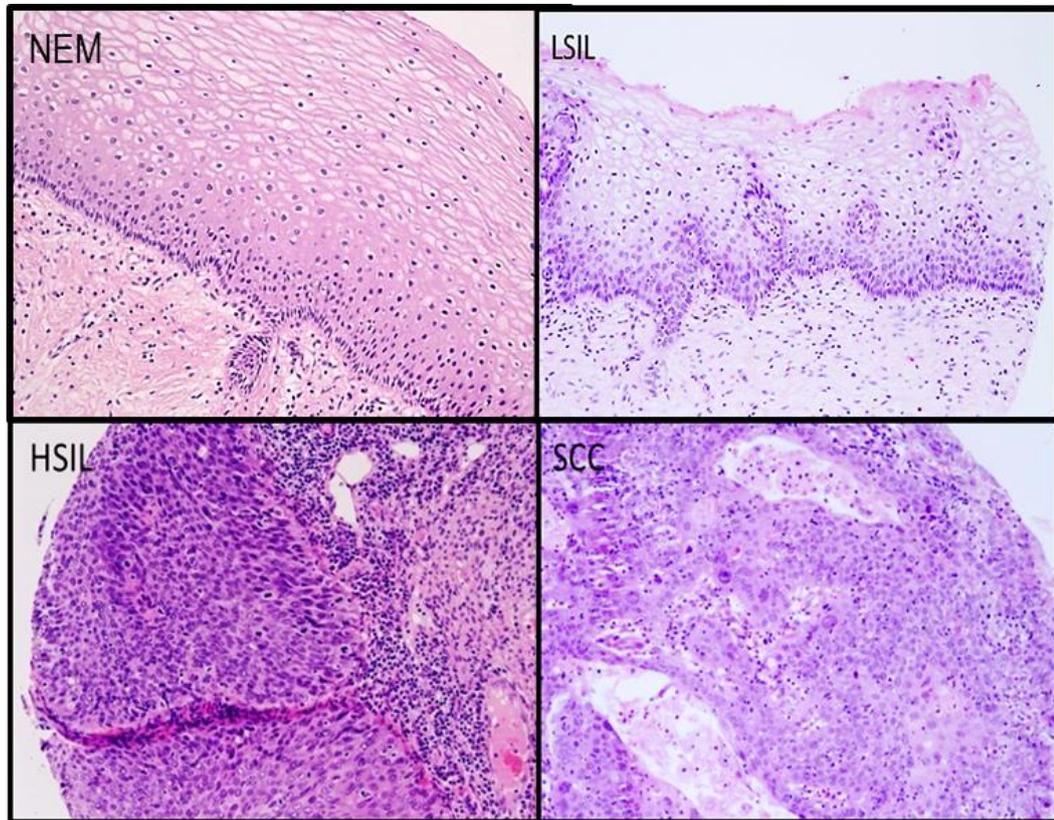


Figure 1.7 H&E Staining of Various Grade Cervical Epithelium

Images from cervical samples representing various grades of squamous intraepithelial lesions. No evidence of malignancy (NEM) is shown in addition to low and high grade squamous intraepithelial lesions and squamous cell carcinoma. LSIL displays abnormal immature cells in the basal third of the epithelium with HSIL showing diffuse atypia and immature cells inhabiting the entire depth of the epithelium. Malignant cells invading through the basement membrane, with complete loss of epithelial structure and a large number of proliferating cells is observed with SCC. Sections were stained with haematoxylin and eosin with magnification shown at 100X (Orrù 2012).

1.6 The HPV Genome

Human Papillomaviruses are a group of small non-enveloped double stranded DNA viruses with circular genomes of approximately 7,900 base pairs. However, despite this small size, their molecular biology is complex. The HPV genome is organised into three domains: a long control region (a non-coding region also referred to as the upstream regulatory region [URR]), an early region and a late region (Figure 1.8 and 1.9) (Gravitt & Shah 2004). Each genome contains eight major open reading frames (ORFs), six of which are located in the early region (E1, E2, E4, E5, E6, and E7) with two in the late region (L1, L2) (Zheng & Baker 2006).

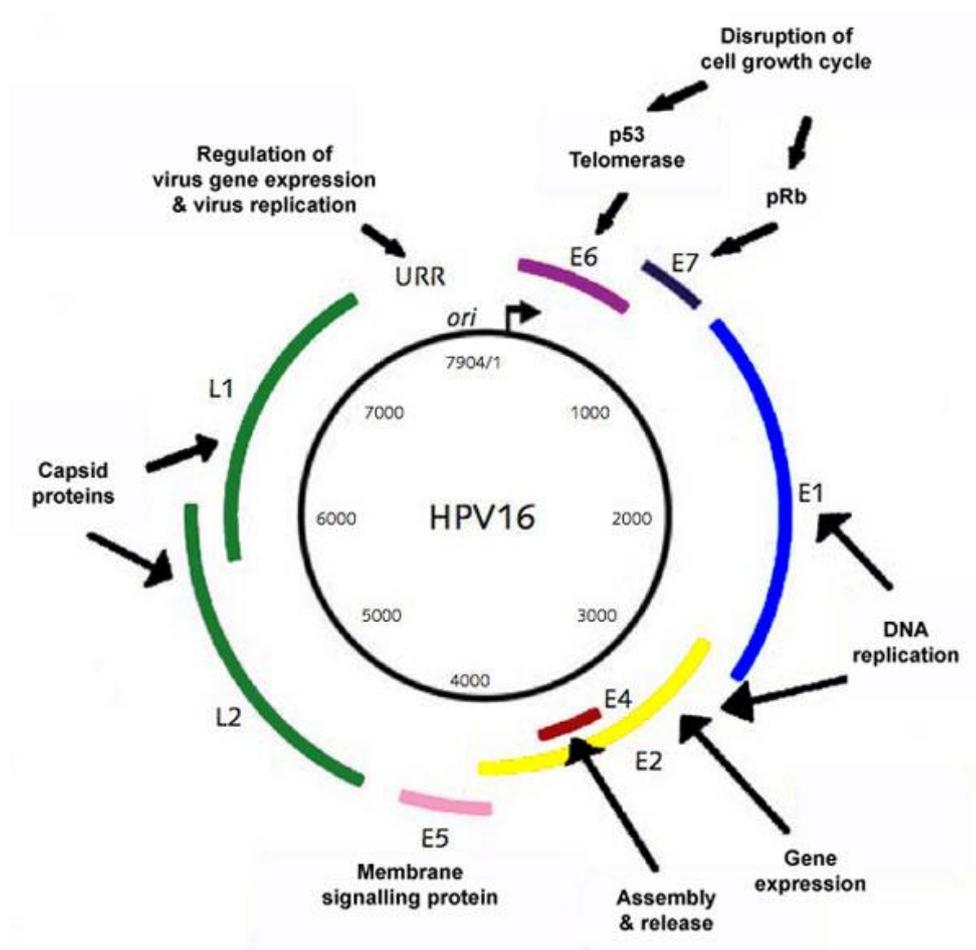


Figure 1.8 The HPV-16 Circular, Double Stranded Genome (Sharma et al. 2013).

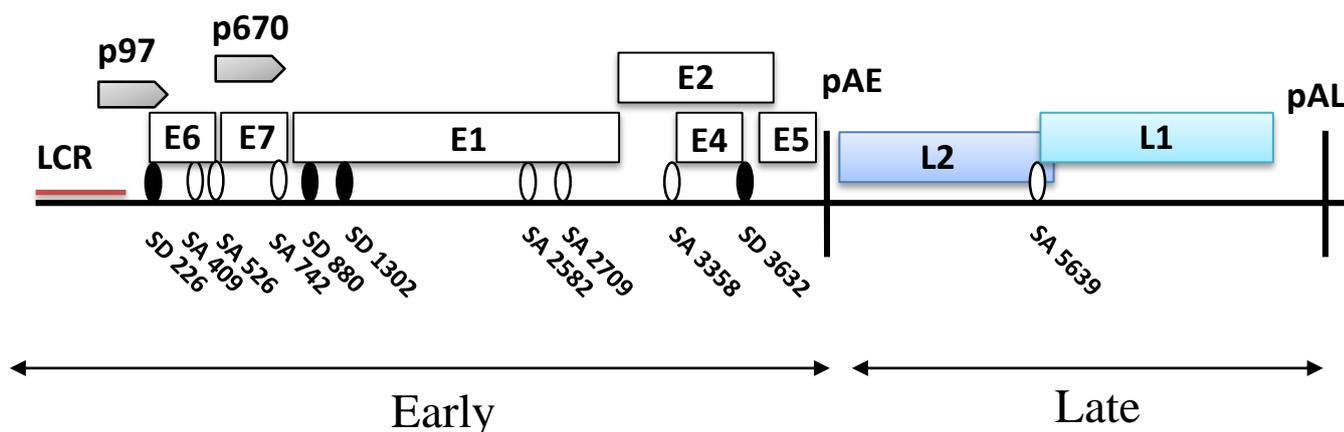


Figure 1.9 Linear Representation of the HPV-16 Genome

Boxes indicate protein-coding regions, with E representing the early genes and L representing the late genes. The long control region (LCR) is also shown as are the early and late viral promoters (p97 and p670) and the early and late polyadenylation signals (pAE and pAL). Black circles indicate splice donors (5' splice sites) while white circles indicate splice acceptors (3' splice sites). Numbers refer to nucleotide positions in the HPV-16 sequence (Orrù 2012).

The proteins of the early region are regulatory in function and are expressed immediately after initial infection of the host cell. They play roles in various biological processes including HPV genome replication and transcription, cell signalling and apoptosis control as well as immune modulation and structural modification of the infected cell (Graham 2010). The viral capsid is composed of the major and minor late gene proteins, termed L1 and L2, respectively. These capsid proteins are essential for the transmission of the virus and its survival in the environment (Graham 2010). The function of each HPV protein is summarised in Table 1.2. The life cycle of HPV is specifically associated with epithelial differentiation, with L1 and L2 proteins detected only in the upper layers of the terminally differentiated infected epithelium (Schwartz 2000). The products of these late genes are highly immunogenic and it has been suggested that suppression of these products in the early stage of the life cycle may prevent detection of the virus by the immune system, leading to persistence of infection. Expression of HPV genes is

complex and is tightly regulated by differentiation-dependent transcription and RNA processing events. The inhibition of the late genes may be attributed to certain RNA elements in the late region which are suggested to regulate various RNA processing events including mRNA stability, splicing and translation (Schwartz 2000; Rush et al. 2005). Alternative splicing is a fundamental mechanism in the control of gene expression, with specific splicing enhancers and suppressors utilised to promote or suppress the recognition of the 5' and 3' splice sites, thus regulating gene expression (Zheng 2004). It is believed that premature induction of late gene expression, through intervening in the regulation of posttranscriptional steps such as splicing, could result in detection of the HPV virus by the host. This in turn would lead to successive clearance of infection. Therefore, identification of mechanisms to modulate late gene expression would be a worthwhile investigation.

Open Reading Frame	Protein Functions
E1	Viral Replication
E2	Regulates viral transcription and replication
E4	Interacts with cytoskeletal proteins
E5	Downregulation of MHC class 1 molecules
E6	Oncoprotein, binds to tumor suppressor protein p53
E7	Oncoprotein, binds tumor suppressor protein retinoblastoma (Rb)
L1	Major viral capsid protein
L2	Minor viral capsid protein

Table 1.2 Summary of HPV Genes and Corresponding Functions (Tristram & Fiander 2007).

1.7 HPV Integration

HPV gains entry to the basal cell layer of the cervical epithelium as a result of epithelial erosions or mucosal ulcerations in the transformation zone at the squamocolumnar junction, thus giving rise to infection. In order for the virus to attach to the cells, it must first exploit the L1 protein, using it to bind to different receptors on the cell surface. Studies have shown that glycosaminoglycans (GAGs), in particular heparan sulfate proteoglycans (HSPGs), can be utilised to mediate the initial attachment of virus particles to the cells. These HSPGs are located on the basement membrane and are exposed following trauma to the epithelium (Joyce et al. 1999; Schiller et al. 2010). Additional investigations have also identified that HPV can enter a cell via interaction with $\alpha 6$ integrin, the main receptor of HPV-16 (Yoon et al. 2001). Once attachment has taken place, the virus gains entry to the host cell via L2 dependant, clathrin-mediated endocytosis (Schiller et al. 2010). The virus then takes advantage of the cell's replication machinery to reproduce its genome multiple times, with a low viral load of approximately 50 copies per infected cell. The viral episomes and cellular DNA replicate concurrently (Stubenrauch & Laimins 1999).

In uninfected epithelium, basal cells exit the cell cycle soon after migrating into the suprabasal cell layers and undergo a process of terminal differentiation (Doorbar 2005). However, when the infected basal cells replicate, the viral DNA is split between the 2 daughter cells. One of these cells migrates to the suprabasal layer where differentiation occurs while the other daughter cell remains in the basal layer and continues to proliferate. Only low levels of the early E1 and E2 proteins, together with the early oncogenes E6 and E7 are expressed at this point, while late genes expression is suppressed (Stubenrauch & Laimins 1999). When the cell migrates to the upper layers of the epithelium, the viruses, as well as the cells, proliferate. However, the virus waits until it's host cells have begun to differentiate and have safely escaped immune surveillance before it switches to late replication and gene expression (Sakakibara et al. 2013).

Once the host cell reaches the S-phase of the cell cycle, the virus can commence the process of differentiation, late viral gene expression and viral genome amplification. The HPV begins to replicate its genome to approximately 1000 copies, utilising the E6 and E7 growth promoters to stimulate the host cell to this point (Doorbar 2005). Expression of the late genes L1 and L2 is also induced, which, as stated previously, encode the capsid proteins. These late phase proteins encapsidate newly synthesised viral genomes forming virions, which are then released from the uppermost layers of the epithelium. These shed viruses have the ability to initiate a new infection (Florin et al. 2002). HPV gene expression throughout the different layers of cervical epithelium is demonstrated in Figure 1.10.

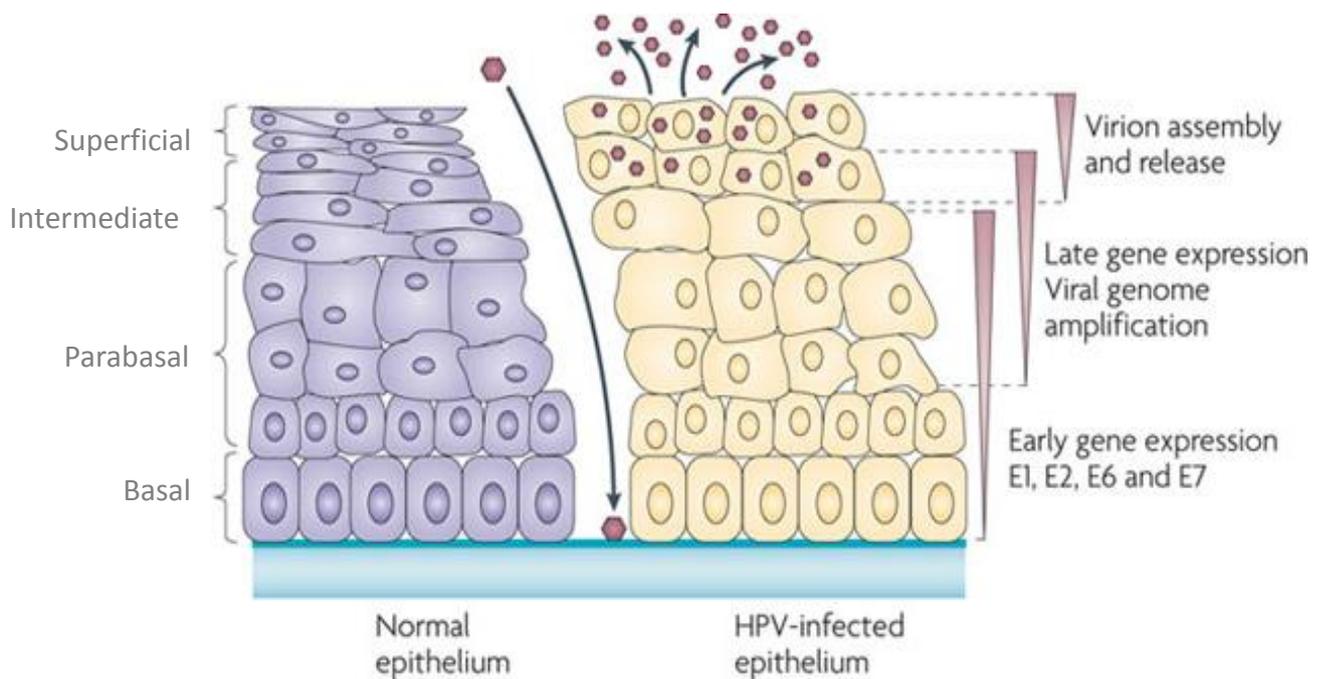


Figure 1.10 The Life Cycle of Human Papillomaviruses

Uninfected epithelium is shown on the left and HPV-infected epithelium is shown on the right. HPV gains entry to the basal cell layer of the cervical epithelium through micro-abrasions. Once initial infection has occurred, the early HPV genes E1, E2, E6 and E7 are expressed and the viral DNA replicates from episomal DNA. As the viral genome migrates towards the upper epithelium, it is further replicated and the late genes, L1 and L2, as well as E4, are expressed. These L1 and L2 proteins allow the viral DNA to become enclosed into capsids and form virions which are shed from the cell (Moody & Laimins 2010).

1.8 E1 and E2 Proteins

The E1 and E2 proteins are pivotal for transcriptional regulation, replication and segregation of papillomaviral DNA, a function which has been characterised via genetic studies (Lusky & Botchan 1985) and replication assays (Ustav & Stenlund 1991). Both proteins unite at the viral origin of replication (ori), which contains binding sites for the two proteins. This forms an E1-E2-ori complex which is fundamental for initiation of DNA replication (Berg & Stenlund 1997). The HPV E1 protein is the largest and arguably most complex protein encoded by HPV and acts as the 'initiator' protein for viral DNA replication. It is an ATP-dependent DNA helicase and is the only enzyme encoded by papillomaviruses (Hughes & Romanos 1993). This protein plays an essential role in the maintenance of the viral genome as an episome during the life cycle of the virus (Egawa et al. 2012). It also creates key novel interactions between the viral origin of replication and the cellular DNA replication machinery via multiple protein-protein and protein-nucleic acid interactions (Bergvall et al. 2014). E1 is expressed at very low levels, with binding to the viral ori very weak, requiring the presence of E2 to be proficiently increased. The E2 protein is a multifunctional regulatory protein associated with transcription and replication of the viral genome (Longworth & Laimins 2004). It is encoded by all papillomaviruses and expressed at early and intermediate stages of the viral life cycle. These E2 proteins are DNA-binding proteins with specific sequences that bind to 12bp motifs located primarily within the URR of the viral genomes (Mcbride 2013). The E2 protein has a regulatory role in the transcription of the E6 and E7 viral genes and has been shown to negatively regulate E6 and E7 transcription through repression of the viral early promoter (Thierry & Yaniv 1987). Therefore disruption of E2 results in transcriptional activation of the E6 and E7 oncogenes in cervical cancer, with over-expression of these oncoproteins thought to encourage the development of neoplasia (Cricca et al. 2009). In addition to the down-regulation of the E6 and E7 genes, accumulation of high levels of E2 during the HPV-16 life cycle has been shown to

induce the expression of the late HPV genes L1 and L2. Induction of HPV-16 late gene expression by E2 occurs by inhibiting polyadenylation at the early polyA (pAE) signal on the viral genome (Johansson et al. 2012). Further investigation has demonstrated additional intrinsic properties of the E2 protein that could contribute to cell transformation. These oncogenic features rely on their ability to induce abnormal mitoses, resulting in either a loss or surplus of DNA (Bellanger et al. 2011). E2 has also been associated with improvement of the efficiency of genome encapsidation during natural infection (Zhao et al. 2000).

1.9 E4 and E5 Proteins

Although E4 proteins are found at relatively low levels during the early phase of viral infection, a significant increase in expression is noted during the late phase, with the E4 protein the most abundantly expressed HPV protein in the differentiating cells of the upper layers of the stratified epithelium (Raj et al. 2004). E4 proteins possess an array of activities, such as the ability to bind to the cytokeratin network. A reason for this function may be the potential for the virus to exit the cell on cytokeratin reorganisation (Doorbar et al. 1991). The E4 proteins have also been demonstrated to induce apoptosis through association with the mitochondria (Raj et al. 2004). Furthermore, E4 can participate in arresting cells in the G₂ phase of the cell cycle, thereby averting progression of the cells into mitosis (Davy et al. 2002). Although E4 is part of the early region, it is expressed later in the virus life cycle (Stern et al. 2001) and may be accompanied by E5, which is also present in the late stage of infection. E5 proteins are membrane proteins primarily located in the endoplasmic reticulum, Golgi apparatus and nuclear membrane (Conrad et al. 1993). HPV E5 has been shown to interact with growth factor receptors such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and the colony stimulating factor-1 receptor (CSF1R) as well as gap junction proteins (Hwang et al. 1995). The E5 protein is also considered to play a role during the initiation of neoplasia (D.-H. Yang et al. 2003) and appears to inhibit programmed cell death (Bubb et al. 1988). However,

the open reading frame coding for E5 is often deleted in cervical carcinoma cells (Schwarz et al. 1985), thus signifying that E5 has no essential role in maintaining the malignant phenotype (Malik 2005).

1.10 E6 and E7 Proteins

The E6 and E7 proteins play a key role in the oncogenic properties of human papillomaviruses. These proteins work by forming specific complexes with tumour suppressors, in turn inhibiting their activity. The E6 proteins of high-risk HPV types have the potential to combine with the p53 tumour suppressor protein, a protein that controls responses to various incidents of cellular stress and initiates pathways for DNA repair, cell cycle arrest and apoptosis, depending on the type and extent of damage incurred (Howie et al. 2009). The formation of a complex comprising of E6, p53 and the cellular ubiquitination enzyme E6-AP promotes the degradation of the p53's oncosuppressive functions, a process which is ATP-dependent and involves the ubiquitin-dependent protease system (Scheffner et al. 1993). This process results in considerably diminished levels of p53 in cervical carcinoma cell lines, with the normal response to DNA damage by the protein abolished (Matlashewski et al. 1986; Kesisis et al. 1993). The E7 protein inhibits the retinoblastoma protein (pRb). pRb tumour suppressor proteins are negative cell-cycle regulators and function by blocking the activity of transcription factors, in particular E2F, thus preventing cell division (Dyson et al. 1989). One of the primary roles of pRb protein is to inhibit the expression of replication enzyme genes by binding to the E2F-family of transcription factors (Lipinski & Jacks 1999). However, pRb destruction results in the release of these E2F factors in their transcriptionally active forms and the subsequent activation of genes promoting cell proliferation (Chellappan et al. 1992). High-risk E7 proteins bind to pRB with a higher affinity than E7 proteins derived from low-risk HPVs, with a stronger affinity for the under-phosphorylated, "active" form of pRB (Münger et al. 1992). The

combined functional inactivation of p53 and pRb by the E6 and E7 oncoproteins, respectively, make cells susceptible for uncontrolled division and consequently cervical carcinogenesis.

1.11 L1 and L2 Proteins

The viral capsid, or outer shell, of HPV consists of a major and minor capsid protein. These proteins are the products of late gene expression and are referred to as the L1 and L2 proteins respectively. They are expressed in the upper layers of infected epithelium once viral genome amplification is complete (Ozbun et al. 1998). These L1 and L2 proteins play critical roles in mediating efficient virus infectivity and are essential for functions such as virus transmission, transfer and survival in the environment. The L1 protein is the principal structural element of the HPV virion, with 360 copies of the protein organised into 72 capsomeres (Becker et al. 2004). L1 provides viral entry ability via interaction with heparan sulfate proteoglycan (Giroglou et al. 2001; Joyce et al. 1999) and subsequent internalisation by endocytosis, a process which is clathrin-dependent (Bousarghin et al. 2003). L2 is the minor virion component with the 72 capsomere shell composed of approximately 12 copies of the protein (Becker et al. 2004). L2 has an important role in transferring viral DNA to the nucleus, following disassembly of the HPV particles. In addition to L1 and L2, studies have also suggested that E2 is necessary for the assembly of infectious virions in the upper epithelial layers (Day et al. 1998). As previously discussed, HPV late gene expression is found only in terminally differentiated epithelial cells, with the production of the L1 and L2 structural proteins strongly suppressed in the lower layers of the infected epithelium. As these L1 and L2 proteins are highly immunogenic it has been suggested that suppression of these products may prevent detection of the virus by the immune system, leading to persistence of infection.

1.12 HPV Vaccination

Cervarix (GlaxoSmithKline, Middlesex, UK) and Gardasil (Merck, NJ, USA) are two prophylactic HPV vaccines designed primarily for the prevention of cervical cancer. These vaccines are based on the HPV L1 major capsid protein, which has the ability to assemble into virus-like particles (VLPs). These VLPs closely resemble native HPV particles and include the conformational epitopes that generate strong virus-neutralising antibodies and consequently prevent HPV infection (Figure 1.11) (Ma et al. 2010). These VLPs contain no viral DNA, therefore they cannot infect cells, reproduce or cause disease. The antibody-mediated response elicited by these vaccines are type-restricted. Cervarix responds to HPV types 16, 18, 31, 33 and 45, which are responsible for 82% of cervical cancers globally, as well as adenocarcinoma for which we cannot adequately screen (Schwarz 2009). Gardasil is effective against HPV types 16, 18 and 31, responsible for squamous cell carcinoma and HPV types 6 and 11 which are the causative agents of genital warts and respiratory papillomatosis. Data states that Cervarix's efficacy is proven for 7.3 years (Schwarz 2009) and Gardasil's for 5 years (Romanowski 2011), figures which are imperative to assess the impact of the vaccines in question.

This year, a third vaccine against cervical cancer has also been approved. Gardasil 9 (Merck, NJ, USA) protects against 9 types of HPV, greater than that offered by Gardasil and Cervarix. This new vaccine will guard against approximately 90% of cervical cancers, while still providing protection against genital warts (Centers for Disease Control and Prevention 2015). The development of these vaccines has been a milestone for the prevention of cervical cancer, with another promising method involving the L2 minor capsid protein, which is highly conserved across HPV genotypes, currently underway. However, although multimeric L2 shows robust antibody responses in preclinical models against multiple HPV types, it is not as immunogenic as the VLPs (Ma et al. 2010). While these vaccines act as preventative agents, they exert no therapeutic qualities. It would also require years of vaccination before any

significant impact would be made on cervical cancer rates due to the high prevalence of HPV infections and slow rate of cervical carcinogenesis. Furthermore, since its introduction, the HPV vaccine has been a topic of controversy, with a number of questions being posed regarding its safety. Although studies suggest that the benefits of HPV vaccination far outweigh the risks, numerous cases of serious adverse reactions in response to the vaccine have been reported (Hawkes et al. 2013). This emphasises the urgent need for further investigation into safe protective and therapeutic agents against HPV-associated lesions and cervical cancer.

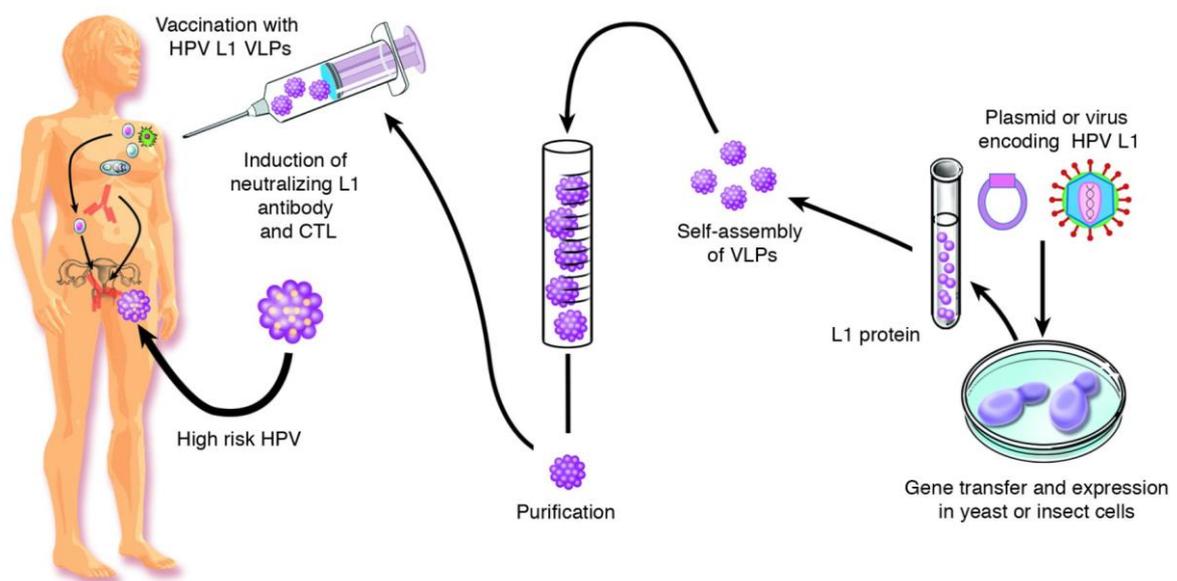


Figure 1.11 Vaccination Against HPV Infection Utilising HPV L1 VLPs

Recombinant L1 capsid protein self-assembles to form VLPs, which are particularly potent at inducing neutralising antibodies. As they do not possess any viral nucleic acid, they lack the ability to induce infection. VLP vaccines have shown potential for prevention of HPV infection and HPV-associated cervical cancer. The cells shown in the vaccinated subject include dendritic cells that present antigen to helper T cells and B cells which stimulate the generation of antibodies, which are capable of neutralising the virus (Berzofsky et al. 2004).

In addition to its use in vaccines, expression of the L1 capsid protein can also be utilised in a prognostic capacity. Immunohistochemical evaluation of HPV L1 positivity on Pap smears has shown disease progression in 9% of L1+ patients and 26% of L1- patients with CIN1 and CIN2 lesions (Origoni et al. 2013). HPV L1 positivity is more highly associated with low-risk HPV

subtypes than high-risk HPV subtype and cytological diagnosis has discovered that a higher expression rate of L1 is observed in LSILs than in HSILs and cervical cancers (Lee et al. 2008). For these reasons, it is reasonable to suggest that L1 capsid expression may be related to a favourable disease prognosis.

1.13 HPV DNA Integration

High-risk HPV DNA is integrated into the host genome in the majority of invasive cervical carcinomas, whereas the viral genome is maintained as an episome in preinvasive cervical lesions. The opposite occurs with low-risk HPV types, where integration is rare as is the potential to cause carcinomas (Burd 2003). It is the genetic alteration caused by high-risk HPV integration which is most likely a contributing factor to tumour progression, with integration transferring selective growth advantage to affected cells (Kessis et al. 1993). One significant result of HPV integration is the loss of the viral E2 gene, a transcriptional repressor of the E6 and E7 transforming genes. This in turn leads to an elevated and uncontrolled expression of E6 and E7, a factor which contributes greatly to the malignant transformation of the host cells and tumour formation (Bosch et al. 1992). Therefore, HPV DNA integration is determined to have a significant effect on cellular proliferation and the carcinogenic process.

1.14 Regulation of Gene Expression

Gene expression is the method by which genetic instructions are used to synthesise gene products. The process of gene expression involves two key stages i.e. transcription and translation. Transcription involves the production of messenger RNA (mRNA) from DNA by the enzyme RNA polymerase and the processing of the resulting mRNA molecule. Translation refers to the subsequent use of this mRNA to direct protein synthesis, and the successive post-translational processing of the protein molecule (Strachan & Read 1999). Gene regulation is a fundamental function employed by cells to increase their versatility and adaptability. Gene

expression can be controlled at several different stages. The majority of mechanisms that regulate gene expression do so by controlling transcription. Examples include regulation of the rate of transcription and regulation of the processing of RNA molecules, for example employing alternative splicing to produce multiple protein products from a single gene. The stability of mRNA molecules can also be affected, in addition to the regulation of mRNA translation efficiency, stability and localization (Ward & Cooper 2010). In eukaryotic cells, the first step in transcription is the formation of pre-mRNA from a DNA template. This pre-mRNA must undergo three major processing events, referred to as capping, splicing and polyadenylation, before it can become a mature and stable mRNA and be exported to the translation machinery in the cytoplasm (Mandel et al. 2008) (Figure 1.12).

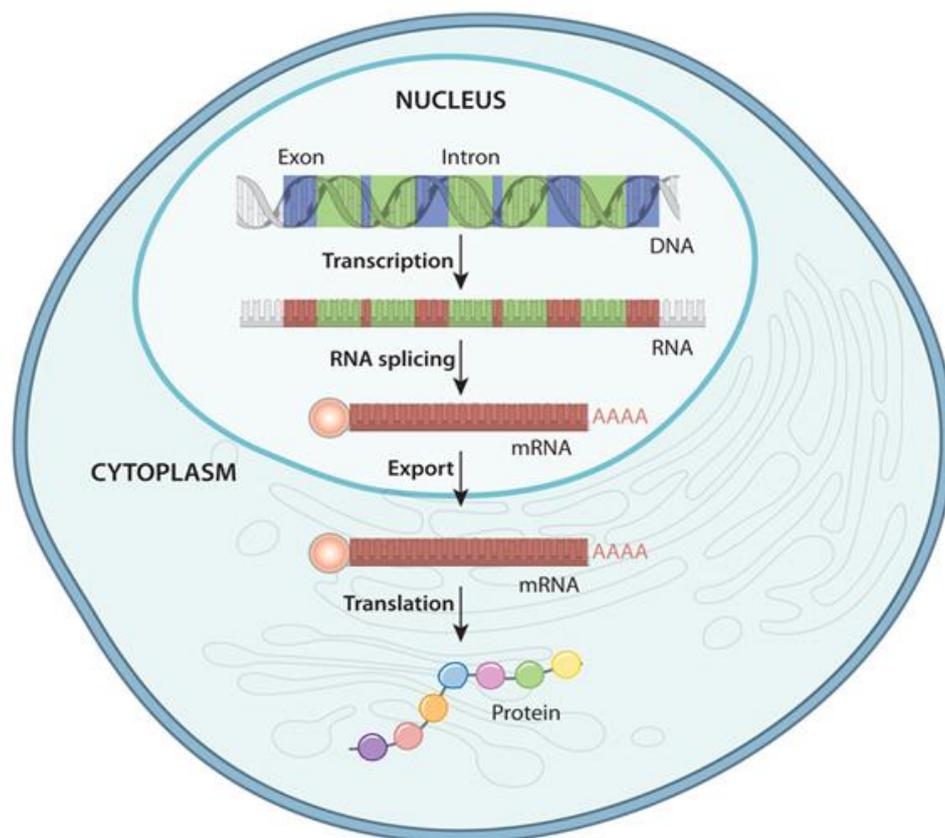


Figure 1.12 mRNA Processing

The processing of pre-mRNA includes capping, splicing and polyadenylation before eventual construction of a protein (Nature 2014).

- **Capping** involves the addition of 7-methyl guanosine groups (mRNA "cap") to the 5' ends of the newly synthesized pre-mRNA. This occurs once approximately 20-30 nucleotides of the molecule have been transcribed and requires removal of the terminal 5' phosphate, which is achieved with the aid of a phosphatase enzyme. The process of capping converts the 5' end to a 3' end by 5'-5' linkage, protecting the mRNA from 5' exonuclease, which degrades foreign RNA. The newly formed complex assists with the binding of ribosomes to the mRNA during translation and also aids in the protection of the mRNA from premature degradation (Decroly et al. 2012).
- **Splicing** is a modification of pre-mRNA in which introns, the noncoding regions of RNA, are removed with remaining exons left to form a continuous strand (Will & Lührmann 2011). The areas at which introns are removed from primary transcripts are referred to as splice sites which are found at the 5' and 3' ends of introns. Splicing occurs in several steps and is catalysed by a spliceosome, a large protein complex composed of small nuclear ribonucleoproteins (snRNPs). The spliceosome functions by binding to the splice sites on either end of the intron, looping the intron into a structure referred to as a lariat and subsequently cleaving it off. The remaining ends of the exons are then joined together, the edited RNA and intron are released and the spliceosome disassembles (Figure 1.13) (Cooper 2000).

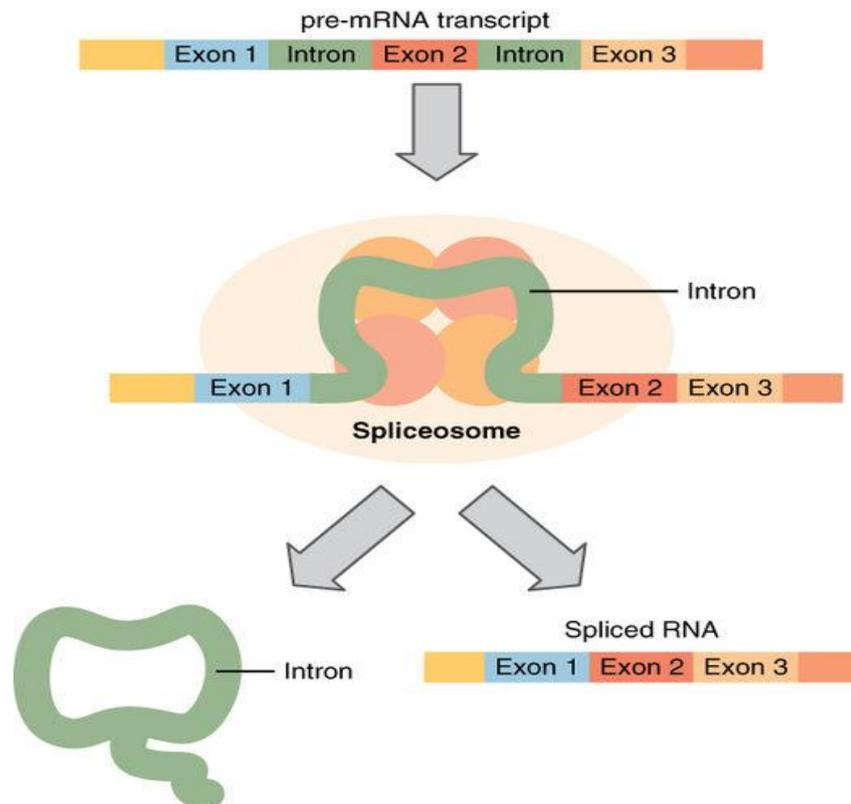


Figure 1.13 Splicing

The figure illustrates the exons and introns in pre-mRNA and the formation of mature mRNA through the removal of noncoding introns as occurs with splicing (OpenStax 2014).

- **Polyadenylation** is a method utilised in gene regulation in which a sequence of adenosine ribonucleotides are added to the 3' end of a spliced mRNA to form a poly (A) tail. The primary transcript is cleaved at the polyadenylation signal sequence, an AAUAAA sequence, by the cleavage and polyadenylation specificity factor (CPSF). The poly (A) tail is a useful tool in the protection of mRNA from digestion with nuclease and greatly increases the efficiency of translation (Birnstiel et al. 1985).

- **Alternative splicing** of mRNA precursors provides a significant means of genetic control and is a crucial step in the expression of the majority of genes, with analysis indicating that 92-94% of human genes undergo alternative splicing (Wang et al. 2008). The process involves exons or portions of exons or noncoding regions within a pre-mRNA transcript becoming differentially joined or excluded, resulting in multiple protein isoforms being encoded by a single gene (Nancy et al. 2015). As well as providing an opportunity for gene regulation, this practice of alternative splicing increases the informational diversity and functional capacity of a gene during post-transcriptional processing. Six methods of alternative splicing have been described: constitutive, exon skipping, alternative donor site, alternative acceptor site, mutually exclusive exons and intron retention (Figure 1.14). Viruses competently exploit alternative splicing to order to produce many functional mRNAs from small genomes. Defects in alternative splicing have been linked to numerous genetic diseases including muscular dystrophy, Alzheimer's disease and cancer. Interestingly, a complex pattern of alternatively spliced and polyadenylated mRNAs is observed during the HPV life cycle (Li, Johansson, et al. 2013). During alternative splicing, *cis*-acting regulatory elements in the mRNA sequence determine which coding sequences are retained and which coding sequences are spliced out. These *cis*-acting regulatory elements have been identified in the coding regions and the late 3' untranslated regions (UTRs) of several papillomaviruses (Graham 2010). It is proposed that these elements employ different RNA-based mechanisms to regulate viral late gene expression. Research has also been carried out investigating the link between alternative splicing and cancer, with many splicing factors up-regulated in cancer cells (Faustino & Cooper 2003; Philips & Cooper 2000). For this reason, further examination of the mechanisms which influence splicing and alternative splicing would be highly beneficial.

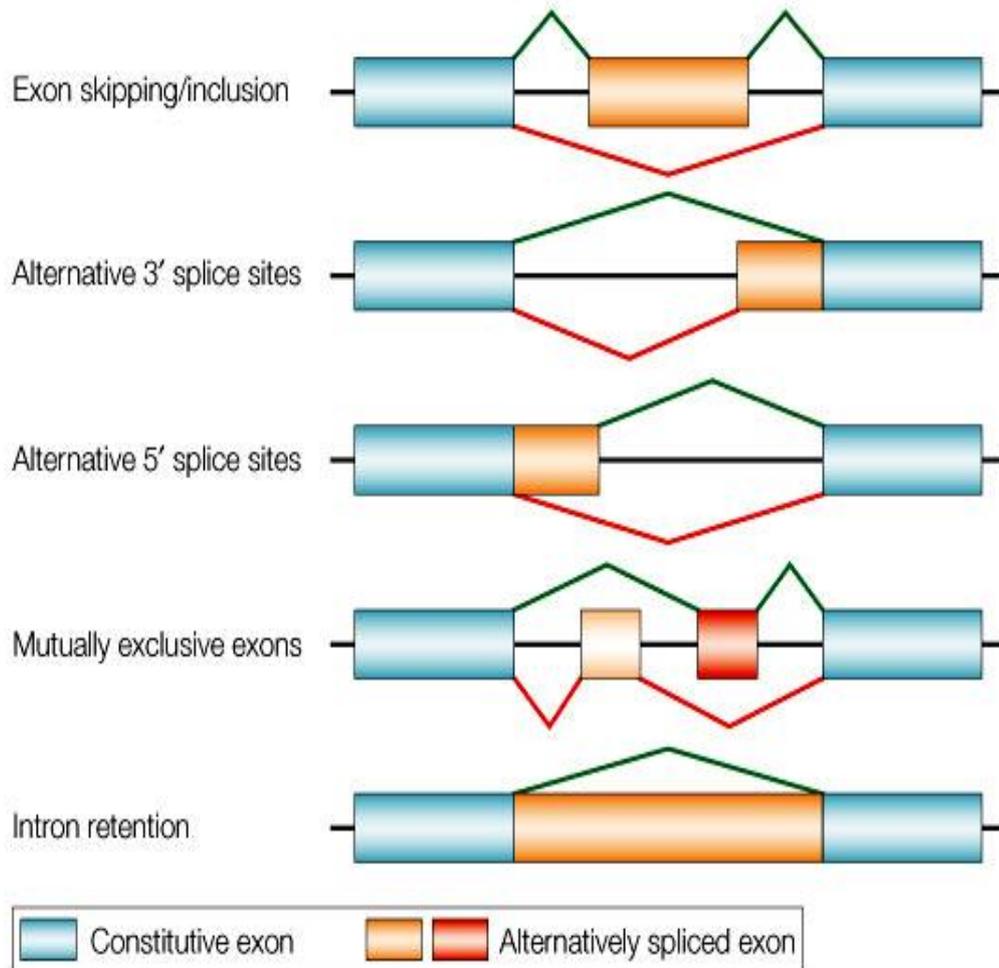


Figure 1.14 Alternative Splicing

The diagram shows different types of alternative splicing including exon inclusion or skipping, alternative splice-site selection, mutually exclusive exons and intron retention (Cartegni et al. 2002).

1.15 Regulation of HPV Late Gene Expression

As previously discussed, expression of the human papillomavirus capsid genes, L1 and L2, as well as amplification of viral DNA and virion assembly occur only in the terminally differentiated layers of infected epithelium. Furthermore, it has also been established that HPV-16 late genes are not expressed in cervical cancer containing HPV-16 DNA (Doorbar 2005). Therefore, as cervical cancer cells do not express the late viral mRNAs or proteins, it can be speculated that inhibition of late gene expression is a prerequisite for cancer progression.

During transcription all mRNAs are regulated through the use of splicing and polyadenylation signals. In relation to HPV-16, 20 different transcripts have been identified, 14 of which are produced from the early promoter to the early polyadenylation signal (Zheng & Baker 2006). Eleven splice sites have also been identified, 10 located in the early region and only 1 in the late region, as displayed in Figure 1.15 (Doorbar et al. 1990). E4 mRNA is one of the most abundant HPV-16 mRNAs produced and is generated from splice donor (SD880) to splice acceptor (SA3358). The most efficient splice site utilised by HPV-16 is this major 3'-splice site SA3358, which is involved in the production of E4, E6, E7, L1 and L2. Late mRNAs are transcribed from the late promoter and are thought to be spliced either from SD880 to SA3358 and from SD3632 to SA5639 or directly from SD880 to SA5639. The SD3632 and SA5639 are used exclusively by the late mRNAs and the presence of an adjacent splicing silencer that actively suppresses the use of these splice sites has been shown to inhibit late gene expression (Zhao et al. 2007; Rush et al. 2005). *In vivo*, HPV-16 splicing between SD880 and SA3358 has been determined as the most-common splicing event in both low and high grade cervical lesions (Schmitt et al. 2010), suggesting that SA3358 plays a significant role during a productive HPV-16 infection and is also likely to be important for pathogenesis. Alternative splicing appears crucial for the production of L1, since the 3' end of L2 and the 5' end of L1 overlap. As mentioned, late viral mRNAs are expressed only in differentiating cells and it has been demonstrated that posttranscriptional events are highly involved in late gene regulation (Zheng & Baker 2006). In contrast to the inhibitory factors of late gene expression, overexpression of certain proteins, such as adenovirus E4orf4 (E4orf4), polypyrimidine tract binding protein (PTB) and alternative splicing factor/splicing factor 2 (ASF/SF2) have been indicated as inducers of late gene expression (Somberg & Schwartz 2010; Somberg et al. 2009; Somberg et al. 2008). Additionally, studies have shown that protein kinase C (PKC) activation is essential for the efficient completion of the late phase of the HPV life cycle *in vitro* (Meyers

et al. 1992). These proteins are a source of specific interest, as, should a method be determined to induce their expression, late gene products could be subsequently up-regulated.

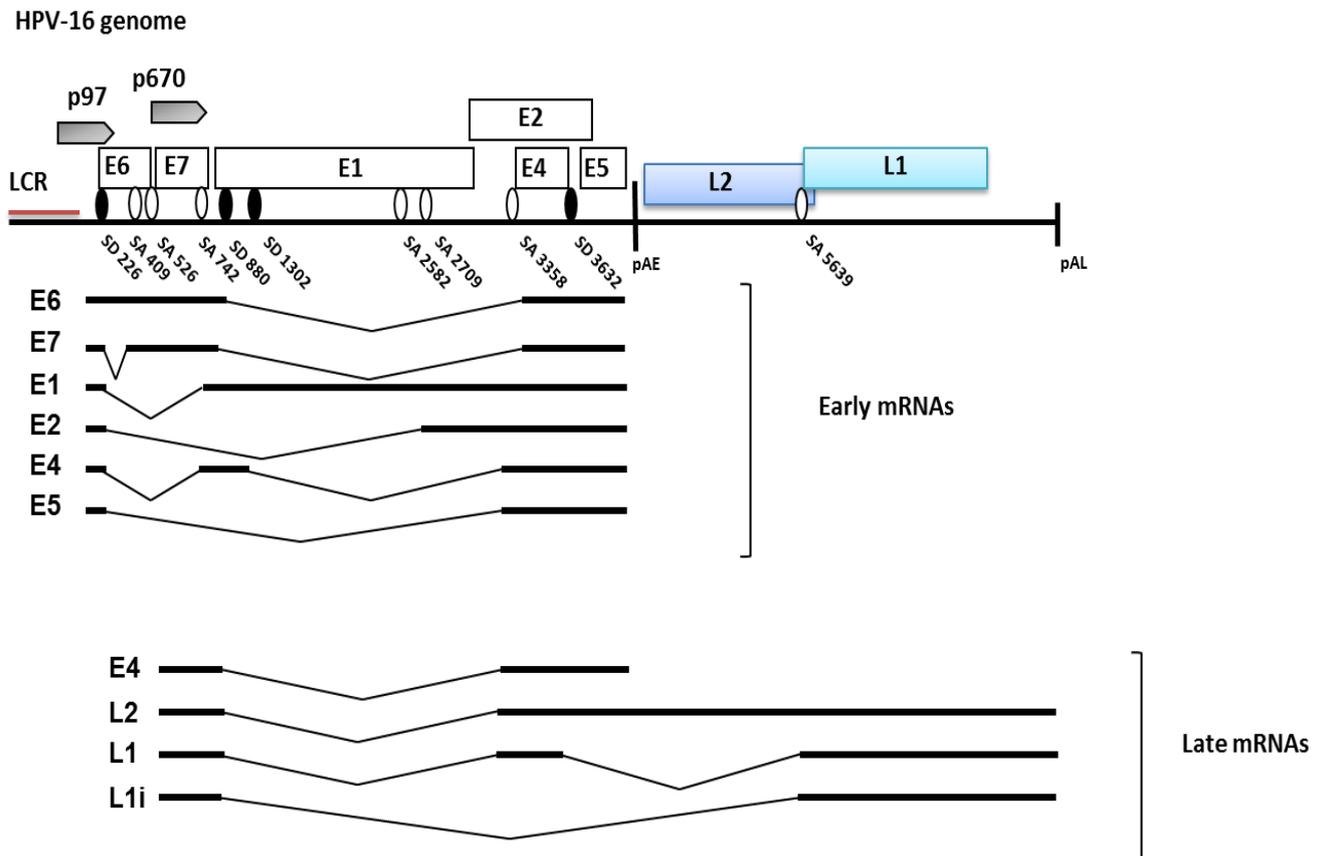


Figure 1.15 Splicing and Gene Regulation in HPV

The viral genome encodes the early (E) genes, designated E1–E7, and the late (L) genes L1 and L2. The non-protein-coding sequence between the L1 stop codon and the E6 AUG is termed the long control region (LCR) and contains the origin of DNA replication (ori) in addition to the early viral promoter, p97. The late promoter, p670, which is differentiation-dependent, is found in the E7 coding region. Two polyadenylation signals are present in the HPV genome. The early polyadenylation signal (pAE) is located downstream of E5 and preceded by the early 3' untranslated region (eUTR). The late polyadenylation signal (pAL) is located downstream of L1 and preceded by the late 3' untranslated region (lUTR). Known 5' splice sites (splice donor 226 (SD226), SD880, SD1302 and SD3632) and known 3' splice sites (splice acceptor 409 (SA409), SA526, SA742, SA2582, SA2709, SA3358 and SA5639) are indicated as are early and late mRNAs (Orrù 2012).

1.16 Protein Involved in Splicing

1.16.1 SR Proteins

The SR protein family consists of a group of structurally related proteins which play a crucial role in pre-mRNA splicing. As well as this, they are also important in the regulation of alternative splicing and post-splicing activities, including mRNA nuclear export, nonsense-mediated decay and mRNA translation. These proteins are referred to as SR proteins as they contain a protein domain with long repeats of serine and arginine amino acid residues, whose standard abbreviations are "S" and "R" respectively. As these SR proteins have a wide number of roles, they are deemed significant in the regulation of mRNA metabolism (Long & Caceres 2009). The SR protein family is encoded by nine genes, namely *ASF/SF2*, *SC35*, *SRp20*, *SRp40*, *SRp55*, *SRp75*, *SRp30c*, *9G8*, and *SRp54* with *ASF/SF2* and *SC35* two of the best characterised. SR proteins are identified by their ability to interact simultaneously with RNA and other protein components through an RNA recognition motif (RRM) and through a domain rich in arginine and serine residues, referred to as the RS domain (Shepard & Hertel 2009).

i) ASF/SF2

Alternative splicing factor 1 (ASF1)/pre-mRNA-splicing factor SF2 (SF2) or ASF1/SF2, also known as serine/arginine-rich splicing factor 1 (SRSF1), is an essential sequence-specific splicing factor involved in pre-mRNA splicing. It is approximately 33 kDa in size and binds to pre-mRNA transcripts and components of the spliceosome. It has the potential to either activate or repress splicing depending on the location of the pre-mRNA binding site. ASF/SF2 promotes recruitment of U1 snRNP to 5' splice sites (Kohtz et al. 1994) and can also help bridge 5' and 3' splice sites (Wu & Maniatis 1993). It has been discovered that the splicing factor ASF/SF2 is a proto-oncogene and is up-regulated in various human tumours (Karni et al. 2007)

including cervical carcinoma (Fay et al. 2009). It has also been noted that the most frequently used 3'-splice site on the HPV-16 genome, SA3358, is dependent on ASF/SF2. SA3358 is used to produce HPV-16 early mRNAs encoding E4, E5, E6 and E7 and late mRNAs encoding L1 and L2 (Li, Cardoso Palacios, et al. 2013). ASF/SF2 regulates SA3358, specifically binding to exonic sequences located between SA3358 and SD3632. In particular ASF/SF2 enhances splicing to SA3358 and inhibits usage of the splice donor SD3632. High levels of ASF/SF2 may also down-regulate expression of E2 (Somberg & Schwartz 2010). As a means of investigating modulation of splicing factor expression, valproic acid has been shown to increase the expression of ASF/SF2 (Harahap et al. 2012).

ii) SC35

Serine/arginine-rich splicing factor 2, or the SC35 protein is necessary for the splicing of pre-mRNA. SC35 regulates alternative splicing in a concentration-dependent manner both *in vitro* and *in vivo* (Sureau et al. 2001). It is essential for formation of the earliest ATP-dependent splicing complex and possesses the ability to interact with spliceosomal components bound to both the 5'- and 3'-splice sites during spliceosome assembly (Kim et al. 2011). It is required for ATP-dependent interactions of both U1 and U2 snRNPs with pre-mRNA and affects transcriptional elongation in a gene-specific manner (Lin et al. 2008). Additionally, it has also been deduced that overexpression of SC35 in HeLa cells results in a significant decrease of endogenous SC35 mRNA levels together with alterations in the abundance of SC35 alternatively spliced mRNAs (Sureau et al. 2001).

1.16.2 hnRNP Proteins

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are complexes of RNA and protein which are located in the nucleus during both gene transcription and post-transcriptional modification of the newly synthesized pre-mRNA. These proteins have molecular masses of 34-120 kDa and play a critical role in regulating gene expression. They are involved in the processing of heterogeneous nuclear RNAs (hnRNAs) into mature mRNAs as well as DNA repair and telomere regulation (Piñol-Roma & Dreyfuss 1993). These RNA-binding proteins are among the most abundant proteins in the nucleus, with their presence, when bound to pre-mRNA, indicative of the fact that processing is still underway. hnRNPs bind to pre-mRNA with sequence specificity to high-affinity binding sites consisting of sequences that code for 5'- and 3' splice sites, polyadenylation elements and the polypyrimidine tract (PPT) (Ghetti et al. 1992).

i) hnRNP A2/B1

hnRNP A2/B1 proteins belong to the hnRNPs A/B family; RNA-binding proteins imperative for alternative splicing. They are among the most abundant pre-mRNA binding proteins of vertebrates and play significant roles in RNA processing, mRNA trafficking, and telomere maintenance (He & Smith 2009). In most tissues, they are located in the nucleus; however, in the squamous epithelium of the skin and oesophagus, A2 is also distributed in the cytoplasm (Kamma et al. 1999). hnRNP A2/B1 proteins are up-regulated in several cancers and can act as oncogenes when up-regulated (Shilo et al. 2014). Studies have shown that hnRNP A2/B1 is overexpressed in glioblastomas and is correlated with poor prognosis, while patients with deletions of the *HNRNPA2B1* gene show better prognosis than average (Golan-Gerstl et al. 2011). In relation to HPV-16, investigations have shown that the 5' splice site SD3632 is negatively regulated by two AUAGUA motifs located immediately upstream of SD3632 (Li, Johansson, et al. 2013). These sites interact specifically with members of the hnRNP D family and hnRNP A2/B1. It is concluded that hnRNP D proteins and hnRNP A2/B1 inhibit SD3632,

with results demonstrating that knock-down of these factors induced HPV-16 late gene expression, whereas overexpression of hnRNP A2/B1 further suppressed HPV-16 late gene expression (Li, Johansson, et al. 2013). Further studies have shown that hnRNP A2/B1 expression in human neuroblastoma cell lines is down-regulated by retinoic acid (Liang et al. 2011).

ii) PTB

Polypyrimidine tract-binding protein (PTB), also referred to as hnRNP I, is a 57 kDa, ubiquitous RNA-binding protein that binds to the pyrimidine tract typically found near the 3' end of introns (Pérez et al. 1997). It has the ability to shuttle between the nucleus and the cytoplasm, a characteristic which must be carefully regulated as the processes which require PTB are dependent on its location (Sawicka et al. 2008). PTB has a regulatory role in alternative splicing and functions in a large number of diverse cellular processes such as polyadenylation, mRNA stability and translation initiation (Castelo-Branco et al. 2004; Wollerton et al. 2004). Fay et al. have recently shown that PTB is highly up-regulated in cervical cancer cells while it is down-regulated in the superficial layers of the cervical epithelium (Fay et al. 2009). Studies have also demonstrated that overexpression of polypyrimidine tract binding protein induces HPV-16 late gene expression. PTB has the potential to activate SD3632, the only 5' splice site on the HPV-16 genome that is used exclusively by late mRNAs. PTB interferes with splicing inhibitory sequences located immediately upstream and downstream of this SD3632 site, consequently activating late gene expression (Somberg et al. 2008). It has also been demonstrated that the small molecule drug tannic acid, increases the expression of *PTBP1* mRNA, the gene which encodes PTB, in a dose-dependent manner (Bian et al. 2009).

1.16.3 Protein Kinase C

Protein kinase C (PKC) isoforms consist of a family of lipid-activated enzymes that are associated with a wide range of cellular functions (Steinberg 2008). Protein kinase C is particularly important in differentiation, with the various PKC isoforms expressed in keratinocytes having distinct functions in keratinocyte differentiation (Yang et al. 2003; Ohba et al. 1998; Dlugosz & Yuspa 1993). The tumour-promoting phorbol ester TPA has the potential to activate PKC (Niedel et al. 1983). Stimulation of protein kinase C by TPA is due to the fact that TPA is an analog of diacylglycerol, the natural activator of PKC. Studies have indicated that PKC activation is essential for the efficient completion of the late phase of the HPV life cycle *in vitro* (Meyers et al. 1992), most likely through regulating the expression of late genes via post-transcriptional mechanisms (Hummel et al. 1995; Terhune et al. 1999). Hummel et al. have demonstrated regulation of HPV-31b late gene expression by PKC-dependent changes in post-transcriptional RNA processing while additional studies have shown that HPV-31 genome amplification in the intermediate phase of the life cycle is dependent on PKC activity (Bodily & Meyers 2005). Further research has also indicated that the PKC pathway is an important regulator of differentiation-dependent HPV-31 replication and transcription (Bodily et al. 2006).

1.17 Transient and Stable Transfection

Transfection is the process by which nucleic acids are artificially introduced into mammalian cells, a procedure first described by Vaheri and Pagano in 1965. Transfection is an influential and invaluable tool used widely in biomedical research to study and control gene expression. Through the process of selectively enhancing or inhibiting the expression of a certain gene, transfection permits investigation of gene function and offers insights into the role of certain genes in a variety of biological processes and diseases (Kim & Eberwine 2010). Cloned genes can be transfected into cells and be utilised for biochemical characterisation and mutational analyses as well as for the investigation of gene regulatory elements, the effects of gene expression on cell growth and specific protein production. Meanwhile transfection of RNA can be used to induce protein expression, or to repress its expression through antisense or RNA interference (RNAi) procedures. Methods of transfection can be broadly classified into biologically, chemically and physically mediated methods. Biological methods typically employ a viral vector, most commonly based on adenoviruses, to deliver nucleic acid into a target cell. This process is also known as transduction (Pfeifer & Verma 2001) and tends to have a high transfection efficiency although cytotoxicity is possible. Chemical methods are the most widely employed in contemporary research and were the first to be utilised in the introduction of foreign genes into mammalian cells (Schenborn & Goiffon 2000). These methods facilitate entry of foreign DNA into the target cell by forming positively charged complexes with the foreign DNA that is attracted to the negatively charged cell membrane (Kim & Eberwine 2010). Various different chemical transfection techniques are utilised including calcium phosphate and cationic lipid as well as GeneJuice® Transfection Reagent, which is based on a nontoxic cellular protein and a small amount of a novel polyamine. This reagent is optimised for maximal transfection efficiency, ease of use and minimal cytotoxicity. Physical transfection methods include electroporation, biolistic particle delivery and direct injection

(Kim & Eberwine 2010). Genetic material which has been introduced into the cells exist in either a stable or transient manner, depending on the nature of the material (Recillas-Targa 2006) (Figure 1.16). Products introduced in stable transfection are integrated into the host genome and commonly possess a marker gene for selection, referred to as a transgene. These cells can sustain transgene expression even after host cells replicate (Glover et al. 2005). Meanwhile, transiently transfected genes are only expressed for a limited period of time, usually up to 72 hours and are not integrated into the genome (Recillas-Targa 2006).

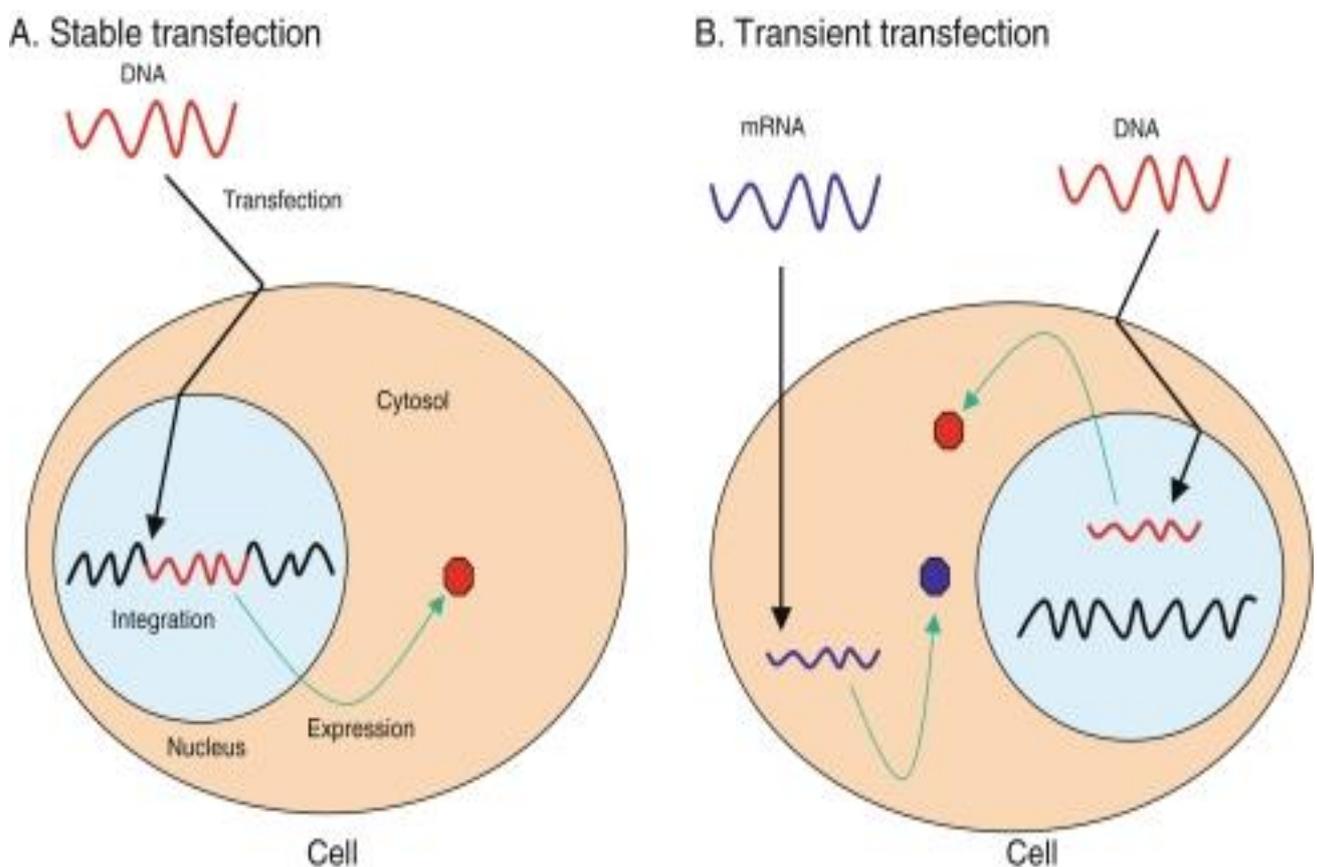


Figure 1.16 Stable and Transient Transfection

A) Stable Transfection: Foreign DNA (*red wave*) is delivered to the nucleus by passage through the cell and nuclear membranes. Foreign DNA is integrated into the host genome (*black wave*) and sustainably expressed.

B) Transient transfection: Foreign DNA is delivered into the nucleus but is not integrated into the genome. Foreign mRNA (*blue wave*) may also be transfected and translated in a transient transfection. Hexagons shown are expressed proteins from transfected nucleic acids while the black arrows indicate the delivery of foreign nucleic acids (Kim & Eberwine 2010).

The choice of transfection is dependent on the objective of the experiment. The generation of stably transfected cells begins with transient transfection. Foreign DNA is delivered into the nucleus for both processes but in stable transfection a minority of these transfected cells integrate the exogenous nucleic acid into the chromosomal DNA. To aid in the identification of stable transfection, a selectable marker is co-expressed with the gene of interest, allowing cells with the new gene to be recognised. As this foreign gene becomes part of the host gene it is consequently replicated. This is the hallmark of stable transfection, with replicated cells also expressing the new gene, thus generating a stably transfected cell line. Foreign DNA integrates in the chromosomal DNA randomly at one or very few sites (Murnane et al. 1990) with site integration having a significant influence on the transcription rate of the gene of interest (Wurm 2004). Cellular DNA in the nucleus is combined with proteins for a multitude of reasons, including protection of the DNA from damage, control of gene expression and DNA replication and to ensure it fits correctly within the nucleus. This compact structure is referred to as chromatin. There are two varieties of chromatin: 'open' euchromatin and 'compacted' heterochromatin (van Steensel 2011). Euchromatin consists of DNA associated with several nucleosomes, which are composed of proteins called histones. Histones have short sequences of DNA wrapped around them loosely, allowing RNA polymerase and gene regulatory proteins to bind to DNA sequences, initiating gene transcription (van Steensel 2011). In heterochromatin, the DNA is tightly wound which impedes the binding of protein factors and RNA polymerase complexes to the DNA, thus inhibiting gene expression. Heterochromatin is only found in eukaryotes. The integration site of transfected DNA is therefore crucial. If the transfected DNA becomes integrated into heterochromatin, expression of the target gene is unlikely, while integration into cellular euchromatin is much more likely to yield target gene expression. 'Integrational hotspots' is the name given to regions of the endogenous genome in which DNA integration is likely to occur (Woychik & Alagramam 1998). Although integration

of foreign DNA into the cellular genome generally does not cause damage to its integrity, detrimental outcomes sporadically occur such as deleterious rearrangement of the endogenous DNA at the site of integration, potentially leading to deletions, duplications and translocations which can interfere with coding sequences (Hamada et al. 1993; Covarrubias et al. 1987; Woychik & Alagramam 1998). The successful development of genome manipulation and establishment of stable cell lines is a noteworthy achievement, allowing for investigations into therapeutic mechanisms through large scale recombinant protein production and analysis. For use in this study, stable cell lines were generated with specific reporter plasmids based on the HPV-16 genome integrated in the cellular genome.

1.18 Establishment of HPV-16 Reporter Cell Lines

Inhibition of HPV-16 late gene expression in the early stage of the life cycle may be a prerequisite for persistence of infection. Therefore, it is speculated that activation of L1 and L2 late gene expression in persistently infected cells could alert the host immune system to the presence of the virus, potentially clearing the infection. In order to investigate factors affecting the regulation of HPV-16 late gene expression, previous work was performed in which stable cell lines containing reporter plasmids for the L1 gene were created. The 2 plasmids utilised were pBEL and pBELM (Zhao et al. 2004). Both of these plasmids carry viral early and late genes, except E6 and E7, with the weak viral promoters replaced by the strong human cytomegalovirus (CMV) immediate early promoter. Early and late splice sites are also present in the plasmid, allowing for the examination of the splicing events that can occur in HPV-16. Similar to the HPV-16 genome during an infection, pBEL transfected into proliferating cells express high level of the early genes, primarily E4, whereas expression of late genes is undetectable. In pBELM however, the splicing silencer elements adjacent to the late 3' splice site SA5639 (located in the L1 coding region) have been mutated. This mutation reduces silencing on the SA5639 splice acceptor therefore activating late gene expression (Zhao et al. 2004). In order to detect the HPV-16 L1 gene, the plasmids pBEL and pBELM were modified by replacing the L1 late gene with the easily detectable reporter gene chloramphenicol acetyltransferase (CAT) (Orrù 2012). The structure of the pBELCAT and pBELMCAT plasmids is displayed in Figure 1.17.

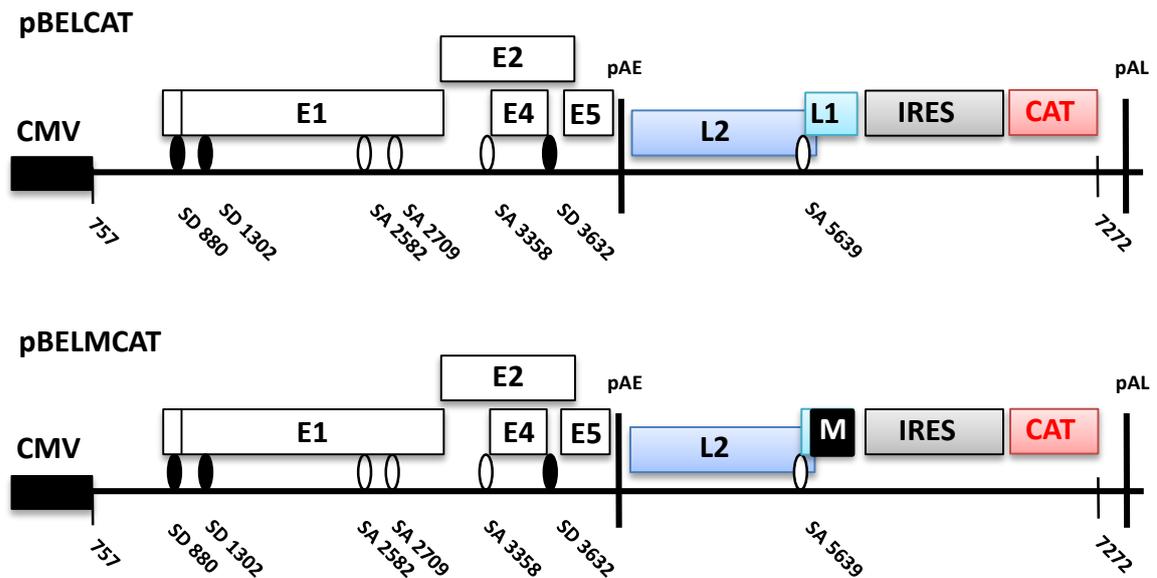


Figure 1.17 Structure of the pBELCAT and pBELMCAT Plasmids (Orrù et al. 2012).

Studies performed on the stable cell lines transfected with these pBELCAT and pBELMCAT plasmids identified that both lines are functional and express detectable levels of CAT, with the CAT reporter mimicking the expression patterns of the L1 gene in the intact viral genome (Orrù 2012). These cell lines therefore provide an excellent means of investigating late gene expression. With regards to this study, the effects of specific small molecule drugs as potential inducers of late gene expression can be easily analysed through treatment of the cell lines and analysis of CAT expression utilising the CAT ELISA assay. pBELMCAT31 and pBELCAT67 were selected for use as representatives of reporter cell lines containing a plasmid with and without a mutation. The pBELMCAT31 and pBELCAT67 cell lines show high and low CAT expression levels respectively as displayed in Figure 1.18.

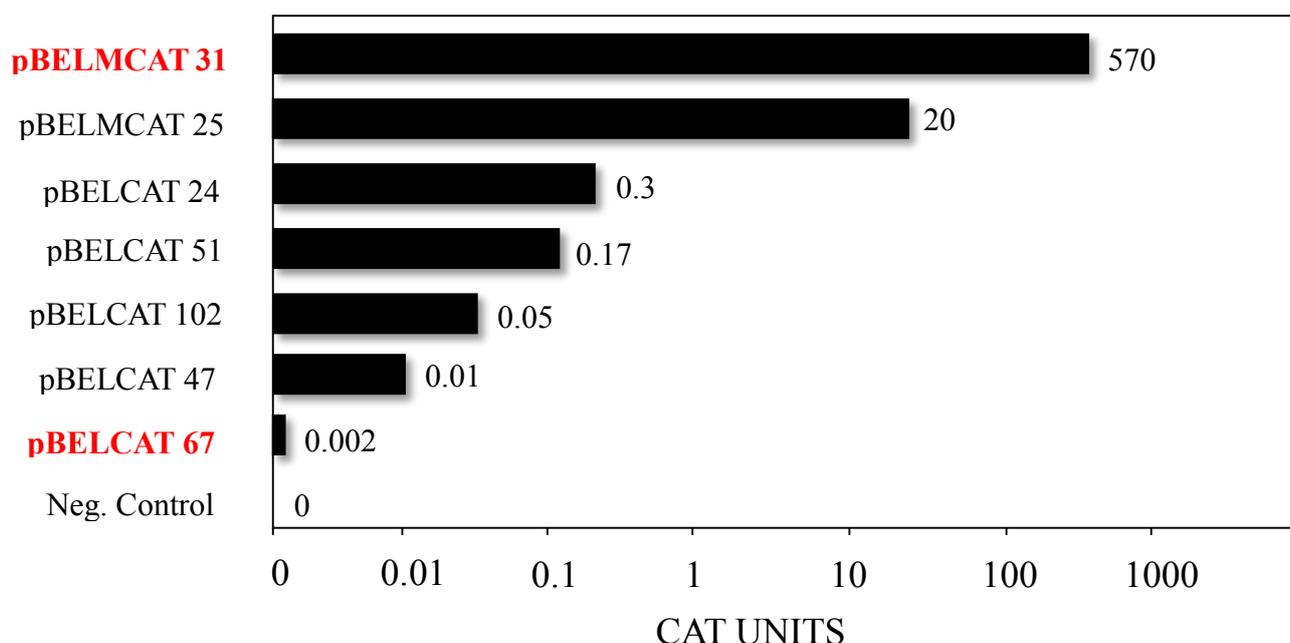


Figure 1.18 Expression of CAT in pBELCAT- and pBELMCAT- Derived Stable Cell Lines

The graph indicates high levels of CAT expressed by pBELMCAT31 with minimal expression by pBELCAT67 (Orrù et al. 2012).

1.19 Small Molecule Drugs Utilised in this Study

i) TPA

TPA (12-O-tetradecanoylphorbol-13-acetate), also called PMA (phorbol-12-myristate-13-acetate), is a small molecule drug and the most commonly used phorbol ester. TPA is a potent tumour promoter and is employed to activate protein kinase C (PKC), a signal transduction enzyme with numerous effects on cells and tissues. PKC activation is essential for the efficient completion of the late phase of the HPV life cycle *in vitro* (Meyers et al. 1992), possibly by regulating the expression of late genes via post-transcriptional mechanisms (Hummel et al. 1995). Investigations have been carried out to explore the therapeutic potential of TPA in patients with a variety of malignant and non-malignant diseases. TPA has been shown to cause differentiation of cells of the human leukaemia cell line HL60 to non-dividing macrophage-like cells, with these differentiated cells cytotoxic for tumour cells (including parent, untreated

HL60 cells) *in vitro* (Weinberg 1981). Additional studies have also identified TPA as a therapeutic drug for patients with leukaemia (Strair et al. 2002; Han et al. 1998). Furthermore, research performed has demonstrated that TPA transactivates the epidermal growth factor receptor (EGFR) and increases cell proliferation by activating the PKC δ /c-Src pathway in glioblastomas. (Amos et al. 2005).

ii) Tannic Acid

Tannic acid (TA) belongs to the class of hydrolysable tannins and is the most commonly used standard for quantitation of tannins. TA has been identified as a potential anticancer agent. There is evidence that tannic acid inhibits the cytokine CXCL12 together with its receptor CXCR4, powerful mediators of metastasis, a function which may contribute to its antitumour properties (Chen et al. 2003). It has also been shown that apoptotic activity is increased in breast cancer and prostate cancer cells in response to exposure to tannin extracts (Losso et al. 2004; Bawadi et al. 2005) with investigations showing that breast cancer cells expressing the estrogen receptor are more susceptible to the effects of TA (Booth et al. 2013). It has also been reported that tannic acid increases the levels of polypyrimidine tract binding (PTB) protein by activating its promoter region (Bian et al. 2009). PTB has previously been characterised with the ability to induce HPV-16 late gene expression by interfering with cellular factors that interact with the inhibitory sequences (Somberg et al. 2008).

iii) Valproic Acid

Valproic acid (VPA) is an analogue of valeric acid, found naturally in valerian and initially used as an organic solvent. It is a histone deacetylase inhibitor and has therapeutic use as an anticonvulsant and mood stabilising drug, particularly useful in the treatment of epilepsy, bipolar disorder, schizophrenia and in the prevention of migraine headaches (Yatham 2004; Jeavons & Clark 1974). Studies have also revealed that VPA is of therapeutic benefit for patients with myelodysplastic syndromes (Kuendgen et al. 2004) in addition to displaying potent antitumour effects in a selection of *in vitro* and *in vivo* systems (Duenas-Gonzalez et al. 2008). VPA has been identified with potential as a treatment for various cancers such as multiple myeloma (Schwartz et al. 2007) and breast cancer (Munster et al. 2007). It has also been shown to induce apoptosis in HeLa cervical cancer cells through the inhibition of Akt1 and Akt2 gene expression. VPA impedes Akt1 and Akt2, essential pro-survival factors in cell proliferation, leading to deactivation and consequent apoptotic cell death (Chen et al. 2006). Furthermore, VPA has been shown to impact the expression of a number of splicing factors such as ASF/SF2 and hnRNPA1, increasing ASF/SF2 and decreasing hnRNPA1 levels (Harahap et al. 2012). Similar studies have shown that various SR and SR-like splicing factors (ASF/SF2, SRp20 and Htra2- β 1) are up-regulated by VPA, which may have important implications for disorders affected by alternative splicing. Notably, the most commonly used 3' splice site on the HPV-16 genome, SA3358, is dependent on ASF/SF2 with this splice site utilised to produce late mRNAs encoding L1 and L2 (Li, Cardoso Palacios, et al. 2013).

iv) Retinoic Acid

Retinoic acid (RA) is a vitamin A (retinol) derivative that mediates the functions of vitamin A required for growth and development. It exists as several isomers *in vivo* including all-*trans*, 13-*cis* and 9-*cis* RA (Krysta et al. 2013). Retinoic acid is essential for the regulation of epithelial cell differentiation and has been proven to be an inhibitor of carcinogenesis. RA acts by blocking the promotion of initiated or transformed cells through three mechanisms: induction of apoptosis, the arrest of further growth of abnormal cells and induction of abnormal cells to differentiate back to normal (Siddikuzzaman et al. 2011). Retinoids are effective chemopreventive agents against certain types of cancer including skin, head and neck, breast and liver (Hansen et al. 2000). Research has demonstrated that retinoic acid can induce differentiation and terminal cell division of leukemic promyelocytes and be utilised as a therapeutic agent in acute promyelocytic leukaemia (Koeffler 1983). RA has also been shown to decrease expression of the HPV proteins E6 and E7, subsequently restoring the tumour-suppressive roles of pRB and p53 proteins. Therefore, retinoids may have a potential therapeutic role in the management of CIN (Pirisi et al. 1992). Treatment with RA was also revealed to down-regulate the expression of hnRNP A2/B1 (Liang et al. 2011), an RNA binding protein which inhibits the HPV-16 splice site SD3632. Significantly, knock-down of hnRNP A2/B1 has been proven to induce HPV-16 late gene expression (Li, Johansson, et al. 2013). Furthermore, RA also plays a role in the immune response, with studies showing that RA promotes the proliferation and activation of NKT cells indirectly *in vitro* by increasing CD1d expression in APCs (Chen & Ross 2015). As well as this, RA can differentially modulate the production of effector cytokines by NKT cells in hepatitis, signifying a potential role for RA as a therapeutic drug in the protection against liver damage by various agents (Lee et al. 2012). The intracellular effects of RA are mediated through two classes of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RxRs). Both receptor classes contain three

subtypes, namely α , β and γ , each of which are encoded by a distinct gene (Chambon 1994). The three RAR types have a strong affinity for all-*trans* and 9-*cis* isomers of retinoic acid while all RXR types have demonstrated strong specificity for the 9-*cis* isomers only (Myga-Nowak et al. 2011). RARs and RXRs are ligand-dependent transcription factors and function primarily as RXR-RAR heterodimers. After ligand binding, activated receptors bind to *cis*-acting DNA sequences called retinoic acid response elements (RAREs) located in the promoter regions of target genes and induce transcription (Xu et al. 1999). In the absence of ligand, RARs have the potential to repress transcription (Wolffe 1997). Interestingly, the level of RAR expression may vary during the development of cancer. Alterations in the *RAR α* and *RAR β* genes and their expression is linked to several diseases including acute promyelocytic leukaemia and hepatocellular carcinoma (Sano et al. 2003; Ferrucci et al. 1997). *RAR β* in particular has been demonstrated to play a significant role in mediating the anticancer effect of retinoids in numerous cancer cells. Expression of *RAR β* is strongly up-regulated by RA treatment, through a RARE (β RARE) present in its promoter, which is activated by RAR-RXR heterodimers (de Thé et al. 1990; Valcárcel et al. 1994). Additionally, alterations in *RAR β* gene expression can give rise to unusually low mRNA levels and loss of ligand inducibility, features identified in several human cancers and tumour derived cell lines, including cervical cancer (Geisen et al. 2000). One particular study has demonstrated that in normal cervical cells, basal *RAR β* mRNA levels are high and can be induced further by RA treatment while conversely, in the cervical carcinoma cells, the basal *RAR β* mRNA levels are low and not inducible or only slightly inducible by RA. Furthermore, the same study also shows that the RA-dependent increase of *RAR β* mRNA levels is mediated by *RAR α* (Geisen et al. 1997). The potential influence of each small molecule drug on late gene expression is summarised in Figure 1.19.

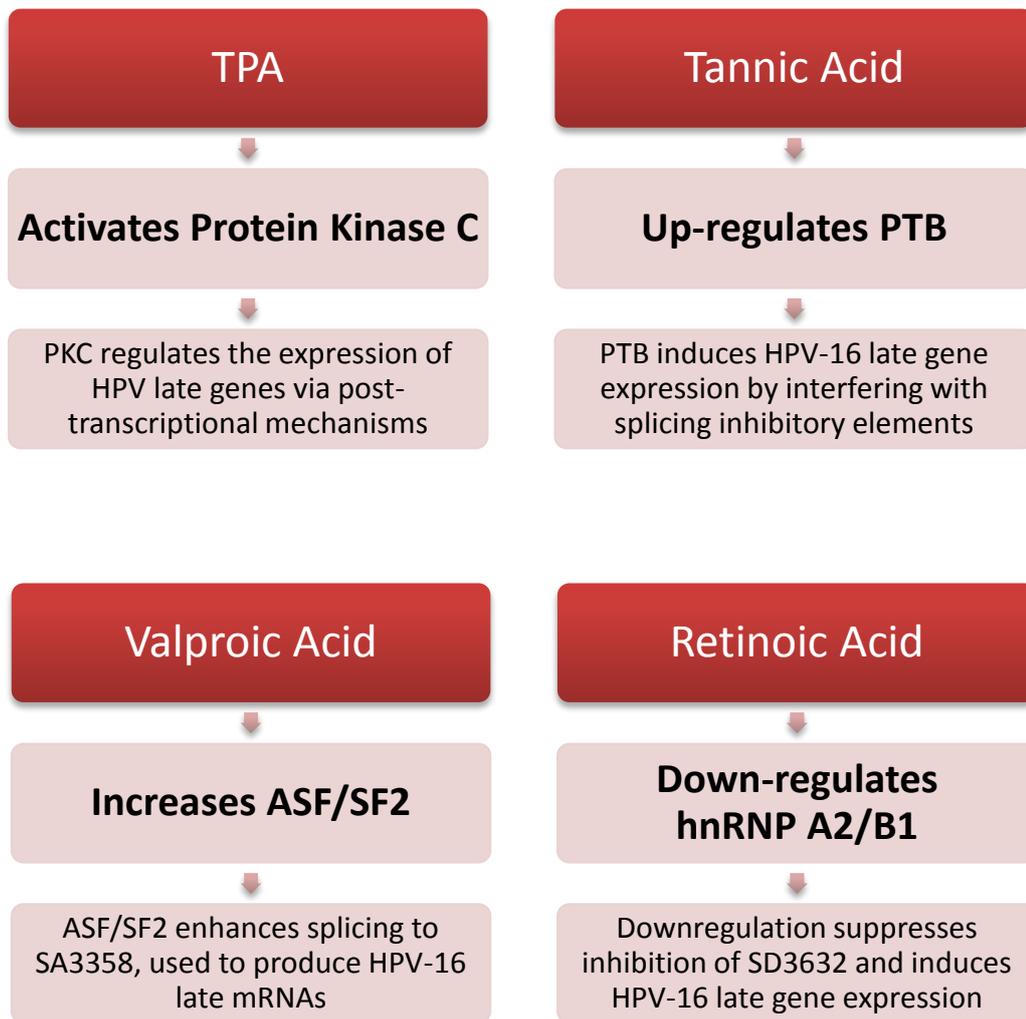


Figure 1.19 The Influence of Small Molecule Drugs on Factors Associated with Late Gene Expression

1.20 The Immune System

The immune system has a pivotal function in the response to HPV infection, as indicated by the effects induced by the highly immunogenic HPV late genes L1 and L2. The immune system, a complex system comprising of a network of different organs, tissues, cells and proteins, works to protect and defend the human body from harmful influences (Figure 1.20). The components of the immune system are connected via the blood and lymphatic circulatory systems with organs such as the bone marrow, thymus, spleen, lymph nodes and mucosa-associated lymphoid tissues occupied in the manufacturing, maturation, differentiation, proliferation and storage of immune cells (Elsabahy & Wooley 2013). Through an intricate series of steps known as the immune response, the immune system has the ability to attack invading pathogens which may have the potential to cause disease. When working efficiently, the immune system can differentiate self from non-self; recognising a variety of threats such as viruses, bacteria and parasites and distinguishing them from the healthy tissue of the body. The substances which activate the immune system are referred to as antigens. These antigens are macromolecules that elicit an immune response in the body. Antigens can be proteins, polysaccharides or lipids and when recognised by special receptors on the defence cells, stimulate an immune reaction. The defence against invading antigens is divided into two general types of immune reactions, namely the reactions of innate immunity and reactions of adaptive immunity. Although they vary in terms of response times and specificity, both aspects are equally important (Janeway et al. 2001).

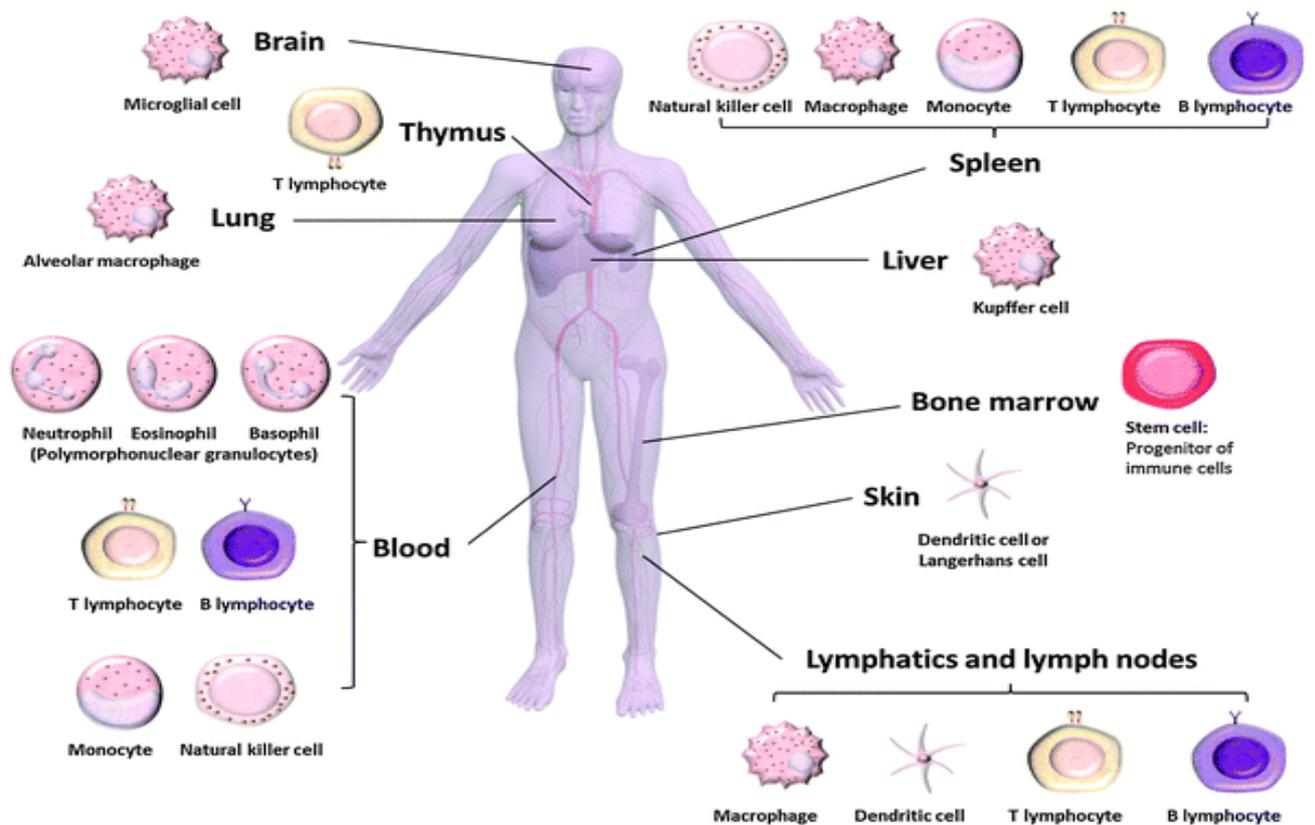


Figure 1.20 The Immune System

The human body displaying the organs of the immune system and the distribution of the various immune cells (Elsabagy & Wooley 2013).

1.21 Innate Immune System

The innate immune system is an evolutionary system that is constantly present and ever ready to provide a general defence against invading pathogens. This system does not require antigen specificity and has the potential to induce a generic immune response against a variety of organisms. However, it is lacking the ability to demonstrate immunological memory and does not confer long-lasting protection to the host. The main components of this nonspecific system include physical epithelial barriers, phagocytic leukocytes, dendritic cells (DC), natural killer (NK) cells and circulating plasma proteins (Clark & Kupper 2005). Research has established that the innate immune response plays an essential role in the clearing of HPV, acting as the first line of defence against infection. Innate immune cells including Langerhans cells (the

dendritic cells of the skin and mucosa), NK cells, natural killer T (NKT) cells and keratinocytes are all crucial for the promotion of an efficient adaptive immune response against HPV. These cells have the capacity to stimulate a cytokine-mediated pro-inflammatory process, therefore linking the innate with the adaptive immune response (Amador-Molina et al. 2013). Keratinocytes, the cells primarily infected by HPV, possess the ability to secrete cytokines including transforming growth factor- β (TGF- β), tumour necrosis factor- α (TNF- α) and interferons (IFNs). TGF- β inhibits viral growth in normal cervical cells while TNF- α may have an antiproliferative effect on HPV-16 infected cells through cell cycle arrest (Mendoza et al. 2008; Scott et al. 2001). TNF- α is also shown to repress expression of E6 and E7 HPV proteins (Kyo et al. 1994), which are crucial for malignant transformation of infected cells (Hawley-Nelson et al. 1989). Furthermore, the direct elimination of HPV-infected cells can be carried out by NK cells, through either granule-dependent cytotoxicity or the apoptosis pathway in the target cell (Sutlu & Alici 2009). Significantly, HPVs possess the ability to evade the immune response, mainly through the action of the previously mentioned E6 and E7 proteins. Viral mechanisms of immune evasion include the modulation of cytokine expression, alteration of antigen presentation and down-regulation of IFN-pathways and adherence molecules (Kanodia et al. 2007). As successful infection by HPV is dependent on this immune evasion, studies have suggested that the stimulation of the innate immune response through strong adjuvants is a promising therapeutic strategy for disrupting the evasion mechanisms utilised by HPV (Amador-Molina et al. 2013).

1.22 Adaptive Immune System

In comparison to the innate immune response, adaptive immunity is involved in the implementation of a specific immune reaction and reacts only with the organism which induced its response. This highly specialised system includes aspects of both humoral immunity and cell-mediated immunity. B cells mediate the humoral immune response and on activation differentiate into antibody-secreting plasma or effector B cells (Alberts et al. 2002). Antibodies are imperative tools in the identification and neutralisation of foreign pathogens and possess the notable ability to combine with the antigen that triggered its production. Conversely, T cells regulate the cell-mediated immune response. Antigen-specific cytotoxic T-lymphocytes have the ability to induce apoptosis in cells presenting epitopes of foreign antigen on their surface (Janeway et al. 2001b). As well as this, protection by cellular immunity employs the activation of macrophages and natural killer (NK) cells, enabling them to destroy invading pathogens. It also involves stimulation of cells to secrete cytokines that influence the function of other cells and help coordinate an appropriate immune response (Dinarello 2007). Although this adaptive system needs some time to react to invading pathogens, adaptive immunity creates immunological memory after the initial response to a specific pathogen, resulting in an enhanced reaction to subsequent encounters with that same pathogen (Janeway et al. 2001c). Adaptive immunity plays a pivotal role against HPV infection. In relation to the cell-mediated adaptive immune response, there are two phases involved; the recognition of the antigen and the response to the antigen. In the recognition phase, Langerhans cells (LCs) are the major antigen presenting cells (APCs) utilised for presentation of the antigen to the naïve T cells, with studies showing that a depletion of these LC cells is associated with enhanced HPV survival, extended infection and potential malignancy (Memar et al. 1995; Matthews et al. 2003; Mendoza et al. 2008). T-helper cells (CD4+) set the cytokine milieu and therefore determine the direction of the immune response. IFN- γ and interleukin-12 (IL-12) are

required for the differentiation of the naïve lymphocyte to a Th1 response, which produces IFN- γ , IL-2, IL-10, TNF- α and lymphotoxin and leads to the activation of cell-mediated immunity. Conversely, IL-4 and IL-2 are essential for the Th2 phenotype, resulting in the production of cytokines such as IL-5, IL-10, IL-13 and IL-25, contributing to the development of humoral immune response (Steele et al. 2005; Zhu & Paul 2008). CD8⁺ cytotoxic T cells are the primary agents in eliciting antigen-specific immunity and recognise the antigens with the assistance of MHC class I. Cell-mediated immunity plays a critical role in the clearance of the HPV lesion with the majority of T-cell activation caused by HPV E6 and E7 proteins (Deligeoroglou et al. 2013). The humoral immune response is mediated by B cells, which neutralise and opsonise viral agents. Stimulation of this response is caused by APCs and the Th2 cytokine pattern as previously mentioned. The antibodies against HPV target mainly the L1 capsid protein although weak antibodies directed against E2, E6, E7, and L2 have also been detected (Deligeoroglou et al. 2013). However, antibodies can only attach to HPV that are free in the body and not those that have already infected the cell (Stanley 2006). As previously discussed, HPV vaccinations have been created which elicit an antibody-mediated immune response. Interestingly, studies have discovered that upon completion of a 3-dose regimen with a quadrivalent HPV L1 virus-like particle vaccine, anti-HPV 6, 11, 16 and 18 antibody levels were 10-104-fold higher than those in natural infection (Villa et al. 2006).

Despite their differences, the innate and adaptive immune responses do not function independently of one another but complement each other in any reaction against a potentially harmful pathogen. One specific means of bridging the gap between innate and adaptive immunity is through a class of lymphocyte entitled NKT cells. Although identified as potent activators of the immune system, the role of NKT cells in HPV-infected lesions has yet to be discovered.

1.23 NKT Cells

Natural killer T cells (NKT cells) are a small subset of CD3+ lymphocytes that express surface markers characteristic of both T cells and natural killer cells. These NKT cells possess attributes of both innate and adaptive immunity and can therefore mediate efficient and amplified immune responses (Berzofsky & Terabe 2008) (Figure 1.21). Furthermore, NKT cells have both immune enhancing and immunosuppressive roles in the body (Robertson et al. 2014).

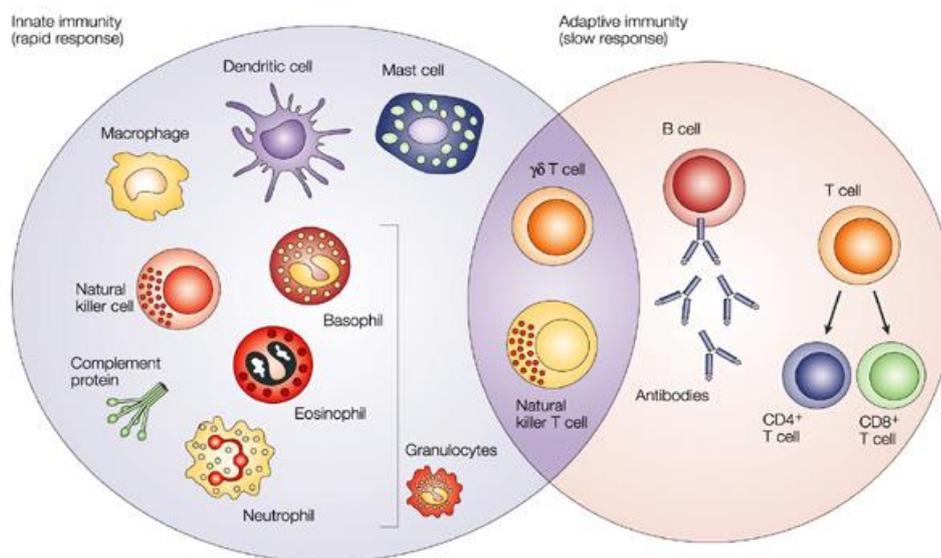


Figure 1.21 Natural Killer T Cells Span the Interface of Innate and Adaptive Immunity (Dranoff 2004).

In a similar fashion to innate immune cells, NKT cells are rapid responders when the immune system is activated, and help recruit other cells into action. NKT cells activate transcription of cytokine genes during early development in the thymus, providing the ability to produce cytokines promptly on activation (Stetson et al. 2003). Upon antigenic stimulation, NKT cells have the potential to produce an array of cytokines including IFN- γ , IL-2, IL-4, IL-10, IL-13, IL-17, IL-21 and IL-22, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF- α (Parekh et al. 2013). The nature and class of the antigen-specific T cell response that ensues is determined by the cytokines produced (Terabe & Berzofsky 2008). Remarkably, NKT

cells can simultaneously secrete Th1/pro-inflammatory and Th2/anti-inflammatory cytokines (Figure 1.22). Within 2-4 hours of initial stimulation, NKT cells secrete copious amounts of IL-4 and IL-13, promoting T helper 2 (Th2) immunity and IFN- γ , promoting T helper 1 (Th1) immunity. This in turn facilitates *in vivo* priming of antigen-specific immunity and stimulates downstream activation of dendritic cells, NK cells, B cells, and conventional T cells (Carnaud et al. 1999; Galli et al. 2003). As these cytokines determine which immune cells are activated, NKT cells are imperative for guiding the adaptive immune system in the desired direction. With regard to the effector functions of NKT cells, research has shown that these cells can mediate both protective and regulatory immunologic functions, such as anti-tumour responses, protection against pathogens, maintenance of transplant tolerance and inhibition of autoimmunity (Taniguchi et al. 2003). Loss of NKT cells has detrimental consequences on the immune response, with studies suggesting that the deficiency of CD1d-restricted NKT cells in HIV-1 infected patients could have numerous pathologic effects, including impaired tumour immunity and compromised immune responses against opportunistic infections (Moll et al. 2006). Additionally, research utilising CD1d gene knockout (GKO) mice, which lack CD1d-restricted NKT cells, indicate that these cells play an important role in immunity to herpesvirus. GKO mice displayed amplified morbidity, enhanced spread of the virus in the nervous system and significantly diminished clearance of virus from the skin and nervous system (Grubor-Bauk et al. 2003). NKT cells can be grouped into several subsets but the most commonly described group is the type 1, CD1d-restricted, invariant NKT (iNKT) subset. Although iNKT cells constitute only approximately 0.1% of all peripheral blood T cells in humans, they are proven to have a significant impact in many disease settings far beyond their strength in number (Terabe & Berzofsky 2008).

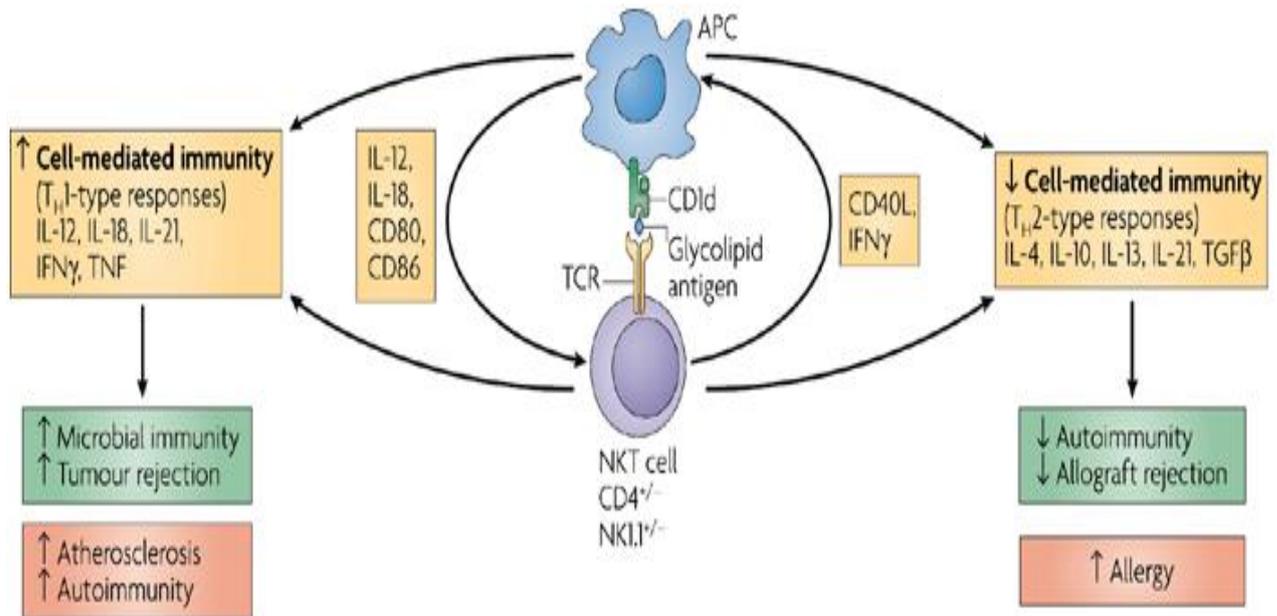


Figure 1.22 iNKT Cytokine Production

A series of events ensues once NKT cells recognise a glycolipid antigen presented by CD1d, including the production of cytokines and co-stimulatory molecules resulting in the activation of antigen-presenting cells as well as NK, T and B cells. The release of these cytokines induces a broad range of diverse effects, ranging from enhanced cell-mediated immunity (Th1-type responses) to suppressed cell-mediated immunity (Th2-type responses). Shown in green are the responses beneficial to the host, while the actions indicated in red demonstrate the detrimental effects (Godfrey & Berzins 2007).

1.24 Invariant Natural Killer T Cells

NKT cells are similar to NK cells in that they share expression of certain cell surface antigens, such as the NK-associated receptor, CD161, both in humans (NKR-P1A) and in mice (NK1.1) (Godfrey & Kronenberg 2004; Tarazona et al. 2003). However NKT cells also express a TCR which is absent on NK cells, therefore establishing NKT cells as specialised T cells.

The T cell receptor (TCR) is a molecule found on the surface of T lymphocytes and is responsible for recognising antigens bound to major histocompatibility complex (MHC) molecules on antigen presenting cells. The TCR repertoire of human NKT cells consists of an invariant V α 24-J α 18 alpha chain together with a diverse V β 11 beta chain, giving rise to the name invariant NKT cells (iNKT cells). This expression of V α 24-J α 18 in humans is a unique

iNKT cell signature (Fujii et al. 2013). It is standard practice that iNKT cells be identified via flow cytometry, simply by co-expression of TCR-V α 24 and TCR-V β 11 or through the use of the monoclonal antibody (mAb) 6B11, which has the ability to recognise the invariant CDR3 loop of the TCR α chain (Metelitsa 2004). Like true T cells, the antigen-specific TCR on iNKT cells allows them to recognise both self and foreign antigens. However their TCR does not interact with peptide antigen presented by classical MHC molecules, but instead provides the immune system with a unique mechanism for identifying lipid and glycolipid antigens which go undetected by conventional T cells. These lipids and glycolipids are presented to iNKT cells by a nonclassical MHC-like antigen presenting molecule called CD1d, as shown in Figure 1.23. iNKT cells are CD1d-restricted (Matsuda et al. 2008).

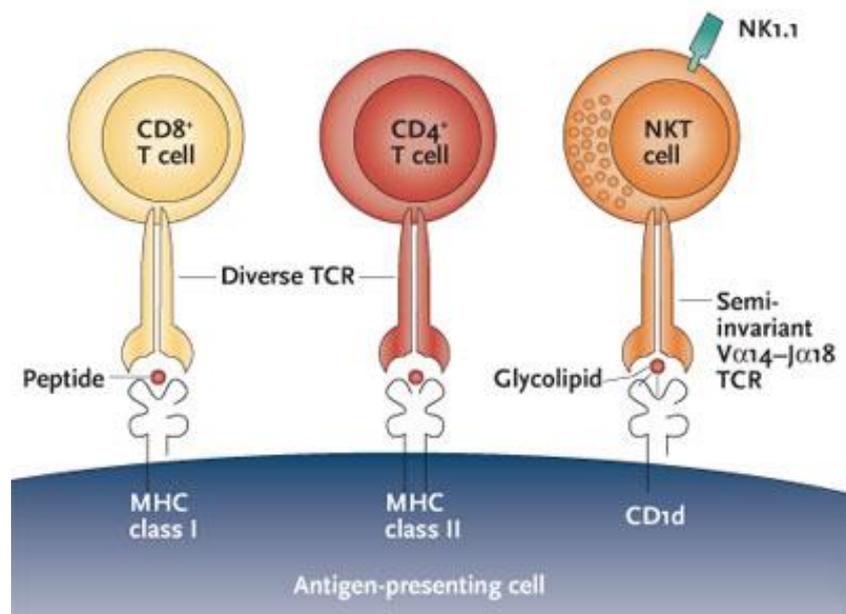


Figure 1.23 T Cell and NKT Cell Antigen Recognition

In comparison to the CD8⁺ and CD4⁺ T cells, which are shown to display diverse TCRs recognising peptide antigens, the NKT cells display an invariant TCR which is employed to recognise glycolipid antigens (Van Kaer 2005).

1.25 CD1d

CD1d is an MHC-like molecule, utilised to present glycolipid and lipid molecules to iNKT cells. It is detected on several cells of the body, including the majority of thymocytes, peripheral lymphocytes, epithelial cells in the gastrointestinal tract and hepatocytes (Bleicher et al. 1990). CD1d is not only important for antigen presentation but also plays a pivotal role in the development of iNKT cells, with studies discovering that mice lacking CD1d are severely depleted in their NKT cell pools (Chen et al. 1997). Similar in structure to MHC class I, the CD1 glycoprotein consists of a 45 kDa heavy chain noncovalently associated with beta-2-microglobulin (β 2m), forming a heterodimer that is expressed on the cell surface of the antigen-presenting cell (APC) (Chen et al. 1997). Newly assembled CD1d- β 2m complexes contain endoplasmic reticulum (ER)-derived endogenous lipid antigen, which upon arrival in the endosomal pathway is exchanged for antigenic lipids (Subleski et al. 2011). These endogenous lipids are believed to stabilise the CD1/ β 2m complexes until exchange for antigenic lipids is achieved (De Silva et al. 2002). Initial self-lipid binding by CD1d molecules occurs in the ER and is mediated by the ER-resident lipid transfer protein microsomal triglyceride transfer protein (MTP). During assembly in the ER, MTP lipidates CD1d in a step that is critical for CD1d to present both endogenous (ER-loaded) and exogenous (endosomal or surface-loaded) antigens to CD1d-restricted NKT cells (Kaser et al. 2008). On stimulation, CD1d molecules are loaded with antigenic lipid antigen in late endosomes/lysosomes. However, as a result of their bio-physical properties, lipid antigens require a number of mechanisms to control their uptake into cells, extraction from membranes and eventual loading onto CD1d (Salio et al. 2010). Some complex lipids also require processing by resident lysosomal lipases and glycosidases, such as α -Galactosidase A, mannosidase and hexosaminidase, which are shown to be involved in the trimming of glycolipid antigens (Zhou et al. 2004; Prigozy et al. 2001). Furthermore, lipids have a tendency to insert into the lysosomal

membrane bilayer and therefore require assistance from helper molecules to facilitate lipid extraction from the membranes and loading onto CD1d. Sphingolipid activator proteins (SAPs) such as saposins, the GM2-activator protein and the Niemann-Pick C2 protein (NPC2) are crucial for the loading of lipid antigen onto CD1d molecules (Kang & Cresswell 2004; Zhou 2004). After binding, the antigenic lipid-CD1d- β 2m complexes relocate to the cell surface and present to iNKT cells (Sillé et al. 2009).

1.26 α -GalCer

The best characterized CD1d ligand is the synthetic glycolipid α -Galactosyl Ceramide (α -GalCer), a compound originally derived from marine sponge (Hayakawa et al. 2003). α -GalCer is deemed the most efficient antigen for the activation of the majority of iNKT cells (Godfrey & Kronenberg 2004). It functions by firstly binding to the CD1d molecule on antigen presenting cells and subsequently combining with the TCR of the iNKT cell. This in turn induces activation of both iNKT cells and various other immune-competent cells as well as the rapid production of regulatory and proinflammatory cytokines (Figure 1.24), therefore bestowing α -GalCer with therapeutic potential. Research has been performed utilising synthetic α -GalCer, or its variants, in mouse models to prevent tumour metastases, to reduce autoimmunity in experimental autoimmune encephalomyelitis (EAE) and diabetes models and to enhance the responses to viral and parasitic infections (Godfrey & Kronenberg 2004). α -GalCer has also been investigated in numerous phase I cancer clinical trials with the aim of elucidating whether the antitumour effects of iNKT cells obtained in mice are also observed in humans (Giaccone et al. 2002; Ishikawa et al. 2005; Chang et al. 2005). As well as being the first antigen discovered that could bind to the CD1d molecule and activate NKT cells, (Kawano 1997) α -GalCer was also the first antigen used to load CD1d tetramers for the detection of NKT cells (Sidobre & Kronenberg 2002). While α -GalCer is one molecule that has been determined to strongly stimulate NKT cells, endogenous antigens can also stimulate NKT cells, however their

activation capacity is lower than that of α -GalCer (Mattner et al. 2005). Although HPV does not have NKT-stimulating glycolipids, HPV infection may have potential to modify the profile of endogenous glycolipids, which can in turn be presented to and activate iNKT cells (Amador-Molina et al. 2013). Since HPV is a local infection, understanding the contribution of iNKT cells in infected cervical tissue is essential to identify the factors involved in HPV clearance.

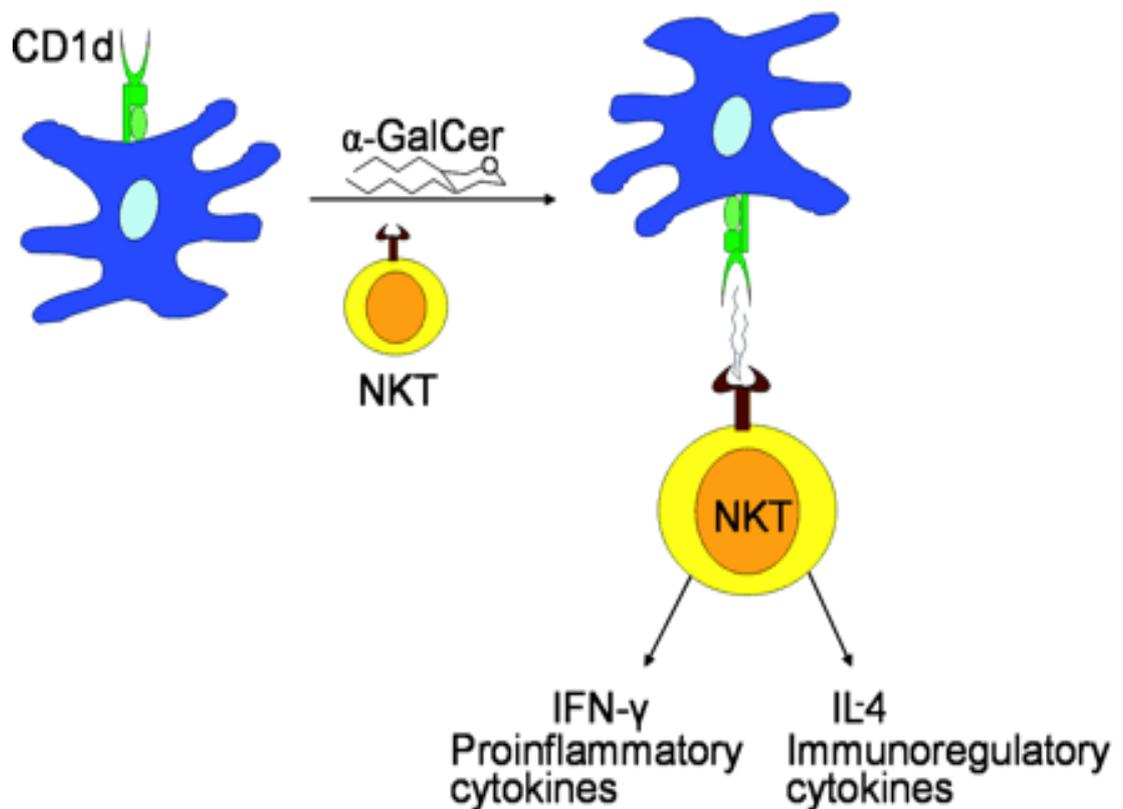


Figure 1.24 NKT Cells Reactive to CD1d-Bound α -GalCer

Stimulation of NKT cells by CD1d-bound α -GalCer results in the production of copious amounts of cytokines such as IL-4 and IFN- γ (Wu et al. 2005).

1.27 iNKT Subsets - Cytokine Production

In the same way as conventional T cells develop, iNKTs expand in the thymus from CD4⁺ CD8⁺ thymocytes. Expression of the iNKT TCR is selected by reactivity with CD1d-presented endogenous lipid, which directs cells to the iNKT lineage (Juno et al. 2012). In both mice and humans, iNKT cells can be CD4⁺CD8⁻ or CD4⁻CD8⁻ double negative (DN), while humans possess an additional CD4⁻CD8⁺ subset (Gumperz et al. 2002). In humans, this broad classification of CD4⁺ and CD4⁻ subsets provides an important functional distinction, due to the fact that CD4⁺ NKT cells make both Th1 and Th2 cytokines (such as IFN- γ , TNF, IL-4, IL-10, IL-13), whereas CD4⁻ NKT cells primarily make Th1 cytokines (IFN- γ and TNF) (Lee 2002; Godfrey & Kronenberg 2004). Studies performed have identified these subsets of iNKT cells and determined their unique cytokine production and cytotoxicity capabilities. Both CD4⁺CD8⁻ and CD4⁻CD8⁻ subsets produce high levels of IL-4 and IFN- γ , in comparison to CD8⁺ iNKT cells (Hammond et al. 1999). Additional research utilising CD1d tetramers has confirmed that CD4⁻ iNKT cells have the potential to selectively produce the Th1 cytokines IFN- γ and TNF- α and up-regulate perforin after exposure to IL-2 or IL-12. In comparison, CD4⁺ iNKT cells can produce both Th1 and Th2 cytokines as well as up-regulate perforin as a result of stimulation with phorbol myristate acetate and ionomycin, but not IL-2 or IL-12. Antigenic stimulation of these iNKT cells resulted in cytokine production while exposure to inflammatory factors enhanced perforin expression (Gumperz et al. 2002; Tarazona et al. 2003). iNKT cells have been implicated as immunosuppressive cells in certain systems, primarily through their production of the Th2-type cytokines or IL-10, while in other systems, they appear to promote enhanced cell-mediated immunity via production of Th1-type cytokines (Wilson & Delovitch 2003; Godfrey & Kronenberg 2004).

1.28 Detection of iNKT Cells

Further to the use of TCR-V α 24, TCR-V β 11 and 6B11 antibodies, previously mentioned as standard mechanisms for the detection of iNKT cells by flow cytometry, the recent development of CD1d- α -GalCer tetramers has made it possible to uniquely and explicitly characterise iNKT cells, again through flow cytometry (Gumperz et al. 2002; Sidobre & Kronenberg 2002). The tetramer combines four CD1d molecules and a fluorescent label, which is recognised by the TCR of the iNKT cell. As well as this, they are loaded with α -GalCer to improve staining efficiency as shown in Figure 1.25.

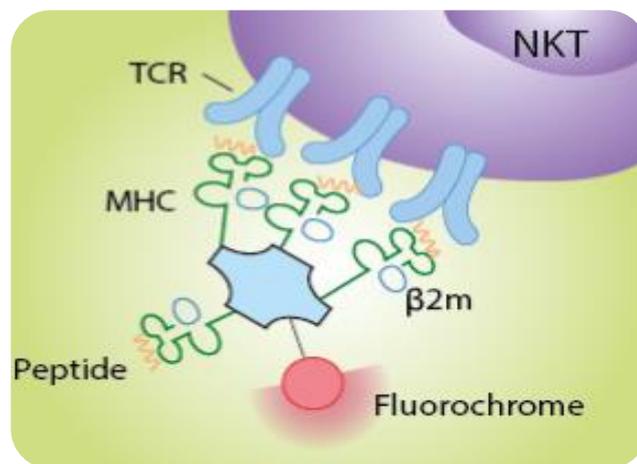


Figure 1.25 CD1d Tetramers

CD1d tetramers consist of four CD1d molecules joined together and loaded with α -GalCer. They are fluorescently labelled and useful for NKT cell identification (MBL International 2014).

The invention of these tetramers has greatly facilitated the identification of iNKT cells, although in humans, the tetramer must be teamed with an additional antibody to provide a diagnostic service for type I NKT cells. This is due to the fact that some type II NKT cells, despite lacking the invariant TCR, may still respond to α -GalCer. Therefore the combination of the CD1d tetramer with anti-V α 24 would provide a more definitive means of identification (Terabe &

Berzofsky 2008). Despite the success of iNKT cell identification in cell suspensions, there is currently no available method for the detection of iNKT cells in tissue sections, a technique which would be an invaluable tool for further research into this cell population. An interesting possibility is the potential of the 6B11 flow cytometry approved antibody (obtained from eBioscience) for iNKT identification in tissue sections through immunohistochemical analysis.

1.29 iNKT Cell Distribution

Type I NKT cells are detected where conventional lymphocytes normally reside, although the proportion of iNKT cells alter in a tissue-specific manner. The distribution of iNKT cells in human tissue is under evaluation, however it is particularly difficult owing to their low frequency (0.01%-1%) amongst peripheral blood T cells, as well as the lack of a single, defining cell-surface marker specific for iNKT cells (Godfrey et al. 2000). Extensive analysis has however been performed in mouse models. While the distribution of iNKT cells is similar between humans and mice, the frequency varies significantly, with far fewer cells present in the human body. Although usually found only at trace levels in many organs, these iNKT cells are enriched in the liver, accounting for 30–50% of hepatic T cells in mouse liver lymphocytes. This is in comparison to approximately 0.5% of the total T cell population in the human liver, the highest concentration of iNKT cells found in the human body (Kenna et al. 2003). Other locations where iNKT cells are most frequently distributed, as a percentage of mature T cells in mice, include the bone marrow (20–30%) and thymus (10–20% of mature T cells, but only 0.3–0.5% of total thymocytes), spleen (3%), lymph node (0.3%), blood (4%) and lung (7%) (Godfrey et al. 2000; Tarazona et al. 2003). As well as this, it has also been proven that iNKT cells are highly enriched in human and murine adipose tissue. Studies performed on TCR expression by human omental tissue have shown that frequencies as high as 50% express the V α 24J α 18 TCR chain found on iNKT cells. This high iNKT frequency was established by flow cytometry using the 6B11 mAb and confirmed in some samples by detecting co-expression of

the V α 24 and V β 11 chains and using the α -GalCer-loaded CD1d tetramer (Lynch et al. 2009). Research has also shown that the proportion of iNKT cells in the human intestine is <0.4% of all T cells and that they are located mainly in the lamina propria. Although the population is low, this study provides evidence for a role for iNKT cells and CD1d expression in intestinal mucosal immunity and inflammation (Wingender & Kronenberg 2008). Significantly, studies have also demonstrated the presence of innate cellular defences including NKT cells in the epithelial layers of the lower female genital tract (Kaul & Hirbod 2010), while further investigations identify iNKT cells as crucial regulatory cells in cutaneous tissue, a role which is dependent on CD1d recognition and IFN- γ production (Matarollo et al. 2009). Additionally, the population of circulating V α 24⁺ V β 11⁺ NKT cells also varies with certain types of tumours, with the number of circulating V α 24⁺ NKT cells significantly decreased in patients with colon cancer, head and neck cancer, breast cancer, renal cell cancer and melanoma (Tan et al. 2010). To help understand the contribution of iNKT cells in infected cervical tissue and cervical carcinoma, further characterisation of iNKT cells presence in genital mucosa and in particular cervical epithelium is required.

1.30 Role of iNKT Cells in Tumour Immunity

iNKT cells induce an adjuvant effect on antitumour immunity by activating other antitumour cytolytic cells primarily through the Th1 cytokine cascades (Tan et al. 2010). Investigations imply that NKT cells may not necessarily kill the tumour cells directly, but instead may recruit and promote a response by downstream effectors in an IFN γ -dependent manner. NK cell and cytotoxic T lymphocyte activity is enhanced through α -GalCer-stimulated NKT cells, with both effector cell types playing a role in the antitumour response (Smyth et al. 2002). However, other research performed has suggested that human iNKT cells also possess their own cytotoxic characteristics, which may be used to stimulate direct lysis of various tumour cells lines. Numerous cytotoxic molecules such as perforin, granzyme, Fas ligand and tumour necrosis

factor-related apoptosis inducing ligand (TRAIL) are expressed by iNKT cells, all of which have the potential to activate cytotoxicity (Smyth 2002; Kawano et al. 1999). Additionally, studies have shown that long-term administration of soluble α -GalCer, spanning the time of tumour initiation, has the potential to inhibit primary tumour formation in three different mouse models (Hayakawa et al. 2003). Further examinations have also been carried out wherein the administration of exogenous IL-12 (Cui 1997) or α -GalCer (Kawano 1997) *in vivo* strongly implicate a vital role for NKT cells in tumour immunity, with the antitumour activity mediated by IL-12 resembling that of α -GalCer. It has also been shown that IL-12 induces production of perforin in these cells (Kawamura et al. 1998). Additional work investigating the role of iNKT cells in protection from spontaneous tumours initiated by the chemical carcinogen methylcolanthrene (MCA) was also performed. Impaired protection in V α 14 deficient mice indicated that NKT cells are crucial in natural immune responses to certain spontaneous tumours, the first finding of NKT antitumour activity in the absence of exogenously administered IL-12 or α -GalCer (Smyth et al. 2000). In other murine studies, NKT cells have again been shown to play a critical role in the cytotoxicity against tumour cell lines. Expanded CD8⁺ NKT cells, which are CD1d independent and produce Th1 cytokines such as IFN- γ and TNF α have been demonstrated to display potent *in vitro* cytotoxicity and provide lifesaving protection from an otherwise fatal tumour challenge (Baker 2001). Conversely, it has been indicated that CD1d dependent CD4⁺ NKT cells, together with IL-13, possibly produced by the NKT cells, have the ability to down-regulate tumour immunosurveillance leading to tumour progression (Terabe et al. 2000). Therefore, as it remains uncertain whether iNKT cells always mediate antitumour immunity, further evaluation is required to elucidate the immune contributions to the outcomes of various tumour types. Significantly, the potency of the anti-tumour effect mediated by NKT cells may also be impacted by the type, complexity and composition of the tumour microenvironment, with the presence of cell surface proteins,

suppressor cells, infiltrating blood vessels and varying cytokine patterns all influencing the response (Schiavoni et al. 2013).

1.31 Role of iNKT cells in HPV Infection

Studies performed on immunodeficient or immunocompetent individuals have indicated that the immune system has a critical role in the success or failure of spontaneous clearance of HPV (Monnier-Benoit et al. 2006). Although the function and distribution of iNKT cells in HPV-infected lesions has not yet been fully investigated, it has been discovered that the expression of CD1d, the MHC-like glycoprotein that presents lipid antigen to iNKT cells, is significantly suppressed in HPV-infected tissue (Miura et al. 2010). This reduced expression of CD1d may contribute to viral immune evasion, by preventing iNKT cell activation. CD1d has been demonstrated to be down-regulated in HPV-positive cells both *in vivo* and *in vitro* (Miura et al. 2010). Research has established that CD1d immunoreactivity is strong in HPV-negative normal cervical epithelium but absent in the presence of both high-risk and low-risk HPV types, namely HPV16-positive CIN1 and HPV6-positive condyloma lesions. The hypothesis for the decreased cell surface expression of CD1d in HPV-infected cells is the inhibition of calnexin folding capabilities by HPV E5 (Miura et al. 2010). This CD1d down-regulation allows the infecting virus to evade the protective immunological surveillance of the host and establish persistent infection at the primary transmission site. Therefore, despite the unclear role of NKT cells in HPV infections, reduced CD1d expression provides important indirect evidence of immune evasion mechanisms developed by HPV to avoid the protective role of iNKT cells during early stages of infection. From this, it can be speculated that the diminished levels of CD1d may have a negative impact on the population of iNKT cells in HPV-infected cervical tissue. However, at present, there is no available method for the detection of iNKT cells in tissue samples to investigate this hypothesis further. Should a novel method be established it would prove an invaluable tool for future iNKT cell exploration.

1.32 Flow Cytometry

At present, the only means of investigating iNKT cell distribution in the body is through flow cytometry. Flow cytometry is a useful tool which provides rapid analysis of multiple characteristics of single cells. To perform flow cytometric analysis, prepared single cell or particle suspensions are required. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA while antibodies may also be conjugated to fluorescent dyes which can bind to specific proteins on the cells. Fluorescein isothiocyanate (FITC), Texas Red and phycoerythrin (PE) are the most common fluorescent dyes used for flow cytometry (Titus et al. 1982). The cell suspension is aspirated into a flow cell, where they are surrounded by a fluid stream and move individually through a focused laser beam. When labelled cells are passed through this light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths. Emitted light is scattered in all directions and collected via optics that direct the light to a series of filters and dichroic mirrors that isolate particular wavelength bands (Brown & Wittwer 2000). The light signals are detected, with data stored, analysed and displayed through an associated computer system. Electronic gating permits separation of the total cell population into individual components, allowing for specific analysis. Physical properties, such as size (represented by forward angle light scatter) and internal complexity (represented by side angle scatter) can be determined by flow cytometry and be used to identify certain cell populations (Shapiro 2005). Flow cytometry facilitates analysis of a large numbers of cells in a short period of time (>1,000/sec), providing statistically valid information about cell populations in a quick and efficient manner. However, to fully elucidate the presence and distribution of iNKT cells in tissue sections, additional methods of detection must be developed.

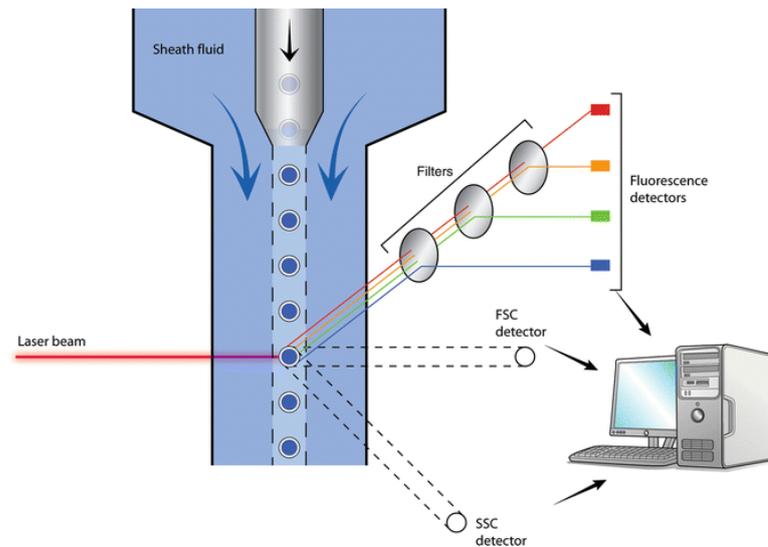


Figure 1.26 Flow Cytometric Analysis

This diagram of flow cytometry indicates focusing of the fluid sheath, the laser beam and the fluorescence, forward scatter (FSC) and side scatter (SSC) detectors. Signals detected are analysed on the associated computer system (Czader 2012) .

1.33 Immunohistochemistry

Immunohistochemistry (IHC) is performed by combining immunological and chemical reactions, with the fundamental concept behind IHC the demonstration of antigens within tissue sections by means of specific antibodies (Ramos-Vara 2005). This technique is considered a highly sensitive and specific method and is widely utilised to identify the distribution and localisation of antigens. The principle of detecting tissue antigens using a direct fluorescence protocol was first described in pioneering publications by Albert H. Coons and his colleagues (Coons et al. 1941). Following this, as a means of improving detection sensitivity, variations of the fluorescent protocol were established, such as the use of horseradish peroxidase and the avidin-biotin complex, however the original procedure has remained the same since its invention.

Current practice for IHC involves incubation of the primary antibody on tissue sections which have been pretreated to encourage optimal antibody binding. Essential pre-treatments to the

staining process include blocking steps for endogenous activities and antigen retrieval. Antigen retrieval is carried out to counteract alterations in protein biochemistry caused by the fixation process and offers numerous advantages to the staining process (Shi et al. 1991). Although essential for the preservation of tissue morphology, fixation may result in the epitope of interest becoming masked by cross linking of peptides. The antibody is therefore unable to bind and the IHC technique will be unsuccessful. To enable efficient antibody binding, two methods employed for antigen retrieval include protease-induced epitope retrieval (PIER) and heat-induced epitope retrieval (HIER). The use of enzyme in PIER allows cleavage of the peptides that are masking the epitope, however its success rate is low with potential to damage both tissue morphology and the antigen of interest. On the other hand, HIER has proved a very effective means of antigen retrieval, working by reversing some cross-links and allowing for restoration of secondary or tertiary structure of the epitope. Time, temperature, buffer and pH are all crucial factors in the successful optimisation of the HIER protocol, with citrate buffer, EDTA buffer or Tris-EDTA buffer the most widely employed for the enhancement of antibody staining (Krenacs et al. 2010). Visualising of the antibody-antigen interaction can then be accomplished by several methods including immunoperoxidase staining, in which the antibody is conjugated to a peroxidase enzyme, catalysing a colour-producing reaction or through immunofluorescent staining, in which the antibody is associated with a light emitting fluorophore, such as fluorescein, rhodamine or Texas Red.

1.34 Avidin-Biotin Complex Method

The novel invention of a new immunohistochemical method was described in 1981 with the generation of the Avidin-Biotin Complex (ABC) method (Hsu et al. 1981). The principle of this ABC method is based on the strong affinity of avidin, a large glycoprotein, for the low molecular weight vitamin biotin. Avidin contains 4 binding sites for biotin, while the biotin molecule is easily conjugated to antibodies and enzymes. There are three layers to the technique, with the first step the incubation of the tissue section with unlabelled primary antibody, followed by addition of a biotinylated secondary antibody which binds to this primary antibody. The final step in the process involves the introduction of an avidin-biotin-peroxidase complex. Binding of this complex to the biotin associated with the secondary antibody results in a high staining intensity, with a substrate such as diaminobenzidine (DAB) used to produce the colourmetric end product (Figure 1.27). The ABC method is an excellent technique for the identification of specific antigens in tissue sections.

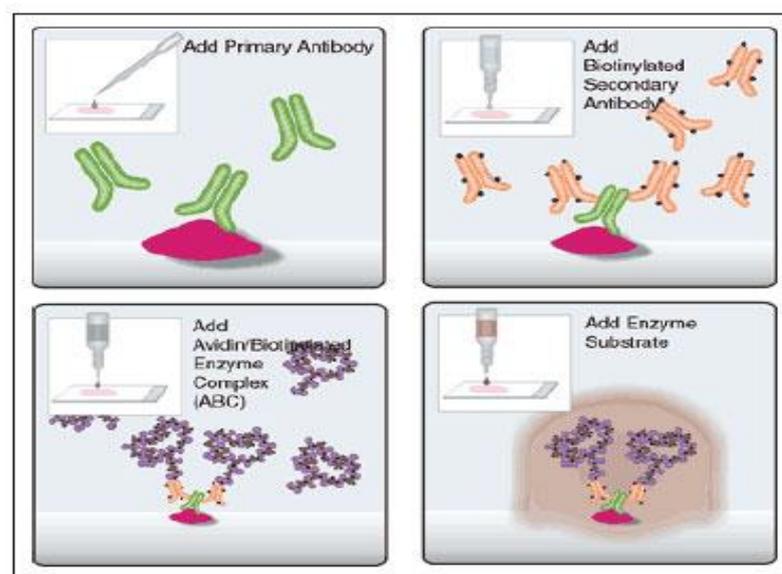


Figure 1.27 Avidin-Biotin Complex Method

Illustration of the ABC staining method, in which the secondary antibody, which is conjugated to biotin, acts as a link between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex (Meiring et al. 2011).

1.35 Tissue Microarrays

The tissue microarray (TMA) is a recent innovation in the field of pathology. These microarrays are composed of several small representative tissue samples from hundreds of different cases assembled on a single histologic slide, allowing high throughput analysis of multiple specimens at the same time. The technique was first described by Battifora in 1986, who created a method of embedding 100 or more different tissue samples in a normal-sized paraffin block called a multitumour tissue block (MTTB). He utilised a “sausage block” method in which 1mm thick ‘rods’ of different tissues were wrapped in a sheet of small intestine before embedding in a paraffin block and cutting sections for examination (Battifora 1986). The array format was then conceived by Wan and colleagues in 1987 (Wan et al. 1987). Currently employed tissue microarrays are paraffin blocks created utilising cylindrical tissue cores extracted from different paraffin donor blocks and re-embedded into a single recipient (microarray) block at specific array coordinates (Figure 1.28). Through the development of this technique, we now have the ability to analyse up to 1000 or more tissue samples from a single paraffin block. This pioneering process permits simultaneous analysis of molecular targets at DNA, mRNA and protein levels under identical, standardised conditions on a single glass slide (Jawhar 2009). Other advantages of tissue microarrays include amplification of a scarce resource, reduced assay volume, decreased time and cost and preservation of original tissue blocks. This practical tool can be utilised in various streams of biomedical research and is imperative for the identification of new diagnostic and prognostic markers and targets in human cancers.

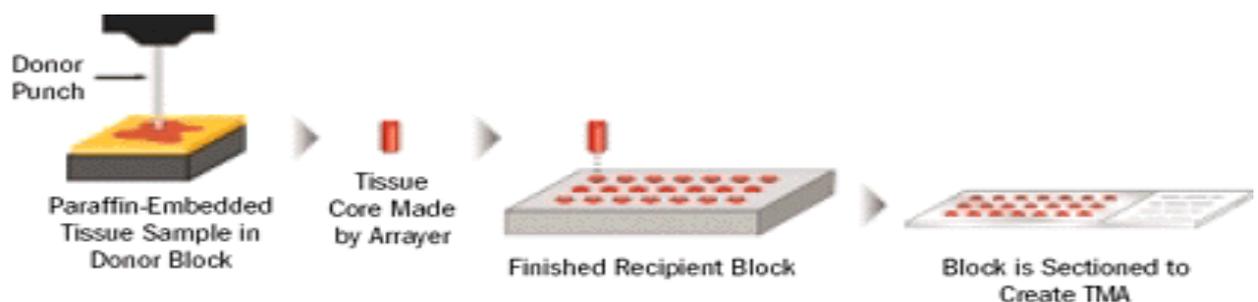


Figure 1.28 Creation of a Tissue Microarray (Schneider 2004).

1.36 Hypothesis and Aims of this Study

Human papillomaviruses are found in 99.7% of all cervical cancers, with HPV-16 alone responsible for approximately 50% of cases. Late gene expression of HPV-16 is found only in terminally differentiated epithelial cells and is completely absent in cervical cancer containing HPV-16. As the products of these late genes L1 and L2 are highly immunogenic, it has been suggested that suppression of these products may prevent detection of the virus by the immune system, leading to persistence of infection. Therefore, if expression of these proteins in the lower layers of the cervical epithelium could be induced, it may lead to clearance of the virus. The focus of this study is on the regulation of the HPV-16 L1 late gene and the identification of small molecule drugs which may up-regulate its expression. Furthermore, there is also interest in the immune response to HPV infection, in particular the role of iNKT cells. iNKT cells are proven potent activators of the immune system and have a predominantly protective function. However CD1d, which is utilised to present lipid antigen to iNKTs, is down-regulated in HPV-infected cells and may represent a mechanism to evade protective immunological surveillance by iNKT cells. As there is currently no available method to detect iNKT cells in tissue sections, both presence and function of iNKT cells in cervical epithelium is currently unknown. The goal of this research is to establish a novel method for iNKT cell detection in tissue, which would prove an invaluable tool for future research into this unique cell population.

Overarching Aim

To examine the regulation HPV-16 gene expression, with principal importance placed on late gene expression and small molecule drugs capable of modulating late gene expression. Additionally, it is proposed to generate an innovative protocol for the detection of iNKT cells in human tissue, with this method subsequently used to enumerate iNKT cells in HPV-infected cervical cancer samples.

Individual Aims

- Culture 2 stable reporter cell lines, pBELCAT67 and pBELMCAT31, in which the L1 gene has been replaced by the easily measurable CAT reporter gene. Both cell lines have previously been shown to express detectable levels of CAT, with inactivation of splicing silencers enhancing CAT expression in pBELM.
- Identify the effects of selected small molecule drugs on late gene expression and their potential to treat persistent HPV infection.
- Examine the expression of any putative small molecule drug targets that arise from the molecular analysis.
- Create a cell block utilising a pure population of iNKT cells for use as a positive control in iNKT identification methods.
- Examine the viability of various antibodies as a specific technique for the detection of these iNKT cells.
- Optimise this antibody detection method and investigate iNKT cell presence in human cervical tissue.

2. Chapter 2 - Materials and Methods

2.1 Investigation of Late Gene Regulation

2.1.1 Culture of Stable Cell Lines

Stably transfected HeLa cells lines, pBELCAT67 and pBELMCAT31, were previously created by Beatrice Orrù in Dublin Institute of Technology utilising the pBELCAT and pBELMCAT reporter plasmids (Figure 2.1). Transfection was carried out by employing GeneJuice® Transfection reagent according to the protocol of the manufacturer (Novagen) and the CAT ELISA assay was utilised to confirm successful transfection and detectable levels of CAT expression (Orrù 2012). In this study, the pBELCAT67 and pBELMCAT31 stably transfected cell lines were cultured in RPMI-1640 complete cell culture medium containing 2mmol/l L-glutamine (Lonza), 10% foetal bovine calf serum (Sigma), and 40 U penicillin/streptomycin (Sigma) in 75cm² flasks at 37°C in 5% CO₂ until fully confluent, before being trypsinised with 2% trypsin diluted in 0.02% EDTA (Sigma) (appendix) for 10 minutes at 37°C.

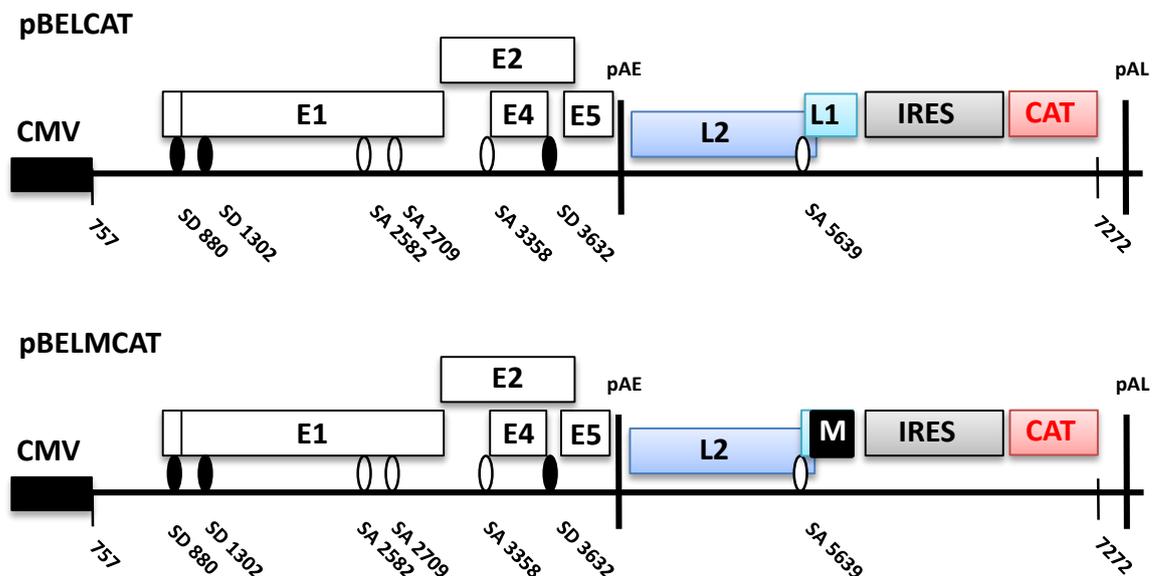


Figure 2.1 Structure of the pBELCAT and pBELMCAT Reporter Plasmids (Orrù et al. 2012).

2.1.2 Treatment of Cell Lines with Small Molecules Drugs

pBELCAT67 and pBELMCAT31 cells were plated at a density of 2×10^5 cells per individual well in 6 well plates containing 2mls of RPMI-1640 complete cell culture medium. Following incubation for 24 hours at 37°C in 5% CO_2 , the medium was replaced with 2mls of fresh complete cell culture medium together with varying concentrations of selected small molecule drugs; TPA (phorbol 12-myristate 13-acetate, Sigma), valproic acid (Sigma), tannic acid (Sigma) and retinoic acid (Sigma) (Table 2.1). For treatment with drug combinations, a control of $0.5\mu\text{l}$ H_2O was also included. The concentrations of TPA, valproic acid and tannic acid were selected based on previous research performed by Orrù on HPV-16 late gene expression, while the concentrations of retinoic acid were chosen as a result of previous work carried out employing retinoic acid for cell differentiation (Orrù 2012; Edwards & McBurney 1983).

Cells were incubated in the presence of each drug for 24 hours at 37°C in 5% CO_2 , following which the effects on CAT expression were analysed by the CAT ELISA assay.

Small Molecule Drug	Concentrations	Solubility
<i>TPA</i>	0ng/ml, 400ng/ml, 800ng/ml	DMSO
<i>Valproic Acid</i>	0mM, 0.5mM, 100mM	H_2O
<i>Tannic Acid</i>	$0\mu\text{M}$, $1\mu\text{M}$, $10\mu\text{M}$	H_2O
<i>Retinoic Acid</i>	10^{-5}M – 10^{-9}M	DMSO

Table 2.1 Concentrations and Solubility of Small Molecule Drugs

2.1.3 CAT ELISA Assay

Cells were harvested 24 hours after incubation with the selected small molecule drugs and CAT levels were determined using a CAT ELISA assay kit (Roche). The CAT ELISA is based on the sandwich ELISA principle. The first step involved the lysis of the transfected cells using 1ml of lysis buffer, after which the cell extract was centrifuged at max speed for 10 min at 4°C .

Following on from this, 200µls of the cell extract, which contains the CAT enzymes, were added to the wells of the microplate provided and incubated in the dark at 37°C in 5% CO₂ for 1 hour. These wells have been precoated with a polyclonal antibody to CAT (anti-CAT). All CAT present in the cell extracts consequently attaches to the anti-CAT antibody that is bound to the plate surface. The wells were then rinsed 5 times with a washing buffer, after which a digoxigenin-labeled antibody to CAT (anti-CAT-DIG) was added and incubated in the dark at 37°C in 5% CO₂ for 1 hour. This anti-CAT-DIG binds to any CAT enzyme present. Washing was repeated and an antibody to digoxigenin, which is conjugated to peroxidase (anti-DIG-POD), was introduced. Incubation was performed as before followed by another washing step. Finally, the peroxidase substrate ABTS was added and incubated for 20 minutes at room temperature. The peroxidase enzyme catalyses the cleavage of the substrate, yielding a coloured reaction product. Absorbance was then measured at 405nm using a 96-well microplate ELISA reader (Labsystems Multiskan Plus), with readings obtained directly correlated to the level of CAT present in the cell extracts.

2.1.4 Data Analysis and Statistics

GraphPad Prism 6 was employed for the construction of line graphs, with CAT ELISA data represented as fold-change. GraphPad Prism 6 was also used to perform statistical analysis on the CAT ELISA results, with the significance of changes in CAT expression determined utilising an unpaired, 2 tailed t-test.

2.2 Investigation of Retinoic Acid Receptors

2.2.1 Tissue Microarray Analysis

Anti-retinoic acid receptor alpha (RAR α) antibody (abcam) and anti-retinoic acid receptor beta (RAR β) antibody (abcam) were used to determine the expression of specific RARs in cervical tissue microarray (TMA) sections. These TMAs were previously created in Dublin Institute of

Technology by Ciaran Cunniffe. Each TMA contains 12 cases of cervical carcinoma, 12 cases of HSIL, 12 cases of LSIL and 4 cases of tissue showing no evidence of malignancy (NEM), giving a total of 40 cases per TMA. Each case was sampled in triplicate giving a total of 120 cores per TMA (Cunniffe 2014). The TMA also contains liver and adipose cores for orientation purposes. Conditions for use of the RAR antibodies were first optimised followed by H&E and immunoperoxidase staining of the TMA sections.

2.2.2 Creation of Cell Line Blocks

Cell blocks were created utilising pBELMCAT31, pBELCAT67 (HPV+) and C33A (HPV-) cell lines for additional RAR analysis. Each cell line was cultured in RPMI-1640 complete cell culture medium in 75cm² flasks at 37°C in 5% CO₂ until fully confluent, before being trypsinised with 2% trypsin diluted in 0.02% EDTA for 10 minutes at 37°C. Cell blocks were then created as described in Section 2.4.1, with 3x10⁶ cells utilised per block and sections were subsequently stained with H&E and anti-RAR antibodies.

2.2.3 Haematoxylin and Eosin (H&E) Staining

Sections were cut at a thickness of 5µm using a standard histology microtome (Lecia) and placed on SuperFrostPlus glass slides (Thermo Fisher) before melting in the oven for 2 hours at 60°C. Dewaxing was then carried out in baths of xylene (2), absolute alcohol (2) and spirit (1) (appendix) for 5 minutes each before rinsing in distilled water. Sections were covered in Harris haematoxylin (VWR Chemicals) for 5 minutes before being placed under running water for 5 minutes to blue the nuclei. Differentiation was then performed using an acid-alcohol solution (appendix) for 2 seconds, following which the slides were immediately washed in water for 1 minute. Sections were then placed under a 1% eosin solution for 1 minute before being washed in water for 1 minute. On completion, the sections were dehydrated by placing in spirit for 30 seconds and baths of absolute alcohol (2) and xylene (2) for 5 minutes each. The

sections were then mounted with DPX and left to dry on a flat surface before examination under the light microscope (Olympus BX51).

2.2.4 Avidin-Biotin Complex Immunoperoxidase Staining

Sections were cut as before and melted in the oven for 2 hours at 60°C. Dewaxing was carried out in baths of xylene (2), absolute alcohol (2) and spirit (1) for 5 minutes each before rinsing in distilled water. Heat induced epitope retrieval (HIER) was then performed. For HIER, 500mls of 10mmol/l citrate buffer (appendix) at pH6 was placed in a staining dish and moved to a water bath preheated to 90°C. Once the buffer reached this same temperature, dewaxed sections were immersed and incubated for 20 minutes. The staining dish was then removed to room temperature and slides were allowed to cool. The slides were rinsed in distilled water and tissue sections were surrounded with a hydrophobic barrier using a barrier pen. Blocking of endogenous peroxidases was carried out by treating sections with 3% hydrogen peroxide in methanol (appendix) for 5 minutes. Sections were then washed in phosphate buffered saline (PBS) (appendix) 3 times, before being treated with the Vectastain Elite ABC kit (Vector laboratories). Normal horse serum, diluted 1:200 in PBS, was firstly applied to the sections for 15 minutes to block non-specific staining between the primary antibodies and the tissue. Slides were then drained and approximately 250µl of specific primary antibody was applied and incubated at room temperature for 1 hour. Following 3 washes with PBS, the biotinylated secondary antibody, which was diluted 1:100 in PBS, was applied for 15 minutes. Sections were rinsed 3 times in PBS prior to treatment with the ABC reagent (diluted 1:100 in PBS) for 15 minutes and were again rinsed in PBS. Peroxidase labelling was visualised using 0.2% 2, 4-diaminobenzidine (DAB) (Sigma) (appendix) diluted in PBS and 0.03% hydrogen peroxide (Sigma). Sections were lightly counterstained by application of Mayer's haematoxylin (appendix) for 40 seconds and blued in distilled water before dehydration in spirit for 30 seconds and baths of absolute alcohol (2) and xylene (2) for 5 minutes each. Finally, sections

were coverslipped in DPX and left to dry before examination under the light microscope (Olympus BX51).

2.3 iNKT Cell Identification by Flow Cytometry

2.3.1 *Ex vivo* Expansion of iNKT Cells

iNKT cells were expanded *ex vivo* and kindly donated by the Institute of Molecular Medicine, Trinity College Dublin, St. James's Hospital, Dublin. Expansion of these cells was carried out as per O'Reilly et al. from peripheral blood mononuclear cells (PBMC), which were prepared from unselected buffy coat packs by density gradient centrifugation over Lymphoprep (Nycomed Pharma). iNKT cells were enriched from PBMC by magnetic bead separation using 6B11 coated magnetic beads (Miltenyi Biotec) and purified by sorting of CD3⁺V α 24⁺V β 11⁺ cells using a Cell Sorter (MoFlo™ XDP Cell Sorter-Beckman Coulter). The sorted iNKT cells were then expanded by culturing in iNKT cell medium (RPMI 1640 containing 0.05mM L-glutamine, 10% HyClone foetal calf serum, 1% penicillin-streptomycin, 1% fungizone 25mM HEPES, 50 μ M 2-mercaptoethanol, 1mM sodium pyruvate, 1% non-essential amino acids mixture and 1% essential amino acids mixture; Gibco and Thermo-Scientific) together with 1 μ g/ml of the stimulating agents phytohemagglutinin-P (Sigma-Aldrich) and 250U/ml IL-2 (R&D Systems) in the presence of an excess (2×10^5) of irradiated allogeneic PBMC prepared from two donors. After 24 hours and again after 48 hours, medium was replaced with fresh iNKT cell medium containing 250U/ml IL-2. Prior to experimental use, cells were expanded for a minimum of 3 weeks (O'Reilly et al. 2011).

2.3.2 Flow Cytometric Analysis of Expanded iNKT cells

Flow cytometry was performed on 4 healthy PBMC samples and 1 sample of iNKT cells expanded from a healthy patient as previously described. Selection of iNKT cells was carried out via a fluorescein isothiocyanate (FITC) labelled 6B11 antibody (BD Pharmingen) teamed with a phycoerythrin (PE) labelled CD3 antibody (BD Pharmingen), a T cell marker (Table 2.2). The cell samples were defrosted and resuspended in 10mls of cell culture medium before centrifugation at 836 x g for 8minutes to pellet cells and remove the dimethyl sulfoxide (DMSO) cryoprotectant. Cell pellets were then washed in 10mls of PBS and centrifugation was repeated. After discarding the supernatant, the PBMC cell pellets were resuspended in 2mls of cell culture medium and the iNKT cells in 5mls of cell culture medium due to their higher concentration. Cell counts were performed by creating a 1:20 dilution of the cell suspensions in Ethidium Bromide Acridine Orange (EBAO) solution and the volume required to obtain 1×10^6 cells was determined. Cell suspensions were then placed in the incubator at 37°C in 5% CO₂ for 2 hours before flow cytometry was carried out. The appropriate predetermined volume of cells required for each sample was placed in falcon tubes together with 2mls of PBA wash buffer (PBS containing 0.1% bovine serum albumin) (appendix) and vortexed before centrifugation at 604 x g for 7minutes. Supernatants were removed and the cell pellets were resuspended in 200µls of PBA per test performed i.e. PBMC sample 1 (200 µl x 4 tests), PBMC sample 2, 3, 4 and iNKT cell sample (200 µl x 1 test). A Fluorescence Minus One (FMO) control was also utilised to properly interpret the data obtained. The FMO control contains all the fluochromes in a panel, except for the one that is being measured. This allows acknowledgement of any spread of fluochromes into the unlabelled channel and permits correct gating of the cells (Table 2.3). Cells and PBA were vortexed and 200µls of each suspension was added to 10µls of the antibody required as demonstrated in Table 2.3. Tubes were vortexed and incubated in the dark at 37°C in 5% CO₂ for 15 minutes. 2mls of PBA was

then added to each tube and vortexed again, before centrifugation at 604 x g for 7 minutes. Supernatants were discarded and each pellet was resuspended in 400µls of PBA. Flow cytometry was then performed on the FACS Calibur flow cytometer (Becton Dickinson) as per analyser guidelines with data evaluated using the flow cytometry data analysis software FlowJo.

Specifications	FITC-6B11 Antibody	PE-CD3 Antibody
<i>Host</i>	Mouse	Mouse
<i>Isotype</i>	IgG1, kappa	IgG1, kappa
<i>Reactivity</i>	Human	Human
<i>Company</i>	BD Pharmingen	BD Pharmingen
<i>Reported Applications</i>	Flow Cytometric Analysis	Flow Cytometric Analysis

Table 2.2 FITC-6B11 and PE-CD3 Antibody Specifications

Tube	Sample	Sample Volume	Antibody	Antibody Volume
<i>1</i>	PBMC 1	200 µl of 1x10 ⁶ cells	Unstained	-
<i>2</i>	PBMC 1	200 µl of 1x10 ⁶ cells	FITC- 6B11 (FMO Control)	10µl
<i>3</i>	PBMC 1	200 µl of 1x10 ⁶ cells	PE-CD3 (FMO Control)	10µl
<i>4</i>	PBMC 1	200 µl of 1x10 ⁶ cells	FITC-6B11/ PE-CD3	10µl of each
<i>5</i>	PBMC 2	200 µl of 1x10 ⁶ cells	FITC-6B11/ PE-CD3	10µl of each
<i>6</i>	PBMC 3	200 µl of 1x10 ⁶ cells	FITC-6B11/ PE-CD3	10µl of each
<i>7</i>	PBMC 4	200 µl of 1x10 ⁶ cells	FITC-6B11/ PE-CD3	10µl of each
<i>8</i>	iNKT Cells	200 µl of 1x10 ⁶ cells	FITC-6B11/ PE-CD3	10µl of each

Table 2.3 Cell Samples and Antibodies Required for Flow Cytometric Analysis

2.4 iNKT Cell Identification in Tissue

The prospect of utilising 6B11 to detect iNKT cells in tissue sections was performed through the creation of iNKT cell blocks. However, Jurkat T cell blocks were first employed for use, allowing optimisation of the protocol before eventual examination of the precious iNKT cells. Additionally, frozen cell sections and cytopins were also produced. H&E staining and immunocytochemistry were then performed on all sections.

2.4.1 Cell Block Creation

i) Jurkat Cell Line

Jurkat cells were grown in RPMI-1640 cell culture medium containing 2mmol/l L-glutamine (Lonza), 10% foetal calf serum (Sigma) and 40 U penicillin/streptomycin (Sigma) in 75cm² flasks at 37°C in 5% CO₂. Once fully confluent these cells were utilised for the creation of the Jurkat cell block.

ii) iNKT Cell Line

The iNKT cells utilised for the creation of the iNKT cell block were kindly donated from Vincent O'Reilly in the Institute of Molecular Medicine, having been expanded *ex vivo* as described in section 2.3.1.

iii) Cell Block Preparation

The optimal concentration of cells required were first washed by resuspending in 5mls of PBS, followed by centrifugation at 353 x g for 7minutes (3x10⁶ Jurkat cells and 15x10⁶ iNKT cells gave an effective concentration of cells per block). The supernatant was then discarded and cells were fixed by resuspending in 5mls of formalin for 2hours. Following this, cells were pelleted by centrifugation at 836 x g for 10minutes and supernatant was removed. Cells were washed in 5mls of PBS and centrifuged again for 10minutes. To aid in the transfer of the cell

pellet to a 1.5ml Eppendorf tube, cells were resuspended in 1ml of PBS. Following the addition of the cell suspension to the Eppendorf tube, centrifugation was repeated at 425 x g for 10minutes and the supernatant was discarded. The cell pellet was then resuspended in 1ml of 2% liquid agar (UltraPure agarose from Invitrogen/ bacteriological agar from Lab M) at 65°C. Resuspension was rapidly performed as agarose may solidify in the pipette tip. The agar-cell pellet was allowed to solidify at 4°C for 1 hour. The agar cone was then carefully removed from the Eppendorf tube and divided laterally in half. The two agar pieces were placed in a Tissue-Tek cassette together with a sponge and processed overnight in an automated tissue processor (Leica) under standard conditions for surgical biopsies. The agar pieces were then embedded in a paraffin wax block (Figure 2.2).

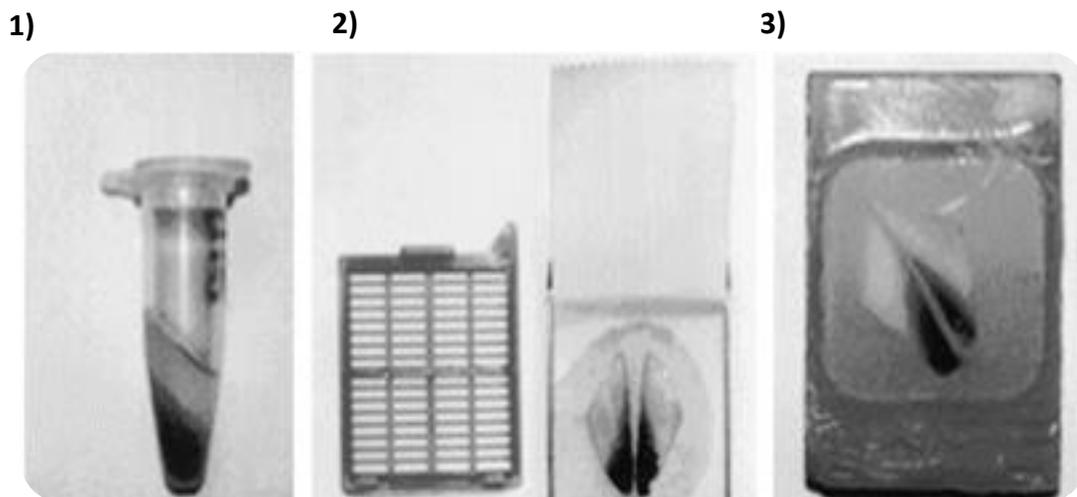


Figure 2.2 Creation of an Agar Cell Block

Images left to right show: **1)** Cell pellet solidified in agar. **2)** Agar cone divided laterally in half and placed in cassette for processing. **3)** Agar cone processed into paraffin wax block (Adapted from Kerstens et al. 2000).

2.4.2 Frozen Cell Block Creation

iNKT cells were resuspended at a concentration of 10×10^6 in Tissue-Tek O.C.T compound which provides a specimen matrix for cryostat sectioning. The suspension was then placed in a cryomould, frozen in liquid nitrogen to form a lozenge and allowed to solidify. This was then removed and mounted immediately onto a chuck before sections were cut on the cryostat and stained (Leica).

2.4.3 Cytospin Preparation

An iNKT cell suspension of approximately 0.2×10^6 cells/ml was first prepared in complete medium for cytopsin creation. Slides were then labelled and mounted with a paper pad and plastic cuvette before being placed in a metal holder. 200µls of the cell suspension was then added into each cuvette and spun at $72 \times g$ for 3 minutes in the specialised cytopsin centrifuge (Shandon). The holder was then carefully removed from the centrifuge and the slide, together with the paper and cuvette, was extracted without disarranging. The cuvette and paper were then detached without damaging the fresh cytopsin. The area around the cytocentrifuged cells was marked with a hydrophobic pen prior to fixation. Cells were fixed in precooled 1:1 acetone:methanol for 15 minutes at -20°C before staining.

2.4.4 Immunocytochemistry

i) Avidin-Biotin Complex Immunoperoxidase Staining

Cell block and frozen sections together with the cytopsin were stained using the avidin-biotin complex immunoperoxidase method as previously described in section 2.2.4. Conditions were first optimised utilising an anti-CD45 antibody (Dako) prior to staining with the 6B11 antibody (eBioscience). Immunofluorescent staining was also performed on the cell block sections.

ii) Immunofluorescent Staining

Sections were cut at a thickness of 5µm using a standard histology microtome (Lecia) and placed on SuperFrostPlus glass slides (Thermo Fisher) before melting in the oven for 2 hours at 60°C. Dewaxing was carried out in baths of xylene (2), absolute alcohol (2) and spirit (1) for 5 minutes each before rinsing in distilled water. HIER was then performed using 500mls of 10mmol/l citrate buffer at pH6, preheated to 90°C in a water bath. Dewaxed sections were immersed in the buffer and incubated for 20minutes before removal to room temperature. Slides were allowed to cool and were then rinsed in distilled water before tissue sections were surrounded with a hydrophobic barrier using a barrier pen. Blocking of endogenous peroxidases was carried out by treating sections with 3% hydrogen peroxide in methanol for 5 minutes. Sections were then washed in phosphate buffered saline (PBS) 3 times. Normal horse serum, diluted 1:200 in PBS, was firstly applied to the sections for 15 minutes. Slides were then drained and approximately 250µl of primary antibody was applied and incubated at room temperature for 1 hour. Following 3 washes with PBS, the biotinylated secondary antibody, which was diluted 1:100 in PBS, was applied for an additional 15 minutes. Sections were rinsed 3 times in PBS prior to treatment with fluorescein streptavidin (Vector) for 30 minutes. Slides were again rinsed and a specialised mounting medium was applied. For imaging utilising the fluorescent microscope, Vectashield mounting medium with 4', 6-diamidino-2-phenylindole (DAPI) nuclear counterstain (Vector) was utilised, while for confocal microscopy, Vectashield mounting medium with propidium iodide (PI) nuclear counter stain (Vector) was applied. Fluorescent microscopy was performed on an Olympus BX51 microscope with a fluorescence illuminator, together with the X-Cite 120Q excitation light source. Confocal microscopy was carried out on a Zeiss LSM 510 confocal laser scanning microscope, which allows for precise regulation of wavelength and excitation intensity. Confocal microscopy also

reduces or eliminates background information away from the focal plane, resulting in high quality image capture.

2.4.5 Automated Staining for 6B11 Antibody

Automated staining was completed in Our Lady's Children's Hospital, Crumlin, Dublin 12, Ireland and the Royal College of Surgeons in Ireland (RCSI), St Stephens Green, Dublin 2, Ireland. Automated staining was performed using the OptiView DAB IHC detection kit (Roche Ventana) on VENTANA automated slide stainers. Normal, healthy tonsillar, liver, gastric, breast, colon, skin and appendix tissue sections were analysed, all of which were obtained from the laboratory in which the staining was performed. Sections from the iNKT cell block created using the UltraPure agar were also stained as well as an additional iNKT cell block produced using bacteriological agar (Lab M). Pre-treatment was performed with cell conditioning solution (CC1, Ventana) while a 1:30 dilution of the 6B11 antibody was utilised.

3. Chapter 3 - Results: Investigation of Late Gene Regulation

3.1 Treatment of Stable Cell Lines with Small Molecule Drugs

Two stable cell lines, pBELCAT and pBELMCAT were previously created containing reporter plasmids based on the HPV-16 genome (Orrù 2012). In these plasmids, the L1 late gene is replaced with chloramphenicol acetyltransferase (CAT), a functional surrogate marker for late gene expression. Additionally, in pBELM, the splicing silencer elements adjacent to the SA5639 are mutated to reduce the silencing on this splice acceptor, therefore activating late gene expression (Zhao et al. 2004). Previous evaluation by Orrù et al. identifies that the pBELCAT and pBELMCAT reporters are functional and have considerable potential for use in future studies intended to examine L1 gene expression.

As a result of this discovery, the initial aims of this investigation were as follows:

- Utilise these stable cell lines, in particular pBELCAT67 and pBELMCAT31, for treatment with an array of small molecule drugs at varying concentrations.
- Determine the potential of these drugs to modulate CAT expression and therefore late gene expression.
- Identify specific drugs or drug combinations as candidates for the treatment of HPV infections.
- Examine the expression of any putative small molecule drug targets that arise from the molecular analysis.

To perform this analysis, the main objectives were therefore to:

- Culture the chosen stable cell lines until appropriate confluency was achieved and plate into 6-well plates as explained in materials and methods.
- Incubate these cells with pre-determined concentrations of the small molecule drugs.

- Analyse CAT expression levels using the CAT ELISA technique.
- Investigate RAR presence in various grade cervical cancer lesions through TMA immunoperoxidase staining

Multiple tests were performed on each drug investigated and all tests were carried out on a 1:100 dilution of pBELMCAT31 cell extract due to exceedingly high levels of CAT initially obtained. Averages of the absorbance readings were calculated, with data represented as fold change in all graphs created. Results shown are indicative of the 1:100 pBELMCAT31 dilutions. The significance of changes in CAT expression was determined utilising an unpaired, 2 tailed t-test with a p value <0.05 deemed significant, while 'n' numbers are indicative of the number of tests performed per experiment.

i) TPA

TPA (phorbol 12-myristate 13-acetate) was selected for investigation due to previous studies demonstrating the ability of this small molecule drug to induce HPV-31 late gene expression (Meyers et al. 1992). From this, the pBELMCAT31 and pBELCAT67 cell lines were treated with TPA in order to investigate the drug's potential to induce HPV-16 late gene expression. Following incubation of the cells in the presence of the selected drug concentrations, CAT presence was investigated utilising the CAT ELISA technique. On examination of Figure 3.1, a dose-dependent increase of CAT is observed, with a maximum 2.61 fold induction of CAT expression in the presence of 400ng/ml TPA and a maximum 3.78 fold induction of CAT expression with 800ng/ml TPA in the pBELMCAT31 cell line. Statistical significance was confirmed, with p values <0.05 obtained between 0ng/ml-400ng/ml and 0ng/ml- 800ng/ml. On inspection of the effects of TPA on the pBELCAT67 cell line, no change in CAT expression was observed, with only slight fluctuations in fold change noted (Figure 3.1).

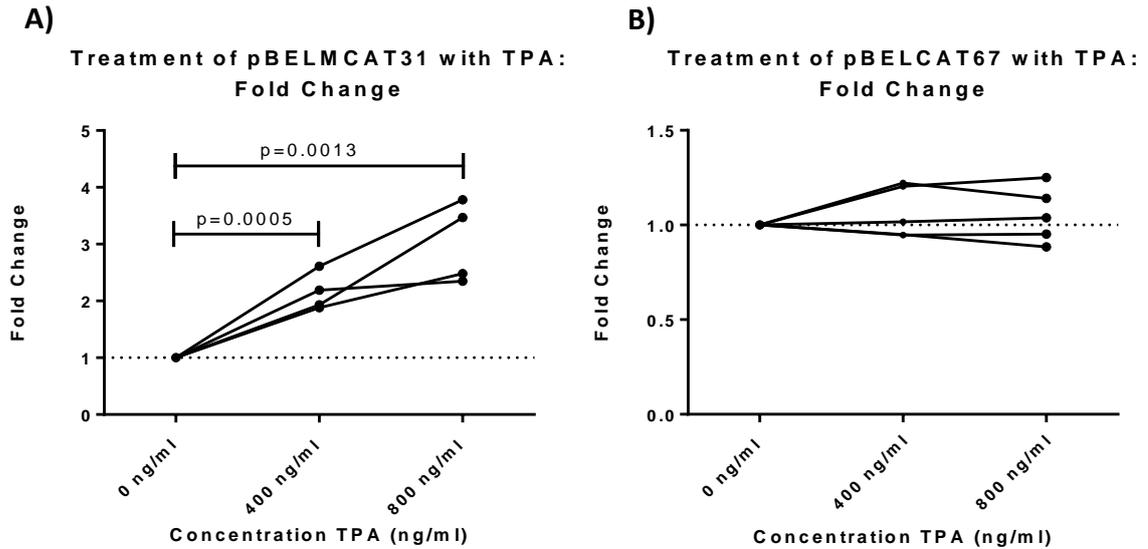


Figure 3.1 Treatment of pBELMCAT31 and pBELCAT67 with TPA

A) Induction of CAT in a dose-dependent manner by TPA in the pBELMCAT31 cell line is detected. **B)** No significant increase in CAT levels by TPA is observed in the pBELCAT67 cell line. n=4 with statistical significance confirmed when $p < 0.05$.

ii) Tannic Acid

Investigation into the potential of tannic acid as an inducer of late gene expression was also performed on pBELMCAT31 and pBELCAT67 transfected cell lines. Previous studies have suggested that an increase in polypyrimidine tract binding protein levels may be required for the activation of HPV-16 late gene expression during the viral life cycle (Somberg et al. 2008). Furthermore, it has also been demonstrated that tannic acid increases the expression of PTB in a dose-dependent manner (Bian et al. 2009). Therefore, it was speculated that tannic acid may have the potential to directly induce late gene expression through the up-regulation of PTB. The effects of tannic acid on CAT regulation and consequently late gene expression in the 2 stable cell lines was determined in the same manner as TPA utilising the CAT ELISA technique. As can be observed in Figure 3.2, no significant increase in CAT expression was detected in either cell line, with fold change fluctuating only slightly.

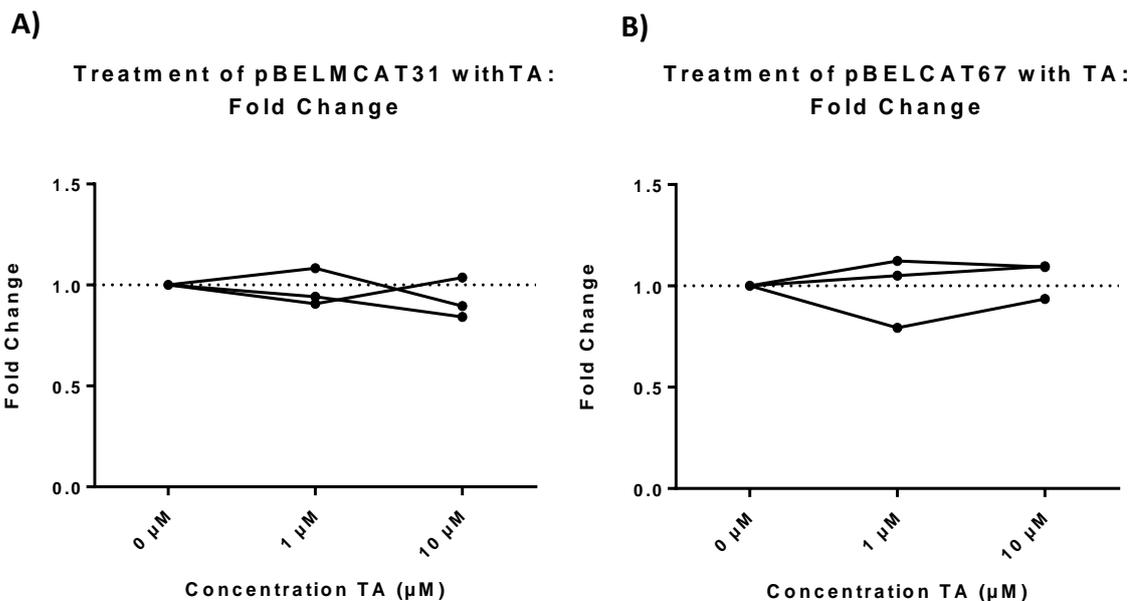


Figure 3.2 Treatment of pBELMCAT31 and pBELCAT67 with TA

No significant increase of CAT expression by TA is observed in the pBELMCAT31 cell line (A) or the pBELCAT67 cell line (B). n=3.

iii) Valproic Acid

Previous investigations have shown that VPA increases expression of ASF/SF2 (Harahap et al. 2012), a protein proven to regulate splicing of HPV and induce CAT expression (Somberg & Schwartz 2010). From this, it was decided to perform an examination on the direct effect of VPA on CAT expression and therefore determine its possible impact on the induction of HPV late gene expression. The influence of VPA on CAT expression was again established utilising the CAT ELISA. Results obtained show that, on treatment of the pBELMCAT31 cell line with VPA, a low concentration of the drug minimally decreases CAT expression while a concentration of 100mM induces a drastically decline in CAT. These changes in CAT expression between both 0mM-100mM and 0.5mM-100mM were indicated as statistically significant. On treatment of pBELCAT67 with VPA, CAT expression is shown to remain constant (Figure 3.3).

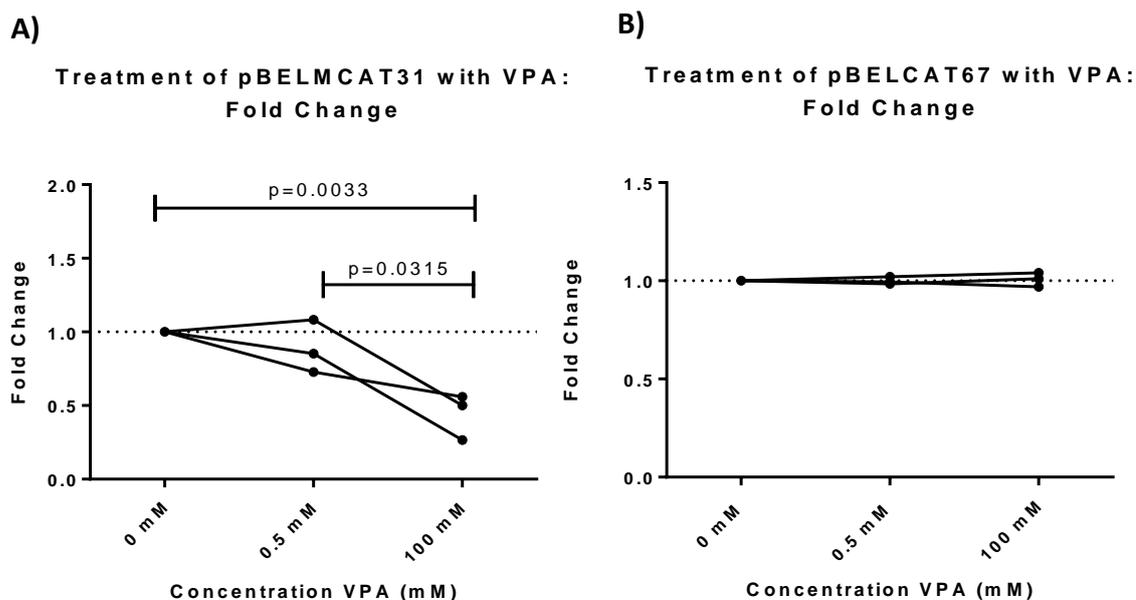


Figure 3.3 Treatment of pBELMCAT31 and pBELCAT67 with VPA

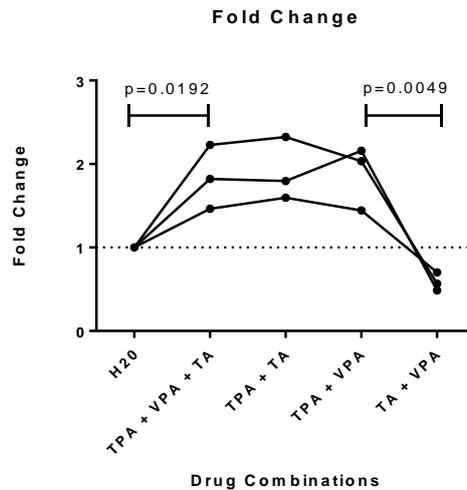
A) A significant decrease in CAT expression is observed in pBELMCAT31 on treatment with VPA, particularly with 100mM of the drug. **B)** No variation in CAT expression is observed in pBELCAT67 with VPA treatment. n=3 with statistical significance confirmed when $p < 0.05$.

iv) TPA, Tannic Acid and Valproic Acid Combined

The results acquired from the treatment of both the pBELMCAT31 and pBELCAT67 cell lines with TPA, TA and VPA individually, indicate that TPA is the only potential inducer of late gene expression. As an additional means of analysis, treatment of both cell lines with a combination of all 3 drugs was completed, speculating that using multiple substances simultaneously may have a greater cumulative effect on cellular factors linked to splicing and therefore significantly increase CAT expression levels. The concentrations selected for use were 800ng/ml TPA, 10 μ M TA and 0.5mM VPA, with these concentrations believed to be most likely to induce any significant impact on late gene expression based on previous results (Orrù 2012). Treatment of the pBELMCAT31 cell line showed a 1.5-2.3 fold induction of CAT expression with all drug combinations, excluding the TA&VPA combination (Figure 3.4). Results reasoned to be statistically significant are also indicated in Figure 3.4, with significance

shown only on the increase of CAT expression between H₂O and the TPA/TA/VPA combination (i.e. the addition of TPA) and on the decrease of CAT expression between TPA/VPA and TA/VPA (i.e. the absence of TPA). Furthermore, no noteworthy effect on CAT expression is observed on treatment of the pBELCAT67 cell line with any of the drug combinations tested (Figure 3.4).

A) Treatment of pBELMCAT31 with Drug Combinations:



B) Treatment of pBELCAT67 with Drug Combinations:

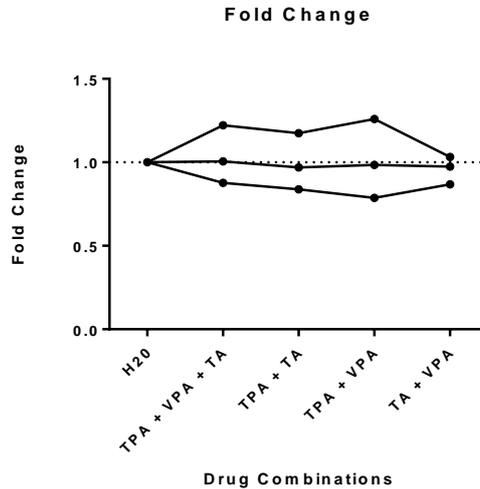
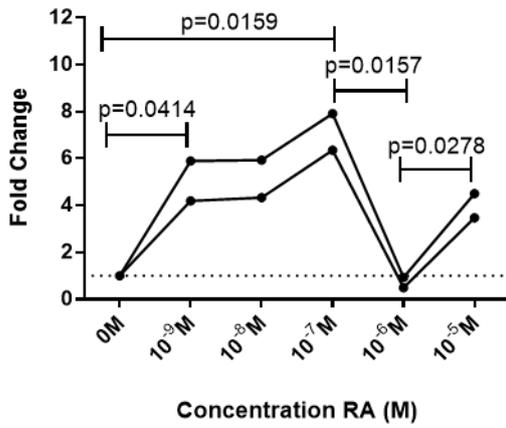


Figure 3.4 Treatment of pBELMCAT31 and pBELCAT67 with Drug Combinations
A) An increase in CAT expression is observed in pBELMCAT31 in the presence of TPA, with a decrease shown only in the absence of TPA. **B)** No significant variation in CAT expression is noted in pBELCAT67. n=3 with statistical significance confirmed when $p < 0.05$.

v) Retinoic Acid

The possibility of retinoic acid acting as an inducer of late gene expression was also selected for investigation. Previous work has determined that hnRNP A2/B1 expression level is down-regulated by RA (Liang et al. 2011) with knockdown of hnRNP A2/B1 proven to induce HPV-16 late gene expression (Li, Johansson, et al. 2013). Therefore, we proposed that RA may have substantial potential to induce late gene expression through inhibition of hnRNP A2/B1 and thus be proposed as an innovative treatment option for HPV infection. RA was investigated at a range of 10^{-5} M- 10^{-9} M, concentrations selected based on previous work which indicates RA as an inducer of teratocarcinoma cell differentiation (Edwards & McBurney 1983). Repeat analysis of TPA in the role of a control was also performed, as TPA has previously been determined to successfully induce CAT expression on the pBELMCAT31 cell line. As can be observed in Figure 3.5, CAT expression in pBELMCAT31 is induced significantly with lower concentrations of RA. A maximum fold induction of 7.9 is seen on treatment with 10^{-7} M of RA, with the increase in CAT expression between 0 M- 10^{-9} M and 0 M- 10^{-7} M deemed statistically significant. However, a decrease in CAT expression is shown at a concentration of 10^{-6} M. One possible suggestion for this decrease may be linked to the volume of DMSO utilised in the creation of this drug concentration, a solvent which may be toxic to cells in high amounts. CAT levels increase again with 10^{-5} M of the drug, with this fluctuation also determined as statistically significant. On treatment of the pBELCAT67 cell line, CAT expression levels were unaffected, with fold change remaining constant (Figure 3.5).

A)
Treatment of pBELMCAT31 with RA:
Fold Change



B)
Treatment of pBELCAT67 with RA:
Fold Change

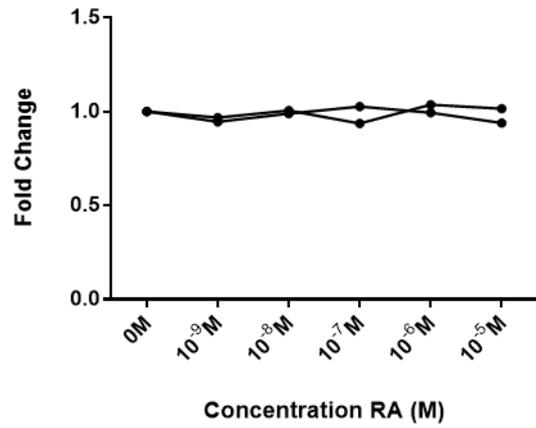


Figure 3.5 Treatment of pBELMCAT31 and pBELCAT67 with RA

A) A significant increase in CAT expression is observed with lower concentrations of RA in pBELMCAT31 with a decrease in CAT shown with higher RA concentrations. **B)** No variation in CAT expression is observed in pBELCAT67. n=2 with statistical significance confirmed when $p < 0.05$.

3.2 Investigation of Retinoic Acid Receptor Expression in Cervical Tissue

3.2.1 Tissue Microarray Staining

i) Antibody Optimisation

Following the discovery that retinoic acid has definite potential to induce HPV-16 late gene expression on the pBELMCAT31 cell line, it was decided to perform further investigation on the drug's mechanism of action. Retinoic acid exerts its biological effects by binding to specific nuclear retinoid receptors. These receptors are members of the steroid hormone gene superfamily and are ligand-activated transcription-modulating proteins (Xu et al. 1999). The nuclear retinoid receptors are divided into retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which are further subdivided into α , β , and γ subtypes (Chambon 1996). Retinoic acid receptors (RARs) function as heterodimers with retinoid X receptors (RXRs) to regulate cell growth and survival (Altucci et al. 2007). In response to retinoid binding, RAR/RXR heterodimers undergo significant conformational changes and coordinate the transcription of specific gene networks (Bastien & Rochette-Egly 2004). Generally speaking, three of the retinoid receptors (RAR α , RXR α and RXR β) have ubiquitous expression patterns, with the remaining three (RAR β , RAR γ and RXR γ) showing complex, tissue-specific expression (Dollé 2009). Interestingly, altered expression of these nuclear retinoid receptors has the potential to cause carcinogenesis (de Thé 1996; Xu et al. 1999). Regarding the cervix, both ectocervical and endocervical epithelia have been reported to express RARs (Darwiche et al. 1994) with altered expression of RARs, specifically loss of RAR- β expression, previously demonstrated in cervical cancer cell lines (Geisen et al. 1997; Bartsch et al. 1992). Geisen et al demonstrated that in normal cervical cells, basal RAR β mRNA levels are high and can be induced further by RA treatment while conversely, in the cervical carcinoma cells, the basal RAR β mRNA levels are low and only slightly inducible by RA, if at all. The RA-dependent increase of RAR β mRNA levels was also shown to be mediated by RAR α . Furthermore, an

additional study has shown that expression of all three RARs is found in normal cervical epithelium, while their levels decrease in premalignant lesions, including CIN1, CIN2, and CIN3 (Xu et al. 1999). Due to the fact that nuclear retinoid receptors are the ultimate mediators of retinoic acid activity, we decided to further identify the presence of selected retinoic acid receptors in cervical tissue and their possible significance to the induction of HPV late gene expression. Utilising anti-RAR α and anti-RAR β antibodies, alterations in receptor presence across normal cervical tissue, LSIL, HSIL and SCC was determined using previously created cervical TMAs. TMA staining was performed following antibody optimisation, which was carried out on a variety of tissue sections including bladder, gallbladder, breast and cervix to identify a suitable positive control. Anti-RAR α and anti-RAR β antibodies were investigated at a range of 1:25-1:100 while negative controls were obtained using PBS in place of the primary antibody. Antigen retrieval was achieved through heat-induced epitope retrieval utilising a 10mmol/l citrate buffer at pH6. Optimised antibody conditions are shown in Table 3.1

Optimised Antibody	Dilution	Optimised Retrieval Method	Optimised Positive Control
<i>RARα</i>	1:25	Citrate Buffer- 90°C Water Bath	Bladder
<i>RARβ</i>	1:50	Citrate Buffer- 90°C Water Bath	Bladder

Table 3.1 Conditions for Optimisation of Anti-RAR α and Anti-RAR β Antibodies

Positive and negative bladder tissue sections are displayed in Figure 3.6. The bladder sections clearly indicate staining of the epithelium with both RAR α and RAR β antibodies expressed. There is no staining of the stromal cells observed on treatment with anti-RAR α while anti-RAR β displays strong stromal expression. Additionally, weak or absent expression of both receptors is noted in the basal layers of the epithelium. A negative control is also displayed.

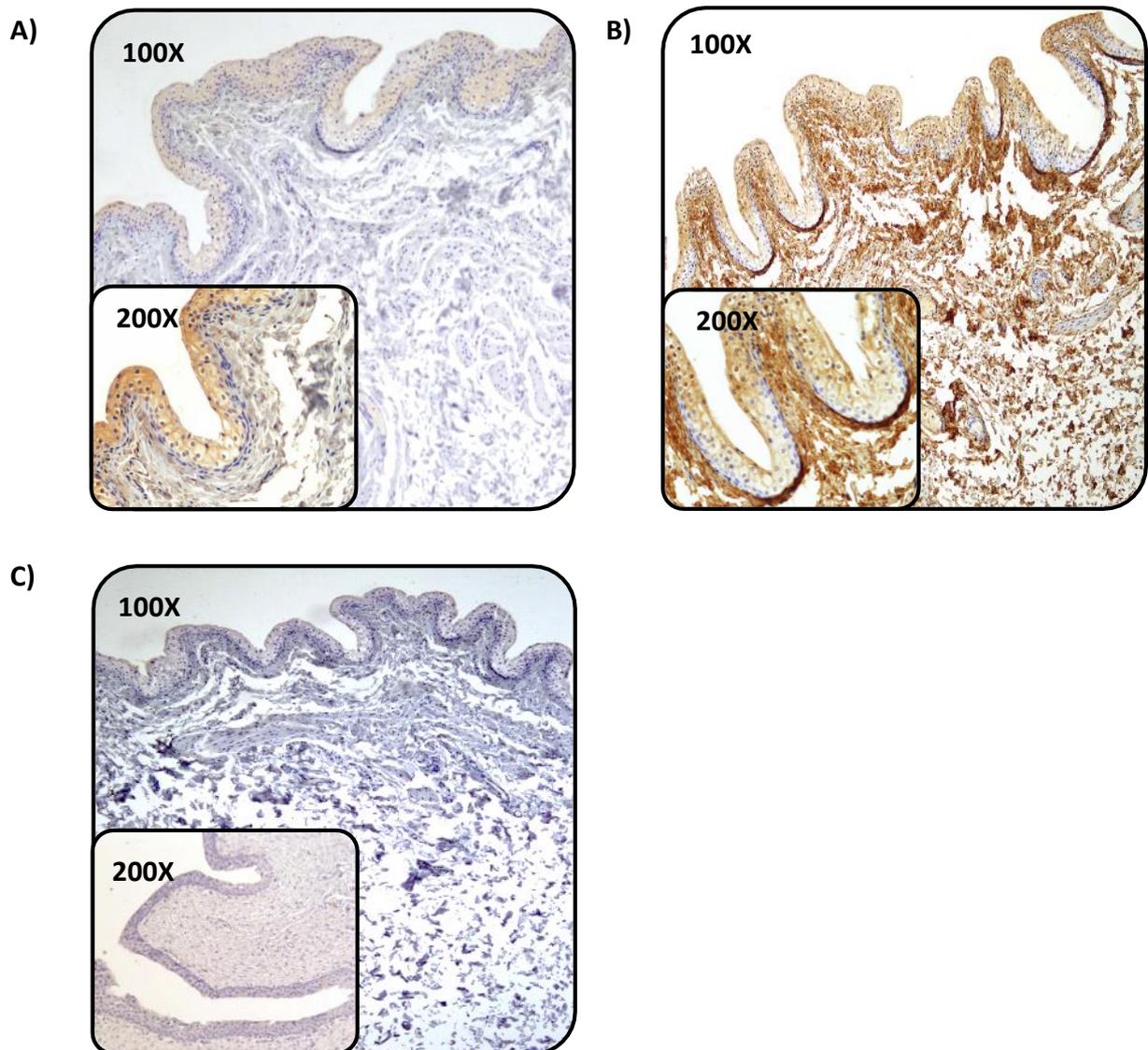


Figure 3.6 Bladder Control Tissue Staining with Anti-RAR Antibodies

A) Bladder tissue stained with a 1:25 dilution of anti-RAR α antibody. **B)** Bladder tissue stained with a 1:50 dilution anti-RAR β antibody. **C)** Negative control bladder tissue.

ii) Cervical TMA Analysis

Employing these optimised conditions for RAR α and RAR β investigation, immunoperoxidase staining was performed on the TMA block. Analysis of the TMA sections provides a complete, standardised, time-efficient mechanism for the investigation of these retinoic acid receptors across various grade cervical cancer lesions. The TMA utilised contains 12 cases of cervical carcinoma, 12 cases of HSIL, 12 cases of LSIL and 4 cases of tissue showing no evidence of malignancy (NEM), resulting in 40 cases per TMA. Each case was sampled in triplicate, therefore giving a total of 120 cores per TMA. Staining was performed as previously described, using a 1:25 dilution of anti-RAR α antibody and a 1:50 dilution anti-RAR β antibody with antigen retrieval performed through HIER. TMA sections were then carefully analysed under the light microscope. Figure 3.7 displays low power magnification of the different grade cervical tissue sections stained with H&E, anti-RAR α antibody and anti-RAR β antibody for a broad comparison of receptor expression. Higher power magnification of anti-RAR α and anti-RAR β staining is demonstrated in Figure 3.8. Images shown are representative of the staining obtained across all TMA cores (n=120) with a consistent pattern in each specific grade of cervical lesion identified throughout. Staining observed indicates the following:

- **Normal Cervical Tissue:** RAR α and RAR β are both expressed in the upper layers of the epithelium in tissue showing no evidence of malignancy. RAR α appears to be weakly expressed while RAR β displays strong expression. No positive staining is observed in the basal layer with either the anti-RAR α or the anti-RAR β antibody.
- **LSIL:** There is complete absence of RAR α and RAR β expression in the epithelium of LSIL sections. Strong stromal expression of RAR β is however observed, with distinctly negative epithelium.

- **HSIL:** Staining with both anti-RAR α and anti-RAR β is again completely absent in the epithelial layers of HSIL. Stromal expression of RAR β is observed with staining in the lesion edge artefactual.
- **SCC:** Complete absence of RAR α and RAR β expression is again noted, with neoplastic cells predominantly negative.

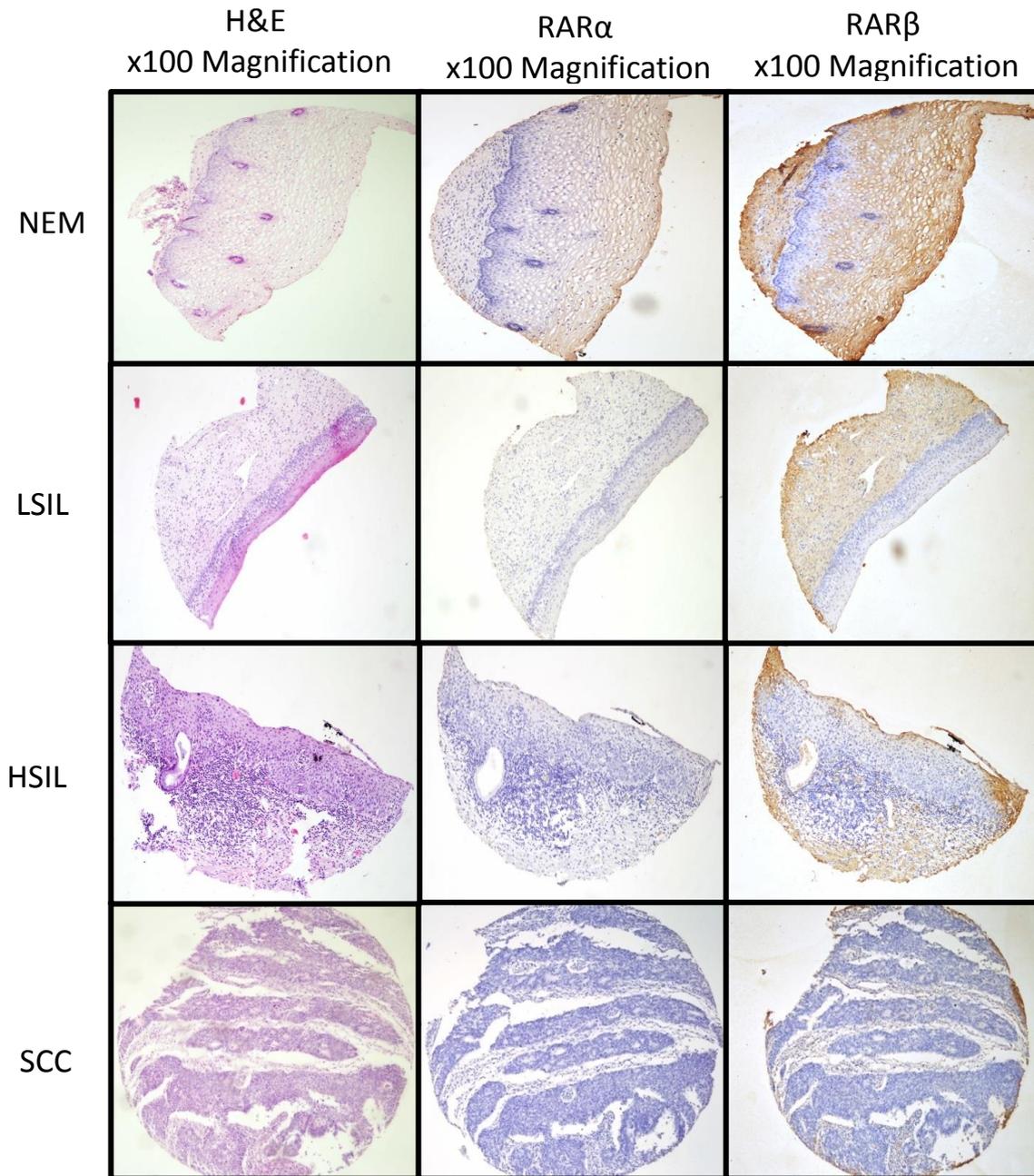


Figure 3.7 Tissue Microarray Staining with RAR Antibodies

H&E staining together with RAR α and RAR β expression as detected by immunoperoxidase staining in tissue showing no evidence of malignancy (NEM), low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL) and squamous cell carcinoma (SCC). A 1:25 dilution of RAR α and a 1:50 dilution of RAR β were utilised.

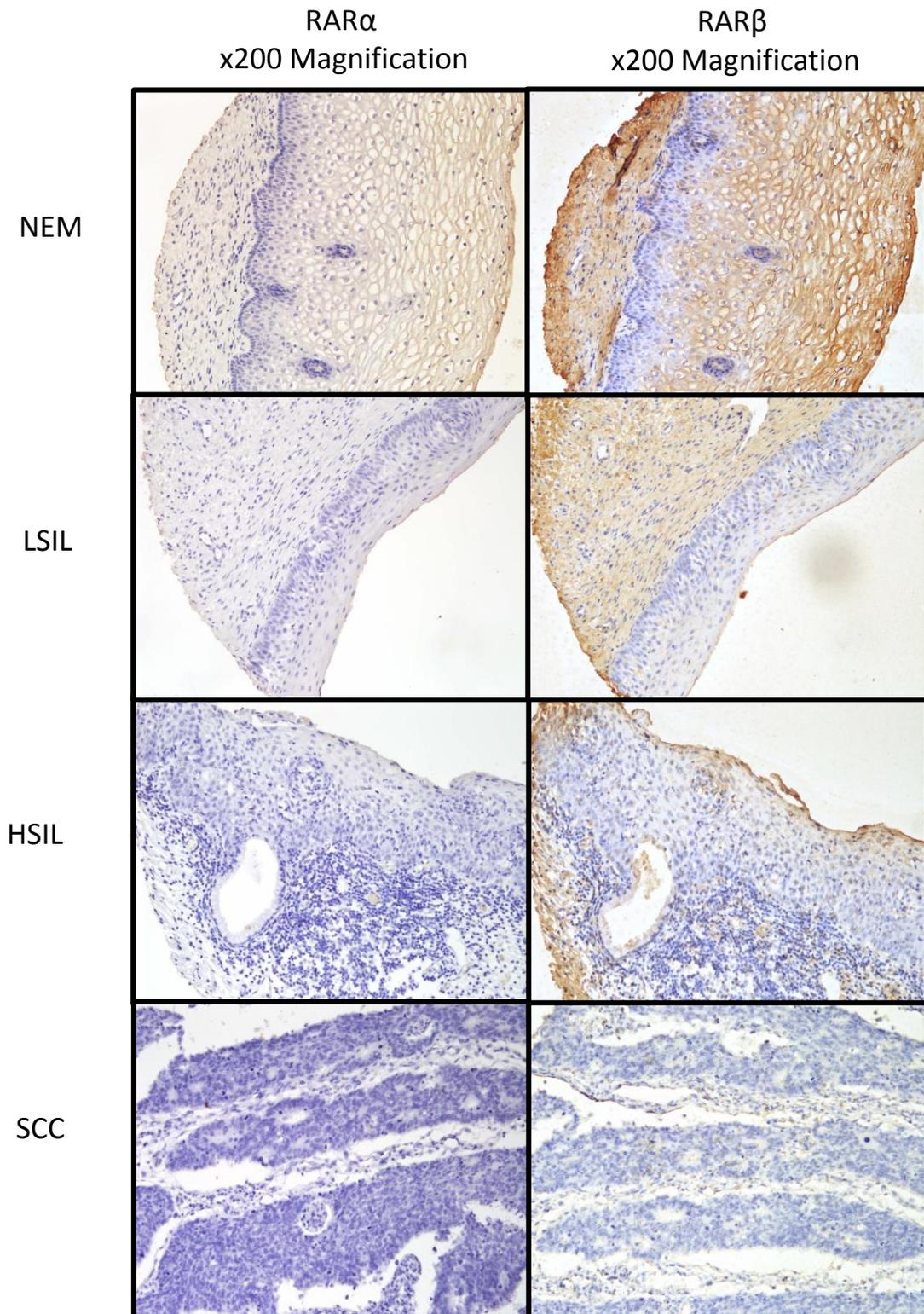


Figure 3.8 Tissue Microarray Staining with RAR Antibodies: High Power Images

RAR α and RAR β expression as detected by immunoperoxidase staining in tissue showing no evidence of malignancy (NEM), low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL) and squamous cell carcinoma (SCC). A 1:25 dilution of RAR α and a 1:50 dilution of RAR β were utilised.

On examination of the TMA sections, it was observed that individual intraepithelial mononuclear cells appear positive with anti-RAR β in cervical tissue sections as shown in Figure 3.9. This may be of interest for future examination of immune cell presence and also have potential to act as an internal positive control when staining with the anti-RAR β antibody. Furthermore, adipose tissue also appeared positive on staining with both the anti-RAR α and anti-RAR β antibodies (Figure 3.10). This incidental finding could be of importance for further research and have a possible use in the investigation and identification of adipose tissue.

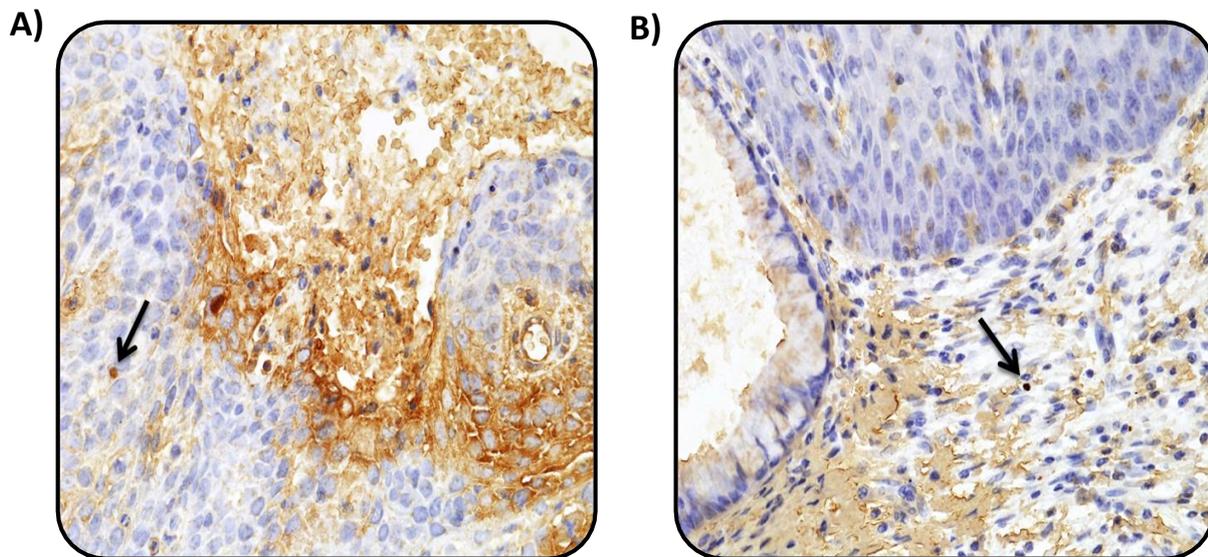


Figure 3.9 Intraepithelial Immune Cell Staining with RAR β Antibody

A) CIN1 tissue section stained with a 1:50 dilution of RAR β antibody.

B) Cervical tumour section stained with a 1:50 dilution of RAR β antibody.

Black arrows indicate the presence of mononuclear cells with magnification shown at 400X.

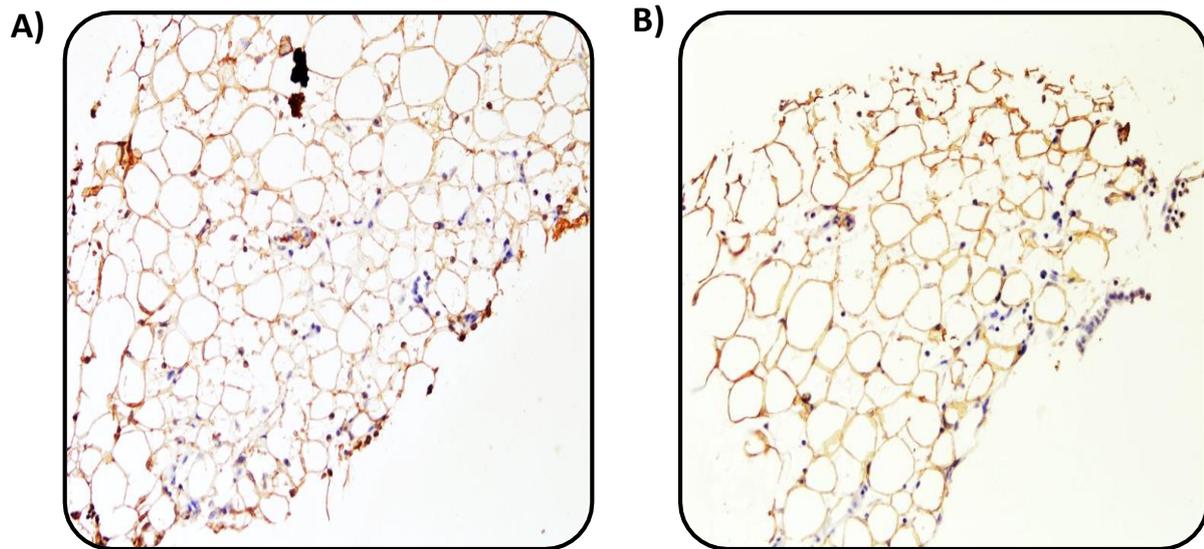


Figure 3.10 Adipose Tissue Staining with RAR Antibodies

A) Adipose Tissue stained with a 1:25 dilution of RAR α antibody.

B) Adipose tissue stained with a 1:50 dilution of RAR β antibody. Magnifications shown are at 200X.

3.2.2 Cell Block Staining

For additional analysis of retinoic acid receptor presence, cell blocks were created using pBELMCAT31 and pBELCAT67 cells, the HPV-16 reporter cell lines previously utilised in our molecular investigations. Furthermore, an additional cell block was formed using the C33A cell line, a cervical cancer cell line that does not contain any HPV material. Sections from all three blocks were cut and stained using anti-RAR α and anti-RAR β antibodies as previously described. Conditions were replicated with a 1:25 dilution of anti-RAR α and a 1:50 dilution of anti-RAR β utilised. As displayed in Figure 3.11, both the pBELMCAT31 and pBELCAT67 cell lines display positive staining with both the RAR α and RAR β antibodies. As before, RAR α staining is weak while RAR β appears to be highly expressed. No positive staining was observed with RAR α in the C33A cell line, however RAR β was again strongly expressed. These results indicate that cells of cervical cancer lineage express RAR β with expression of RAR α shown in HPV-infected cell lines only.

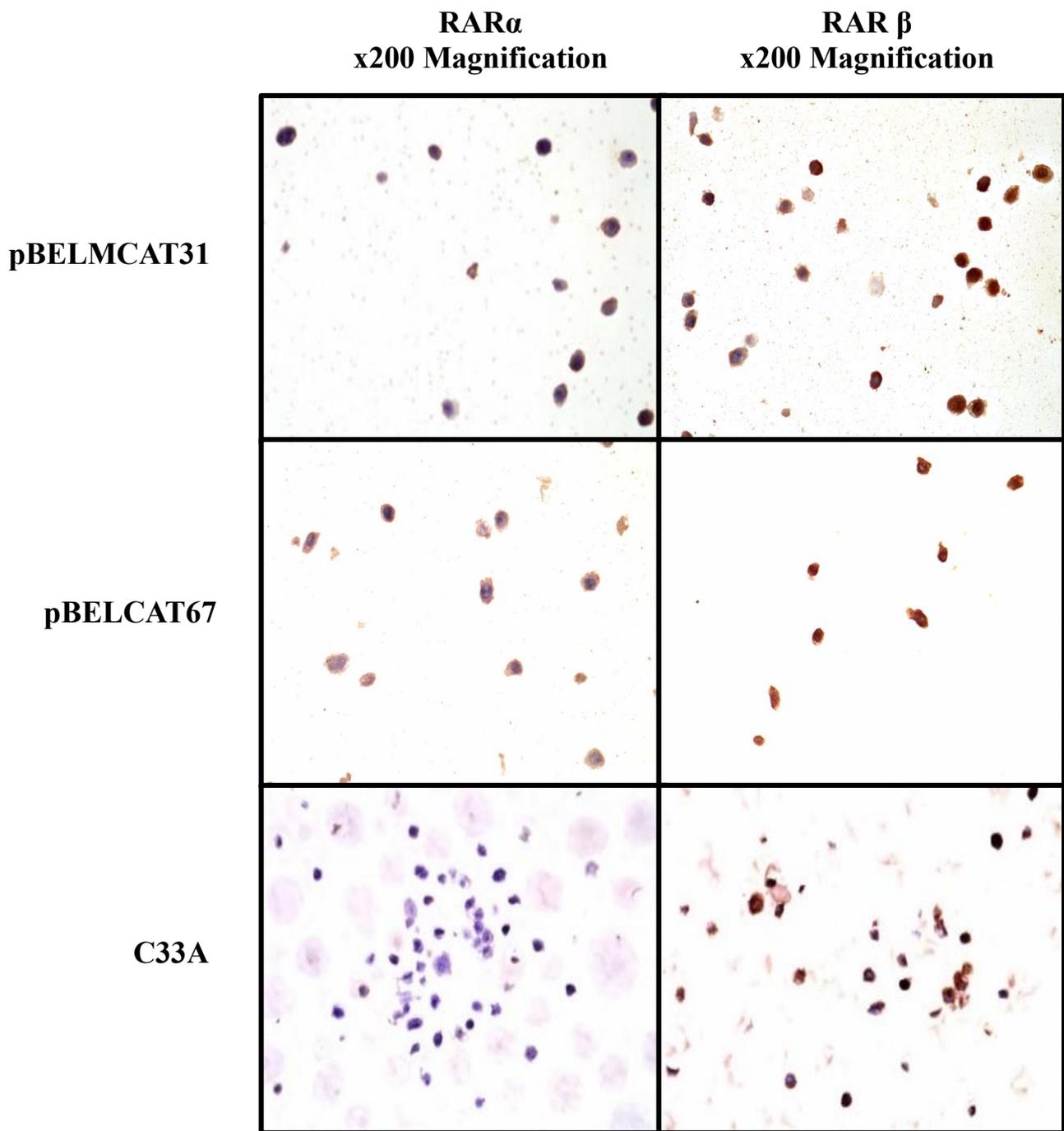


Figure 3.11 Cell Block Staining with RAR Antibodies

RAR α and RAR β expression as detected by immunoperoxidase staining in pBELMCAT31, pBELCAT67 and C33A cell block sections.

4. Chapter 4 - Results: Identification of iNKT Cells

iNKT cells are a relatively recently defined lymphocyte population and play a pivotal role in the immune response despite their small numbers in the body. iNKT cells are proven potent activators of the immune system, forming a bridge between the innate and adaptive immune systems and acting primarily in a protective manner. They are also found to be copious producers of cytokines, a function which defines the nature and quality of the antigen-specific T cell response that ensues (Terabe & Berzofsky 2008). iNKT cells require CD1d for their activity; an MHC-like glycoprotein utilised to present glycolipid and lipid molecules to the iNKT cells. However, studies have shown down-regulation of CD1d in HPV-infected cells, a mechanism which may provide a means to evade the protective immunological surveillance by iNKT cells (Miura et al. 2010). Investigation into the consequence of this CD1d down-regulation on the iNKT cell population is restricted as there is currently no available method to detect iNKT cells in tissue sections.

Therefore, the primary aims of the immunological research were to:

- Identify a pioneering protocol for the detection of iNKT cells in human tissue.
- Utilise this method to subsequently enumerate iNKT cells in HPV-infected cervical cancer samples.

To perform this research, the principal objectives were to:

- Validate the efficacy of the 6B11 antibody for iNKT cell identification through flow cytometry.
- Create a cell block containing a pure population of iNKT cells for use as a positive control.
- Optimise the 6B11 antibody for iNKT cell identification in both iNKT cell blocks and paraffin wax-embedded cervical tissue samples.

4.1 Flow Cytometry

iNKT cells were previously expanded *ex vivo* as per O'Reilly et al and kindly donated for our use. To investigate the effectiveness of the 6B11 antibody in identifying V α 24-J α 18 TCR expressing iNKT cells, flow cytometry was performed. The technique was carried out on 4 healthy PBMC samples together with the expanded population of iNKT cells, utilising a FITC labelled 6B11 antibody in conjunction with a PE labelled CD3 antibody. This process allowed for the calculation of the percentage iNKT cells per total lymphocyte population and per expanded iNKT cell population. Furthermore it capably confirmed the working potential of the 6B11 antibody. Successful analysis was accomplished, with the number of iNKTs per population PBMCs calculated at a range of 0.81%-2.43% while the purity of the expanded iNKT's was 92.7% (Figure 4.1).

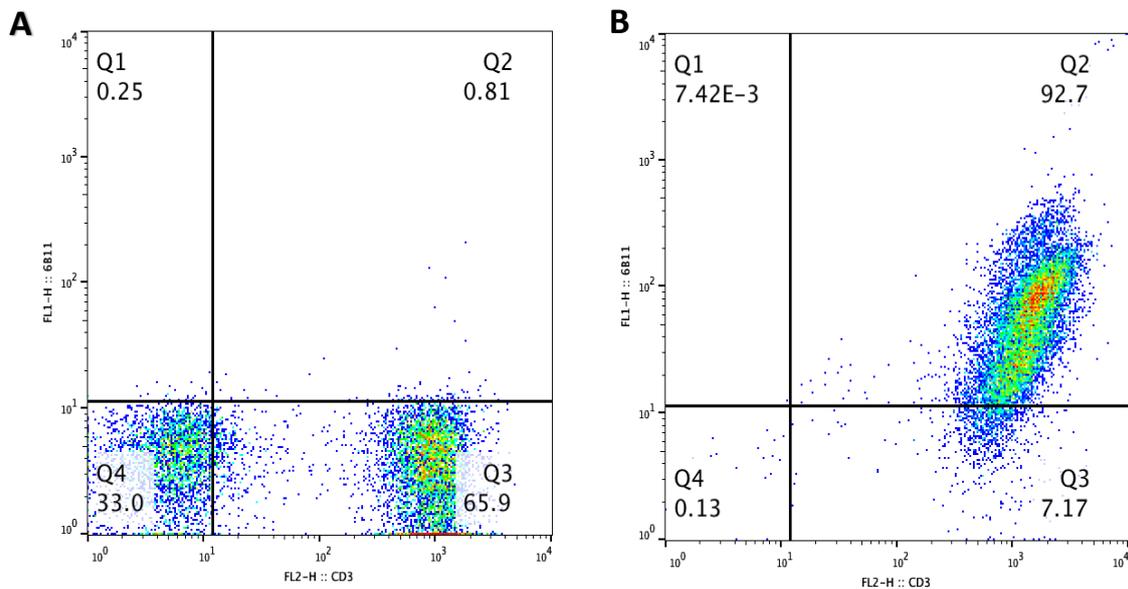


Figure 4.1 Flow Cytometric Analysis of iNKT Cells

A) PBMC Sample: % iNKTs (CD3⁺6B11⁺) of total lymphocyte population is 0.81%.

B) iNKT Cell Sample: % iNKTs (CD3⁺6B11⁺) of total lymphocyte population is 92.7%.

4.2 Immunohistochemical Analysis of Cell Block Sections

Following confirmation by flow cytometry that the 6B11 antibody is an appropriate method of identifying iNKT cells in suspension, it was decided to investigate the prospect of utilising this antibody to detect iNKT cells in human tissue. In order to explore its working potential in tissue sections, a cell block composed of a pure population of iNKT cells was created to be used in the capacity of a positive control. Prior to the use of the precious iNKT cells, Jurkat cells derived from a T lymphocyte cell line were first cultured and utilised to create an agar cell block. This Jurkat cell block proved an invaluable tool for the optimisation of both effective cell block creation and successful staining of these unique cell block sections. Analysis of the iNKT cell block sections was consequently performed.

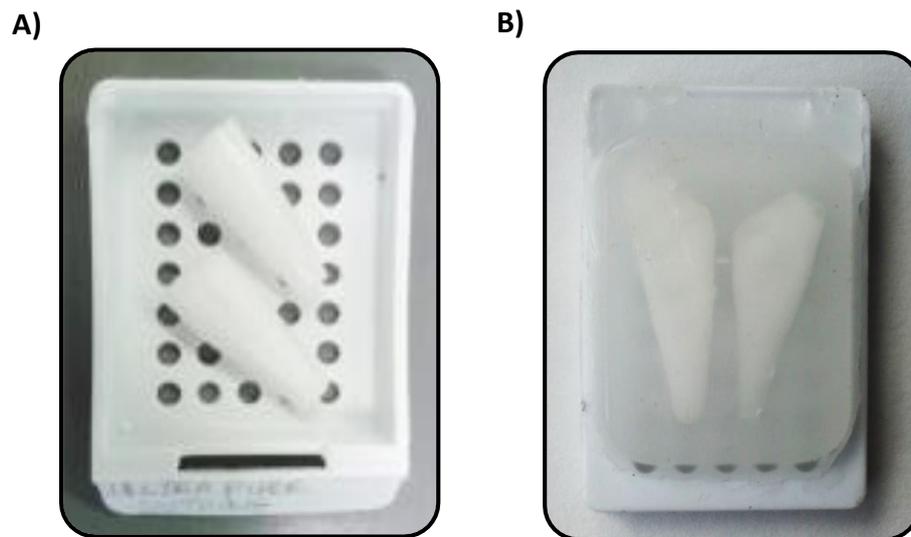


Figure 4.2 iNKT Cell Block Creation

A) Agar iNKT cell pellets after overnight processing prior to embedding in paraffin wax block.
B) Completed iNKT cell block ready for sectioning.

4.2.1 Optimisation of Cell Block Staining

From this Jurkat cell block, sections were first cut and stained using the H&E protocol to determine cell presence (Figure 4.3). Once an adequate cell concentration for further analysis was confirmed, it was decided to evaluate the suitability of these sections for immunostaining. Optimised conditions were first established through immunoperoxidase staining of intestinal tissue and Jurkat sections using a CD45 common leucocyte antibody, prior to the eventual use of the precious 6B11 antibody. Antigen retrieval was carried out through HIER using 10mmol/l citrate buffer at pH6 while a 1:100 dilution of anti-CD45 primary antibody was deemed sufficient for effective staining. Positive staining of leucocytes was observed in various areas of the intestinal tissue sections using the light microscope (Figure 4.4), allowing these established staining conditions to be replicated on the Jurkat cell block sections. Staining of the Jurkat cells proved successful with clear membranous staining of the cells displayed in Figure 4.5.

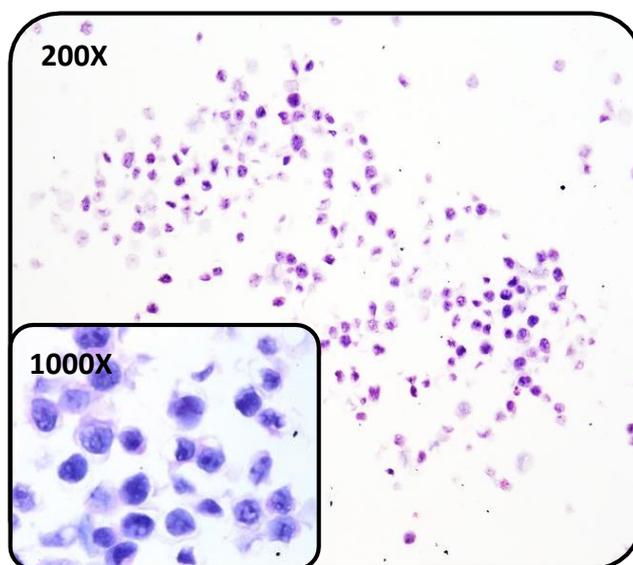


Figure 4.3 H&E Staining of Jurkat Cell Block Sections
Staining of Jurkat cells with haematoxylin and eosin.

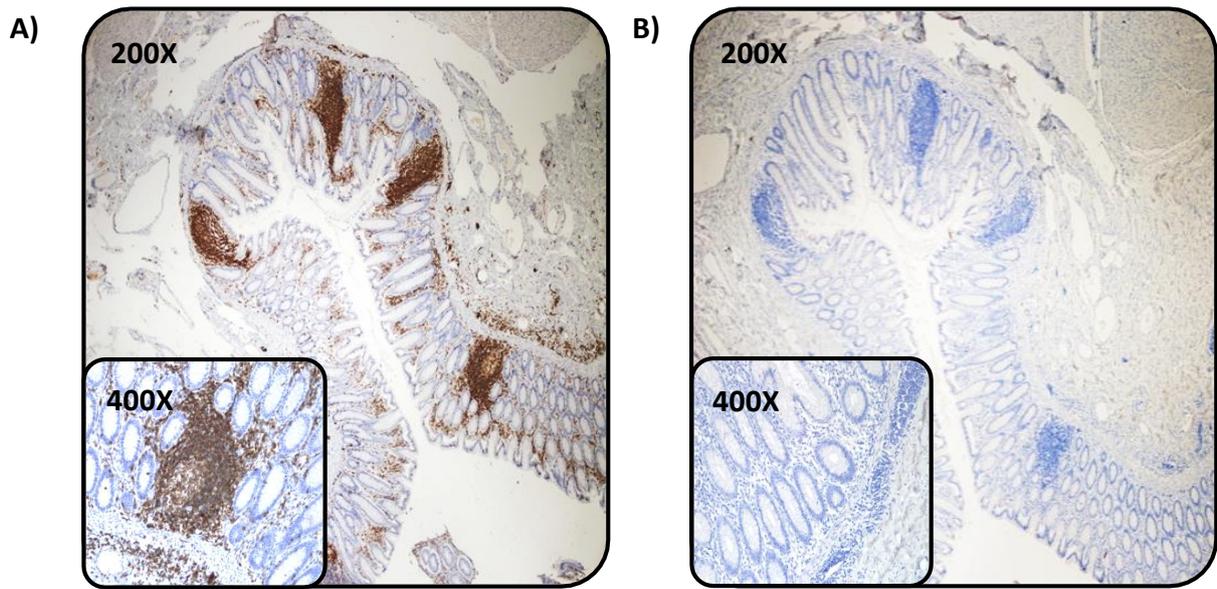


Figure 4.4 Immunoperoxidase Staining of Intestinal Tissue with Anti-CD45

A) Intestinal tissue stained with a 1:100 dilution of the anti-CD45 antibody.

B) Intestinal tissue negative control.

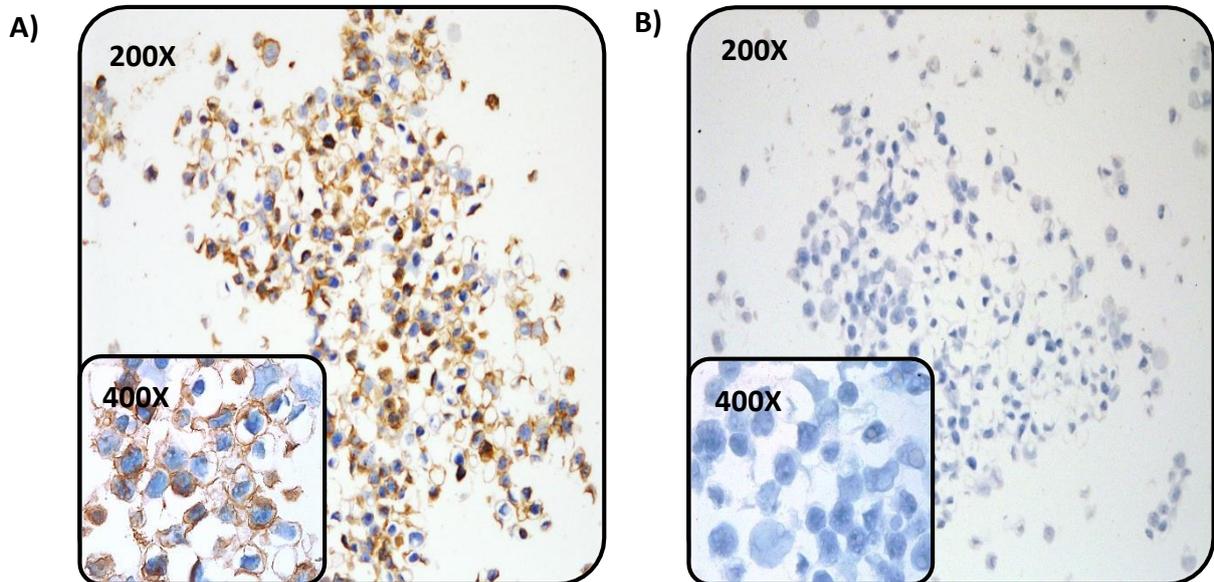


Figure 4.5 Immunoperoxidase Staining of Jurkat Cell Block Sections with Anti-CD45

A) Jurkat cell block section stained with a 1:100 dilution of anti-CD45 antibody.

B) Jurkat cell block section negative control.

In addition to the immunoperoxidase staining, immunofluorescent staining was also performed on the Jurkat cell block sections to obtain enhanced results, with fluorescein streptavidin the fluorescent conjugate of choice and DAPI nuclear counterstain employed for examination under the fluorescent microscope (Figure 4.6). Staining again proved a success, with clear membranous staining of the jurkat cells observed and high quality fluorescent images obtained.

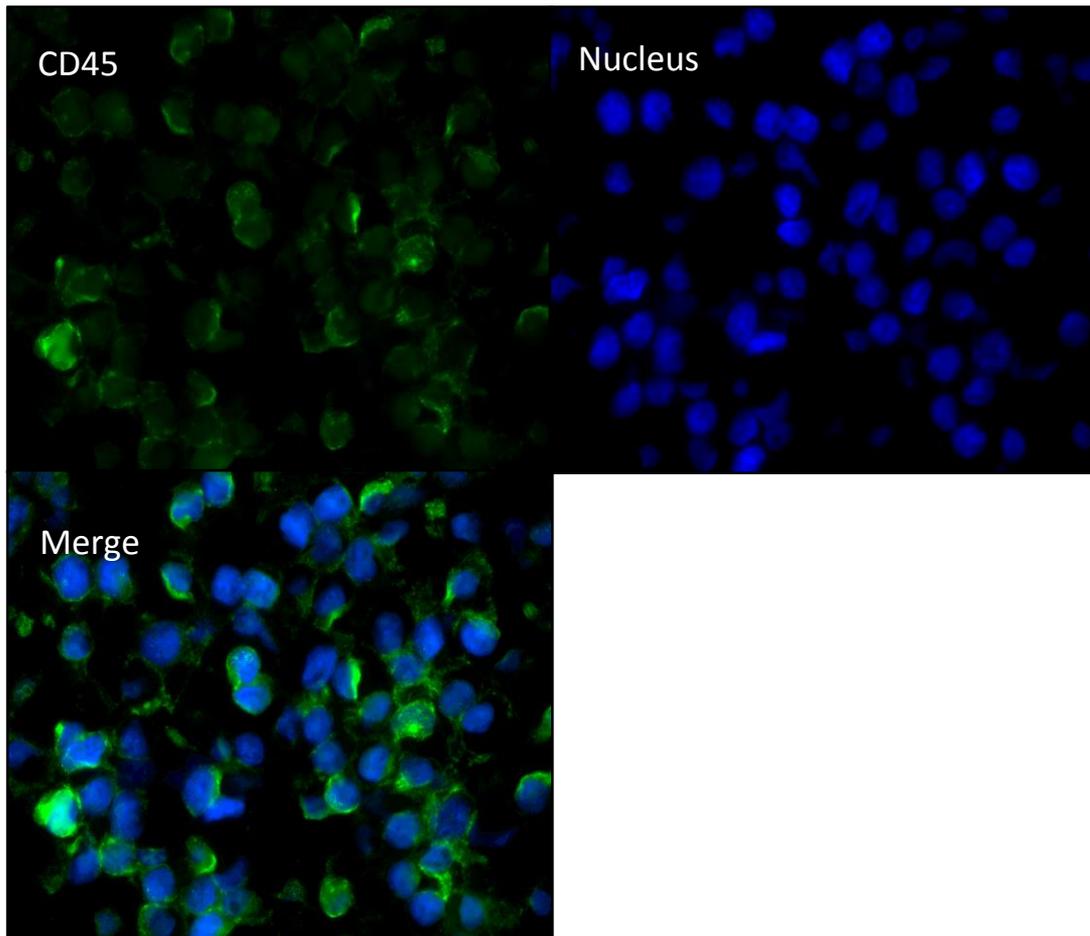


Figure 4.6 Immunofluorescent Staining of Jurkat Cell Block Sections with Anti-CD45 Jurkat cell block sections stained with a 1:100 dilution of anti-CD45 antibody teamed with fluorescein streptavidin and DAPI nuclear counter stain. Magnification is at 400X on the fluorescent microscope.

4.2.2 iNKT Cell Staining with Anti-CD45 Antibody

As a result of the efficacious examination performed on the Jurkat cell block sections, iNKT cells could then be considered for investigation. The iNKT cell block was produced using an identical protocol to that followed for the Jurkat cells, although a higher concentration of 15×10^6 iNKT cells was required to provide an effective concentration for analysis. Initial determination of cell presence was performed as before using the H&E technique (Figure 4.7).

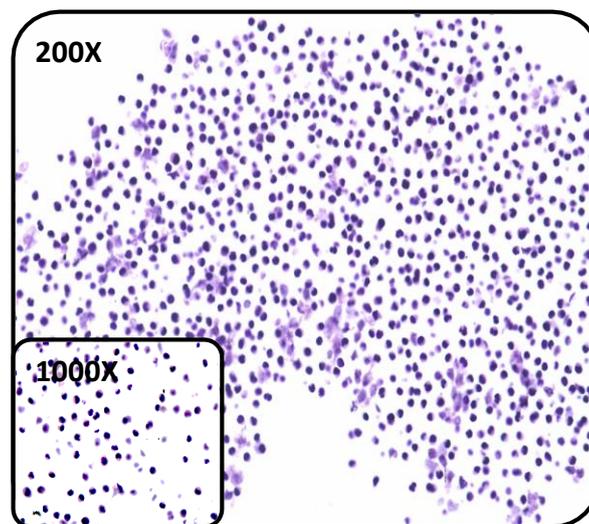
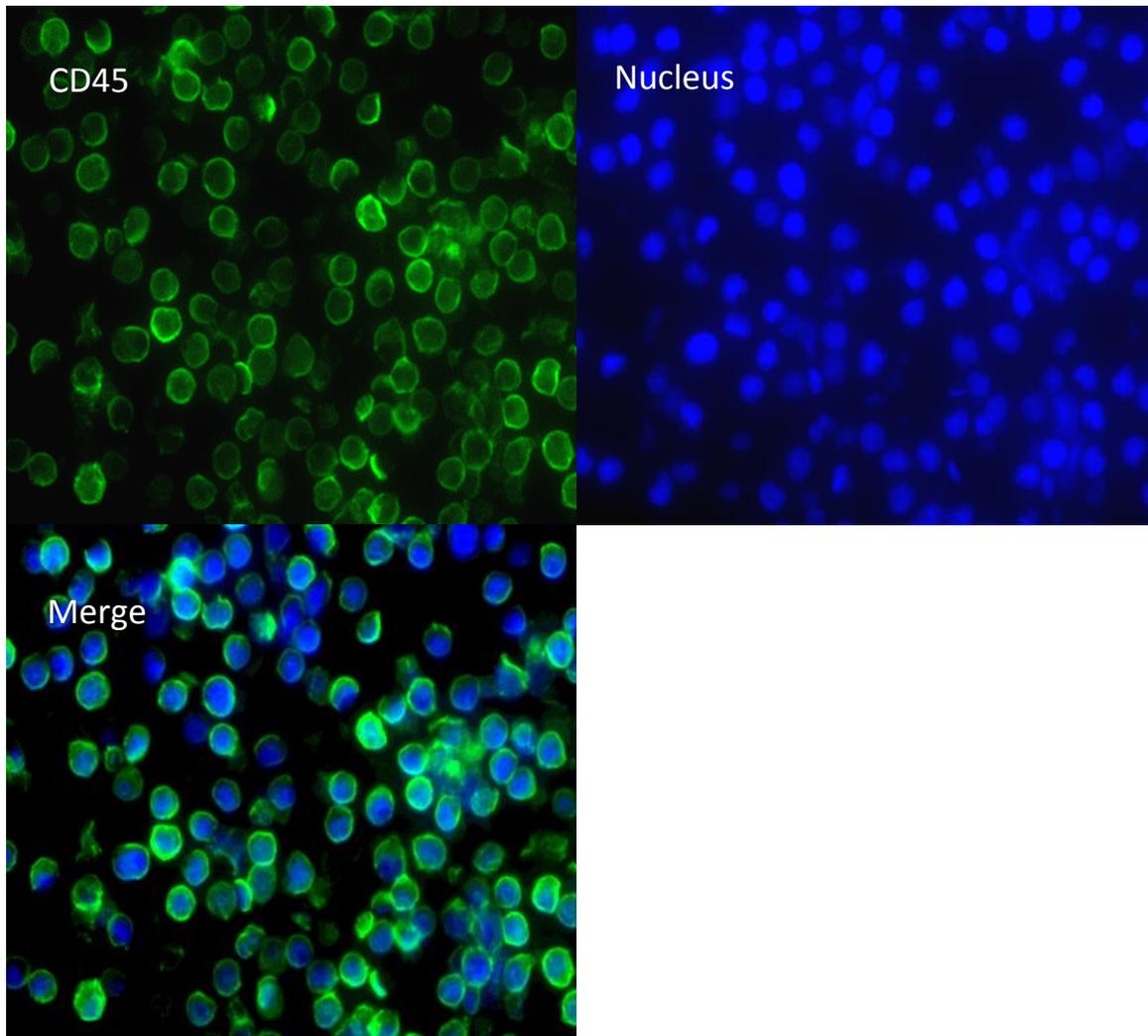


Figure 4.7 H&E Staining of iNKT Cell Block Sections

Staining of iNKT cell block sections with haematoxylin and eosin.

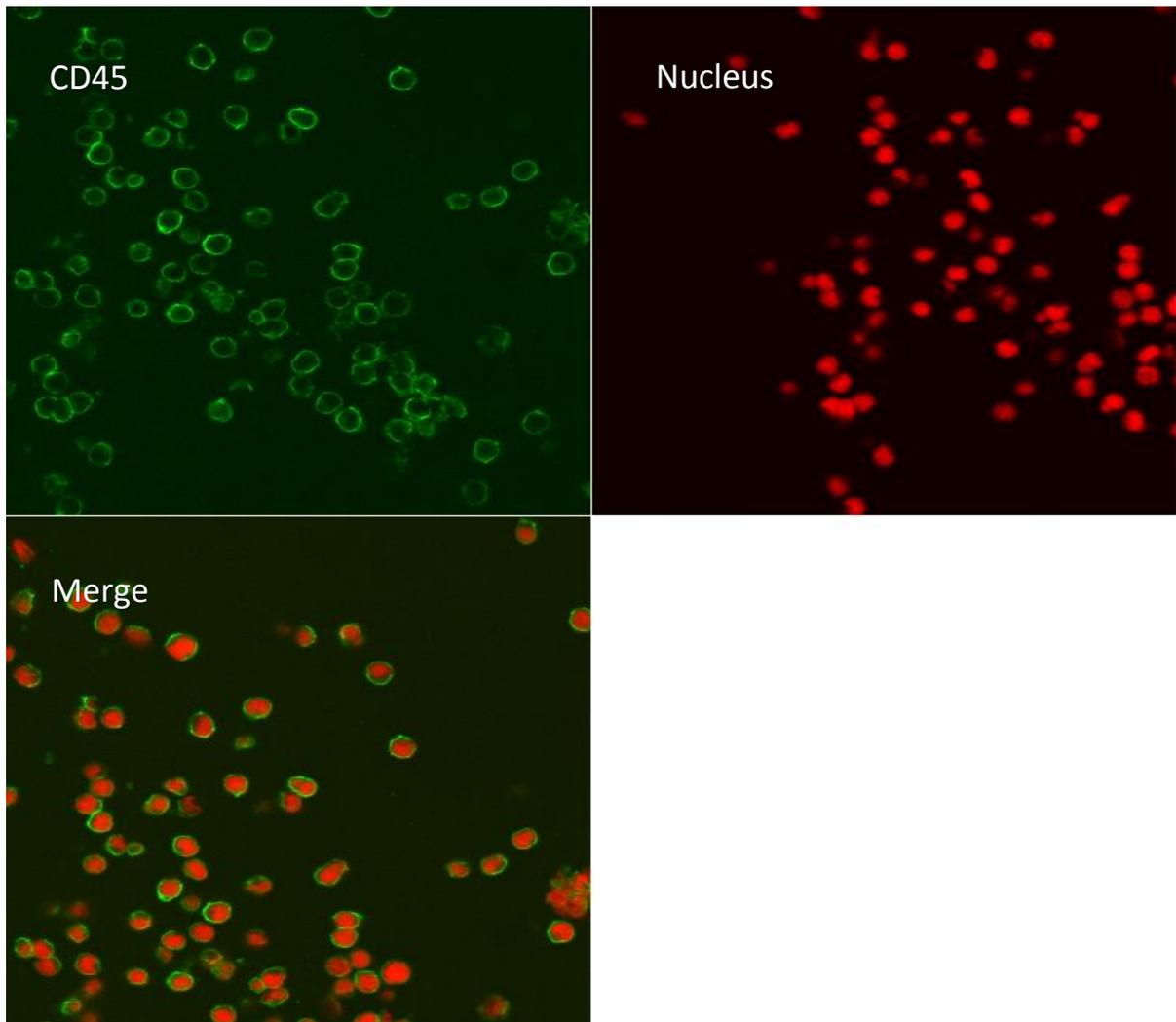
Once a sufficient cell concentration was identified, immunofluorescent examination of the iNKT cells was carried out. It was decided to proceed with immunofluorescent staining only as it provided a far higher quality result compared to immunoperoxidase analysis. A 1:100 dilution of the anti-CD45 antibody was first utilised to determine iNKT cell antigenicity and suitability for future staining with 6B11. For fluorescent microscopy, fluorescein streptavidin was again used with DAPI counterstain as before. Additionally, confocal microscopy was also selected for use in order to obtain greater quality images with increased detail at a higher resolution. For confocal imaging, PI was employed as the counterstain of choice. The investigation proved

successful, with clear membranous staining of the iNKT cells observed utilising the anti-CD45 antibody (Figure 4.8), while the images obtained using the confocal microscope displayed more distinct results with reduced background interference (Figure 4.9).



**Figure 4.8 Immunofluorescent Staining of iNKT Cells with Anti-CD45:
Fluorescent Microscopy**

Staining of iNKT cell block sections with a 1:100 dilution of anti-CD45 antibody teamed with fluorescein streptavidin and DAPI nuclear counter stain. Magnification is 400X on the fluorescent microscope.



**Figure 4.9 Immunofluorescent Staining of iNKT Cells with Anti-CD45:
Confocal Microscopy**

Staining of iNKT cell block sections with a 1:100 dilution of anti-CD45 antibody teamed with fluorescein streptavidin and PI nuclear counter stain. Magnification is 400X on the confocal microscope.

4.2.3 iNKT Cell Staining with 6B11 Antibody

Following the successful staining of the iNKT cell block sections with anti-CD45 antibody, the staining potential of 6B11 was finally investigated. Immunofluorescent staining was performed as previously described, with HIER carried out using citrate buffer at pH6 and a 1:100 dilution of 6B11 initially assessed together with fluorescein streptavidin as a fluorescent conjugate. PI

was the nuclear counterstain of choice and images were obtained using the confocal microscope. However, as displayed in Figure 4.10, no positive staining was observed. Although the staining with PI indicates the nucleus and therefore cell presence, no fluorescence is seen with the 6B11 antibody.

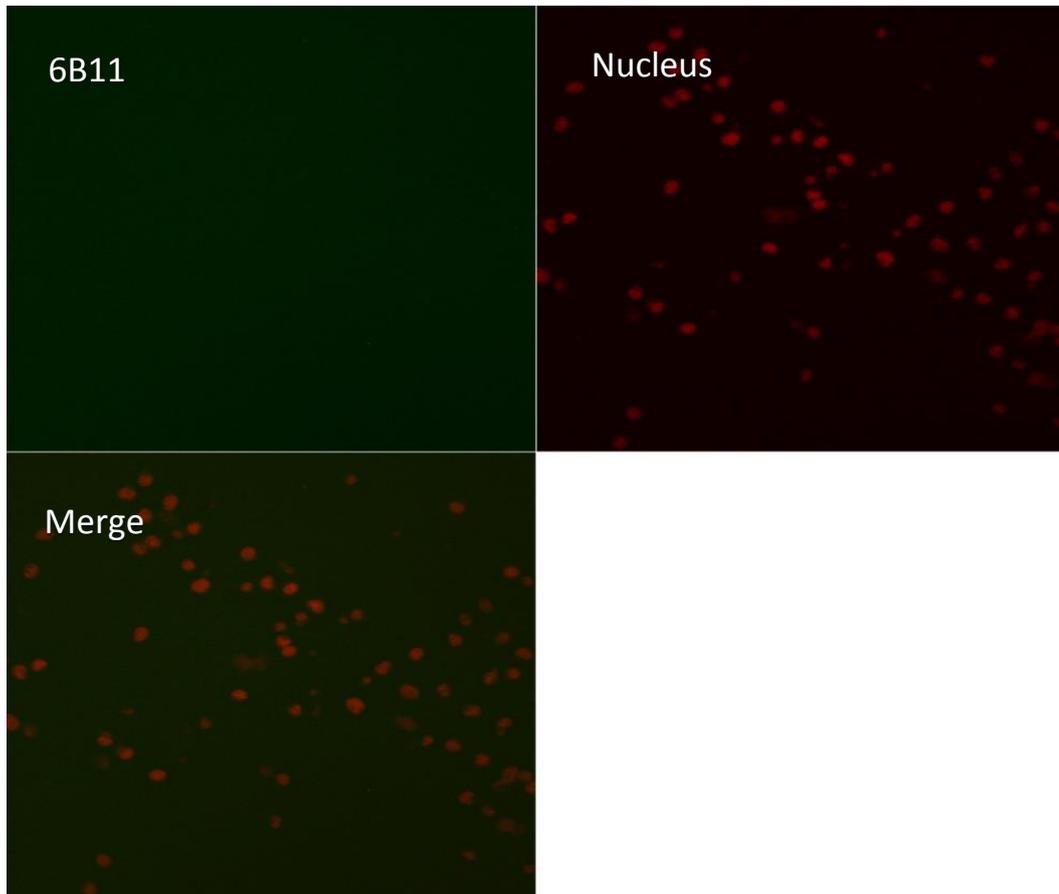


Figure 4.10 Immunofluorescent Staining of iNKT Cells with 6B11 Antibody
Staining of iNKT cell block sections with a 1:100 dilution of the 6B11 antibody teamed with fluorescein streptavidin and PI nuclear counter stain. Magnification is 400X on the confocal microscope.

Following on from this disappointing result, it was decided to explore additional avenues for the investigation of 6B11. Frozen sections and cytopins were produced and the possibility of automated staining as a standardised method of optimising 6B11 staining in tissue sections was also reviewed.

4.3 iNKT Cell Frozen Section Analysis

Frozen iNKT cell sections were created as an alternative means of investigating the staining potential of the 6B11 antibody. The frozen cell block was created as described in materials and methods by resuspending iNKT cells in O.C.T compound before freezing in liquid nitrogen and cutting sections immediately on the cryostat. As per analysis of the paraffin embedded iNKT cell block, a H&E stain was first performed to identify cell presence, followed by immunostaining. A 1:100 dilution of anti-CD45 antibody was employed for immunoperoxidase staining with strong positive staining observed, thus clarifying the cells were intact and viable for analysis (Figure 4.11 A). This was followed by immunoperoxidase staining using a 1:30 dilution of 6B11, a lower concentration to that previously employed to increase the likelihood of achieving a positive result. However, as shown in Figure 4.11 B, no distinct staining of the iNKT cells was detected.

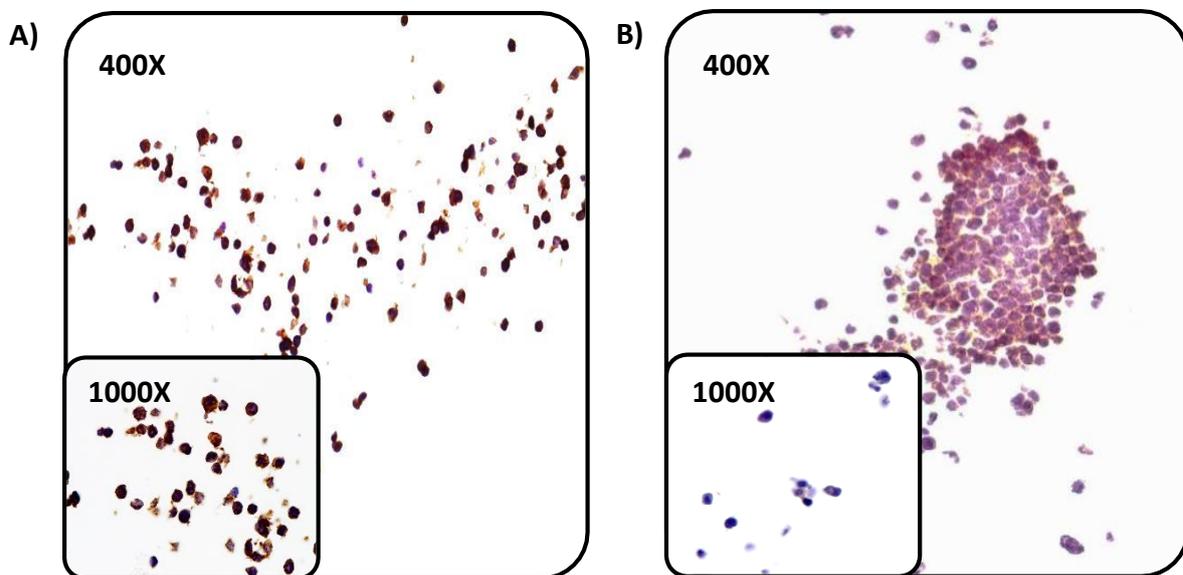


Figure 4.11 iNKT Cell Frozen Sections

A) iNKT cell frozen sections stained with a 1:100 dilution of CD45. **B)** iNKT cell frozen sections stained with a 1:30 dilution of 6B11.

4.4 iNKT Cell Cytospin Analysis

In order to fully explore the staining potential of the 6B11 antibody, iNKT cytopins were also generated. A cytopsin is a unique cytology method that is specifically designed to concentrate a single layer of cells onto a clearly defined area of a glass slide. Once these cytopins were created using the specialised cytopsin centrifuge, it allowed for further analysis of the iNKT cells through immunocytochemistry. The cytopins also provided a technique for 6B11 antibody investigation without any possible disruption to iNKT cells by processing. Following H&E staining to confirm cell presence, preliminary immunoperoxidase staining of the cytopins was performed utilising a 1:100 dilution of the anti-CD45 antibody to clarify cell antigenicity. This was followed by immunoperoxidase staining using a 1:30 dilution of 6B11, with images captured on the light microscope. A strong positive result in the presence of anti-CD45 was obtained but with no significant staining on treatment with 6B11 (Figure 4.12).

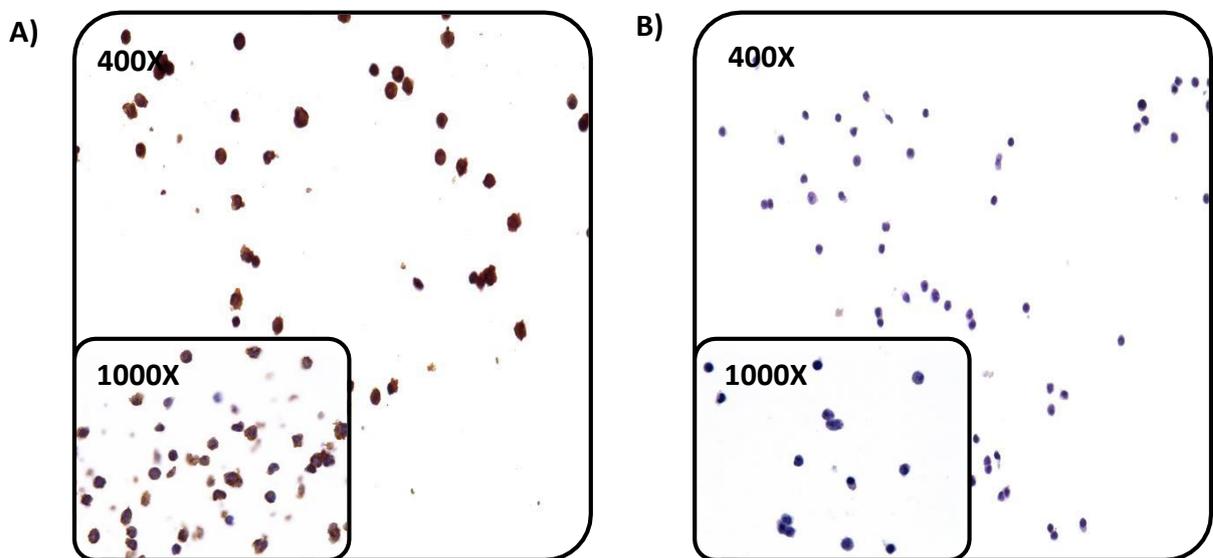


Figure 4.12 iNKT Cell Cytospins

A) iNKT cytopsin stained with 1:100 dilution of CD45. **B)** iNKT cytopsin stained with 1:30 dilution of 6B11.

4.5 Automated Analysis of 6B11

Automated staining was selected for use to fully elucidate the potential of 6B11 for iNKT identification in tissue sections. By employing automated staining, a standardised protocol was utilised, therefore any discrepancies developed through manual staining could be eliminated. An array of tissue sections were investigated including tonsil, liver, gastric, breast, colon, skin and appendix, all of which was performed on VENTANA automated slide stainers. In addition to the iNKT cell blocks created using UltraPure agar, a second iNKT cell block was generated utilising bacteriological agar. This was carried out to investigate any possible advantage of bacteriological agar, as it had been suggested that the UltraPure agar sections may be too hydrophobic for efficient binding of reagents. Sections from each block were stained on the automated stainer, therefore allowing the proficiency of both agars to be identified. Pre-treatment was performed on all sections using the Ventana cell conditioning solution CC1. This solution is capable of disrupting the covalent bonds formed by formalin in tissue, consequently allowing renaturation of protein molecules and increasing antibody accessibility. Pre-treatment also increases antibody binding significantly and improves signal to noise ratios. Immunoperoxidase staining was carried out using a 1:30 dilution of the 6B11 antibody on all sections and results were analysed on the light microscope. Disappointingly, no positive staining was observed in liver sections, despite the population of iNKT cells being most abundant in this tissue type (data not shown). Positive staining was however identified on specific cells in tonsillar and gastric tissue as demonstrated in Figure 4.13. This distinct staining of individual cells corresponds with the low population of iNKT cells found in the human body. Although no staining was observed on iNKT cells sections from either agar block (Figure 4.14), the positive result in the tonsillar and gastric tissue may be a significant breakthrough for the identification of iNKT cells in human tissue and indicates substantial potential for future investigation.

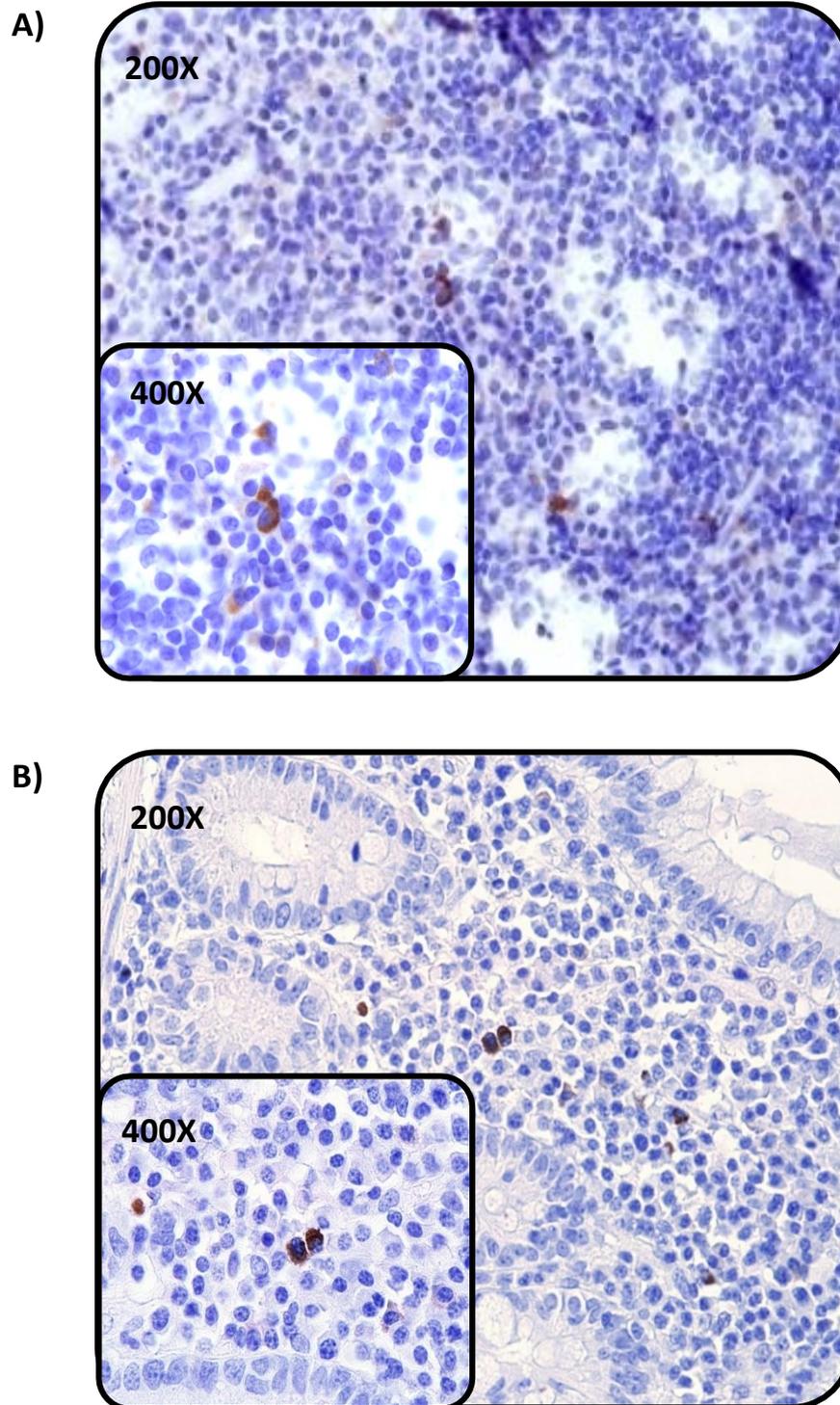


Figure 4.13 Automated Staining of Human Tissue Sections using 6B11 Antibody

A) Tonsillar tissue stained with a 1:30 dilution 6B11 antibody. B) Gastric tissue stained with a 1:30 dilution of the 6B11 antibody.

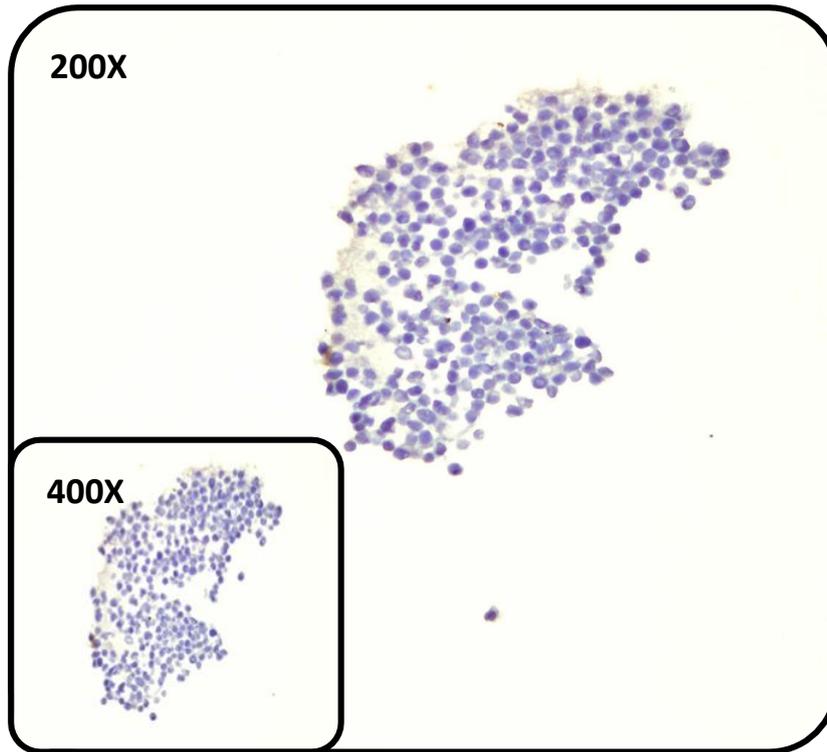


Figure 4.14 Automated Staining of iNKT Cells Sections
iNKT cells in UltraPure agar stained with a 1:30 dilution of the 6B11 antibody.

5. Chapter 5 - Discussion

Human papillomaviruses (HPVs) are ubiquitous, sexually transmitted viruses. HPV is the most common sexually transmitted infection in women (Aral et al. 2006) with more than 70% of the female population infected by this virus in their lifetime (Syrjänen et al. 1990). Although the majority of infections are usually transient and asymptomatic, resolving spontaneously without causing disease, untreated persistent infection may lead to precancerous lesions, potentially progressing to cervical cancer (Juckett et al. 2010). Cervical cancer is the second most prevalent cancer in women worldwide, with HPV infection found in 99.7% of all cases and the most common high-risk type HPV-16 present in approximately 50% (Walboomers et al. 1999; Bosch et al. 2008). Consequently, it has been determined that persistent infection with high-risk HPV is a necessary prerequisite for the development of cervical cancer and its immediate precursor lesion CIN3 (Saslow et al. 2012). Despite the high incidence of HPV infection and its associated malignant diseases, there is no effective antiviral agent available for therapy at present. Current treatment for high grade CIN is limited to cryotherapy, laser treatment and most commonly surgical excision. Loop electrosurgical excision procedure (LEEP), also referred to as large loop excision of the transformation zone (LLETZ) is a procedure accomplished using a high-frequency alternating current (radiofrequency) and thin wire loop electrodes (Apgar et al. 1992). This technique has many advantages including low cost, high success rate and ease of use. It can also be performed using only local anaesthetic, producing quality specimens for cytologic evaluation and carrying a low risk of affecting childbearing ability (Mayeaux & Harper 1993). In addition to LEEP, prophylactic HPV vaccines (Gardasil and Cervarix) have been shown to be effective in the prevention against HPV infection, however they lack any therapeutic efficacy against pre-existing HPV infection or pre-malignant lesions (Harper 2009). Therefore, the detection of additional therapeutic agents against HPV infection and preventative methods to eradicate HPV-related malignancies without surgical

manipulation would be a ground-breaking discovery. Investigation into the regulation of HPV gene expression and the role of specific cellular proteins in the HPV life-cycle may assist in this development.

Human papillomaviruses infect epithelial cells and depend on epithelial differentiation for completion of their life cycle (Doorbar 2005). Although the early HPV genes are expressed throughout the cervical epithelium, production of the L1 and L2 late genes is restricted to terminally differentiated keratinocytes, in the upper layers of the epithelium (zur Hausen 1996). As the L1 and L2 structural proteins are highly immunogenic and can induce an immune response, it has been suggested that suppression of these proteins in the lower layers of the epithelium allows the virus to escape the host's immune surveillance, resulting in persistence of infection (Scheurer et al. 2005). Subsequently, it is speculated that activation of L1 and L2 late gene expression in the persistently infected cells would expose the HPV-infected cells for recognition by the host's immune system.

One aim of our study was to elucidate the mechanism of HPV-16 gene regulation, with our main objective to investigate an array of small molecule drugs and their capabilities of modulating HPV-16 late gene expression. This was performed by treating two stable cell lines with the small molecule drugs in question. The cell lines utilised were previously created for HPV late gene research utilising 2 reporter plasmids pBEL and pBELM, separately introduced into HeLa cells. Similar to the HPV-16 genome during an infection, pBEL transfected into proliferating cells expresses high levels of the early genes whereas expression of late genes is undetectable. In pBELM, the splicing silencer elements adjacent to the splice site SA5639 have been mutated to reduce the silencing on this splice acceptor therefore activating late gene expression (Zhao et al. 2004). In order to detect the HPV-16 L1 gene, the plasmids pBEL and pBELM were modified by replacing the L1 late gene with the easily detectable reporter gene chloramphenicol acetyltransferase (CAT) (Orrù 2012). Evaluation of CAT expression levels in

both the pBELCAT and pBELMCAT stable cell lines confirmed that pBELCAT, as expected, produced very little CAT whereas pBELMCAT (in which the mutation that reduces the negative regulation on the splice site present in the late region is introduced), efficiently produced CAT (Orrù 2012). This result established pBELCAT and pBELMCAT derived stable cell lines as functional tools for L1 late gene investigation. Employing the pBELMCAT31 cell line and the pBELCAT67 cell line, which produce considerably high and low levels of CAT respectively, analysis of selected small molecule drugs was undertaken in our investigation. Small molecules have the ability to associate with, or bind to, a protein utilising specific mechanisms and may also modulate the protein's function through activation or inactivation. These small molecule drugs are most commonly synthesised using chemical reactions. They are well-characterised and can be easily purified and analysed with routine laboratory tests. The discovery that all-*trans* retinoic acid is an effective inducer for attaining complete remission in patients with acute promyelocytic leukaemia established the concept of utilising small molecule drugs in cancer therapy (Huang et al. 1988). Several small molecules are now widely recognised as drugs for the treatment of a variety of cancer types (Collins & Workman 2006). Studies are also ongoing towards the identification of small molecule drugs that specifically target and inhibit vital HPV protein functions and viral-host protein interactions (D'Abramo & Archambault 2011). The development of a novel antiviral agent for the treatment of HPV infection would be a remarkable discovery. For this reason, it was of interest to us to investigate an array of small molecules that have previously been determined to interfere with cellular proteins involved in the regulation of HPV-16 late gene expression.

The first molecule employed for investigation was TPA. TPA has been proven to activate protein kinase C (Blumberg 1988), with studies showing that activators of protein kinase C, including TPA, have the ability to induce HPV-31b late gene expression (Hummel et al. 1995; Meyers et al. 1992). Therefore we wished to determine TPA's potential to induce HPV-16 late

gene expression. Utilising the CAT ELISA assay, the influence of TPA on CAT concentration, and in turn L1 late gene expression was determined. Although no significant effect on CAT expression was detected in the pBELCAT67 cell line, a dose-dependent increase in CAT was observed in pBELMCAT31 across multiple experiments. Our results demonstrate that a concentration of 800ng/ml of TPA has the potential to induce a maximum 3.78 fold induction of CAT expression on the pBELMCAT31 cell line, a discovery which was deemed statistically significant. This finding indicates that TPA has the ability to induce late gene expression, potentially through the activation of protein kinase C. Research has determined that PKC is predominantly expressed in squamous epithelia or epithelia from which squamous cell carcinomas arise, with particular isoforms playing a crucial role in the signalling of cell differentiation (Kashiwagi et al. 2002; Dlugosz & Yuspa 1993). Although the mechanism of action by which PKC could up-regulate the HPV promotor is not clearly defined, it is suggested that PKC may directly phosphorylate and activate specific transcription factors involved in the regulation of the HPV late promotor (Tommasino 2011). Based on the clear increase in L1 expression illustrated in our results, TPA can be most definitely deemed a potential candidate as a novel drug for treatment of HPV infection. However, the physiological relevance of the dose administered is a factor which must be considered. Previous phase I clinical trials have been undertaken on patients with relapsed or refractory malignancies. The starting dose utilised in these studies was 0.063 mg/m², with 0.125 mg/m² established as the maximal dose of TPA tolerated (Schaar et al. 2006). Further investigations have also been performed in which 0.063 mg/m² TPA was again administered, with the amount of TPA-like activity in blood ranging from 0.31-5.3 ng/ml immediately after the infusion and from undetectable to 3.6 ng/ml 2 hours later (Strair et al. 2002). It has also been determined that the extent of the adverse effects associated with TPA correlates with the dose received, with effects observed much milder in conjunction with lower levels of the drug (Han et al. 1998). Although these investigations

confirm the feasibility of TPA administration to humans, the concentrations utilised are far lower than that in our study. Therefore additional research is required into the *in vivo* effects of TPA and the most tolerable therapeutic dose.

The increase of CAT expression in pBELMCAT31 on treatment with TPA reflects results from previous investigations carried out by Orrù et al. However, these earlier findings also demonstrated an increase of CAT expression in pBELCAT67. Although the base levels of CAT expression in pBELCAT67 were shown to be notably low, induction on treatment with various small molecules drugs including TPA, TA and VPA was proven (Orrù 2012). Therefore, the absence of late gene induction in pBELCAT67 on treatment with TPA and indeed all drugs tested in our investigation indicates a potential problem with this cell line. As its behaviour did not coincide with previous analysis, additional examination of this cell line should be performed before further use. RT-PCR may be used to determine that the integrated pBELCAT plasmids are producing the expected early and late mRNAs, thus confirming the integrity of this stable reporter cell line.

Tannic acid has previously been attributed with the ability to increase the expression of polypyrimidine tract-binding protein in a dose-dependent manner (Bian et al. 2009), a protein which is linked to the activation of HPV-16 late gene expression during the viral life cycle (Somberg et al. 2008). Therefore, we wished to investigate the direct effect of TA on HPV-16 late gene expression. However, our results showed no remarkable influence on CAT expression in either pBELMCAT31 or pBELCAT67 on treatment with TA. Fold change remained relatively constant with no significant fluctuations observed. Contrary to previous results obtained by Orrù et al, our findings suggest no potential for the use of tannic acid as an inducer of late gene expression.

The effect of valproic acid on late gene expression was also investigated. Research has shown that VPA increases expression of ASF/SF2 (Harahap et al. 2012), a protein proven to regulate

splicing of HPV and induce CAT expression (Somberg & Schwartz 2010). We therefore speculated that through treatment of the stable cell lines we could replicate this influence of VPA on ASF/SF2 and consequently upregulate HPV-16 late gene expression. On treatment of the pBELMCAT31 cell line with 0.5mM VPA, no significant change in CAT expression was observed, while a high concentration of 100mM caused a substantial decrease in CAT expression. Meanwhile, there was no important influence noted on treatment of the pBELCAT67 cell line. Similarly to TA, we can conclude that our results show no potential for VPA as a novel inducer of HPV late gene expression.

To investigate the possible synergistic effects of combinations of TPA, TA and VPA on late gene expression, the pBELMCAT31 and pBELCAT67 cell lines were treated with a combination of the three drugs at previously defined optimal concentrations. As before, no noteworthy induction of CAT expression was observed on the pBELCAT67 cell line. However, the effects detected on pBELMCAT31 treatment reinforce our earlier analysis. The results obtained indicate an increase in CAT expression with combinations in which TPA was present and a decrease in CAT expression in its absence. This implies that TPA alone is the only molecule in this combination capable of significant CAT induction on the pBELMCAT31 cell line and consequently the only evidential inducer of late gene expression. Future investigations should therefore focus predominantly on the role of TPA as a potential treatment for persistent HPV infection.

We also explored the impact of retinoic acid on late gene expression. RA was selected for investigation as it is known to down-regulate the expression of hnRNP A2/B1 (Liang et al. 2011). hnRNP A2/B1 is an RNA binding protein up-regulated in many tumour cell lines. Studies have also demonstrated that knock-down of hnRNP A2/B1 induces HPV-16 late gene expression, while overexpression of the protein further suppressed HPV-16 late gene expression (Li, Johansson, et al. 2013). We therefore questioned whether treatment of the stable

cells lines with RA would induce HPV-16 late gene expression, potentially through the down-regulation of hnRNP A2/B1. The concentrations of retinoic acid selected for investigation were previously utilised in studies to induce the differentiation of teratocarcinoma cells in culture (Edwards & McBurney 1983). Results obtained in our investigation proved highly significant, with a remarkable increase in CAT expression observed on treatment of the pBELMCAT31 cell line with lower concentrations of RA. A dose-dependent increase in CAT expression was observed with concentrations of 10^{-9} M- 10^{-7} M RA, with a 6.4 and 7.9 fold induction of CAT expression detected on treatment with 10^{-7} M of the drug in 2 individual experiments. In the presence of the 10^{-6} M and to a lesser extent 10^{-5} M RA, a decrease in CAT expression was noted. However, this may be linked to the volume of DMSO utilised in the creation of these drug aliquots. DMSO has the potential to be toxic to cells when utilised in excessive volumes, therefore the increased levels present with the higher concentrations of the drug may have contributed to the abundant decline in CAT. For future analysis, the viability of the cells following incubation with high volumes of DMSO should be determined prior to performance of the CAT ELISA. As before, treatment of pBELCAT67 with RA had no impact on CAT expression. However, due to the significant increase of CAT expression with pBELCAT31, it can be concluded that retinoic acid has the potential to act as a novel drug in the up-regulation of late gene expression and consequently has promise as a treatment for persistent HPV infection. This may be as a result of retinoic acid down-regulating hnRNP A2/B1 expression. The hnRNP A2/B1 protein is widely recognised as a splicing factor that binds to splicing silencers and inhibits splicing to both 5'- and 3'- splice sites (Bilodeau et al. 2001; Hutchison et al. 2002). Specific research indicates that hnRNP A2/B1 has an inhibitory effect on the HPV-16 late 5'-splice SD3632. The late 5'-splice site SD3632 is employed exclusively for production of spliced L1 mRNAs, however it is negatively regulated by two AUAGUA motifs located immediately upstream of SD3632. As these sites interact specifically with hnRNP A2/B1,

knock-down of this protein has been proven to induce HPV-16 late gene expression (Li, Johansson, et al. 2013). The negative influence of hnRNP A2/B1 is further exposed by its high expression in cervical cancer cell. Therefore, it can most definitely be suggested that retinoic acid has a future for the induction of late gene expression through the possible down-modulation of hnRNP A2/B1.

Clinical trials previously carried out to investigate the potential of all-*trans* retinoic acid as a differentiation therapy for acute promyelocytic leukaemia (APL) utilised 45 mg/m² per day for 90 days, with results confirming that this concentration of RA has the ability to induce remission in this most severe form of leukaemia (Castaigne et al. 1990). Additional studies administered oral all-*trans* RA at a dose of 60-80 mg per day to patients with APL, with a higher dosage (100-120 mg/d) given to certain patients who relapsed. All patients who received RA as a single agent responded to the drug, with only some mild side effects observed. These side effects were usually alleviated following treatment and after the dose of RA was reduced (Chen et al. 1991). Therefore, although the concentrations utilised in our study were less than this, the lowest therapeutic dose of RA would be recommended for *in vivo* administration in order to avoid any adverse events.

Retinoic acid exerts its biological effects by binding to specific nuclear retinoid receptors. For this reason, we decided to investigate receptor presence in various grade cervical lesions. Previous investigations carried out on retinoic acid receptor presence in cervical epithelium discovered a loss of RAR- β expression in cervical cancer cell lines. Basal cell RAR β mRNA levels were shown to be high in normal cervical cells and further induced on addition of retinoic acid, while basal RAR β mRNA levels were revealed to be low in the cervical carcinoma cells, and were either not induced or only slightly induced by treatment with retinoic acid (Geisen et al. 1997). The results obtained by Geisen et al. indicated that the abnormal down-regulation of *RAR β* gene expression may be an essential step in the multifactorial process of cervical

carcinogenesis (Geisen et al. 1997). Additionally, Geisen et al. also publicised that the ligand-dependent activation of *RAR β* gene transcription is specifically triggered by *RAR α* . Interestingly, it was also noted that the unusually low *RAR β* gene expression in tumour cells did not apply to *RAR α* , with *RAR α* levels similar to those of normal cells (Geisen et al. 1997). Utilising anti-*RAR α* and anti-*RAR β* antibodies, our aim was to identify the alterations in receptor presence in various grade cervical tissue. TMA slides containing cores from tissue showing no evidence of malignancy, low grade squamous intraepithelial lesions, high grade squamous intraepithelial lesions and squamous cell carcinoma were analysed for receptor presence. Results obtained showed that *RAR α* and *RAR β* are both expressed in the upper layers of the epithelium in normal cervical tissue. While *RAR α* appeared to be weakly expressed, strong staining in the presence of anti-*RAR β* was displayed. Interestingly, no positive staining was observed in the basal layer with either the anti-*RAR α* or the anti-*RAR β* antibody. In the LSIL, HSIL and SCC sections, a distinct absence of *RAR α* and *RAR β* expression was observed in the epithelium, with strong stromal expression of *RAR β* displayed in LSIL and HSIL. This loss of *RAR β* expression in precancerous and cancerous lesions was somewhat expected, as previous studies have also identified a similar pattern of expression in cervical cancer cell lines. However, in contrast to the findings by Geisen et al, in which *RAR α* presence in cervical cancer cells were comparable to those in normal cells, our results display a complete loss of *RAR α* in the epithelial layers of cervical lesions. Further studies have however elucidated that RARs are expressed in normal cervical epithelia but that their expression is reduced in cervical lesions, including CIN1, CIN2 and CIN3 ,with loss of *RAR α* in 55.8% and *RAR β* in 64.7% of cases (Xu et al. 1999).

On analysis of the various cores present in our TMA sections, we determined an almost uniform loss of expression of both receptors across the various grade lesions. This distinction in expression levels between normal and infected epithelium may indicate a role for retinoic

acid receptors as biomarkers in clinical cervical cancer chemoprevention trials. The influence of RAR expression levels on the induction of HPV late gene expression by retinoic acid is an area which requires additional examination.

The loss of RAR α and RAR β expression in the epithelium of cervical cancer lesions may possibly correlate with HPV L1 capsid protein suppression. Previous research has demonstrated a decrease in L1 presence across cervical cancer lesions, with one study identifying that the L1 was positive in 63.6 % of low-grade CINs and 9.1 % of high-grade CINs with no expression observed in cervical SCC (Izadi-Mood et al. 2014). Furthermore, studies have shown that failure to detect L1 in cervical tissue correlates with progression of the lesion, as results indicate reduced expression of L1 capsid proteins in HPV positive HSIL with no significant reduction of L1 expression in HPV positive LSIL (Balan et al. 2009). Immunocytochemical detection of the HPV L1 capsid protein therefore has prognostic value for cervical lesions. From our findings, the clear loss of RAR α and RAR β expression in cervical neoplastic tissue may also have potential to be utilised in a similar capacity.

The presence of RAR α and RAR β on individual cells from cervical cancer lineage was investigated through cell block staining. pBELMCAT31 and pBELCAT67 HPV-infected cell lines showed positive staining with both the RAR α and RAR β antibodies while C33A, a cervical carcinoma cell line which does not contain any HPV copies, showed staining with RAR β only. This is an interesting observation considering expression of both receptors was previously shown to be lost in both premalignant and malignant lesions. The significance of this finding also calls for further exploration. Furthermore, on inspection of the TMA sections, it was also observed that individual intraepithelial mononuclear cells stained positive with anti-RAR β in cervical tissue sections. This incidental finding has potential to act as an internal positive control when staining sections with the anti-RAR β antibody. Additionally, adipose tissue also appeared positive on staining with both the anti-RAR α and anti-RAR β antibodies.

This again may be of use in a control capacity with both RAR antibodies prospects for future investigation and identification of adipose tissue.

A further aim of our study was to investigate the immune response to HPV infection, with particular emphasis on the role of iNKT cells. iNKT cells are a subset of T lymphocytes which are characterised by expression of a TCR comprised of V α 24-J α 18 paired with V β 11. iNKT cells recognise glycolipid and lipid antigens presented by CD1d, a non-classical MHC class I-like molecule, with ligation of the iNKT cell TCR leading to rapid and copious secretion of Th1 and Th2 cytokines (Bendelac et al. 2007). iNKT cells play a central role in regulating immune responses and are recognised to form an important link between the innate and adaptive immune systems. Though the iNKT cell population ranges only between 0.01%–1% of all CD3+ lymphocytes in humans, their activity is imperative in many disease settings including infectious disease, allergy, autoimmunity and tumour surveillance (Juno et al. 2012). Providing an interesting link between the molecular investigation and iNKT cell analysis is the proven effects of retinoic acid on NKT cell activity. Research shows that RA can differentially regulate the secretion of numerous effector cytokines by NKT cells in hepatitis (Lee et al. 2012) and also down-modulate IFN- γ expression by activated NKT cells (Chang & Hou 2015). Furthermore, retinoic acid has been previously identified with the ability to promote the proliferation and activation of NKT cells indirectly *in vitro* by increasing CD1d expression on antigen presenting cells (Chen & Ross 2015). This study by Chen & Ross speculates that the level of CD1d on antigen presenting cells may influence their capacity for antigen presentation, consequently affecting the efficacy of NKT cells. Although the role of iNKT cells in HPV-infected lesions has yet to be uncovered, studies have revealed that expression of CD1d is significantly suppressed in HPV-infected tissue (Miura et al. 2010). This reduced expression of CD1d indicates a vital strategy of immune evasion developed by HPV to elude the protective functions of iNKT cells in the early stages of infection. The decrease in CD1d levels would

inevitably impact upon the population of iNKT cells in HPV-infected cervical tissue. However, since there is currently no available method for the detection of iNKT cells in human tissue sections; their number in cervical tissue is unknown. Our objective therefore was to create a novel protocol for the detection of iNKT cells in human tissue, with this method ultimately employed to enumerate iNKT cells in HPV-infected cervical cancer samples. Should a method of identification in tissue be determined, it would have a significant impact on future iNKT cell research. The antibody selected for iNKT cell identification was 6B11, a novel monoclonal antibody generated for the precise detection of human iNKT cells. 6B11 is specific for the invariant CDR3 loop of the human V α 24J α 18 TCR α chain and has the capacity to be used unaccompanied to recognise all T cells expressing this α chain as well as in combination with anti-V α 24, anti-V β 11 or anti-CD3 for highly specific and sensitive iNKT cell detection (Montoya et al. 2007). As this antibody is used exclusively for iNKT cell characterisation via flow cytometry, its efficacy was first confirmed through this method. Successful analysis of 4 PBMC samples as well as an expanded population of iNKT cells was achieved, with the percentage of iNKTs per population PBMC's calculated at 0.81%- 2.43% and the percentage per population of expanded iNKT's calculated at 92.7%, corresponding with previous findings. We therefore established the 6B11 antibody as a competent means of specific iNKT cell analysis. Following on from this, our focus was on the exploitation of this 6B11 antibody for use in tissue section staining. Prior to immediate investigation in human tissue sections, we decided to first create our own cell block. This block contained a pure population of iNKT cells and was an innovative tool for the optimisation of immunohistochemistry. The protocol utilised called for fixation of cells in formalin and suspension in agar before wax embedding. However, as the gold standard for immunohistochemistry is the use of paraffin-embedded tissue sections, we were aware that staining conditions may not be identically replicated. Optimisation was first performed by creating a Jurkat T cell block and staining with anti-CD45 antibody, a common

leucocyte marker, limiting the use of the more precious iNKT cells and 6B11 antibody until an optimised protocol was achieved. Images obtained showed a high standard of both immunoperoxidase and immunofluorescent staining, with clear membranous staining of the Jurkat cells observed. iNKT cell blocks were consequently created and immunofluorescent staining was again successfully completed utilising the CD45 antibody. Images were captured on both the fluorescent and confocal microscope with distinct, high quality staining detected. The effective creation of the cell blocks and subsequent successful staining was a noteworthy achievement. Due to the low distribution of iNKT cells in the body and the resultant difficulty of locating a positive control for their depiction in human tissue, the iNKT cell blocks undoubtedly have potential for use in future investigation.

Following on from this, we attempted to stain the iNKT cell block sections with the 6B11 antibody. As this antibody is used exclusively for flow cytometry, it was understood that optimisation for use in immunohistochemistry was an ambitious task. For the first attempt the immunofluorescent technique as again employed, as results previously obtained with anti-CD45 proved very successful and returned higher quality images than those achieved with immunoperoxidase staining. A 1:100 dilution of 6B11 was initially employed for iNKT cell staining of the agar cell block sections, however on examination under the confocal microscope no positive result was detected. At this point, additional means of investigation were then analysed, including frozen cell block sections and iNKT cell cytopins. Our hypothesis was that both of these methods would eliminate any possible interference to iNKT antigenicity which may have been caused to the cell block sections by the processing procedure. The identification of cells in both the frozen sections and the cytopins was first confirmed by anti-CD45 staining. The 6B11 antibody was then investigated, using a 1:30 dilution to increase the possibility of detecting cell presence. However, no positive results were returned.

By implementing examination on the wax-embedded agar iNKT cell block sections, the frozen

iNKT cell sections and the iNKT cytopins, we were confident in the fact that a thorough assessment had been performed and that all immediate avenues had been exhausted. The use of automated staining was then suggested for use. Automated staining provides a standardised technique and ensures that any discrepancies developed through manual staining are eliminated. Furthermore, it also employs more powerful reagents for antigen retrieval, which are proven to significantly increase antibody binding. To provide for a widespread assessment, tonsil, liver, gastric, breast, colon, skin and appendix tissue sections were investigated for iNKT cell presence, again utilising a 1:30 dilution of 6B11. Positive results were obtained using the automated protocol. Distinct cells with lymphocytic morphology were identified in tonsillar and gastric tissue by the immunoperoxidase technique, with high-intensity staining clearly observed on the images obtained. Interestingly, no positive cells were detected in liver sections, a surprising result due to the fact that liver has previously been confirmed as the tissue with the highest concentration of iNKT cells in the human body (Kenna et al. 2003). For future analysis of iNKT cell distribution, more extensive exploration of the liver should be performed. iNKT cell block sections were also evaluated using the 6B11 antibody on the automated stainer. Two cell blocks were created, one utilising UltraPure agar as initially performed and one utilising bacteriological agar. It was suggested that the UltraPure agar sections may create a hydrophobic environment and prevent efficient binding of the staining reagents. However, no positive cells were detected on either cell block type with automated analysis using 6B11, indicating no specific advantage of the bacteriologic agar. Although the lack of positive staining on the iNKT cell block sections was a disappointing result, the discovery of the iNKT cells in the tonsillar and gastric tissue sections is of striking importance and indicates a considerable potential for the 6B11 antibody in the identification of iNKT cells in human tissue sections.

As pBELMCAT31 has been successfully determined as a suitable stable cell line for assessing the effects of small molecule drugs on HPV late gene expression, future work may include the

use of small molecule drug libraries for a more extensive evaluation. A diverse selection of compound libraries containing a variety of drugs are available for purchase, providing a high throughput facility for the validation of new drug discoveries. This would be an interesting investigation and may lead to the detection of additional HPV late gene inducers. As previously discussed, further examination is required on the pBELCAT67 cell line to confirm its integrity before future analysis is carried out.

With regards the potential of 6B11 as a novel means of identifying iNKT cells in human tissue, confirmation that the individual cells identified in the tonsillar and gastric tissue sections are indeed iNKT cells should be performed. This could be completed through the use of CD1d tetramers, which have been developed for the identification and enumeration of CD1d-restricted NKT cells by flow cytometry. Furthermore, if these tetramers showed a similar staining pattern to the 6B11 antibody, they too could be a prospect as an innovative iNKT cell identifier in human tissue sections.

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Appendix

Phosphate Buffered Saline (PBS)

1 PBS tablet (Oxoid) for every 100ml distilled water and autoclave

0.02% EDTA in PBS

0.2g EDTA (BDH)

Make up to 1 litre with PBS (appendix) in a volumetric flask

Trypsin with EDTA

10ml 2.5% Trypsin

10ml 0.02% EDTA (appendix)

Mix by inverting

Cell Lysis Buffer/ Anti-CAT-DIG/ Anti-DIG-POD/ ABTS Peroxidase Substrate

As per manufacturer's instructions

CAT ELISA assay kit (Roche). Catalogue Number: 11363727001

Mayer's Haematoxylin

2g haematoxylin (Merck)

100g aluminium sulphate (BDH)

0.4g Sodium Iodate (EMD Chemicals)

Make up to 2 litres with distilled water and leave overnight

2g citric acid (BDH)

Mix and Boil for 5 minutes

Allow to Cool and filter before use

1% Eosin

1g eosin powder (Merck) dissolved in 100ml water

Spirit (96%)

960ml absolute alcohol (Merck) made up to 1 litre with distilled water

Alcohol (70%)

700ml absolute ethanol (Merck) made up to 1 litre with distilled water

Acid Alcohol Solution (1%)

1ml Hydrochloric acid

100ml 70% ethanol

Mix well

10mmol/l Citrate Buffer (pH6)

2.94g tri-Sodium citrate (BDH)

Add approximately 800ml distilled water

Adjust to pH 6 using 2M NaOH

Make solution up to 1 litre

3% Hydrogen Peroxide in Methanol

1ml 30% hydrogen peroxide (Sigma)

9ml Methanol (Sigma)

0.02% 2, 4 diaminobenzidine (DAB)

Add 16ml PBS into 100mg container of DAB (Sigma)

Mix well and aliquot into desired quantity

Normal Horse Serum/ Biotinylated Secondary Antibody/ ABC reagent

As per manufacturer's instructions

Vectastain[®] Elite ABC kit (Vector laboratories). Catalogue Number: PK-6200

PBA wash buffer

PBS (appendix)

0.1% BSA

0.1% sodium azide (NaN₃)

2% Liquid Agar

2g UltraPure Agar (Invitrogen) / Bacteriological Agar (Lab M)

100ml dH₂O

Boil in microwave until dissolved

Cool to 60°C