Cellular Immune Responses to Human Papillomavirus (HPV) type 16 at the Cervix of Women with HPV-Associated Squamous Intraepithelial Neoplasia

By

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Declaration

I, Michelle Milner, hereby declare that the work on which this thesis is based is original (except where acknowledgements indicate otherwise) and that neither the whole work, nor any part thereof, has been, is being or is to be submitted for another degree in this or any other university.

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Contents

Index to Tables

Index to Figures

Abbreviations List

mM	micro moles
µg	micrograms
mg	milligrams
µl	microlitres
ml	millilitres
min	minutes
hr	hours
kDa	kilo daltons
RT	room temp (24°C)
7AAD	7 Amino Actinomycin D
APC	Allophycocyanin
APC	Antigen Presenting Cells
BCG	*M. Bovis* Bacillus Calmette Guerin
BFA	Brefaldin A
BSA	Bovine Serum Albumin
CANSA	Cansa Association of South Africa
CBA	Cytometric Bead Array
CChr	CyChrome
CIN	Cervical Intraepithelial Neoplasia
CMI	Cell Mediated Immunity
CO	cut off
CTL	Cytotoxic T Lymphocytes
DC	Dendritic Cells
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorter
FCS	Fetal Calf Serum
FITC	Fluorescein
FSC	Forward Scatter
ICC	Intracellular Cytokine
IFNγ	Interferon - gamma
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IL-13	Interleukin - 13
HC	Hybrid Capture
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
LAMP	Lysosomal Associated Membrane Glycoprotein
LC	Langerhans Cells
LCR	Long Control Region
MHC II/I	Major Histocompatability Class I or II
NCI	National Cancer Institute
NK	Natural Killer cells
OD	Optical Density

ORF	Open Reading Frame
PAGE	Polyacrylamide gel electrophoresis
Pap	Papanicolaou
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PMA	Phorbyl Myristate Ester
pRB	protein Retino Blastoma
PV	Papillomavirus
RBC	Red Blood Cells
RLB	Reverse Line Blot
RLU	Relative Light Units
SA-HRP	streptavidin conjugated horse radish peroxidase
SD	Standard Deviation
SEB	Staphyllococcus Enterotoxin B
SEM	Standard Error of the Mean
SSC	Side Scatter
TB	Tuberculosis
TCR	T Cell Receptor
Th1/2/0	T Helper cells type 1, 2 or 0
TNFα	Tumour Necrosis Factor-alpha
TZ	Transformation Zone
VLP	Virus Like Particle
WHO	World Health Organisation
WPBTS	Western Province Blood Transfusion Service

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ABSTRACT

Cervical cancer is the most common cause of cancer-related death in black South African women. Human papillomavirus (HPV) has been found to be a necessary causative agent of cervical cancer and has been reported to be associated with 84% of cervical intraepithelial neoplasia (CIN). HPV type 16 (HPV-16) is the most prevalent HPV type associated CIN and cervical cancer with ~56% of women with cervical disease being infected with HPV 16. Yet studies have shown that 47-85% of CIN regressed, suggesting that perhaps an effective immune response could result in HPV clearance and lesion regression. Since HPV infection does not disseminate and there is no systemic phase of infection, it is hypothesized that local cervical immune responses are important in lesion regression and clearance of HPV infection. There are, however, very few studies of mucosal immune responses to HPV infection. The aim of this study was to determine the type of mucosal immune response elicited by the CD4 and CD8 T cell subsets to HPV infection at the cervix of women diagnosed with varying grades of CIN and to compare these to systemic responses. One hundred women with varying grades of histologically confirmed CIN attending the Groote Schuur Hospital Colposcopy Outpatient Clinic were enrolled into the study. Cervical T cells were isolated from the endocervix/transformation zone of these women using a Digene cytobrush. Of these 100 women, only 33 were found to have suitable cervical cytobrush specimens for analysis of mucosal T cell responses based on CD3+ T cell numbers and absence of red blood cell contamination.

Peripheral and cervical T cells were stimulated with the major capsid protein L1 of HPV-16 which self assembles into virus like particles (VLP) and the major

HPV-16 oncogenic protein E7 and the subsequent production of intracellular cytokines (ICC) was detected through flow cytometry.

Women with CIN 1 consistently had the strongest CD4 IFN-γ (but not necessarily CD8 T cell) responses at the cervix to HPV-16 antigens compared to women with no cervical neoplasia or those with more severe disease (CIN 2/3). This was observed particularly if one focused on women with active HPV-16 infection but also if one looked at the group as a whole (irrespective of the type of HPV causing infection). There was a significant trend towards decreasing Th2 responses (IL-13 production) with increasing disease severity, in cervical CD4 and CD8 T cells to both HPV antigens (L1 and E7) if one looked at the group as a whole. Conversely, this trend was reversed with increasing Th2 responses with increasing disease severity in the women with active HPV-16 infection. When PBMC responses from women with HPV-16 DNA at the cervix were compared with those that were infected with other HPV types, the HPV-16 DNA+ women generally produced a Th1 dominant response (more IFN-γ and less IL-13) which changed to a Th2 dominant response with increasing disease severity (particularly for E7 antigen). In contrast, the HPV-16 negative women (infected with other HPV types) showed a complete reversal of this profile with increasing IFN-γ responses and decreasing IL-13 responses with increasing disease grade. The only cervical immune response that correlated with disease grade in this study was that both CD4 and CD8 T cell IL-13 production decreased with increasing disease severity but this was observed in both women infected with HPV-16 and those infected with other HPV types. Although evidence of HPV-16 specificity is lacking, the results do imply that Th2 dominant responses are associated with a "healthier" disease state and IL-13 responses (possibly driving a protective antibody-mediated response) diminish with increasing disease grade. The

magnitude of Th1 responses elicited by cervical T cells was generally lower than those produced by T cells from peripheral blood. This study shows that the cytobrush method of obtaining cervical lymphocytes combined with intracellular cytokine analysis and flow cytometry is a non-invasive and potentially useful approach to studying immune responses in the genital tract and also suggests various modifications to improve the low numbers of suitable cervical specimens for study.

CHAPTER ONE:

LITERATURE REVIEW

1.1 The Prevalence of Cervical Cancer in the Female Population

Cancer of the cervix is a worldwide problem. It is estimated that in 2002 a total of 493,243 women were diagnosed with cervical cancer (Ferlay et al., 2002). International cancer registries have estimated that it is the second most prevalent cancer disease among women (after breast cancer), making up 16.8% of the total number of cancer cases reported (International Association of Cancer Registries, 2002). Here in South Africa, the statistics are just as alarming. The Cancer Association of South Africa announced in 2002 that cervical cancer has now become the leading cause of cancer related deaths in black African women (CANSA, 2002). This means that South African women are now at higher risk of suffering from cervical cancer than they are of breast cancer. It was also pointed out that of all the women between the ages of 15 and 29 yr who were diagnosed with cancer, 13.4% were suffering with cervical cancer, making it the most common cancer in that age group. Therefore this is an area of growing concern, especially for South Africans, and there is much research being undertaken to understand the disease itself and various other factors, which may either predispose women to, or protect them from, cancer progression.

1.2 Progression from CIN to Cancer of the Cervix

Many clinics in South Africa offer screening to detect early onset of cervical cell abnormalities. The initial screening is usually performed by papanicolaou (pap)

smear, which involves smearing a scraping of the cervical cells onto a glass slide for analysis of cellular abnormality under a microscope (World Health Organisation, 1988). This is referred to as a cytological diagnosis. If unusually high cellular abnormality is detected, the patient is referred for colposcopic or histological diagnosis, since these are both more accurate than pap smears. A colposcopy diagnosis involves washing the cervix with acetic acid and thereafter using a colposcoscope to visualize any white (HPV infected) lesions (WHO, 1988). A histological diagnosis makes use of the microscope to analyse the patients degree of cellular abnormality, but a biopsy specimen (2-4mm of cervical epithelium) is taken and therefore allows determination under the microscrope of the degree to which the epithelium is dysplastic (WHO, 1988). The lesions are graded under the microscrope according to the extent of the abnormal cells; if 1/3 of the epithelium is cytologically abnormal, the lesion will be graded as a cervical intraepithelial neoplasia grade 1 (CIN 1) (NCI Workshop, 1988; Shah and Howley, 1996). As the lesions get progressively worse (and therefore more of the cervical epithelium is classed as abnormal cell growth) the lesions will be graded CIN 2 and CIN 3, depending on the severity of the lesion.

A woman diagnosed with CIN is either immediately treated to prevent progression to cervical cancer or depending on the severity of the lesion, asked to return for a follow up visit at a later stage in order to check the lesion progression. This is due to the fact that not all cervical lesions will progress to cervical cancer. In fact studies have shown that 47-58% of grade 2 and 3, and up to 85% of grade 1 cervical lesions regress naturally (Chan et al., 2003; Iatrakis et al., 2004). Therefore although a woman is diagnosed with CIN this does not mean that the lesion will necessarily become cancerous.

This raises the question then, what other factors play a role in the progression or regression of CIN? Articles published in the interest of increasing public awareness regarding cervical cancer have cited the following as risk factors for progressive worsening of the cervical lesion: sexually transmitted diseases (including Human Papillomavirus [HPV], Human Immunodeficency Virus [HIV], Chlamydia and various Herpesviruses), high sexual activity with multiple partners, high parity, long term use of hormonal contraceptives, socioeconomic and ethnic differences (cervical cancer incidence is higher in less developed countries – although this is probably due to the improved and more accessible screening facilities in well developed countries which allow for earlier detection and treatment of cervical lesions), smoking and an inherited genetic predisposition to cancer (Ho et al., 1998; Well-Connected, 2002; Bosch and de Sanjosé, 2003).

1.3 The Biological Structure of the Cervix

Apart from the above-mentioned factors, the cervix itself is predisposed to abnormal cytological growth in the way that it is biologically structured. The cervix is the meeting point of the columnar endocervical cells from the uterus and the squamous ectocervical cells of the upper vaginal tract (Figure 1.1 and 1.2) (Crompton, 1976; Cartier, 1984). The area where these two very different cell types have to meet in the cervix is labelled the 'Transformation Zone' (TZ), and this is where most of the cervical lesions occur (Shah and Howley, 1996). At the TZ there is a high rate of cell turnover and this is conducive to cellular proliferation and tumour formation.

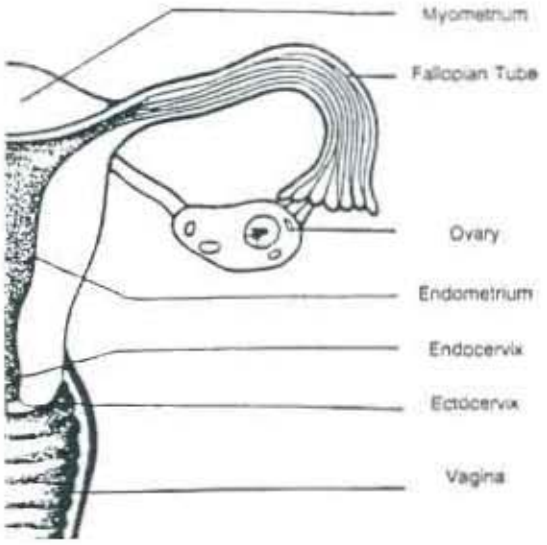


Figure 1.1. Structure of the Female Reproductive System, highlighting the transformation zone, the meeting point of the columnar endocervical and squamous exocervical cells (Taken from Kutteh, 1999).

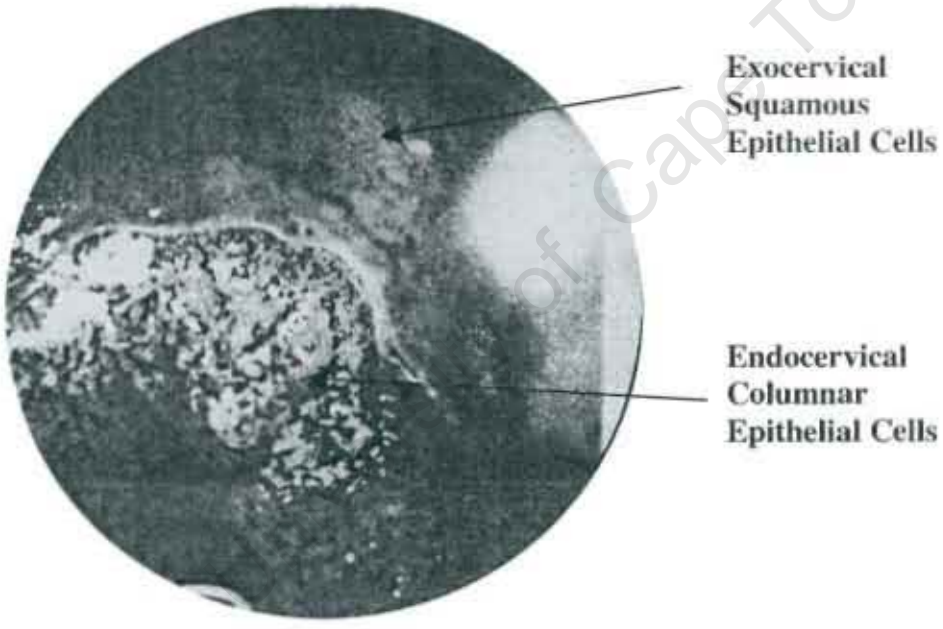


Figure 1.2. Picture of the squamo-columnar junction at the cervix of an 18 year old girl to illustrate the degree to which the two cell types (squamous and columnar epithelial cells) differ in structure (Taken from Crompton, 1974).

1.4 Role of HPV in Cervical Cancer

The greatest risk factor for progression to cervical cancer is Human Papillomavirus (HPV) infection. It has been reported that at least 94% of cervical cancers and greater than 84% of CIN were associated with HPV DNA (Kay et al., 2003; Chan et al., 2003). HPV infection has now been recognized as a necessary causative agent in progression to cervical cancer (Walboomers et al, 1999; Bosch et al, 2002a; Bosch and Sanjosè, 2003).

During a 3 yr longitudinal study of university students, 43% of the participants acquired new HPV infections and of the new infections clearance occurred within the first 12 months in 70% of the women (Ho et al., 1998). Although studies have shown that 70-80% of HPV infections can be cleared (Ho et al., 1998; Evander et al., 1995) other studies have shown that a small percentage (7%) do persist for as long as 5 years (Molano et al., 2003). The persistence of an HPV infection is the most important risk factor for CIN progression (Molano et al., 2003). Another important risk factor is the type of HPV infecting the cervix since not all HPVs have the same oncogenic potential (Schlecht et al., 2003). High-risk HPV infections result in lower rates of CIN regression than infections with low-risk HPV types. The most prominent and persistent HPV type, associated with CIN and cervical cancer, has been shown to be HPV 16 as shown in Figure 1.3 (Bosch et al., 1995; Kay et al., 2003; Molano et al., 2003). CIN regression analyses indicate that without HPV infection patients suffering from CIN had a four fold higher regression rate than patients in whom the lesion was associated with HPV infection (Chan et al., 2003).

When viewed with the knowledge that these studies also reported a mortality rate of approximately 50% for all cervical cancer incidences, the significance of these

statistics and the large of impact HPV infection on the female population can be realized.

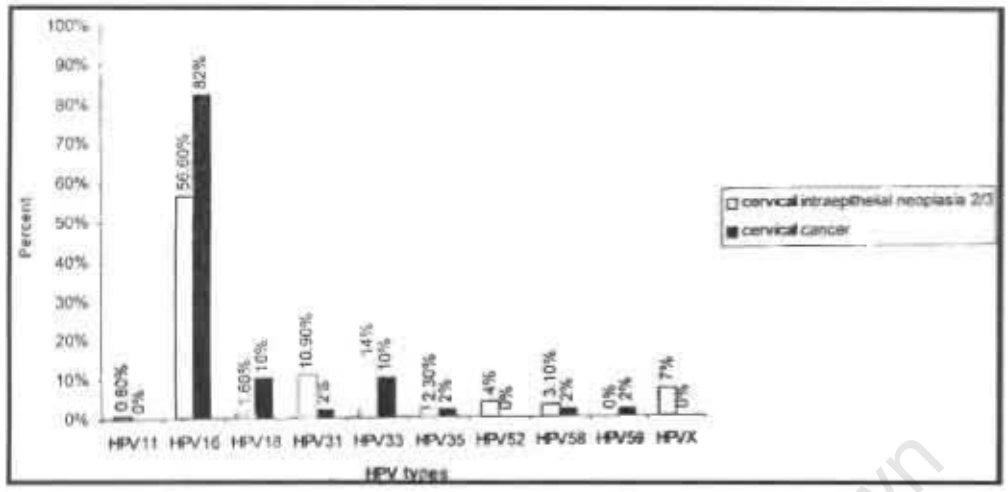


Figure 1.3 Prevalence rates of various HPV types detected in cancers of the cervix or cervical intraepithelial neoplasias (grade 2 and 3) in a study of South African women (Taken from Kay et al., 2003).

1.5 HPV: Properties and Characteristics

Human Papillomavirus is a small non-enveloped double stranded DNA virus of about 8000bp (Howley et al., 1996). It is packaged in an icosahedral capsid composed of two proteins, 80% L1 gene product (major capsid protein) and 20% L2 gene product (minor capsid protein). HPV has a double stranded circular genome. Only one strand serves as the coding template (Figure 1.4) and all the open reading frames (ORFs) are located on that one strand (Howley et al., 1996). The HPV genome consists of a long control region (LCR), which contains the origin of replication and constitutive enhancer elements. These elements are responsive to both host and viral transcription promotion factors, thereby ensuring that the viral genome is translated once it has successfully infected the cell (Cripe et al., 1987). The rest of the genome consists of 8 translationally active ORFs; 6 encode 'early' genes and the other two encode the 'late' genes (L1 and L2). These genes are designated 'late' due to the nature of their transcription, which only occurs at the very end of the viral

lifecycle. Transcription of these genes is delayed, because they are capsid proteins and therefore only need to be synthesized when viral replication is complete (i.e. when large quantities of viral DNA have accumulated) and the viral progeny are ready for packaging and exiting the cell to continue infection of neighbouring cells.

A brief summary of the functions of all the genes encoded in the HPV genome are described in Table 1.1 below. Most studies demonstrating immunogenicity to HPV infection have focused either on the major capsid protein L1 or the oncogenic proteins E6 and E7.

Table 1.1 Description of the genes in the HPV genome and their function.[4]

Gene	Function
E1	Helicase activity to unwind viral DNA and facilitate replication
E2	Viral transcription factor which binds to E1 and the LCR region thereby allowing initiation of viral DNA replication
E4	Participates in virion assembly and interacts with cellular cytoskeleton to collapse cell and release viral particles (Doorbar et al., 1991)
E5	Capable of transforming cells but more importantly upregulates cell membrane growth factors
E6	ONCOGENE – binds p53 and prevents inhibition of cell cycling
E7	ONCOGENE – binds pRB and promotes cell cycling
L1	Major capsid protein
L2	Minor capsid protein

[4](Table modified from Howley, 1996 and Stanley, 2001)

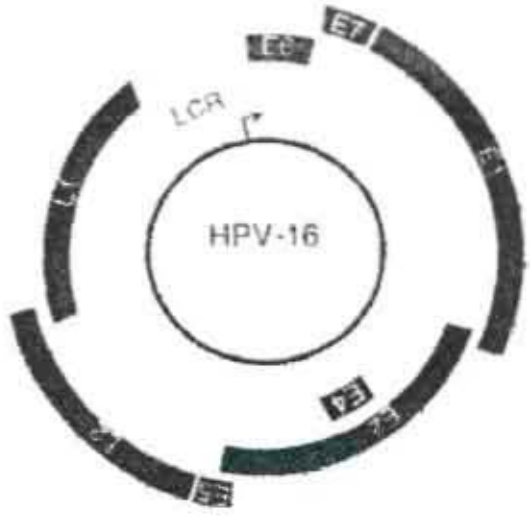


Figure 1.4 Genomic map of Human Papillomavirus (HPV) to indicate the location of the viral genes on the coding sequences (Taken from Munger, 2004).

1.5.1 L1

The HPV L1 gene encodes the major capsid protein, a 55 kDa protein which in combination with the L2 gene forms the 'coat' around the viral DNA to make an infectious viral particle (Favre, 1975). The HPV L1 coat protein is the most common antigen from HPV used for testing antibody and immune cell responsiveness to HPV, since it is the first antigen that the host comes into contact with during HPV infection and it is also the most conserved HPV gene (de Villiers et al., 2004). Yet production of L1 proteins occurs only late in the viral life cycle, due to the dependence of viral transcription on the different stages of the keratinocytes which it infects. It is only at the most terminally differentiated epithelial layers that the L1 protein is produced in abundant quantities in order to facilitate packaging of all the replicated viral DNA, and to allow HPV to further infect the neighbouring epithelial cells (Stanley et al., 2001).

For research purposes it is possible to express the HPV L1 virus like particles (VLP) in recombinant systems following which the L1 particles assemble

automatically into conformationally correct VLPs (Zhang et al., 2000). Therefore HPV L1 VLPs can be successfully used to induce HPV specific antigen responses in host immune cells, allowing assessment of host immune function to HPV infection.

1.5.2 E6 and E7

The oncogenic early genes, E6 and E7, are transcribed into proteins which bestow on HPV the ability to cause abnormal cell proliferation by facilitating immortalisation of the infected cell (Munger et al., 2004). The E6 protein complexes to a host cell protein called p53. The function of p53 is to halt cell cycling if DNA damage has occurred, and thereby facilitate the repair of the DNA (Brenna and Syrjänen, 2003). When E6 binds to p53 it prevents it from performing its vitally important function. The E7 gene product binds the retinoblastoma tumour suppressor protein (pRB), a nuclear protein that regulates gene expression (Brenner and Syrjänen, 2003). In its dephosphorylated state, pRB is a negative regulator of cell growth by inhibiting a cellular transcription factor (E2F-1). E7 preferentially binds the dephosphorylated pRB and therefore allows the cell to continue cycling (Howley et al., 1996).

In non-productive HPV infections (i.e. infections which are not progressing through the epithelial layers, but rather causing abnormal cellular growth of the basal keratinocytes) a large quantity of the early E7 protein accumulates in the basal infected cells as they undergo repeated cell cycling (Middleton et al., 2003). Therefore in late stage CIN and early cervical cancers, E7 proteins are abundant in the basal epithelial layer and these proteins could then induce priming of the immune system in order to elicit a response to the HPV infection.

1.5.3 Diversity of HPV Genotypes

HPV belongs to the *Papillomaviridae* family of which 118 different papillomavirus (PV) types have thus far been sequenced and grouped according to the relatedness of their genome sequences (de Villiers et al., 2004). HPV types are host species and tissue specific. They are primarily grouped as either mucosal or cutaneous depending on the location of their preferred site of infection; mucosal HPV types infect the genital and oro-pharynx area whereas the cutaneous HPV types infect the skin (Frazer, 2004).

It is the mucosal HPV types which cause infections at the cervical mucosal epithelium, but not all of those HPV types are associated with cervical cancer. The mucosal HPV types have been classified as either high risk (HPV type 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 82, 83 and 73) or low risk (HPV type 6, 11, 40, 42, 53, 54, 57, 66 and 84) (Gravitt et al., 1998). The high-risk types are so called since they are more likely to cause cellular transformation and progression to cancerous lesions (zur Hausen, 2002).

There are some functional differences between the high-risk HPV types and the low-risk types. Firstly the E6 protein transcribed off the low risk DNA can not bind the p53 protein and secondly, the E7 proteins from low risk types bind the pRB with ten fold less affinity compared with the high risk types (Werness et al., 1990; Gage et al., 1990). Another difference between the HPV types is that low-risk viral DNA remains extra-chromosomal, whereas the high-risk HPV genomes become integrated into the host cell's chromosome, which allows the increased and persistent expression of the HPV viral genes, thereby leading to HPV persistence and lesion progression (Tjiong, 2001).

Studies have shown that the HPV antigens were entirely type specific and elicited only responses from patients who were previously infected with that HPV type, especially in serum antibody responses (de Gruil et al., 1996, 1998; Gill et al, 1998). This strict type specificity in immune responses to HPV is not absolute since there is some evidence for cross-reactivity of both serum antibodies and T cell responses to HPV types other than the HPV type with which the patient was infected (Höpfl et al., 2000; Kadish et al., 2002). This could imply that immunity to one HPV type is cross-protective against infection by other HPV types, or it could suggest that those patients who had responses to an HPV type that they were not currently infected with, might have had a previous infection with that specific HPV type.

1.5.4 HPV: Infection Mechanism and Viral Lifestyle

Papillomaviruses are host species and tissue specific (Stanley, 2001). Therefore HPV only infects humans and the site of infection (skin or mucosal epithelial cells) depends on the specific HPV type causing infection. Initial infection by HPV is at the basal epithelial cells (Shah et al., 1996). Epithelial cells continuously undergo differentiation until they become terminally differentiated (at the outermost surface of the epithelium) after which they are shed. HPV utilizes the continuous cell division of keratinocytes (subtype of epithelial cells) in order to vegetatively increase the original viral copy number at infection (of 10 copies) to 50 copies per cell (Figure 1.5). HPV is fully dependant on the differentiation stages of the keratinocyte and only in the late stages of differentiation can the virus initiate expression of 'late' genes and exponentially increase the copies of viral DNA in the cell (Oriel, 1971).

This dependence on the differentiation stages of the epithelial keratinocytes has prevented the culturing of HPV in the lab, since it is difficult to make a tissue culture in which the cells gradually become more differentiated. Therefore it is not possible to collect HPV antigens through culturing, it is necessary to express the genes of interest recombinantly, as HPV genes expressed in other vectors.

Eventually, the dead keratinocyte laden with viral particles is sloughed off the epithelium and the infectious viral particles are released and able to infect a new host, or to infect neighbouring cells (Tindle, 2002). Infection with HPV is a long process - approximately 3 weeks from time of infection to virus particle release, since this is the time it takes for a keratinocyte to fully differentiate (Stanley, 2001).

Due to the nature of HPV infection which was described above, it has several advantages over the immune system. Firstly, HPV causes only a localized infection at the outermost periphery of the host where immune cells are less abundant (Tindle, 2002; Frazer, 2003; Stanley 2003). This selective location, combined with the ability of HPV to prevent apoptosis of infected cells thereby preventing inflammation of the infected area, protects HPV antigens from coming into contact with cells of the immune system (Stanley, 2001; Tindle, 2002). Secondly, at the early stages of infection, only low levels of viral transcription occur and it is only once the infected cell is almost fully differentiated that the virus initiates high viral gene expression (Stanley et al., 2001). Finally, studies have shown that viral proteins may modulate the immune response, by interfering with cytokine production or through modulation of antigen presentation, in order to disrupt any immune response that might have been initiated (Frazer et al., 1999; Arany et al., 2002; Woodworth, 2002). It is almost as though HPV was designed to avoid immune surveillance.

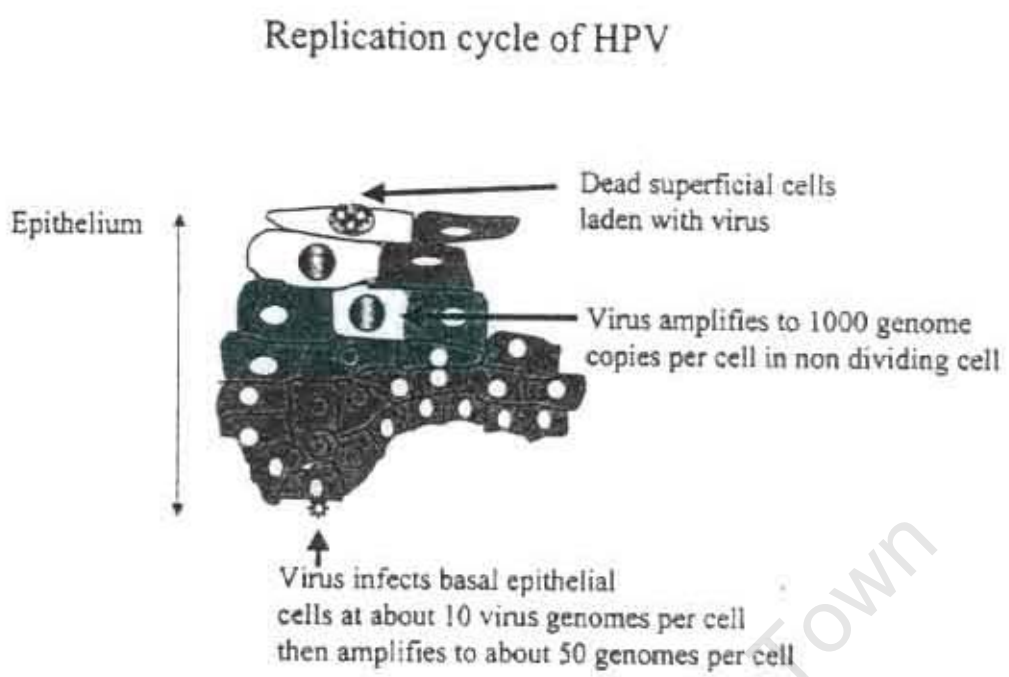


Figure 1.5 The life cycle of the *Papillomavirus* species, illustrating the initial infection occurring in the basal keratinocytes and then the dependance of the viral life cycle on the differentiation stages of the epithelial keratinocytes. It is in the uppermost layers of the skin that the virus begins to transcribe the late genes L1 and L2 in abundance, in order to package viral DNA and form viral progeny. The fully differentiated keratinocyte is laden with HPV viral particles ready to escape the dead cell and infect new basal epithelial cells (taken from Stanley, 2001).

All this information raises the question: what is the involvement of the host's immune system in prevention or clearance of HPV infection and regression of CIN development? The following sections will go into more detail as to the experiments that have been performed to determine the correlates of protection in HPV infection and how these results have improved our knowledge of HPV infection and lesion regression or progression.

1.6 Immune Responses to HPV Infection

Due to the nature of HPV, infection usually remains localized to the original site of entry of the HPV virion into the basal epithelium (Frazer, 2003; Stanley 2003). This is especially so since the various HPV types are highly tissue specific (infecting either skin or mucosal epithelium). Unlike some other viruses, which can be found systemically throughout the body after the initial replication stages have occurred (e.g. HIV), it is unlikely for HPV infection to disseminate and cause a lesion at another site.

One would expect that any immune responses that are elicited by HPV infection at the cervix would only be found locally in the immune cells situated in the mucosal epithelial strata of the cervix. It follows then that one would not expect a strong immune response to the HPV infection in the systemic T cells which patrol the body, circuiting in the blood. Yet many studies have shown that there are significant responses to HPV antigens in the PBMC isolated from the blood of patients suffering with HPV infections.

1.6.1 Antibodies: the mediators of Humoral Immunity

In most viral infections, neutralizing antibodies are the first line of defense to protect the host from the pathogen. Antibodies have been shown to play a very important role in the immunity of the cervix and are an effective protective mechanism in the host's defense against Papillomavirus (PV) infection (reviewed by Stanley, 1997; Kutteh, 1999; Quayle, 2002).

Studies of antibody responses in humans to HPV show clearly that systemic HPV16 specific IgG responses to HPV 16 VLP are associated with HPV16 persistance and late stage CIN lesions whereas systemic IgA HPV16 VLP specific

antibodies seem to be associated to viral clearance (Luxton et al., 1997; Marais et al., 1997; Bontkes et al, 1999; de Gruil et al., 1997, 1999a). Local HPV16 specific antibodies at the cervix were not correlated with viral clearance (Bontkes et al., 1997). These studies showed that patients with HPV16 specific IgG reactivity to HPV VLP L1 in their serum had an increased risk of cervical cancer or late stage CIN 3.

There is also clear evidence that serum HPV specific IgA antibodies to the HPV16 E7 antigen are significantly associated with HPV clearance at the cervix, whereas the HPV specific IgG antibodies to E7 antigen seem to be more representative of previous immune response activity to HPV (de Gruil et al., 1996a, 1996b).

From these results, it is now apparent that using antibodies as a marker for cleared or persistent viral infection depends on which HPV antigen the HPV specific antibodies are reactive against. Antibodies to VLP seem to indicate a persistant HPV infection whereas antibodies to E7 might be the result of an effective immune response (which responded to the virus when it was in the early stages producing E7 in the basal keratinocytes) and therefore perhaps indicates viral clearance.

1.6.2 Cell Mediated Immunity at the Female Reproductive Tract

The host defense system for the female reproductive tract has evolved to be highly advanced, involving sophisticated interaction between many different parts of the immune system. This need has arisen due to the complicated immune requirements placed on the reproductive tract. It must facilitate the entry of 'foreign' sperm cells and allow the attachment and unhindered growth of a foetus. Yet any pathogens which could potentially cause infections must be prevented from accessing the completely sterile uterus and fallopian tubes (Quayle, 2002). The lower genital

tract, comprising the vagina and ectocervical areas, is particularly interesting in that it is able to facilitate the growth of commensal bacteria which in turn prevent the survival of any pathogenic microbes (Hillier, 1999).

1.6.2.1 Antigen Presentation at the Cervix and the Cells Facilitating the Process

At the cervix there are many different cells, some structural (squamous and columnar epithelial cells and keratinocytes) and some for protection. The cells which serve to protect the cervical epithelium include the CD4 and CD8 T cells, antigen presenting cells (APC), B cells and natural killer (NK) cells (Al-Saleh et al., 1998; Bell et al., 1995; Jacobs et al., 2003; Johansson et al., 1999).

In the mucosal epithelium there are two main types of cells that can present foreign antigen to the T cells in order to initiate an immune response; these are langerhans cells and keratinocytes. The langerhans cells (LC) are a subset of immature dendritic cells (DC). Dendritic cells are professional antigen presenting cells (APC) that patrol the mucosal epithelium throughout the body, and after encountering foreign antigen, can initiate a primary immune response by presenting the antigen to T cells. In comparison with the exocervix, the epithelium of the transformation zone (TZ) has decreased numbers of LC and these have been shown to generally have impaired function, which implies that the TZ is highly vulnerable to pathogen challenge (Giannini et al., 2002).

Prior experiments on DC activation using HPV VLP showed that DC successfully bind and take up the VLP, following which induce a Th1 immune response at the site of the infection (Lenz et al., 2001; Rudolf et al., 2001). But the DC subset at the cervix, LC were shown to have significantly reduced functional abilities in comparison to other DC (Fausch et al., 2002). Since LC are the primary APC at the

cervix, these results indicate that there is a serious deficit in the immunity of the cervix in relation to HPV infection.

Keratinocytes (or epithelial cells) are the primary target of HPV infection, therefore it is highly beneficial that these cells are able themselves to present foreign antigens to the T helper cells through the use of the major histocompatibility complex II (MHC II) complexes. Unfortunately, in order to fully induce activation of the T cells, when presenting antigen the connection needs to be strengthened and the activation signals enhanced through the involvement of co-stimulatory molecules (Abbas et al., 1994). Since keratinocytes are not professional antigen presenting cells, they are unlikely to have costimulatory molecules on their surface. Keratinocytes presenting HPV antigens have been shown to induce anergy (a state of antigen tolerance) in T cells and allow lesion progression (Nickoloff and Turka, 1994; Nickoloff et al., 1995). Yet after induction of B7 costimulatory molecule expression, the keratinocytes were able to present effectively to the T cells resulting in an immune response and lesion regression.

The fact that LC and keratinocytes are the most abundant cells presenting HPV antigens at the cervix and both show varying inability to successfully activate T cells to induce immune responses, suggests that there may be even lower numbers of antigen specific T cells to elicit immune responses to pathogens at the cervix than elsewhere in the epithelium. Fortunately there are other cells involved in HPV clearance and lesion regression, for example the natural killer (NK) cells and the macrophages. These cells have both been shown to cause apoptosis of the infected keratinocytes, upregulate adhesion molecules and MHC class I expression and also to secrete IFN-γ and TNF-α to initiate a TH1 immune response (Woodworth, 2002).

1.6.2.2 T cell Responses to HPV Infection

Probably the most important host immune cells involved in viral clearance, found both locally (at the cervix) and peripherally (in the blood) are CD4 T Helper cells and the CD8 Cytotoxic T Lymphocytes (CTL). The importance of both CD4 and CD8 T cell infiltration into areas of HPV infection has been thoroughly investigated. Studies which have looked at HPV associated warts, and analysed the lymphoctes which infiltrated those warts in healthy but HPV infected women, have begun to define the type of immune responses and/or cytokine profiles that are associated with the clearance of an HPV infection (Coleman et al., 1994; Nicholls et al., 2001; Stanley, 2001). Spontaneously-resolving genital warts were compared with those that did not regress, and it was found that the non-regressing warts did not show any immune infiltration while the regressing warts showed an infiltration of CD4 T cells (and CD8 T cells) into both the stroma and epithelium of the lesion (Nicholls et al., 2001). A Th1 dominant response was found in these regressing warts with detectable levels of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-12). These wart infiltrating lymphocytes were activated memory cells, which supports previous evidence that the CTL responses from patients who could clear their HPV infections, lasted for up to 20 months (Nakagawa et al., 2002). These studies suggest that effective immune responses result in immunological memory and long term immune protection.

In response to an attack of the immune system (e.g. invasion of the host by a pathogen) the T cells that recognize the pathogen's foreign antigens will induce cytokine production and/or kill the infected target cell. CD4 T cells produce cytokines that activate local immune cells and recruit other types of immune cells (e.g. phagocytes) to the local area of infection in order to eliminate the pathogen

(Abbas, 1994). Similarly to the CD4 T cell, CD8 T cells are capable of producing cytokines, but the CD8 cells also have the ability to directly kill cells that they recognize are infected (Barry and Bleackley, 2002).

1.6.2.3 Cytotoxic T cell responses to HPV antigens

The actual cytolytic functions mediated by CTLs involve either a granule dependant or a ligand to ligand induced cellular death. The granule dependent death is more complicated, involving the recruitment of pre-formed cytotoxic granules to the cell surface membrane location of the activated T cell receptor and then the exocytosis of the contents of the granule into the immunological synapse between the CTL and the infected cell (Peters et al., 1991). The granules contain compounds that are toxic and cause damage to the contents of a cell, including perforin and granzyme B. Perforin polymerizes and polyperforin complexes create holes in the cell surface membrane which act as ion channels (Abbas, 1994). These holes allow the infiltration of the cell by granzyme B which specifically cleaves a family of caspase protein which subsequently cause damage to the DNA (Barry and Bleackley, 2002). The resultant permeabilisation of the cell membrane and entry of toxic compounds into the cell, induces apoptosis of the infected cell and subsequent death of the pathogen.

The method used for testing the HPV specific cytolytic functional ability of T cells is the chromium release assay (Nakagawa et al., 1997). This assay requires 1-3 week stimulation and culturing of the patient's T cells. Simultaneously antigen presenting target cells have to be prepared, which express HPV gene products (e.g. VLP L1, E6 or E7) and are labeled with radioactive chromium. Upon completion of the culturing period, the target cells are added to the cultured effector cells and the cellular mixture is left to incubate. The amount of chromium which is released during

antigen specific CTL activity is measured as a reflection of the ability of the CD8 T cells to elicit cytotoxic functions against HPV antigens (Nimako et al., 1997). Although the cytolytic activity is a function of CD8 T cells, the responses detected through chromium release assays are unable to clearly differentiate CD8 and CD4 T cell involvement (Nakagawa et al., 1999).

HPV16 specific cytotoxic T lymphoctye responses were found to be significantly higher in disease free women than in those women suffering from CIN (Nakagawa et al., 1996, 1997). Furthermore, in a longitudinal study of 8 HPV16 + women who became HPV negative, CTL responses could be detected in all women up to 20 months after clearance of their HPV infection. In some cases a second detection of HPV infection occurred during follow up but this was cleared within 4 months. It was suggested that this might be evidence of immunological memory against HPV infection (Nakagawa et al., 2002).

1.6.2.4 Proliferation of T cells to HPV antigens

Proliferation assays determine whether cells are responding to the antigen by stimulating the cells in a culture medium to which radioactive thymidine is added for the last part of the stimulation period. As the cells proliferate in response to antigen, they incorporate radioactive thymidine into their daughter cell's DNA strands (Luxton et al., 2003). The relative amount of proliferation is determined by the amount of radioactivity incorporated into the final proliferated cell population. Another method of measuring T cell proliferation is by measurement of IL-2 production by T cells following antigenic stimulation (de Gruil et al., 1998).

Proliferation studies have reported conflicting results. In some studies of patients suffering with CIN, proliferation of PBMC to HPV16 E7 antigens was

significantly associated to HPV clearance and HPV associated lesion regression (Kadish et al., 2002; Höpfl et al., 2003).

Other studies have shown that percentages of responding PBMC were higher in patients with persistent disease than in those patients in whom disease was cleared or fluctuating (Luxton et al., 2003). It was also reported that 75% of women who acquired disease also seemed to acquire a response to the E7 antigen and that increased magnitude and breadth of proliferation responses by PBMC to HPV16 E7 antigen is associated to increased lesion progression at the cervix (de Gruil et al., 1998; Luxton et al., 1996, 2003). It has also been suggested that CIN 1 patients elicited the highest responses to HPV E5 antigen, compared with both patients with more severe disease or less dysplasia at the cervix (Gill et al., 1995).

This phenomenon of proliferation responses to HPV E7 antigen in patients who are diseased but seem unable to clear their disease might indicate that those patient's T cells are proliferating following stimulation by the HPV E7 antigen, but they are not actually eliciting an effective response to clear the virus and induce lesion regression. Another reason for the increased responses to E7 in patients who are suffering with late stage CIN could be due to the fact that in a non-productive HPV infection, the type that leads to abnormal cell proliferation and CIN development, it is the E6 and E7 proteins that are abundantly expressed. Therefore, in HPV-associated CIN there are probably much higher levels of E7 antigen to prime the T cells whereas in HPV infections without CIN, where the virus is progressing with the keratinocyte to a fully differentiated level, there are very low levels of the early E7 gene product and the predominantly expressed antigen is rather the late gene L1 capsid protein (Middleton et al, 2003).

1.6.2.5 Cervical Cancer Patients Have Impaired Immune Responses

Immune cells isolated from the blood of patients with cervical cancer have been shown to exhibit impaired functional abilities in comparison with PBMC from healthy women or patients with CIN (Clerici et al., 1997; Luxton et al., 1996; Höpfl et al., 2000). These studies showed that both the non antigen specific responses to PMA/ionomycin and the HPV specific responses of PBMC were reduced in cervical cancer patients.

Perhaps the lack of T cell responses to HPV specific antigen is due to the state of disease in a cervical cancer patient. Cervical cancer is usually the end result of a long-lasting chronic HPV infection. At that stage only low levels of the HPV antigen are expressed, which could result in T cell anergy, immune tolerance and therefore a lack in the ability to kill infected cells, allowing the tumour cells to proliferate unchecked.

1.6.2.6 CD4 Responses in HPV Immunity: the Th1 versus Th2 Paradigm

T helper cells are CD3+ (T cells), which also display the CD4 co-receptor on their surface. Following stimulation by specific antigen, the T cells secrete soluble cytokine molecules to signal to and induce activation of other immune cells. The T helper cells are divided into three groups; T helper 1 (Th1), T helper 2 (Th2) and Naïve T cells (Th0), each defined by the cytokines they produce. Th1 cells produce primarily inflammatory cytokines (IFN-γ and interleukin-12 [IL-12]) and Th2 cells produce higher levels of IL-4 and IL-5 cytokines (to induce an anti-inflammatory response) (Cousins et al., 2002). It must be noted that both inflammatory and immune inhibitory cytokines can be produced simultaneously by a T cell, therefore the T cell is assigned as Th1 or Th2 depending on which cytokine is predominant. Th0 cells are

called 'naïve' since they haven't yet been exposed to antigen and produce both types of cytokines (Th1 and Th2) in equal amounts due to their undifferentiated state (Openshaw et al., 1995). Therefore a population of T cells is defined to be type 1 if IFN-γ is being produced in the absence (or relative absence) of IL-4.

Studies have shown that a Th1 cytokine response to HPV infection is preferential for clearance of the pathogen and regression of lesions (Al-Saleh et al., 1998; Luxton et al., 1997). Women displaying predominantly Th1 inflammatory cytokines at the cervical mucosa have been shown to be more likely to clear their HPV infections than women with Th2 or Th0 profiles (Luxton et al., 1997; Scott et al., 1999).

Following stimulation with HPV E7 antigen, only CD4 T cells were found to produce IL-2 and not CD8 T cells (de Gruil et al., 1998). Although it was demonstrated that these cells did produce IL-2 following stimulation of PBMC, no IL-2 was detected in cervical biopsies of the patients (de Gruil et al., 1999). Therefore, although the assays and cells used to detect IL-2 differed, it is tempting to speculate that PBMC in these women were capable of producing this cytokine, but cells at the site of pathology were not.

1.6.2.7 Impact of HIV Infection on Progression of HPV associated CIN

Probably the ideal cohort of women in which to study the role of CD4 T cells in HPV lesion progression, would be one in which their CD4 cells are suppressed, for example a cohort of HIV+ women. Currently this is an area of great interest and much research, since HIV infection has been shown to be associated to increased infection with high-risk HPV types. This is particularly true in those high risk women with CD4 counts < 500 cells/mm^2, where rapid progression to late stage CIN and

cervical cancer is clearly noted (Hawes et al, 2003; Schuman et al., 2003; Lee et al, 1999). In HIV+HPV+ women, the time from HPV infection to subsequent CIN and cervical cancer detection is significantly shorter, in comparison with HPV infected but otherwise healthy women (Schuman et al, 2003). This indicates that perhaps the lack of immunocompetent CD4 T cells in HIV+ women allows for rapid progression of the HPV associated lesion and one would therefore deduce that CD4 T cells are integral to the cell mediated immunity (CMI) against HPV infection and lesion progression. But the study by Schuman et al. (2003) revealed that this conclusion might not be correct, by analyzing HIV+HPV+ patients receiving highly active anti-retroviral therapy (HAART) in whom the number of CD4 cells should have increased to > 500 cells/mm^2 and observed that there was no associated decrease in risk of CIN development or progression as the T cell numbers increased. Perhaps this is due in part to functional impairment of the CD4 T cells that survived the infection of the immune system by HIV (McCune, 2001). The above-mentioned studies suggest that CD4 T cells play an important role, but are not exclusively involved in HPV immunity.

1.6.2.8 Cytokines Play an Integral Role in the Progress of HPV Infection

The cytokine microenvironment is a crucial factor when initiating an immune response. At the cervix, it is maintained through the constant release of cytokines from both keratinocytes and the local immune cells (Malejczyk et al, 1997). Most studies have suggested a role for Th1 cytokines in HPV clearance and a role for Th2 cytokines in CIN development (Scott et al, 1999; de Gruil, 1999).

The cytokine profile of tissues from sections of cervical biopsies of normal or diseased women showed that IL-12 (inflammatory) and IL-10 (anti-inflammatory)

cytokine levels were increased in the CIN biopsies as compared to the normal exocervix tissue (Giannini et al., 1998). Yet interestingly the IL-12 levels peaked in low-grade biopsies and began to decrease again in high-grade CIN, which could suggest (since this is an inflammatory cytokine) that in the early CIN 1 lesions, the immune system is attempting to initiate a response to kill the infected tumour cells, but as the CIN progressively worsens the immune system can somehow no longer fight the onslaught and therefore allows (or can no longer prevent) the switch to a Th2 environment. Of importance, they also observed in the study that the region of the cervix where most CIN occur, the transformation zone, was on average associated with higher levels of the immuno-suppressive cytokine, IL-10 (Giannini et al., 1998; Jacobs et al., 2003).

These studies suggest how a skewing of the cytokines towards a Th2 profile does result in a more immunosuppressive and antigen-tolerant cervix and therefore could make the cervical cells more susceptible to infection by pathogens including HPV and less able to prevent persistence of the infection, which could in turn lead to lesion formation and cervical cell dysplasia.

In a longitudinal study to analyse the effects of the cytokines on HPV status, 100% of the women who cleared their HPV infection had a Th1 response at the visit preceding clearance (Scott et al., 1999). None of these women's samples showed any presence of IL-4 (the immunosuppressive cytokine). This situation was not apparent in the HPV negative patients, in which equal percentages of IL-4 and IFN-γ mRNA were detected in the samples. This suggests that the Th2 cytokines might be employed by the immune system once the virus is cleared to then modulate and decrease the inflammatory response in order to prevent excess damage to the surrounding tissues.

Cytokines can have more specific roles in HPV immunity than the broad skewing of the host's immune response between inflammatory and suppressive. Studies have shown that IFN-γ actually reduces HPV gene expression, especially expression of E7 RNA, simultaneously preventing the immortalisation of keratinocytes by interfering with production of the E7 protein. Yet, in defense, HPV E6 and E7 proteins are able to inhibit and decrease the IFN-γ signaling (Woodworth, 2002). TNF-α is also able to perform an anti-viral function; by repressing HPV16 early gene transcription and therefore expression where IFN-γ can not (Kyo et al., 1994).

The anti-inflammatory IL-10 works against the host, increasing HPV16 E7 mRNA levels significantly (by upregulating the transcription rate) thereby enhancing the progression of the CIN (Arany et al, 2002). This is an area for concern since the levels of IL-10 at the cervix were found to increase, with increasing severity of the cervical lesion (Giannini et al, 1998). But the cytokine balance between pro- and anti-inflammatory is a complicated one. In contrast to the many studies that have labelled IL-10 as an anti-inflammatory cytokine, a possible role has been observed for IL-10 in promoting inflammatory responses (Santin et al., 2000). PBMC were incubated with a combination of IL-10 and IL-2 (a growth cytokine and inducer of T cell proliferation) before stimulation. Results were compared with those elicited by PBMC incubated with just IL-2 or IL-10 alone. The results showed both a significant increase in the proliferation ability of the T cells and a significant CTL response when the cells were exposed to specific antigen in the presence of both cytokines in combination. Interestingly, the cells that had been incubated with both IL-2 and IL-10 were also expressing significantly higher levels of IFN-γ.

1.6.2.9 Conclusion of T cell Mediated Immunity to HPV Infection

From previous studies it is evident that a Th1 inflammatory type of cell mediated response at the cervix of HPV infected individuals is a necessary factor in the clearance of the HPV infection and regression of cervical lesion. Yet, there is much confusion surrounding the optimal T cell proliferation response needed to induce lesion regression and HPV clearance. Most published work to date used either the ability of T cells to proliferate in response to HPV antigens (proliferation assay) or the ability of cytotoxic T cells to lyse HPV antigen expressing target cells (Chromium release cytotoxicity assay). The major difficulty with both of these approaches is that it is not possible to determine from the results which T cell subsets are eliciting the strong antigen specific responses, and thereby inducing the disease regression. Therefore it could be that the different studies are finding conflicting results since they are detecting responses from different T cell subsets. Therefore, more techniques for analysing T cell responses need to be developed in order to (i) determine the intracellular cytokine (ICC) responses of the individual T cell subsets (CD4 vs CD8) and (ii) to compare the ability of the T cells to elicit cytotoxic activity as well as ICC production, in response to the HPV16 specific antigens.

Most importantly, many published reports of CMI responses during HPV infection have focused on responses in the peripheral blood of infected or diseased women. It is well recognized that HPV types that infect the genital mucosa (such as HPV-16) do not cause systemic infection but viral replication is localized, highly tissue specific (only infecting basal keratinocytes) and tightly controlled. The value of studies of systemic T cell responses to such a localized infection is questionable without a representative comparison from T cells isolated from the site of pathology, the genital mucosa.

1.7 Objectives of Project

Studies which have used chromium release based cytotoxicity assays have been able to indicate a strong role for CD8 cytotoxic T cells in HPV immunity. Proliferation studies and IL-2 assays also suggest an important role for the T helper cells in clearing HPV infections and associated lesions. Yet none of these studies were able to specifically distinguish between the T cell populations and determine which T cell subset is more involved in inducing a response to the HPV antigens.

Therefore this study proposes to use the technique of flow cytometry, which allows individual analysis of the cytokine profiles and cytotoxic ability of each cell in a sample, thereby allowing accurate and sensitive analysis of the immune responses from T cells and facilitating distinct separation of which responses were elicited by the CD4 or CD8 cells.

Because cervical immunity to genital HPV infections is rarely studied and investigations of this nature add significant value to our collective knowledge of HPV correlates of protection, the major focus of this dissertation was to study cervical T cell responses in women with active HPV infections and HPV-associated cervical disease. This was done using a cervical cytobrush to non-invasively obtain a sample of the cells present at the cervical transformation zone for investigation into cervical T cell responses to HPV. In order to relate these findings back to published reports, all studies on cervical T cell responses to HPV were compared with T cells responses in peripheral blood.

Since HPV-16 is the major high-risk type associated with CIN and cervical cancer in South Africa (Kay et al., 2003), this study focused exclusively on responses to this oncogenic type. Because the major capsid protein L1 and the major oncogenic

protein E7 have demonstrated some degree of immunodominance in published reports, these 2 gene products were selected for this study.

1.7.1 Development of Techniques for Investigating Cervical T cell Responses

Because only a single HPV study (Scott et al., 1999) and a very small number of HIV studies (Musey et al., 1997; 2003; Kaul et al., 2001) have used this approach to investigate T cell responses from the cervix, the first objective was to develop approaches and technology to obtain and functionally assess cervical cytobrush-derived T cell responses (Chapter 2). This involved determining the viability of the cellular sample following collection by cytobrush method. The reliability of various counting methods were compared, to determine which would provide a quick and accurate estimate of the approximate number of T cells per cervical samples. Flow cytometry was also used to develop an accurate method to quantify the actual peripheral blood cell contamination of cervical samples. Finally a statistical model was used to establish the validity of the cervical cellular sample and whether the T cell populations were sufficiently large to be used in the subsequent ICC assay.

1.7.2 Determination of cervical versus peripheral blood T cell intracellular cytokine responses to HPV16 specific antigens

This project used flow cytometry (as described by Passmore et al., 2002) to analyse the Th1 or Th2 responses from cervical and PBMC T cells isolated from women with CIN and genital HPV infections (Chapter 3). The T cells were stimulated with HPV16 specific VLP L1 and E7 in order to induce T cell activation and allow the detection of HPV antigen specific responses. Because immune responses at the genital mucosa are likely to be influenced by a whole variety of host

and pathogen determined factors, the intracellular cytokine T cell responses detected in this study were interpreted in light of these confounding variables. The major factors that were compared in this study are: (i) Impact of HPV type actively infecting the cervical tissues (as determined by Roche Reverse Line Blot); (ii) influence of HPV viral load (as determined by relative light units from Digene Hybrid Capture II); (iii) impact of previous HPV-16 infection (as determined by seropositivity in patients to HPV-16 VLPs); and (iv) effect of cervical inflammation on T cell responses and disease severity (as determined using BD Cytometric Bead Array analysis of cervical washes by flow cytometry).

1.7.3 Determination of the cytotoxic ability of HPV specific T cells

Finally, this project aimed to develop a flow cytometry-based cytotoxicity assay in order to accurately determine the level cytolytic T cell activity in a cervical T cell population in response to HPV16 specific stimulation (Chapter 4). A previous study had reported the use of the molecule CD107a (LAMP 1) to act as a marker of degranulation (Betts et al., 2003). Initially various markers of degranulation (e.g. Perforin, CD107a) were compared and the CD107a based assay was favoured since it yielded optimal results. PBMC T cells isolated from twenty three women with varying grades of cervical disease and HPV-16 infection status were then investigated for CD107a expression (indicative of cytotoxicity) following stimulation with HPV-16 L1 and E7.

CHAPTER TWO: DEVELOPMENT OF TECHNIQUES FOR PROCESSING CERVICAL CELL SAMPLES

2.1 Introduction

It is known that HPV-16 is the most prevalent HPV type associated with CIN and progression to cervical cancer in Western Cape (Kay et al., 2003). HPV preferentially infects cells of the transformation zone of the cervix because this is the zone most rapidly dividing (Crompton, 1976). Therefore, several techniques have been developed to evaluate cells or immune responses directly from the cervix. The most common include cervical biopsy, cervical lavage, weck cel and cervical cytobrush (NCI Workshop, 1989; Snowhite et al., 2002; Musey et al., 1997; 2003). This chapter focused on cervical cytobrush sampling to evaluate mucosal HPV-16-specific T cell responses direct ex vivo.

It was necessary to determine the most efficient method for collecting T cells from the cervical epithelium and transformation zone of patients with cervical disease (CIN), so that these cells would be both competent for use in direct ex vivo functional studies and sufficient in number for the results to be statistically meaningful. Phenotypic characterization by immunohistochemical staining of immune cells at the cervical epithelium have mostly been studied through biopsies of cervical tissues, which were obtained following standard hysterectomy in both healthy and diseased individuals (Bell et al., 1995; Al-Saleh et al., 1998). The major advantage of this approach is that it yields very high numbers of cells for study, but an obvious disadvantage of this approach is that study participants are restricted to those undergoing hysterectomy. This can be avoided by using small tissue biopsy sections (Jacobs et al., 2003), which requires the removal of only a 2-4mm^3 area of the cervical epithelium in order to obtain sufficient cells for further analysis.

The least invasive method for collecting cervical cell samples which makes use of a cone-shaped brush inserted into the cervical os and rotated 360°C in order to dislodge and obtain cells from the cervical epithelium, was described by Musey et al. (1997). This cytobrush method has been successfully utilised in other studies, although further development and refinement of the subsequent processing of the cervical samples is still required (Koelle et al., 2000; Passmore et al., 2002; Milner, 2003).

Factors of importance when collecting cells for subsequent analysis include (i) viability of cellular sample, (ii) the number of mucosal T cells available for analysis and (iii) establishing a reliable screen and cut-off level for T cell number in a sample in order for the subsequent functional assays to be statistically meaningful. The first aim of this study was to develop the cytobrush method of cervical sample collection through optimisation of basic techniques and determination of reliable checks to ensure that samples were sufficient in T cell number and were not contaminated with peripheral blood cells.

2.2 and Methods

ng a Digene Cervical cytobrush sampler

ed according to the method described by Musey inal speculum examination, a Digene Cervical os and gently rotated 360°. The brush was then ube containing 3ml transport media (10% human g/ml streptomycin and 2.5 µg/ml amphotericin B ating microbial organisms). Patients who were reported discharge (and therefore other potential mpact on the immune microenvironment) were e stored on ice and transported to the laboratory

m cytobrush specimens

orush samples were incubated for 15 min in a 37°C samples collected where significantly contaminated ponent of the samples had to be broken down using ny trapped T cells. This was done by the addition of 10µl/ml DTT (500mM Dithiothreitol; Sigma-Aldreich, Germany) and the cells were incubated for a further 15 min. Thereafter, the cervical cytobrush was vigorously rotated against the sides of the tube in order to dislodge all of the cervical cells and a pipette was utilised to flush the media through the cytobrush bristles to ensure all cells were resuspended in the medium. The cytobrush was then discarded and the transport media

containing cervical cytobrush-derived cells was then transferred to a conical bottom 15ml centrifuge tube and centrifuged for 10 min at 200 x g in order to pellet the cells. The supernatant was removed (and stored at –20°C for evaluation of cytokine content), the cells were washed once in 10ml PBS and then finally resuspended in 1ml PBS from which two 50µl aliquots of sample were removed to 2 BD Falcon FACS tubes for the CD3 Screen and determination of RBC contamination (section 2.2.3 and 2.2.4.3 below). The remaining sample containing cervical cells was stored on ice to await further processing.

2.2.3 CD3+ Screen to accurately determine the quantity of T cells in specimens

The technique of flow cytometry enables the study of intracellular cytokines (ICC), allowing sensitive and individual analysis of the cellular phenotype and cytokine contents of each cell in a sample population. Briefly, monoclonal antibodies to markers of interest (e.g. receptors: CD3, CD8 or cytokines: IFNγ, IL-13) are supplied pre-conjugated to fluorochromes. These fluorochromes emit light of specific wavelengths when they become activated through laser beam excitation. The monoclonal antibodies are used to 'stain' the cells in order to determine whether the cells are carrying the markers of interest. Thereafter the samples are analysed on a flow cytometer to allow detection of the antibodies. Flow cytometers are able to channel individual cells through a stream of fluid in the flow cell, which is intersected perpendicularly at one point with a laser beam. As cells pass through that point, the laser beam activates any fluorochromes bound to the cellular surface or the intracellular constituents, and the emitted light is captured by various photo-detectors, which then translate to the computer the relative quantity of each marker on each cell.

Flow cytometery can be used on many different cellular samples to detect many different cellular characteristics or events. In this development part of the study, flow

cytometry was used to determine more accurately the numbers of CD3+ cells in the cervical samples. This was necessary since cervical cytobrush specimens generally yield very low levels of CD3+ T cells for analysis (~10^4 cells/brush; Prakash et al., 2001; Passmore et al., 2002), and the responses I was interested in characterizing were likely to be present at an extremely low frequency (Waldrop et al., 1997). Therefore a minimum level of statistical power and hence cell number had to be determined to allow for statistically meaningful results.

In order to determine the absolute number of T cells present in the cervical sample, cells were stained with a monoclonal directed against CD3. CD3+ T cells in the cervical samples were then evaluated using a Becton Dickinson FACS Caliber Flow cytometer. Briefly, from the 1ml sample of cervical cells, an aliquot of 50µl was removed for CD3+ screening. The cells were incubated in the dark on ice for 15 min in the presence of anti-CD3-APC conjugated antibody (BD Biosciences). The sample was washed with 2ml PBS (FCS) for 5 min at 200x g to remove unconjugated antibody and the cells were finally fixed in 400µl BD CellFix solution (BD Biosciences). The CD3+ screen samples were acquired on a BD FACS Caliber Flow Cytometer within 24 hours of staining and analyzed using BD CellQuest software.

2.2.4 Determination of Red Blood Cell (RBC) contamination of cervical specimens

When using the cervical cytobrush, the disturbance of the epithelium through the brushing can sometimes result in bleeding and hence potential contamination of cervical immune cells from those derived from peripheral blood. Prevention of this potential contamination is another important consideration in cervical specimen collection. This is especially important when cervical T cell responses to HPV are to be compared with the

responses elicited by the peripheral blood T cells, as is the case in this study. The results are not reliable if there is blood contamination of the cervical sample.

Previous studies (Musey et al., 1997; 2003) have excluded cervical samples on the basis of RBC contamination by visually assessing contamination. This study evaluated the usefulness and sensitivity of this visual assessment method compared to an objective quantification method based on glycophorin A (CD235) staining) (Data not shown). CD235 or glycophorin A is expressed on RBC but not on peripheral blood mononuclear cells (BD Pharmingen Technical Catalogue, 2004; Chasis and Mohands, 1992) so may serve as a useful marker for RBC contamination.

2.2.4.1 Sensitivity of CD235 staining for use on cervical specimens

Once the cervical T cell sample had been processed (as described in section 2.2.2), a 50µl aliquot of the cellular sample (which had been resuspended in 1ml PBS) was transferred to a BD FACS Falcon tube and incubated for 15 min at 4°C in the presence of anti-CD235a-CChr antibody. The sample was washed once with 2ml PBS for 10min at 200 x g and fixed with 400µl BD CellFix (BD Biosciences). Percentage contamination of cervical samples with CD235a expressing cells was determined using a BD FACS Caliber Flow Cytometer and the BD CellQuest software.

2.2.5 Trypan Counting method using haemcytometer

The number of T cells in a cervical sample was also quantified using Trypan Blue staining and counting on a Nubaur haemcytometer. Once cervical cytobrush specimens had been processed to release T cells from cervical mucous, a 10µl aliquot of the sample containing cervical cells was stained with 0.4% Trypan Blue stain (Sigma; equal volume of

cells to Trypan stain). The stained cells were placed onto a haemcytometer slide and viewed under a light microscope (100x magnification). The dead or apoptotic cells are permeable to the trypan stain (Shapiro, 1988), therefore these cells will become pigmented with a dark blue colour which clearly distinguishes them from the translucent viable cells (which can not be permeated by the stain).

2.2.6 7AAD Staining to measure cell viability of cervical cells by FACS analysis

Due to the DNA-binding nature of 7-Amino Actinomycin D (7AAD), which intercalates between the guanine and cytosine bases in the DNA of dying cells, this stain is a simple and accurate method for determining the percentage of non-viable cells in a sample (Philpott et al., 1996). The stain is taken up readily by dead cells, faintly by apoptotic cells and is not taken up by healthy living cells (since their membranes are not permeable to the stain). 7AAD is capable of emitting a fluorescence which can be detected through the use of a flow cytometer, therefore on a flow plot of forward scatter (FSC) versus 7AAD fluorescence, the cells are depicted as three distinct populations. The dead population is that which has the highest fluorescence intensity and the healthy living cells are the 7AAD negative population, therefore the percentages of viable cells can easily be determined (Figure 2.2).

7AAD (Sigma) stocks were dissolved in acetone at 5mg/ml and stored at –20°C. Working stocks were made up in PBS with 7AAD at a concentration of 200µg/ml. This test was performed on 50µl of the processed cervical cellular sample which had been stained with monoclonal antibodies to the CD3 marker (anti-CD3-APC). Cells were washed with 2ml of PBS and then resuspended in 400µl of PBS. A volume of 40µl 7AAD stain was added to the 400µl cellular sample and this was incubated for 20min at 4°C

followed by immediate analysis using the BD FACS Caliber Flow Cytometer. Samples have to be analysed within 20min of staining in order to obtain maximum fluorescence readings before the stain begins to fade (Philpott et al., 1996). Unstained cellular sample was used as a negative control.

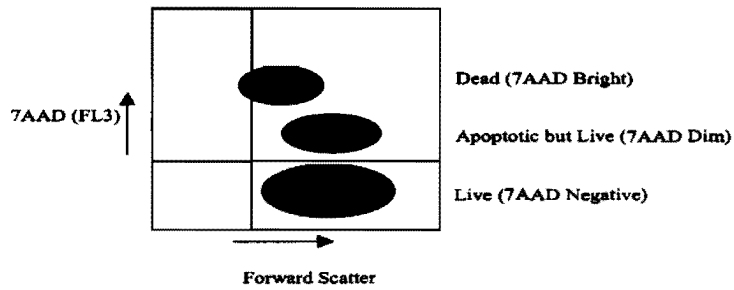


Figure 2.1 Diagram of Facs plot of forward scatter (FSC) vs 7AAD stain showing the three positions of the distinct populations relative to their viability. The uppermost population with brightest 7AAD fluorescence are where the dead cells are expected to sit, below that the apoptotic cells which take up stain but not as readily as dead cells, and finally in the 7AAD negative area, the healthy and viable cellular population.

2.3 Results

2.3.1 Viability of cervical cell sample after collection using cytobrush method

Unlike peripheral blood samples, where the isolated PBMC is made up primarily of T cells, cervical cytobrush samples have a much more varied cellular composition (with the majority of cells being epithelial in origin) where the frequency of T cells in the sample is always low and varies drastically between donors. Since the diverse range of cells found in a cervical sample constitutes the environment in which the T cells will be stimulated *ex vivo*, it was necessary to check the viability of all cells in the cervical samples, since dead cells emit toxins, which at high concentrations could inhibit the function of the T cells.

To determine the viability of the cervical samples, the cells were stained with 7AAD. As shown in Figure 2.2a, the results show that 95% of the CD3+ were viable and excluded 7AAD stain, while 2% of the CD3 population was dead and a further 3% were apoptotic. Similarly, if 7AAD staining was assessed on an ungated plot (Figure 2.2b; representing all cells present in the cervical sample including epithelial cells), then 70% were alive, 11% were apoptotic and 19% were dead. This confirms that the cells collected through the cytobrush technique were viable after processing and supports previously published literature (Prakash et al., 2001).

A. B.

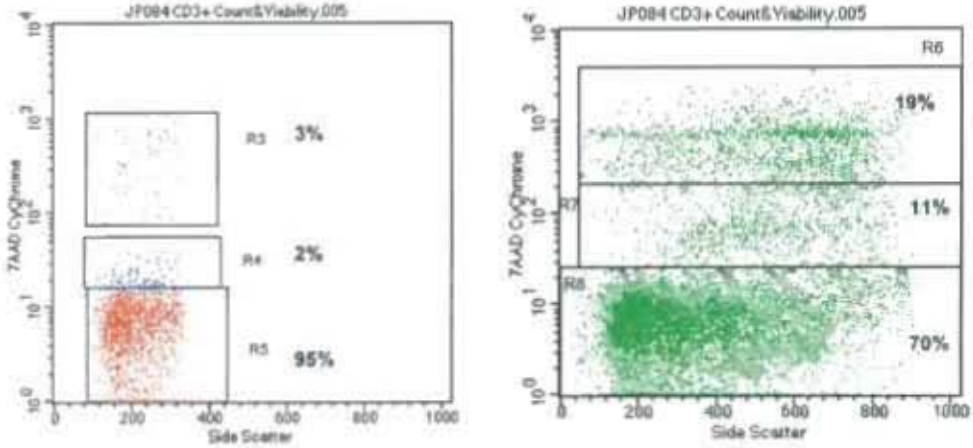


Figure 2.2. Facs plots showing viability of cervical cell sample using 7AAD stain. (A) Forward scatter versus 7AAD (FL2) staining of CD3+ cells. (B) Forward scatter versus 7AAD (FL2) staining of all events. The stain should be taken up mostly by the dead cells (highly fluorescent population; R3 and R6), slightly by the apoptotic cells (middle population; R4 and R7) and not at all by the healthy viable cells (negative population; R5 and R8). The cells were isolated from a cervical cytobrush sample, stained with 7AAD and immediately analysed on a BD FACS Caliber Flow Cytometer using BD CellQuest software. Percentages shown next to each region represent percentages of total CD3+ T cells (A) or all cells (B) that are either live, apoptotic or dead.

2.3.2 Quantity of CD3+ cells obtained using cytobrush technique

2.3.2.1 Reliability of Various Counting Methods

One of the main concerns when collecting cells using the cytobrush method is the variability in the quantity of CD3+ cells recoverable from different patient specimens. Previous studies have shown that the quantity of CD3+ T cells may range from $1,53 \times 10^3$ to $1,53 \times 10^4$ cells (Passmore et al., 2002). Therefore it is absolutely necessary to determine whether there are sufficient numbers of T cells present in the cervical cytobrush specimens for further analysis before initiating the stimulation processes.

The usual method of determining approximate T lymphocyte numbers in the cellular sample is through the trypan counting method with a haemcytometer slide. The number of T cells present in cervical samples was generally so low that they could not be accurately counted on the haemcytometer slide. Based on Trypan staining and manual counting using

a haemocytometer chamber slide, mononuclear cell numbers ranged from 5 x 10^4cells/ml to 4.74 x 10^6cells/ml in all the patients investigated. When one compared the cervical cell concentrations determined by Trypan staining with absolute CD3+ counts on the flow cytometer (Figure 2.3), the results correlated very poorly indicating that Trypan counts could not be reliably used as an estimate of lymphocyte numbers for cervical samples. Furthermore, the abundance of epithelial cells present in the cervical samples (a substantial proportion of which were the same size as mononuclear cells) and the fact that Trypan does not distinguish nuclear morphology, often made accurate differentiation of the mononuclear cells in the population extremely difficult.

It was then decided that a CD3+ "screen", using flow cytometric detection of the fluorochrome labelled CD3+ T cells would be a considerably more accurate representation of the number of T cells in the cervical sample. The numbers of T cells in cervical samples ranged from <100 to 14900 CD3+ T cells per patient sample (~150-fold difference). The mean was 5822 (± 1207; SD) cells analysed per patient sample. The data is represented in graphic form (Figure 2.4), which shows that although there was much variability between the T cell numbers collected from different donors, the CD3+ screen T cells events counted on the flow cytometer were indicative of the actual number of CD3+ T cells in the total sample. Therefore, these results show that although the CD3+ screen might not be accurate enough to be used as a quantitative assessment, it could prove to be a useful qualitative test to check the quantity of T lymphocytes in samples where the number of cells might be too low to be detected by other techniques.

For the purposes of this study, a cutoff was set at >500 CD3+ events in the CD3+ screen which translates into an actual CD3+ count of 4015 cells per patient sample, in order to eliminate samples which had T cell populations that would be too low to yield statistically meaningful results.

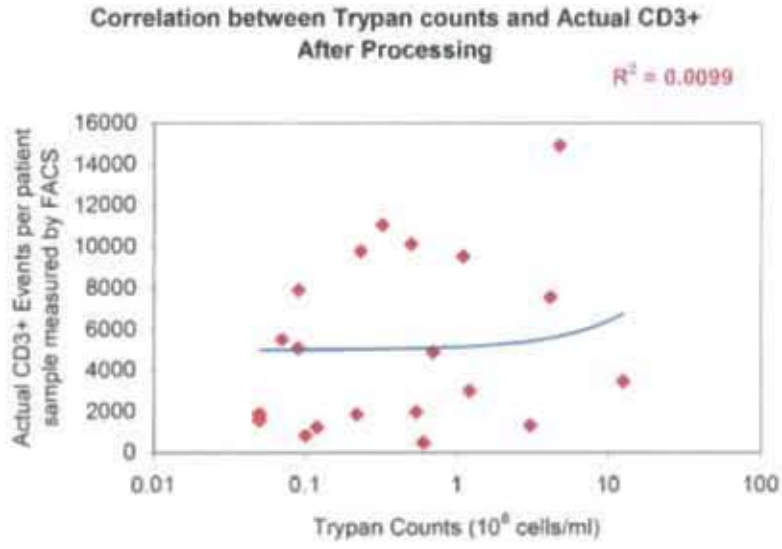
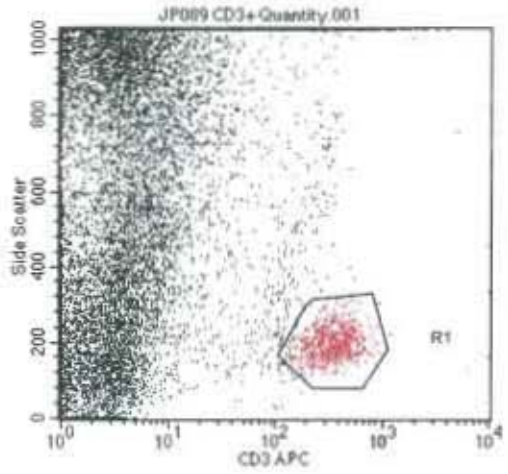


Figure 2.3 Graph showing the correlation of Trypan counts with actual CD3+ events (measured by flow cytometry) to estimate the concentration of T cells in a cervical cell population. Cells were either stained with Trypan blue and counted manually on a haemocytometer using a light microscope or stained with a monoclonal antibody against CD3 and analysed using a BD FACS Caliber flow cytometer. Actual CD3+ events were calculated by determining the number of CD3+ cell events in one quarter of the cervical sample and adjusting for the proportion of total sample.

A.



B

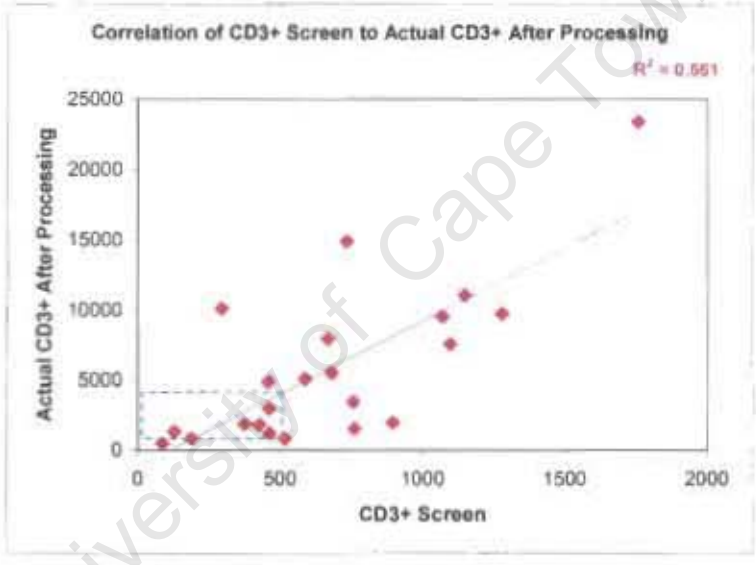


Figure 2.4 CD3+ screening by flow cytometry and correlation with actual CD3+ events after stimulation. (A) Representative flow plot, depicting the gating of a CD3+ lymphocyte population when utilising the CD3+ Screen method to determine accurately the number of T cells in the total specimen population. (B) Graph to depict the correlation between the CD3+ Screens and the Actual Total CD3+ T cells after processing and final analysis. CD3+ Screens were prepared by removing an aliquot of the fully processed cervical sample and staining it with anti-CD3-APC. The blue line represents the 500 cell/CD3 screen cut-off that was established for this study and shows it to correlate 4015 CD3+ cells after stimulation and processing.

2.3.2.2 Validity of cervical cellular sample size for further use in statistical analyses

The analysis of cellular responses to non antigen specific stimuli such as PMA/ionomycin or PHA (which non-specifically elicit a response from most T cells) is relatively easy to analyse on the flow cytometer, since the numbers of stimulated responding T cells is usually large and much higher than the background values from the unstimulated population. In comparison, analysis of a T cell population's responses following exposure to a specific antigen (such as virus like particles (VLP), proteins or individual peptides) is not as simple, since the percentage of T cells which will recognise and respond to that specific antigen are usually relatively low. This type of data capturing is referred to as Rare Event Analysis, which implies that maximal numbers of T cells need to be analysed in order to increase the significance of the low frequency positive responses above the background levels (Waldrop et al., 1997; Roederer et al., 2004).

The repercussions from rare event analysis on a study such as this one, where the numbers of cells per sample is in most cases very limited, can result in insufficient numbers of cells being acquired and therefore lead to data of questionable significance. Therefore it is necessary to determine the minimum number of cells which need to be acquired in order to give an accurate or significant representation of the low frequency antigen-specific T cell responses expected from the cervical region. I have used a spreadsheet based statistical model (kindly provided by Dr Holden Maecker, Becton Dickenson, personnel communication) to calculate the minimum number of events needed to have statistical confidence in the low frequency HPV-specific responses anticipated in the study compared to background (unstimulated) levels of response.

For this study Table 2.1 below was calculated to indicate the minimum events necessary for acceptance of the various differences between predicted percentages of unstimulated and test samples as statistically significant. From the highlighted blocks it is

possible to see that the previously mentioned CD3+ screen cut off of >500 cells (which correlates to a final amount of approximately 1000 T cells per stimulation condition analysis tube) should be sufficient to allow the results obtained to be confidently accepted as significant values. Unfortunately, what is also evident from Table 2.1 is that the difference between background unstimulated values and antigen-specific responses need to be quite high (on average ~ 0.9-1.8%) to have statistical confidence in the data and this is because of the low numbers of cells recoverable by cytobrush. The obvious implications of this would be that many potentially real but lower frequency positive events would have to be ignored in a study such as this.

Table 2.1. Calculated Minimum Number of Events Needed for Rare Event Analysis

%Bckground	%Positive Events in Test Sample	%Difference	Min # Events Required
0.01	0.1	0.09	11673
	0.5	0.49	1822
	0.85	0.84	1044
0.05	0.1	0.05	51561
	0.5	0.45	2329
	0.95	0.9	1056
0.1	1	0.9	1161
	1.95	1.85	1090
	2	1.9	495
1	2	1	2541
	2.7	1.7	1061
	5	4	313

2.4 Conclusion

The initial development of techniques for processing cervical cytobrush samples proved to be highly beneficial to the purposes of this study. By exploring new methods to determine with greater accuracy the number of CD3+ cells in the sample and also the %RBC contamination of the samples, I was better able to make decisions regarding which cellular samples would be of no use in the subsequent intracellular cytokine (ICC) assay and should therefore be discarded.

In studies where the T cells are being isolated in order to undergo *ex vivo* stimulation with various virus specific antigens and the frequency of positive events to those specific antigens is low, the number of events evaluated by flow cytometry need to be as large as possible so that the strength of numbers might lend significance to the few positive events above the background unstimulated population. The most important criteria for this study was a CD3+ event number >500 in the CD3+ screen. This correlated to an approximate quantity of 4000 T cells in the total cervical sample and 1000 T cells per stimulation condition (unstimulated, PMA, VLP-16 and E7). According to a statistical model this number of cells would be sufficient to support differences between the antigen specific stimulation and a background of 0.85% to 1.7% depending on the level of response in the unstimulated sample. The higher the background the greater the antigen specific responses need to be, in order to be considered significant. Following these results, the CD3+ screen method and FACS is recommended as an accurate T cell counting tool.

A technique utilised by many studies in order to maximise the cellular samples potential is *in vivo* culturing of the cells for 1-3 weeks in the presence of stimulatory factors. This method aims to induce proliferation of responding T cells, thereby amplifying their detectable responses to specific antigens. This is a necessary step in studies where the

T cell response to antigen is measured by a proliferation or cytolysis assay, since both of these techniques have low detection rates. In comparison, flow cytometry is much more sensitive in its detection of many different markers simultaneously, at a high rate, with high sensitivity, accuracy and reproducibility. An *in vitro* culturing step was not included in the processing of the cervical samples for this study since the major aim was to attempt to investigate HPV-specific cervical responses directly *ex vivo*. A major concern of *in vitro* expansion is that both phenotypic and functional properties of the T cell populations may change and some T cell subsets may expand preferentially over others (Moscicki et al., 1995). In addition, because the female genital tract is not a sterile environment and many pathogenic and commensal organisms co-exist at the site, long-term culture sterility is another concern. However, it would be very interesting and useful for future studies which also have to rely on the cytobrush collection method to obtain cervical cell samples to include a comparison of direct *ex vivo* with an *in vitro* culturing stage. This could potentially improve both the numbers of responding T cells and the number of women that could be studied.

CHAPTER THREE:

CERVICAL T CELL RESPONSES TO HPV-16 L1 AND E7 IN WOMEN WITH HPV-ASSOCIATED CERVICAL DISEASE

3.1 Introduction

The immunology surrounding HPV infection is an area of research which has recently gained much interest and subsequent insight. The reason for this interest is due to the well-established fact that certain high-risk HPV infections are strongly associated with the development of cervical cancer (Walboomers et al., 1999; Bosch et al., 2002; Bosch and Sanjosé, 2003). Cervical cancer is a fatal disease and it is currently the primary cause of cancer related deaths in black South African women (CANSA, 2003).

The interesting fact is that not all women who become infected with a high risk HPV progress to cervical cancer. Seventy to ninety percent have been shown to clear their HPV infection within 12 to 30 months (Evander et al., 1995; Ho et al., 1998). Those who have more persistent infections are at increased risk of developing cervical disease (Molano et al., 2003; Schlecht et al., 2003). Therefore knowledge regarding the profiles of the immune responses which correlate with HPV associated disease progression and those that are rather associated with HPV clearance and lesion regression are of vital interest to research groups which are attempting to generate effective HPV vaccines.

Previous insight into the immune responses which are elicited by the many different immune cells following exposure to the HPV infection has revealed conflicting evidence in terms of the type of T cell responses that are associated with

protection or disease progression. Investigation into wart infiltrating lymphocytes (Coleman et al., 1994; Nicholls et al., 2001; Stanley, 2001) from healthy but HPV infected women have begun to define the type of immune responses and/or cytokine profiles that are associated with the clearance of an HPV infection in this model. When spontaneously-resolving genital warts were compared with those that did not regress, the non-regressing warts did not show any immune infiltration (Nicholls et al., 2001) while the regressing warts showed an infiltration of CD4 T cells (and CD8 T cells) into both the stroma and epithelium of the lesion. These wart infiltrating lymphocytes were activated memory cells. A Th1 dominant response was found in these regressing warts with detectable levels of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-12). From studies of systemic T cell responses to HPV, most studies used either proliferation assays to measure T cell clonal expansion in response to HPV specific antigens or Chromium-release assays to measure cytotoxic T lymphocyte (CTL) responses (Luxton et al., 1996; Nakagawa et al., 1996). Although both of these methods allow determination of the quantity of HPV specific responses in a patient's T cell population, neither allows the analysis of the actual T cell subtype (CD4 or CD8) eliciting the response. This is important because CD4 T helper cells perform different functions to the CD8 CTLs therefore if only one T cell subtype was responding to the antigen this could result in a less efficient immune response to HPV infection. Importantly, most of the published data on T cell responses to HPV have used cells derived from peripheral blood and not the site of pathology, the cervix. Because HPV causes localized infection and is not cytopathic, it does not disseminate or have a systemic phase of infection (Tindle, 2002; Frazer, 2004), therefore it is obviously best to focus studies on T cells derived from the cervix. For this reason, the

present study will be focusing on a comparison between T cells derived from the cervix and peripheral blood of women with CIN and/or HPV-infection at the cervix.

Although there is evidence showing an association between Th1 responses and HPV lesion regression, the viral antigens targeted by the immune response are poorly defined. Studies in mice have shown that the strongest cell mediated immune (CMI) responses are against E6 and E7 (McLean et al., 1993; Chambers et al., 1994). In clinical studies using PBMC from healthy women and patients with cervical disease, T cell responses were predominantly against L1 (Shepherd and Luxton, 1999). Studies on wart-infiltrating lymphocytes, showed that 75% of HPV-specific T cell responses were against L1 (Hong et al., 1997). Therefore, I have chosen to focus this study on both L1 and E7 HPV-16 antigens.

The aim of this study was to compare the HPV-16 L1 and E7-responsive immune cells derived from cervix to those from peripheral blood; by determining their phenotype (CD4 versus CD8) and their ability to produce cytokine responses to HPV specific stimulus. Of particular interest for the purpose of this study is the ratio of Th1 (IFNγ): Th2 (IL-13) cytokines produced in response to the HPV antigens. In this chapter, the technique of flow cytometry was used, which allows the automated and fast, individual analysis of each cell in a stimulated population. Using these techniques, it was possible to characterise (i) the immune responses to HPV infections, (ii) the cellular subtypes eliciting those responses and (iii) the cervical cytokine microenvironment at the time of specimen collection.

3.2 Materials and Methods

3.2.1 Study Population

This study involved the participation of 100 women between the ages of 18 and 40 who had been referred to the Groote Schuur Colposcopy Clinic following diagnosis by cytology of CIN disease at the cervix. CIN status was subsequently confirmed by Colposcopy and Histology. In accordance with the Research Ethics Committee of the University of Cape Town all participants were fully informed regarding the study and their written consent was obtained. Women who were menstruating, post-menopausal, pregnant or had a thick discharge, any reports or visible vaginal and/or cervical infection (and therefore potential co-infections at the cervix which might skew the immune microenvironment) were excluded from the study.

3.2.2 Procedures for Processing of Donor Samples

From each patient, 3 tubes of Lithium Heparin anti-coagulated peripheral blood (for isolation of PBMC) and 1 tube of coagulated peripheral blood (for detection of serum antibodies) were obtained by venipuncture. In addition, two cervical cytobrush samples were obtained, one was used for isolation of cervical mononuclear cells and the second was used for detection of HPV types and viral load determination (described in more detail below).

3.2.2.1 Extraction of Serum from Clotted Peripheral Blood Sample

Coagulated peripheral blood samples (collected in red capped vacutainer tubes, Beckton Dickinson) were clotted on arrival at the laboratory. In order to extract the serum from the cellular component of the sample, tubes were centrifuged at 280xg for 10 min, which allowed the blood cells to pellet and left a clear serum layer on top. Serum was extracted in 1ml aliquots, transferred to cryotubes and thereafter frozen at -20°C.

3.2.2.2 Isolation of PBMC from Anti-Coagulated Peripheral Blood Sample

Thirty milliletres of blood was collected from each patient into lithium-heparin coated vacutainers, to prevent coagulation of the blood cells. PBMC were isolated from the samples using the FICOLL-Hypaque (Sigma) density centrifugation method, which relies on the weights of the constituents of the blood sample in order to differentiate and separate the mononuclear cells (lymphocytes and monocytes) from the bigger blood cells (erythrocytes and granulotcytes). We used LeucoSep® tubes (Greiner Bio-One) to facilitate faster and more efficient separation of the whole blood through a fixed porous filter disk inside the tube.

LeucoSep® tubes were prepared as per the manufacturer's instructions, by centrifuging 15ml Ficoll (Sigma) through the filter disc at 1000 x g for 30 seconds in a Labofuge 400R centrifuge (Heraeus Instruments). Whole blood was then gently layered over the filter and centrifuged for 10 min at 1000 x g. The PBMC form a distinct 'buffy' layer between the plasma and ficoll as depicted in Figure 3.1. The buffy layer was transferred to a 15ml conical bottom tube and cells were washed twice with 10ml PBS for 10min at 200 x g. The cells were counted using an

automated Coulter Counter (Beckman Coulter, MDI 18) machine and adjusted to $2x10^6$cells/ml.

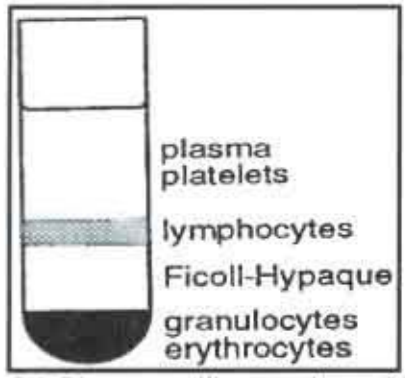


Figure 3.1 Diagram to illustrate the position of the constituents of whole blood following density centrifugation separation on a FICOLL gradient.

3.2.2.3 Collection and Processing of Cervical Cells from Cervi-Brush Sample

The cervical cytobrush sample was collected as described by Musey et al. (1997) with the following modifications. A Digene Cervical Sampler cytobrush was inserted into the cervical os and rotated 360°. The brush was then removed and transferred into a transport tube containing 3ml culturing media, consisting of 10%FCS RPMI and 50U/ml penicillin, 50mg/ml streptomycin and 2.5 µg/ml amphotericin B in order to prevent growth of unwanted contaminating microbial organisms. Samples were stored on ice and transported to the laboratory within four hours of collection.

Upon arrival at the laboratory, cytobrush samples were incubated for 15 min at 37°C. DTT, a mucolytic agent, (500mM Dithiothreitol; Sigma-Aldreich, Germany) was then added to the samples and these were incubated for a further 15 min to allow disintegration of the mucosal constituent of the sample and allow the extraction of the T cells. Thereafter the cervical cytobrush was vigorously rotated against the sides of

the tube in order to dislodge all of the cervical cells and furthermore a pipette was utilised to flush the media around the cytobrush to ensure all cells were resuspended in the medium. Cellular media was then removed to a conical bottom 15ml tube and centrifuged for 10 min at 200 x g in order to pellet the cells. The supernatant was removed and stored in 3 x 1ml cryotubes and kept at –20°C for use in CBA bead assays (section 3.4). Cells were washed once in 10ml PBS with centrifugation at 200 x g for 10min and then resuspended in 1ml PBS from which two 50µl aliquots of sample were removed to 2 BD Falcon FACS tubes. The first aliquot was used for the CD3 "Screen" and 5µl anti-CD3 APC (BD Pharmingen) antibody was added to stain the cells. The second tube was used for screening RBC contamination and 1µl CD235a-CyChrome (BD Pharmingen) antibody. These "screen" tubes were incubated at 4°C for 15 min to allow binding of the antibody to the respective markers before samples were washed with 10ml PBS and fixed in 400µl BD CellFix (BD Pharmingen). These stained cells were then acquired and analysed on the BD FACS Caliber Flow Cytometer the following morning to determine the quantity of CD3 cells in the cervical sample and secondly the red blood cell (RBC) contamination of the cervical sample (Sections 2.2.3 and 2.2.4, Chapter 2).

The rest of the cervical cell sample was washed once again in 10ml PBS, resuspended in 1ml culture media and counted using the trypan haemcytometer method (section 2.1.5). In cases where the cervical T cell population was greater than the required $2x10^6$ cells/ml the cellular concentration was adjusted using culture media.

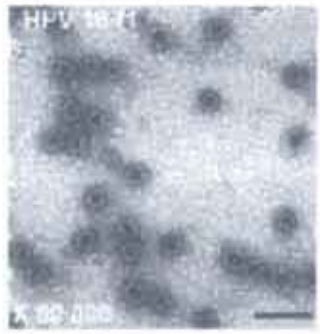
3.2.2.4 Stimulation of cervical and peripheral blood T cells with HPV-16 L1 and E7 antigens

To investigate T cell responses to HPV-16, both HPV16 virus like particles (VLP; made up of L1) and E7 protein were used as immunogens. These two proteins were chosen because the majority of publications from diseased as well as healthy individuals have shown strong T cell responses to either one or both of these HPV proteins (McLean et al., 1993; Chambers et al., 1994; Shepherd and Luxton, 1999; Hong et al., 1997).

3.2.2.4.1 Preparation and quality control of HPV-16 VLP

HPV-16 VLPs were kindly prepared and purified by Eric van de Walt (Dept. Molecular and Cell Biology, University of Cape Town). Briefly, HPV-16 L1 was expressed in Baculovirus cells, isolated using the sonication method (to lyse cells and release VLP) and concentrated to allow extraction from the cellular mixture using centrifugation through caesium chloride density gradients. The VLP were purified through four rounds of dialysis using PBS buffer. VLP-16 preparations were sent through rigorous quality checks: western blots (to confirm reactivity with monoclonals against L1) and Coomassie staining of PAGE gels (to confirm size) (Suzanne Grove, Dept. Molecular and Cell Biology, University of Cape Town), and electron microscopy (Fritz Tiedt, Dept. Medical Virology, University of Cape Town). L1 concentration was determined by Coomassie staining on PAGE (using BSA standards and gel densitometry to calculate concentration) and confirmed by ELISA using V5 (a monoclonal antibody directed against conformational epitopes, kindly provided by Dr Neil Christenson, The Jake Glitten Cancer Research Institute) and J4 (a monoclonal directed against linear epitopes, also provided by Dr Neil Christenson). The ELISA technique used VLP-16 of known concentration (provided by MedImmune) as the standard against which to calculate our In House VLP-16 concentration. Figure 3.1 shows the quality control checks of the preparation of VLP-16 used in this study.

(A)



(B)

(C)

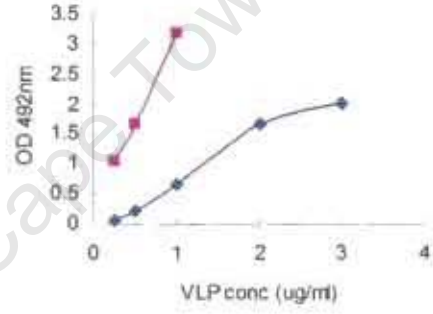


Figure 3.2 An example of the characterization of VLP-16 preparation used in this study. VLP-16 quality and concentration were evaluated by (A) electron microscopy; (B) western blot using monoclonal antibody to detect L1 (arrow indicates position of L1 band); and (C) VLP-16 ELISA using V5 monoclonal antibodies to detect L1 (pink squares indicate V5 detection of MedImmune VLP and blue diamonds indicate reactivity to VLP-16 produced at UCT).

3.2.2.4.2 Preparation and Purification of HPV-16 E7

HPV-16 E7 protein were kindly prepared and purified by Dr Inga Becker (Dept. Molecular and Cell Biology, University of Cape Town). Briefly, HPV 16 E7 gene was amplified with PCR using HPV 16 L2E2E7 gene obtained from John Schiller (Laboratory of Cellular Oncology, National Cancer Institute, Bethesda) as a template. It was cloned into pProEx™ HT Prokaryotic Expression Vector (Life Technologies, GIBCOBRL). Competent DH5α were transformed with the above

DNA and histidine tagged E7 protein was induced with 0.6mM IPTG for 3h at 37°C and purified utilizing the batchwise purification protocol with Ni-NTA resin according to manufactures instructions.

3.2.2.4.3 Stimulation of cervical and PBMC-derived T cells with L1 and E7

Isolated cervical cells and PBMC, adjusted to 2 x 10^6 cells/ml, were transferred in 200µl aliquots into 4 wells (per sample) of a 96-well round-bottom plate. Each sample was stimulated with either (i) no antigen, (ii) the positive control PMA/Ionomycin (PMA 25ng/ml, Sigma; Ionomycin 1µg/ml, Sigma) for 5 hr or HPV16 specific antigens (iii) VLP (10µg/ml) and (iv) E7 antigen (9µg/ml) for 21 hr. The optimal length of stimulation for these HPV specific antigens was determined in a previous study (Milner, 2003). Brefaldin A (10µg/ml, Sigma) was added to the culture from the for the last 3 hours of the 4 hour PMA/ionomycin stimulation and for the last 5 hours of the unstimulated, E7 and L1-stimulations. All stimulations occurred in the presence of co-stimulatory antibodies, anti-CD28 and anti-CD49d (1mg/ml; BD Pharmingen), since previous studies have shown that when antigen is presented by an MHC molecule to the corresponding T cell receptor (TCR), it is vital that the interaction between the two cells is stabilised and supported by various other costimulatory molecules. If the induction of the TCR lacks costimulation it has been shown to result in T cell anergy and subsequently tolerance of that T cell in response to the antigen (Nickoloff et al., 1994, 1995). Therefore it is essential to add antibodies to the co-stimulatory molecules found on the T cells. Cells were incubated at 37°C, 5%CO_2.

3.2.2.5 Staining of stimulated cell populations

Following completion of the staining protocol, cells were thoroughly resuspended and each well transferred to BD Falcon FACS tubes. The cells were washed once with 5% FCS PBS containing 0.01% Azide (Stain Buffer) and then immediately incubated for 10 min at room temperature in 500µl BD Cytofix/Cytoperm solution (BD Pharmingen) which simultaneously fixes the cells and their contents and permeabilises the cellular surface membrane for access of antibodies to intracellular cytokines. It is important that the fixation occurs prior to or at the same time as the permeabilisation in order to prevent the leakage of cellular contents out of the porous cellular membrane.

Fixed cells were pelleted at 200 x g for 5 min and then washed with 2ml 0.1% Saponin (0.01% Azide) (at 200 x g for 5 min). Saponin is a detergent permeabilisation agent which will maintain the porous state of the cellular membrane. Washed cells were resuspended in saponin solution (± 100µl) and the following antibodies (obtained from BD Pharmingen) were added to all tubes: anti-CD3-APC, anti-CD8-FITC, anti-IFN-γ-PE and anti-IL-13-biotin (which needed a secondary streptavidin antibody conjugated to CyChrome for detection). Staining was allowed to occur at 4°C for 30 min protected from light (which could result in bleaching of the fluorochrome dye and reduction of the fluorochrome's intensity). Cells were then washed again in 2ml saponin and then stained with the secondary antibody for IL-13-biotin detection, streptavidin conjugated CyChrome (BD Pharmingen). The streptavidin bound the biotinylated antibody and therefore resulted in amplification of each marker and improved detection of IL-13 production. Percentage CD4 T cell responses were extrapolated from the CD8-CD3+ population.

Stained cells were washed once again with 1ml saponin and thereafter the pellets were fixed in 400µl BD CellFix reagent. Fixed cells were acquired on the BD FACS Caliber Flow Cytometer within 24 hr of staining, and analysed using the BD CellQuest software.

3.2.2.6 Digene Cytobrush for evaluating HPV DNA infection, HPV typing and relative viral load

The second cervical cytobrush specimen was taken using a Digene Cervical Sampler for use in determining the HPV types and the viral load at the cervix of patients participating in the study. The Digene Cervical Sampler includes a Digene Cervical Cytobrush and a tube of Specimen Transport Medium. The cytobrush was inserted into the cervical os and rotated one 360° rotation and then placed into the specimen transport medium and stored at –20°C until they could be processed as described in section 3.3 below. The Digene cytobrush used for detection of HPV infection and typing was always done after the cytobrush used for obtaining cervical T cells to minimize the chance of blood contamination of the first sample.

3.2.3 Testing for active cervical HPV infection, HPV typing and Viral Load Determination

It was obviously important to determine for each patient the HPV types causing infection at the cervix, and to obtain an indication of the quantity of HPV infection present at the cervix (viral load). The experiments to yield these results were the Digene Hybrid Capture® II HPV Test (to determine presence of high risk HPV DNA present at the cervix and an the relative viral load) and the Roche Reverse

Line blot assay (to identify the types of HPV infecting the cervix). The Digene Hybrid Capture II Test was kindly performed by Mr Bruce Allen (Dept. Medical Virology, University of Cape Town) and the Roche Reverse Line Blot assay was kindly performed by Ms Candice Sampson (Medical Virology Department, UCT).

3.2.3.1 Digene Hybrid Capture® II HPV Test

The Digene Hybrid Capture® II Test allows the qualitative determination of how much viral DNA there is in each sample to give an idea of the viral load of each patient. In this study the Digene kit was used to detect DNA from the following high-risk HPV types; 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. All reagents used were supplied by the manufacturer. Briefly, the samples which had been stored at –20°C, were thawed to RT and an equal volume of Denaturation Reagent was added to each sample (including a negative and a positive calibration control). The samples were mixed well by vortexing and this was followed with a 45min incubation at 65°C. The probe to the high-risk HPV DNA types were aliquoted into new eppendorfs (25µl) with 75µl of the denatured sample and thoroughly mixed. The samples were then shaken on a rotary shaker (Thermolyne Maxi-Mix III, Type 65800) at 1100 r.p.m. for 3min to ensure samples were completely homogenous. Thereafter they were incubated at 65°C for 60min. Samples were then transferred to respective wells on the capture microplate and covered with a plate sealer to prevent spilling of the well contents through the next 60 min 1100 r.p.m. mixing period on the rotary shaker. Thereafter the samples were decanted and the plate was blotted well. Detection Reagent 1 was added to each well (75µl), the plate resealed and a further incubation commenced for 30min. After a second decanting of the well contents, the plate was washed 6 times in Wash Buffer and drained for 5min on paper towelling.

Finally each well was immersed in Detection Reagent 2 (75µl) and the plate was allowed to incubate for 15min before the OD was determined through the use of a Luminometer. The OD reading was converted by the computer into a relative light units (RLU) value, which is indicative of the viral load of the patient.

In order to standardise the test so that the samples from one experiment can be compared to those from another independent HC test, the test includes various internal controls. Firstly, the assay includes a positive and negative control, which are performed in triplicate and their results averaged. The positive control mean is used to determine the cut off value in each experiment. The relative light units (RLU) from each sample are then displayed as a ratio, in proportion to the cut off (RLU/CO). This allows standardisation of the experiment. The tests also include a positive and negative calibration control, the OD readings of which must fall within 10% of the means of the controls in order to validate the assay. The RLU/CO results are interpreted as positive if they are greater than or equal to 1 according to the FDA approved interpretation method. This 1 RLU/CO is approximately equivalent to 1pg of HPV DNA per 1ml of sample media (Iftner and Villa, 2003).

3.2.3.2 HPV Consensus PCR and Genotyping utilising Reverse Line Blots

In order to extract and purify the DNA from inside the cervical cells which were collected with the Digene Cervical Sampler, the QIAamp® DNA Mini Kit (QIAGEN) was used. In order to perform HPV genotyping it was necessary to increase the quantity of viral DNA in the purified DNA sample by PCR. The PCR reagents for these experiments were supplied by Roche Molecular Systems, Inc., and the protocols followed were described by Mullis & Faloona (1987).

Reverse line blots (RLB) are a simple and efficient method of determining the actual HPV DNA types in a sample. The technique for using RLB was described by Gravitt et al. (1998). Each genotyping blot is a strip supplied by Roche Molecular Sytems, Inc, which has an array of immobilised oligonucleotide probes set at specific positions relative to an ink reference line. Two of the probes are control probes designated for 2 concentrations of the β-globin PCR product. The rest of the probes are specific for various HPV types. The Roche RLB used in this study detected the following HPV types: high risk HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, MM4, MM7, MM9 and low risk HPV 6, 11, 40, 42, 53, 54, 57, 66, MM8. The principle of the technique is that the amplified DNA will bind the oligonucleotides, which are complementary to that specific HPV type. The positions on the strip where hybridisation has occurred is visualised through labelling the primers (PGMY09/11 which were used to amplify the HPV DNA through PCR, section 3.3.2.2) with biotin and conjugating enzymes to the biotinylated fragment. Those enzymes are then able to break down the substrate which is added to the reaction well and this resulted in the formation of a dye at that position which stains the strip at the position of the hybridised DNA (Figure 3.3).

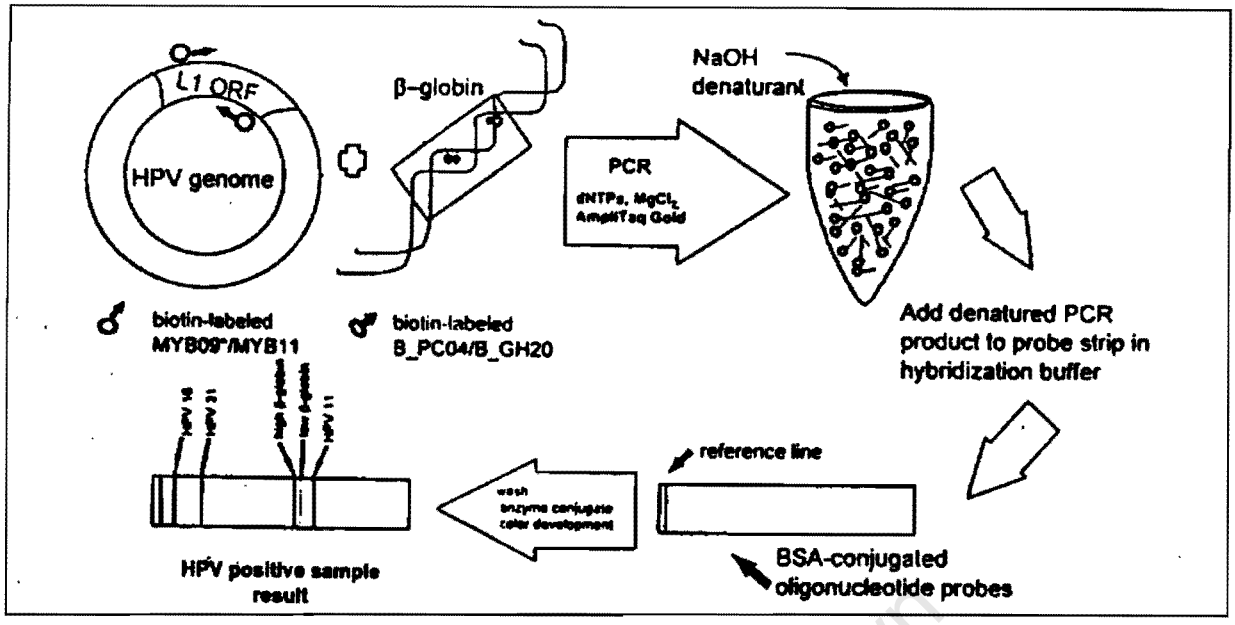


Figure 3.3 Illustrated method depicting the technique of reverse line blot detection of HPV types (taken from Gravitt et al., 1998). Initially HPV DNA is amplified using L1 consensus PCR primers, thereafter the HPV DNA mixture is allowed to bind the corresponding oligonucleotide probes on the RLB strips, and the bound complexes are visualised through the addition of enzymes which cause substrate reactions to produce colour pigment at the position of the DNA complex on the strip.

The methods followed were those described by Gravitt et al. (1998). Initially it was necessary to denature the PCR product (to allow hybridisation to occur between HPV DNA strands and their complementary oligonucleotides) by addition of an equal volume of Denaturation Solution (40µl, Amplicor). HPV genotyping strips (Roche Molecular Systems, Inc.) were prepared by placement into wells of typing tray and addition of 3ml pre-warmed (53°C) hybridisation solution. Thoroughly mixed PCR product was added to each respective well (75µl) and the strips were incubated in 53°C waterbath with shaking for 30min. Vacuum aspiration was utilised to remove the hybridisation solution from each well to allow washing of the strips with 3ml RT wash solution. This washing step involved a brief swirling of the wash solution over the strip to remove any extra hybridisation solution that may have been left behind after the previous aspiration step. Wash solution was removed and the stringency of the hybridisation was tested through another 15 min, 53°C incubation in 3ml of pre-

warmed wash solution (53°C). After careful aspiration of the second wash buffer solution, 3ml of SA-HRP solution was added to each well and the tray was shaken at 70 r.p.m. for 30 min to allow complete conjugation of the horse radish peroxidase (HRP) enzyme to the biotinylated primers which were bound to the strip. The conjugate solution was then aspirated and the wells were rinsed with 3ml RT wash solution as before. Thereafter, two 10min incubations on the platform shaker were performed with strips immersed in 3ml wash solution. After the second removal of wash buffer, the strips were incubated for 5min in 3ml citrate buffer and then immersed in 3ml colour development solution (comprising 4:1; Substrate A to B) for 5min with shaking at 70 r.p.m. Finally strips were thoroughly rinsed with dH_2O and stored in citrate buffer at 4°C to await manual interpretation using the specially made overlay. The overlay was a diagram of the strip which was printed on a transparent sheet and therefore could be placed over each strip, lining up the reference lines and used to determine what positions on the strip had been stained and therefore bound by a specific type of HPV DNA. Interpretation of RLB results is only qualitative and can not give an indication of the actual amount of viral DNA present in each sample since primers do not amplify the viral DNA equally and often have preference for certain viral types (e.g. HPV 16, in which case the HPV 16 position is stained much darker than other positions because it had a much greater amount of biotinylated hybridised DNA and therefore a higher number of conjuagated enzymes to break down the substrate and cause more staining to occur at that position).

3.2.4 Enzyme Linked Immunosorbent Assays (ELISA) to Assess HPV-16 specific antibody (IgG) reactivity to HPV VLPs

It has been shown that the presence of HPV-16 specific antibodies in the serum of individuals could indicate that they have had a previous infection and previous exposure to the HPV antigens (de Gruil, 1996). Therefore in studies of immune responses to HPV it is of great interest as to whether the patients have an antibody response or not. However, looking at serum antibodies is not necessarily a good predictor of HPV infection, as there is a significant time lag between infection and sero-conversion. Serum antibodies specific for HPV-16 L1 have been shown to develop only ~6 to 18 months after infection (Carter et al., 2000), with ~40-60% of women failing to seroconvert at all (Carter et al., 2000; Kirnbauer et al., 1994; Le Cann et al., 1995).

To test for antibodies in serum samples the simplest way is to utilise the method of enzyme linked immunosorbent assays (ELISA). This technique relies on the ability of the antibodies in the serum to bind to a specific antigen which the ELISA plate is pre-coated with. If serum antibodies bind and are 'captured' on the plate, then those antibodies are 'detected' by the addition of a secondary antibody (synthesised by the manufacturer to bind to for example human immunoglobulin) which has been conjugated to an enzyme that is capable of breaking down substrates resulting in either luminescence or colour changes in the well of the plate where the enzyme is located. Accordingly, the greater the amount of light or colour change detected, the higher the concentration of HPV specific antibodies in the patient's serum.

There are many different uses for ELISAs so each one is tailored to the samples that it is testing for. In this experiment the protocols followed were

described by Studentsov et al. (2002). Since the unknown variable is the specificity of the IgG in the serum of the donors (i.e. whether there is an IgG specific to the HPV antigen), HPV VLP (1μg/ml in PBS) was used to coat the of a 96-well flat-bottomed microwell plate overnight at 4°C wells (100μl per well). The following morning the wells were washed twice with PBS and then blocked for 3hr at RT with 200μl blocking solution (0.5% Polyvinyl Alcohol in PBS, ph 7.4). Thereafter the plate was washed 3 times with PBS and serum samples were added to each well (in dulicate) at a 1: 100 dilution with blocking solution. Both control and serum samples were loaded (100μl/well) and the plate was then incubated for 1hr at 37°C in a waterbath. The three control samples are predetermined high, medium and low (negative) responders, obtained from a random batch of sera supplied by the National Healthy Laboratory Service (NHLS) in order to calibrate the experiment and control for interassay variability. Following incubation the plate was washed 6 times and then incubated with 100μl rabbit anti-human IgG secondary antibody (1:6000 dilution in blocking solution with 0.8% polyvinyl pyrrolidone) for 30 min at 37°C. The plate was then washed again and the substrate OPD solution (O-phenylenediamine dihydrochoride, 2mg tablets; Dakocytomation, Denmark) was pipetted into each well (100μl). The secondary antibody was conjugated to the horse radish peroxidase (HRP) enzyme therefore if any of the secondary antibodies had bound there would be a relative amount of HRP in the wells to break down the substrate and create the colour. The enzymatic reaction was stopped after 30min by the addition of 100μl 0.5M H_2SO_4. The OD levels were detected at a wavelength of 492nm and 620nm (reference wavelength) by a VERSAmax ELISA Plate Reader.

Before the results could be interpreted, it was necessary to check the assay's reliability in terms of the calibration controls. The means and ranges of the responses

from the three calibration controls, determined previously through multiple independent ELISA results, were as follows: high OD = 2.5 (3.0 – 2.0), Medium OD = 0.8 (0.94 – 0.62) and Low OD = 0.17 (0.21 – 0.13). Therefore to check that the assay has worked reliably those three controls should have values residing in their respective expected ranges. The seropositivity cutoff levels were calculated by Marais et al. (unpublished data) as 0.42 from the average OD value of children control serum tests against HPV VLP (minus outliers, plus 3 standard deviations). These experiments were kindly carried out by Ms Candice Sampson (Dept. Medical Virology, UCT).

3.2.5 CBA Bead Kit to test for Inflammation at the Cervix

Cervical cell supernatants from 23/33 patients from whom both cervical and PBMC samples had successfully been obtained and analysed, were tested for signs of inflammation at the cervix. This was made possible through the use of a BD Cytometric Bead Array (CBA) Kit (BD Pharmingen), which detects the presence of six cytokines all involved in the mediation of inflammatory responses: IL-6 (costimulator of T cells), IL-8 (pro-inflammatory chemokine), IL-10 (inhibition of phagocytes), IL-1β (activator of T cells and endothelial cells), IL-12 (most potent stimulator and activator of T and NK cells, inducing growth and differentiation into mature functional T cells) and TNF-α (activation of neutrophils, endothelials and T cells) (Abbas, 1994).

The BD CBA kit comprises six bead populations that have distinct fluorescence intensities (in the FL3 detection spectrum). Each set of beads is coated in a single type of capture antibody to bind one specific cytokine. The six bead populations are mixed together and thereafter added to the test sample along with PE-

conjugated capture antibodies (which are able to bind any of the six cytokines if they are bound to the beads) in order to form a sandwich complex attached to the beads. The beads are thereafter analysed using a flow cytometer, which separates the beads based on their FL3 fluorescence intensity and then determines the respective PE (FL2) fluorescence intensity of each of the six populations. The BD CBA Analysis Software provided allows analysis of the final FL2 fluoresence intensities of the bead populations and through the standard curves set by the provided Human Inflammation Cytokine standards of known concentrations, it determines the relative concentrations of each of the six cytokines in the tested sample.

The protocols to perform these experiments were supplied by the manufacturer (BD Biosciences). Briefly, the Human Inflammation Cytokine standards were prepared by doubling dilution from the Top Standard (1/10 dilution of stock) to the final 1:256 dilution. The assay diluent was used as a negative control. Next, the capture beads were prepared by removing a 10µl aliquot of beads for each test to be performed (including standards, samples and a negative control) and mixing together well the aliquots from the six individual bead populations. Aliquots of mixed capture beads (50µl) were then transferred into each experimental BD Falcon FACS tube along with 50µl of PE-Detection Reagent. Thereafter 50µl of each test, standard or control sample was added to each tube and were incubated for 3hr at RT, protected from light.

In parallel to the incubation, the Cytometer Setup beads were prepared. These were three tubes of 50µl of Cytometer Setup Beads which were mixed with (A) no additives, (B) 50µl of FITC Positive Control Detector and (C) 50µl of PE Positive Control Detector. The tubes were incubated at RT for 30min protected from light

exposure, and thereafter resuspended in 450µl (tube A) or 400µl of wash buffer respectively.

Once the test sample staining was complete, the beads were washed with 1ml Wash Buffer at 200x g centrifugation for 5 min. The wash buffer was carefully aspirated using a Gilson pipette and the bead pellet was resuspended in 300µl of Wash Buffer and tubes were immediately analysed using the BD FACS Caliber Flow Cytometer and BD CBA Analysis Software using templates provided by Becton Dickenson.

3.2.6 Statistical Analysis

Where indicated, results were analysed for statistical significance using either the Mann-Whitney U test for unpaired non-parametric data or the Wilcoxon Rank Test for paired non-parametric data (Statistica®).

3.3 Results

3.3.1 Description of women with varying grades of CIN attending the Groote Schuur Hospital Outpatients Colposcopy clinic

3.3.1.1 CIN status of the study participants

This study recruited 100 women who were presenting with abnormal cytology at the cervix and who had been referred to the Groote Schuur Outpatients Colposcopy Clinic run by Dr Lynnette Denny (Dept. Obstetrics and Gynaecology, Groote Schuur Hospital). Of the 100 patients, 9 could not be analysed due to blood contamination of the cervical specimen sample, another 8 could not be sampled when the doctor diagnosed them as they were suffering from micro-invasive carcinoma of the cervix, and the samples from 50 patients had to be discarded due to insufficient cervical T cell numbers (CD3 Screen cut off was set at >500 events, section 2.3.2.1). The remaining 33 patients were suitable for study and complete cervical and peripheral blood samples were obtained. The details of these 33 patients have been listed in Table 3.1. Seven of the thirty three (21.2%) women had histologically confirmed CIN 1, 5/33 (15.2%) had CIN 2 and 9/33 (27.2%) had CIN 3. The remaining 12/33 (36.4%) women were found to be negative for disease at the cervix upon presentation at the Colposcopy clinic. This group of women shall henceforth be referred to as the CIN negative population, but it is very important to remember that these women are not true negative controls due to the fact that they were referred to the colposcopy clinic with a pap smear diagnosis of dysplasia at the cervix. Therefore, they are more likely to be patients in whom the CIN has regressed, than patients in whom no cervical lesion had occurred recently.

In order to prevent peripheral T cell contamination of the cervical samples, any sample which was contaminated with RBC had to be discarded. The cut off for

RBC contamination was set at 30%, which correlated to a contamination of 0.03% white blood cells. The numbers of cervical samples which had to be discarded due to RBC contamination ranged from 1/30 (3.3%) in the group of CIN negative women, 4/14 (28.6%) in the CIN 1 group, 3/14 (21.4%) in the CIN 2 group and 1/16 (6.3%) in the group of women with CIN 3.

It was also necessary to discard samples that had too low T cell yields, therefore a CD3+ screen was implemented. The numbers of cervical specimens discarded due to low T cell quantities from each disease grade group were: 18/30 (60%) in the CIN negative group, 6/14 (42.9%) in the CIN 1 group, 7/14 (50%) in the CIN 2 group and 7/16 (43.8%) in the CIN 3 group. This study did not find that women with increasing disease severity were associated with lower cervical T cell recovery from their cytobrush specimens and were hence more likely to be excluded from the study.

3.3.1.1 Antibody Seropositivity of the study participants

Experiments to determine the serum antibody responses to HPV16 VLP were kindly performed by Ms Candice Sampson, Dept. Medical Virology, UCT. For the purposes of this study, it is appropriate to include this data in order to more comprehensively analyse the immune responses to HPV infection, since seropositivity could indicate previous infection. Seropositivity was detected in 13/33 (39.4%) of the women, with the highest number of antibody responses being detected in the disease free group (7/12; 58.3%) and no responses (0/5) in the CIN 2 group. Interestingly, of the patients who were found to be antibody positive to HPV antigens, 2/13 were currently infected with HPV16 at the cervix, and 11/13 were not infected with HPV16. Of those 11 patients their current HPV status ranged from infection with no

HPV types to infection with 5 HPV types other than HPV16. There were no significant associations between the seropositivity of patients and their subsequent ICC responses to HPV antigens.

Table 3.1 Description of the women recruited into this study showing severity of cervical disease, type of HPV infecting the cervix, viral load and HPV-specific antibody responses

CIN Status	Donor	Age	HPV Types *	Viral Load† (RLU/CO)[a]	Antibody Status*
Neg	JP041	41	-	(0.25)[b]	+
	JP028	34	45	16.93	+
	JP034	28	51	56.03	-
	JP035	25	26,73	(0.25)	-
	JP047	45	**16**, 69, 83	4.44	+
	JP045	29	35	73.46	-
	JP058	30	**16**	39.72	?[d]
	JP061	28	33, 52, 68	709.83	+
	JP010	25	51	(0.33)	+
	JP018	24	6, 33	6.39	+
	JP001	50	33	406.33	+
	JP088	22	**16**	nd[c]	-
Mean (±SD)	N=12	31.8 ± 8.9	11/12 infected with HPV 4/12 infected with multiple types	119.3 ± 229	7/12
CIN 1	JP033	38	59	10.08	+
	JP084	40	56	nd	?
	JP089	35	35, 62	nd	-
	JP091	26	51, 53, 59, 68, 54	nd	+
	JP007	37	-	(0.34)	-
	JP013	23	53, 51, 39	2236.26	+
	JP049	35	**16**, 18, 35, 45, 52, 70, 82, 62, 81	64.49	-
Mean (±SD)	N=7	33.4 ± 6.4	6/7 infected with HPV 4/7 infected with multiple types	577.6 ± 1106	3/7
CIN 2	JP060	31	35	490.63	-
	JP062	35	35	(0.47)	-
	JP008	42	68	11.26	-
	JP011	28	**16**	745.02	?
	JP014	52	35	3.58	?
Mean (±SD)	N=5	37.6 ± 9.6	5/5 infected with HPV 0/5 infected with multiple types	250 ± 347	0/5
CIN 3	JP029	31	18, 35, 39	121.64	-
	JP043	38	**16**, 33	270.79	?
	JP048	38	**16**, 18, 31, 53, 56	25.39	-
	JP055	62	**16**	272.34	-
	JP066	34	31, 33	695.43	+
	JP068	34	-	nd	+
	JP017	36	**16**	80.46	+
	JP002	49	73	2.75	-
	JP005	42	52	231.37	-
Mean (±SD)	N=9	40.4 ± 9.7	8/9 infected with HPV 4/9 infected with multiple types	212.5 ± 222	3/9

[a]RLU/CO = Relative Light Units/Control measured by Hybrid Capture II
[b]Values in brackets are negative for HPV DNA at the cervix in the HC test
[c]nd = not done
[d]? = equivocal
* = data kindly provided by Ms C. Sampson, Dept Medical Virology, UCT
† = data kindly provided by Mr B. Allan, Dept Medical Virology, UCT

3.3.1.3 HPV Genotyping of the study participants

Experiments to determine the HPV genotypes infecting each patient's cervix were kindly performed by Ms Candice Sampson, Dept. Medical Virology, UCT. For the purposes of this study, it was necessary to include this data in order to more comprehensively analyse the immune responses to HPV infection.

Twenty two different types of high and low risk HPV were detected in the 33 women tested through the use of Roche reverse line blot genotyping (Figure 3.4). HPV 16 was the predominant type, found in 9/33 (27%) of the individuals participating in the CIN study. The next most prevalent HPV types were HPV 35 (23%), HPV 33 (17%) and HPV 51 (13%). The number of women with HPV infection (Table 3.1) was not significantly greater with increasing disease severity. There was no correlation between multiple HPV infections and disease grade.

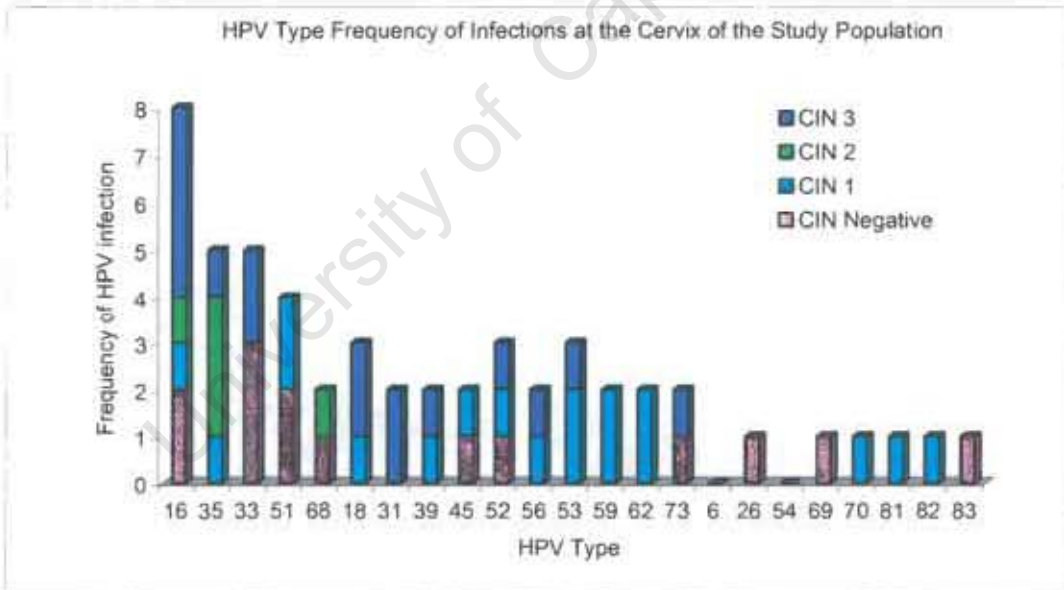


Figure 3.4. The types of HPV found in women attending the Colposcopy clinic. The HPV types were determined through the use of genotyping reverse line blots (RLB) supplied by Roche Diagnostics. HPV DNA was derived from Digene cytobrush sampling of cells from the cervix of women participating in the study.

3.3.1.4 Correlation of cervical disease severity with HPV Viral Load

The Digene Hybrid Capture (HC) ® II HPV Test allows the determination of the relative amount of high risk HPV DNA in the cells isolated from the cervix with a cytobrush. Therefore it gives an indication of the amount of viral DNA and viral replication occurring at the HPV infected cervix. For the purposes of this study, the Hybrid Capture experiment was kindly performed by Mr Bruce Allen, Dept. Medical Virology, UCT. The data depicted previously in Table 3.1 revealed high risk HPV DNA in the cervical cytobrush samples of 23/28 (82.4%) of patients. Three of the five patients who were negative for high risk HPV DNA at the cervix using HC were found to be positive for HPV DNA at the cervix using the PCR and RLB method. This could be due to the fact that if there was a very low copy number of HPV DNA in those cervical cytobrush specimens, it might not have been enough to bind the HC probes, and even if it was detected by the HC probes, the resultant substrate reaction would have been insufficient to change the OD reading to a positive value since HC set a cut off for positivity at 1pg/ml (± 5000 DNA copies). In the RLB method, the HPV DNA has been amplified through a prior PCR step, so there are many more copies of the HPV DNA to bind to the RLB probes and therefore many more complexes capable of increasing the magnitude of the substrate reaction, thereby increasing the detection of the HPV DNA. Also, the HC test does not probe for all the types that can be detected using RLB, therefore, for patient JP035 which had HPV26 and HPV73 at the cervix, the HC results would have been negative since these two HPV types are not detected in HC.

The results from the Digene Hybrid Capture® II HPV test were compared to the stage of CIN disease in the patient group. The data is captured in a box and whisker plot (Figure 3.5). Each box represents the viral load data values for CIN

negative, CIN 1, CIN 2 and CIN 3 patients. The whiskers are representative of the standard error of the results and the mean viral load value for each group is depicted by the center horizontal line drawn in each box. The plot seems to indicate that a higher viral load was associated with CIN 1 patients, but due to the magnitude of the standard error bars and the fact that the mean of all the groups are in close range of one another, it can be concluded that there is no significant correlation between the viral loads and the CIN status. This was also confirmed statistically by overlaying the plot with a regression line, the r value of which was equal to 0.006 (data not shown).

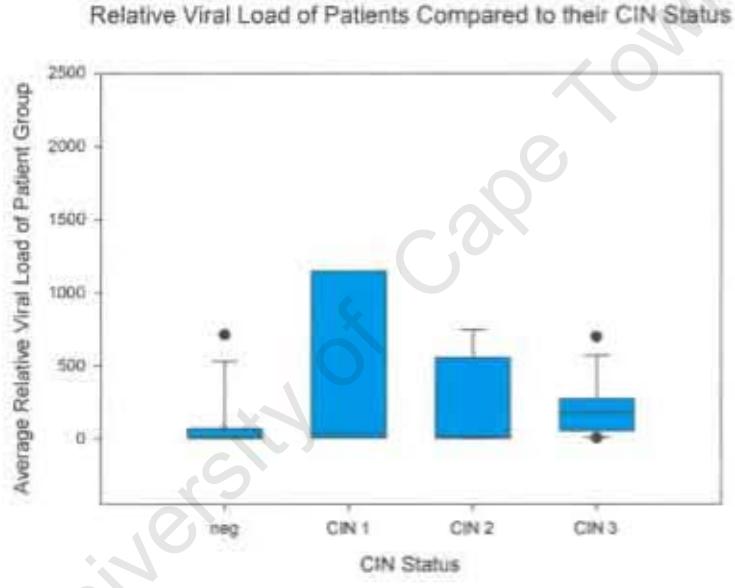


Figure 3.5 Box and whisker plots showing the mean relative viral load according to the disease grade of patients. Viral loads were determined through the Digene Hybrid Capture® II HPV test.

3.3.2 Age of women recruited into the study and T cell recovery from their Cervical Specimens

The mean age of the women in each group showed a non-significant increasing trend from 31.8 ± 8.9 years (mean ± SD) for CIN negative women to 33.4 ± 6.4, 37.6 ± 9.6 and 40.4 ± 9.7 years for CIN 1, 2 and 3 respectively (Table 3.1). Because of the low T cells yields which were obtained from the first 25 patients included in the study (on which no age restriction had been placed), it was decided to exclude women who were either menopausal or post-menopausal (> 40 years of age). Increasing age and menopause is thought to impact negatively on the cellular yield of the cytobrush sample due to the fact that the transformation zone of post menopausal women migrates into the cervical os, and the cervical T cells are therefore less accessible to the cytobrush (Crompton, 1976). Figure 3.6 compares the age of the patient versus the T cell yield, from the CD3+ screens performed on 22 women attending the Groote Schuur Colposcopy Clinics versus their respective age.

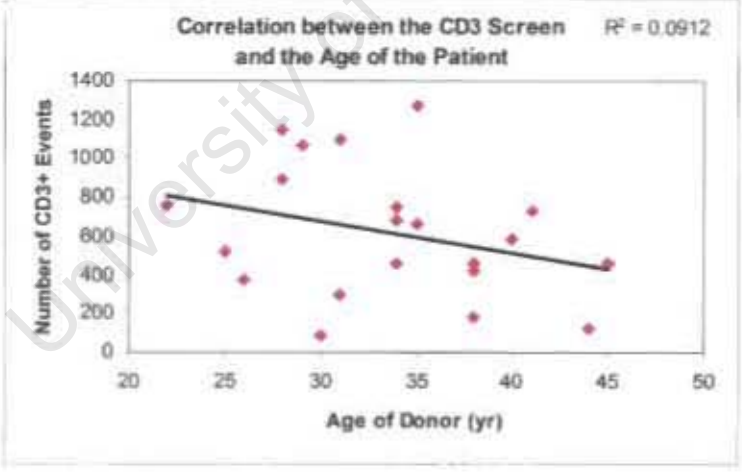


Figure 3.6. Correlation between the total Cervical T cells yielded in a sample and the age of the donor. Processed cervical samples were subjected to a quick CD3+ Screen to determine number of T cells in cervical specimen through the use of flow cytometry. An aliquot of cervical cells were stained with anti-CD3-APC, washed and fixed for analysis on the BD FACS Caliber Flow Cytometer using BD CellQuest software. Each data point represents an individual patient and a trendline has been added to show the correlation between age and numbers of T cells.

3.3.2 Intracellular Cytokine (IFN-γ and IL-13) Production Following Stimulation of Cervical and Peripheral T cells using HPV16 Specific Antigens

3.3.2.1 Individual Patients ICC Responses

Cervical and PBMC T cells from 33 patients were stimulated with HPV16 specific antigens (VLP L1 and E7) and then stained for intracellular IFN-γ and IL-13 cytokines responses. Figure 3.7 shows representative plots from stimulation of donor JP088's cervical cells and PBMC. It is clearly evident that while cervical T cells and PBMC both respond readily to PMA/ionomycin (a non-specific stimulus), the frequency of their responses to HPV antigens is of a much lower magnitude. The raw data from the antigen specific stimulations of all the patients' cervical and peripheral T cells, which was acquired through the flow cytometer, is listed in Table A.1 and A.2 in Appendix A. There were 4 donors whose ICC responses in particular, stood out from the data: JP047 (CIN negative), JP041 (CIN negative), JP049 (CIN 1), and JP043 (CIN 3). Because each donor showed varied background cytokine production, results have been normalized per donor by expressing the percentage response per donor as fold over unstimulated cells; therefore each intracellular cytokine (ICC) percentage has been divided by the percentage of positive events in the unstimulated population from that respective donor. This allowed standardization of the results so that the responses from different individuals could be compared.

The first donor of interest is JP047, from the CIN negative group. This donor had an active HPV 16 infection at the cervix, and HPV16 specific antibodies in their serum. Out of all the donors in the study, the CD4 PBMC T cells from donor JP047 elicited the largest (fold over background) production of IFNγ (9.5 fold and 78.5 fold over background following stimulation with L1 and E7 respectively). The responses to both HPV16 VLP L1 and E7 at the cervix of JP047, however, were definitely

skewed to Th2 cytokine production. The CD4/IL-13 production was 6.03 and 3.88 fold over background and the CD8/IL-13 was 13 and 7.5 fold over background, in response to stimulation with HPV16 VLP L1 or E7 antigen respectively. The IFNγ production in response to L1 or E7 was barely detectable (at less than 0.3 fold over background in both CD4 and CD8 populations).

Another CIN negative patient, JP041, who was not infected with HPV16 at the cervix, but who did have antibody responses to HPV16 VLP, seemed to have remarkably high Th2 responses to the HPV16 VLP L1 antigen at the cervix and PBMC. The PBMC CD4 and CD8 populations produced 11 and 7 times the amount of IL-13 compared to the background unstimulated populations. This was reflected at an even higher fold in the cervical T cells, where CD4 cells produced 75.4 (fold over background) and the CD8 cells 80.78 (fold over background) IL-13 in response to L1 antigen. The cervical T cells from this patient elicited the highest Th2 responses (fold above background) to the HPV16 VLP L1 antigens in comparison with the Th2 responses of all the donors.

JP049, a patient with CIN 1, had the highest number of HPV types infecting the cervical tissues. Nine HPV types were detected through RLB including HPV 16. This patient had good IFNγ responses to HPV16 VLP L1 and E7 in the PBMC, CD4 T cells produced IFN-γ responses of 5 and 6 fold over background (following stimulation with L1 and E7 antigens respectively), and the CD8 T cells produced 5 and 14 fold over background (L1 and E7 antigens respectively). These results were reflected at the cervix, where cervical CD4 cells produced IFNγ responses of 9 and 5 fold over background, although the cervical CD8 cells were less effective than the PBMC CD8+ T cells, producing only 2 fold above background IFNγ when stimulated with L1 and no more IFNγ than the background populations when stimulated with E7.

The only Th2 responses which were greater than 2 fold over background, were those elicited by the PBMC and cervical CD8 T cells in response to E7 stimulation.

Finally, in donor JP043 who was suffering with CIN 3 and had an active HPV16 infection at the cervix, it was interesting to observe that there was remarkably high Th2 cytokine production in the patient's PBMC, but the patient's cervical cells did not elicit any detectable responses (either IFNγ or IL-13 production) above background, following stimulation with the HPV16 specific antigens. The PBMC CD4 cells produced 48 and 46 times the amount of IL-13 detected in the unstimulated population (to L1 and E7 antigens respectively) and the CD8 cells produced 10 and 9 times more IL-13 than the unstimulated population. The CD4 and CD8 cells did produce detectable levels of IFNγ, but these were low in comparison to the IL-13 production (CD4: 3 and 5 fold above background and CD8: 1.5 and 2 fold above background to L1 and E7 respectively). The antibody status of this patient was near the cut-off for positive, which implies that the result was not negative but it could not be reliably considered positive either.

Figure 3.7. Representative FACS plots are shown on the following 2 pages, depicting the populations of CD3+CD8+ and CD3+CD8- (CD4+) T cells from (A) cervical specimens and (B) PBMC specimens of donor JP088 stimulated with PMA/I (5hr; first panel), HPV16 VLP L1 (second panel), HPV16 E7 antigen (third panel) or left unstimulated (21hr; last panel) in the presence of costimulatory molecules and BFA (for the last 5hr). Cells were stained for CD3-APC, CD8-FITC, IFN-γ-PE and IL-13-CyChrome to allow differentiation of Th1 and Th2 responses in the T cells populations. Values shown in each quadrant represent percentages of CD3+ T cells responding to the given stimulus.

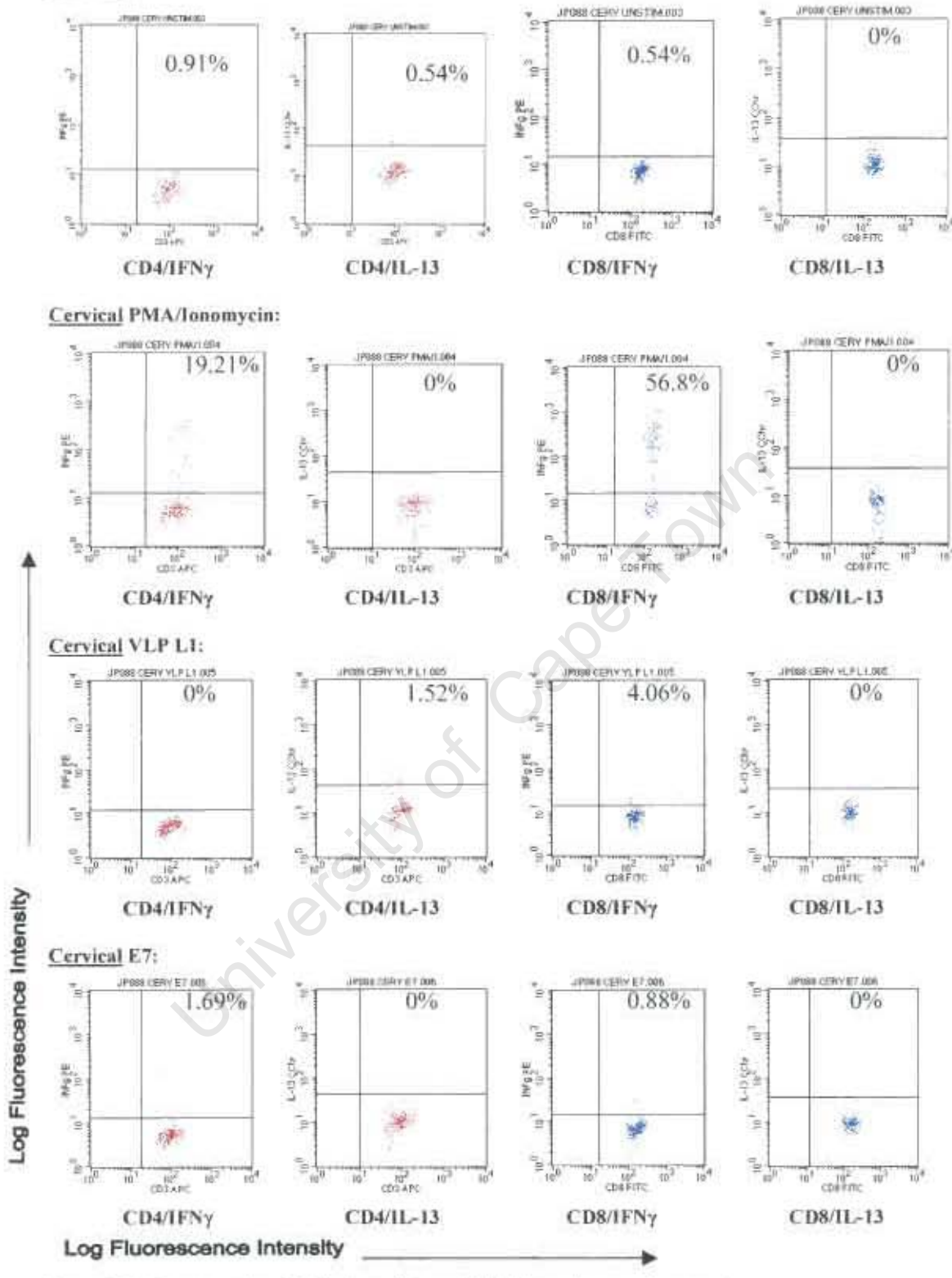


Figure 3.7A Representative FACS plots of Donor JP088 (legend on previous page)

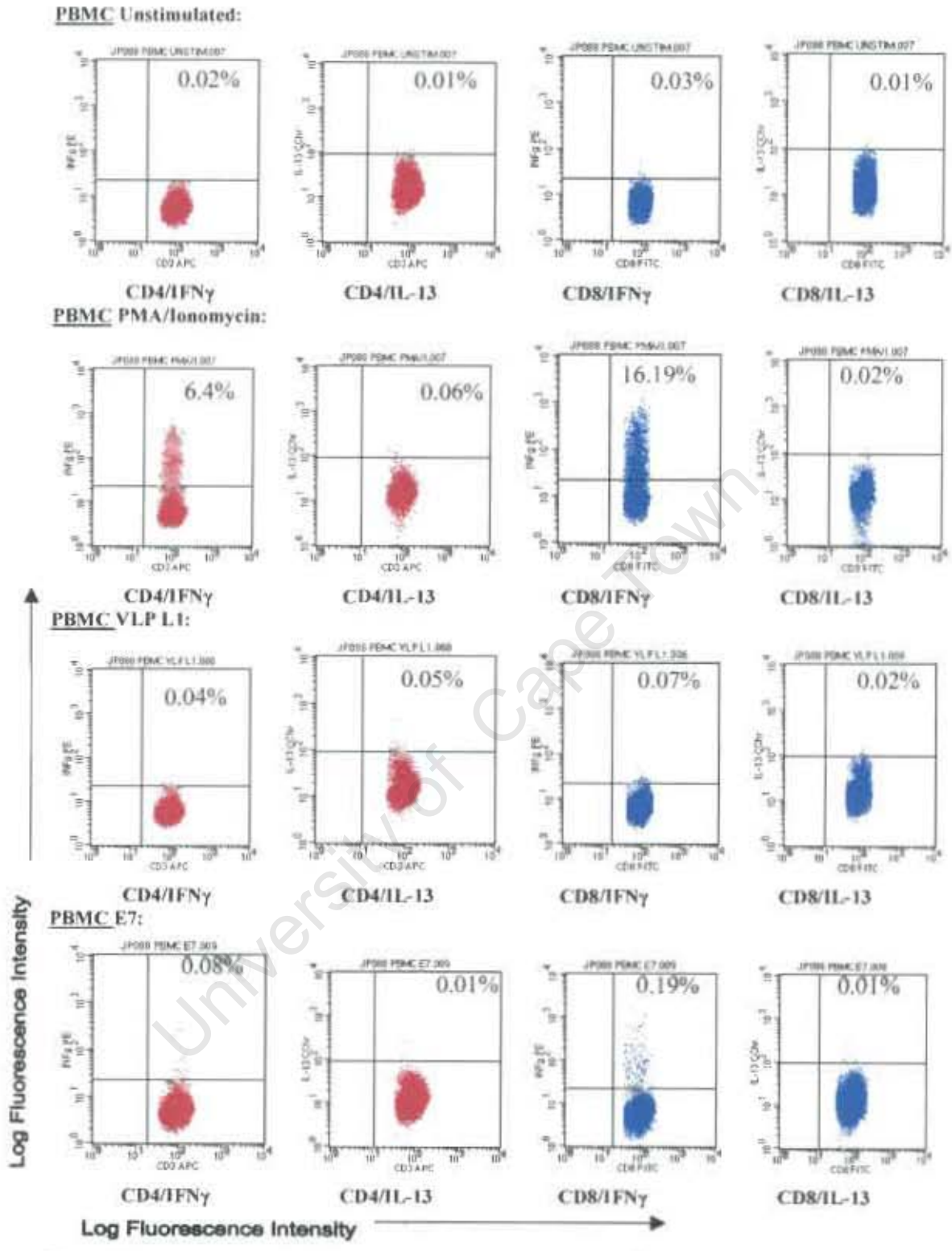


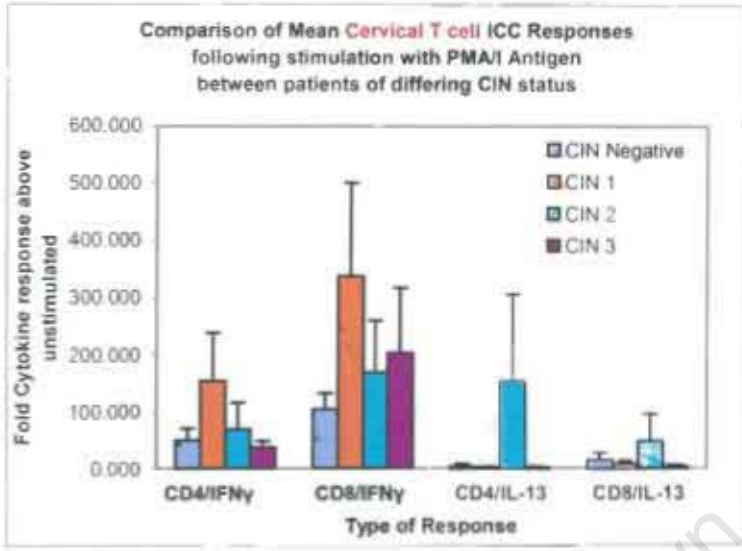
Figure 3.7 B Representative FACS plots of donor JP088 (legend on previous page)

3.3.2.2 Comparison of ICC Responses from all study participants according to disease grade

In a study of this kind, it is of interest whether there are any significant trends between the responses from patients with varying grades of disease. Unfortunately, the number of patients included in the study from whom it was possible to analyse both cervical and PBMC T cells was small and therefore strong significant associations between the various ICC responses and the patients CIN status was limited. Due to the amount of raw data from the 33 patients (listed in Table A.1 and A.2 in Appendix A), the results have been summarised in order to compare the possible trends that could be occurring and which might become more significant in a larger study population.

The PMA/I stimulation was included as a positive control to ensure that the cells were viable and capable of producing immune responses and also to verify that the assay was valid and working correctly. The data shown in Figure 3.8 illustrates that PMA/I stimulation successfully induced Th1 cytokine production in both CD4 and CD8 cells isolated from either the cervix or the peripheral blood. PMA/I is primarily an inducer of Th1 cytokines therefore the results for the Th2 (IL-13) cytokines are far lower than for the Th1. In order to ensure that the lack of detection of IL-13 levels in the positive control was due to it not being produced and not due to assay failure to detect it, a Hick 2 cell line was purchased from Beckton Dickinson Biosiences. This Hick 2 cell line is primed to produce well characterized levels of Th2 cytokines (such as IL-4 and IL-13), therefore the cells were stained and analysed by our standard method and we found that the cytokines were effectively detected and levels of detection compared with those reported by the manufacturer (data not shown). Therefore if IL-13 was being produced it should with confidence be detected.

A. CERVICAL RESPONSES



B. PBMC RESPONSES

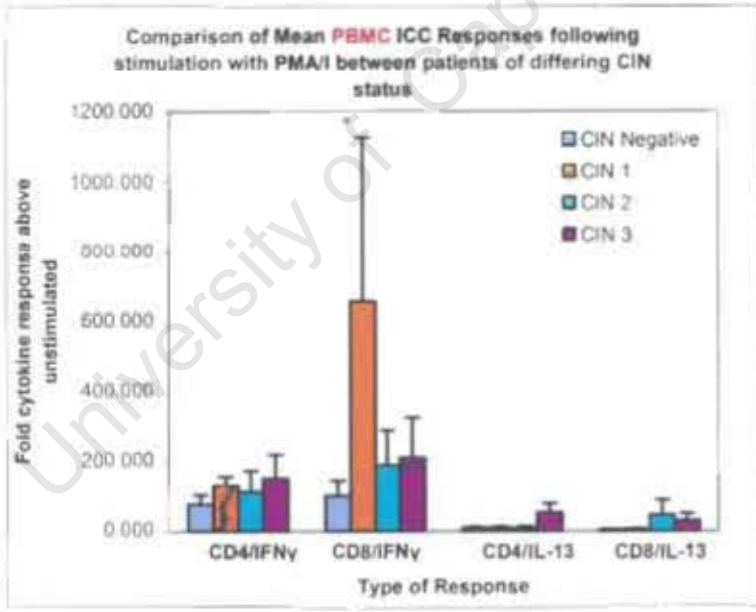


Figure 3.8 Graphs to show successful PMA/I stimulation of T cells from (A) cervical and (B) PBMC samples. Isolated T cells were stimulated with PMA/I in the presence of co-stimulatory molecules and Brefeldin A (BFA) for 5 hr at 37°C, 5%CO_2. Cells were then washed and stained with antibodies to the following markers: CD3-APC, CD8-FITC, IFNγ-PE and IL-13-CyChrome. Each bar on the graph depicts the mean and the standard error of the ICC responses for that disease group (CIN negative, CIN 1, CIN 2 and CIN 3).

Figure 3.9 shows a comparison of the average ICC production by different T cell subsets in response to the HPV specific stimulation conditions (VLP L1 and E7) from the three groups of diseased women and the group of CIN negative women. No significant trend was noticed in terms of cytokine response and disease severity although isolated incidences of significant responses (compared to the negative group) were noted. The relevance of these significant findings is unclear. These figures did indicate that the Th1 responses elicited by the cervical T cells were generally lower than those produced by the blood T cells and this is supported in the CD4+IFNγ+ population where a comparison of the PBMC and cervical data from each patient in the CIN3 group was determined to be significantly different ($p=0.036$; Wilcoxon Ranked test for dependant nonparametric variables). This significance was only noticed in the HPV16 VLP L1 stimulated populations and was not found to be significant in the responses to E7 antigen.

The results in Figure 3.9 also suggest that there might be a trend of decreasing Th2 responses in both PBMC and cervical T cells to both L1 and E7 with disease severity. The interesting difference was that in cervical T cells the largest IL-13 responses were produced by cells collected from CIN negative women, whereas in the PBMC responses, the largest responses were from T cells collected from women with late stage CIN 3 disease. These are both interesting points to acknowledge but significance was only found in one of the comparisons: in responses to VLP L1, the cervical CD4+IL-13+ response was significantly higher in the CIN negative group (3.243 ± 1.376; mean ± SD) than the CIN 3 group (0.741 ± 0.273) ($p=0.04$; Mann Whitney U test comparing the results of independent nonparametric data points).

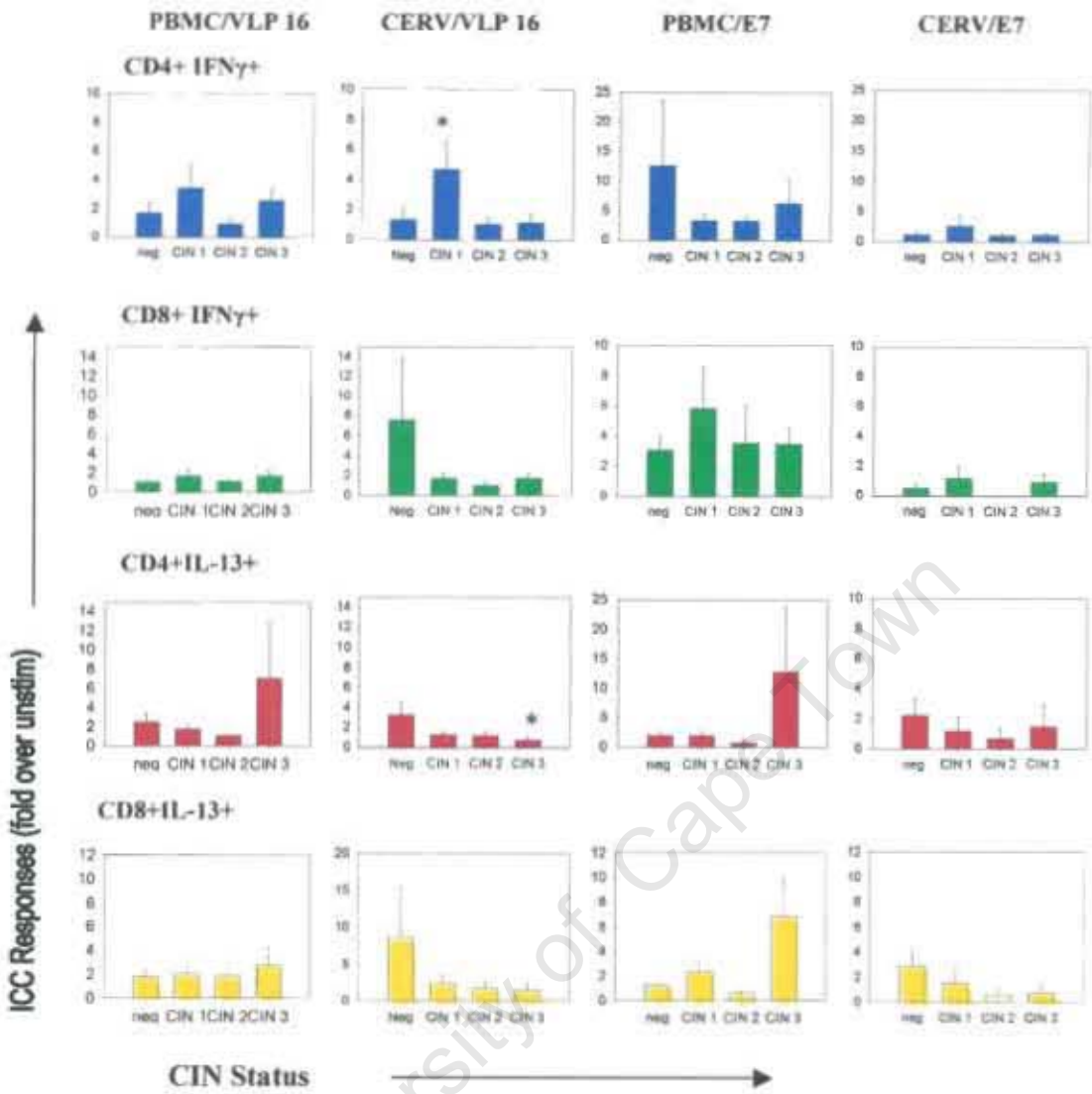


Figure 3.9. Bar graphs to show the mean ICC responses to each stimulation condition in the PBMC (panel 1 and 3) or cervical T cell populations (panel 2 and 4) from all women (irrespective of HPV infection status) compared with disease severity (negative, CIN1, CIN2 and CIN3). T cells were isolated, stimulated with HPV16 specific antigens VLP L1 (panel 1 and 2) and E7 (panel 3 and 4). Cells were then stained with fluorochrome conjugated antibodies to CD3, CD8, IFN-γ and IL-13. Each bar on the graph represents the mean value for ICC responses (fold above background) with standard error bars for each group of women; CIN negative, CIN1, CIN 2 and CIN 3. Significant scores were calculated relative to the CIN negative group, using the Mann Whitney U test for non-parametric data.

3.3.2.3 The impact of active HPV16 infections at the cervix on cervical and peripheral blood immune responses to L1 and E7

The ability of cells to produce T cell HPV antigen specific responses were compared in HPV16 infected individuals and those without HPV16 infection (but potentially suffering from other HPV infections) in order to determine whether the actual presence of an active HPV infection at the cervix might influence the responses of the patients T cells.

Figure 3.10 shows the mean ICC responses (IFNγ and IL-13 production in CD4+ and CD8+ T cells) to the VLP L1 and E7 antigens, in both cervical cells and PBMC irrespective of the disease grade of the patient. The graphs seem to show that the PBMC of HPV16 infected patients elicited higher ICC responses than HPV-16 DNA negative women on average, but this was not statistically significant. The cervical cells from HPV16+ seemed to produce less IL-13 (Th2) responses than the cells from the HPV16 negative women.

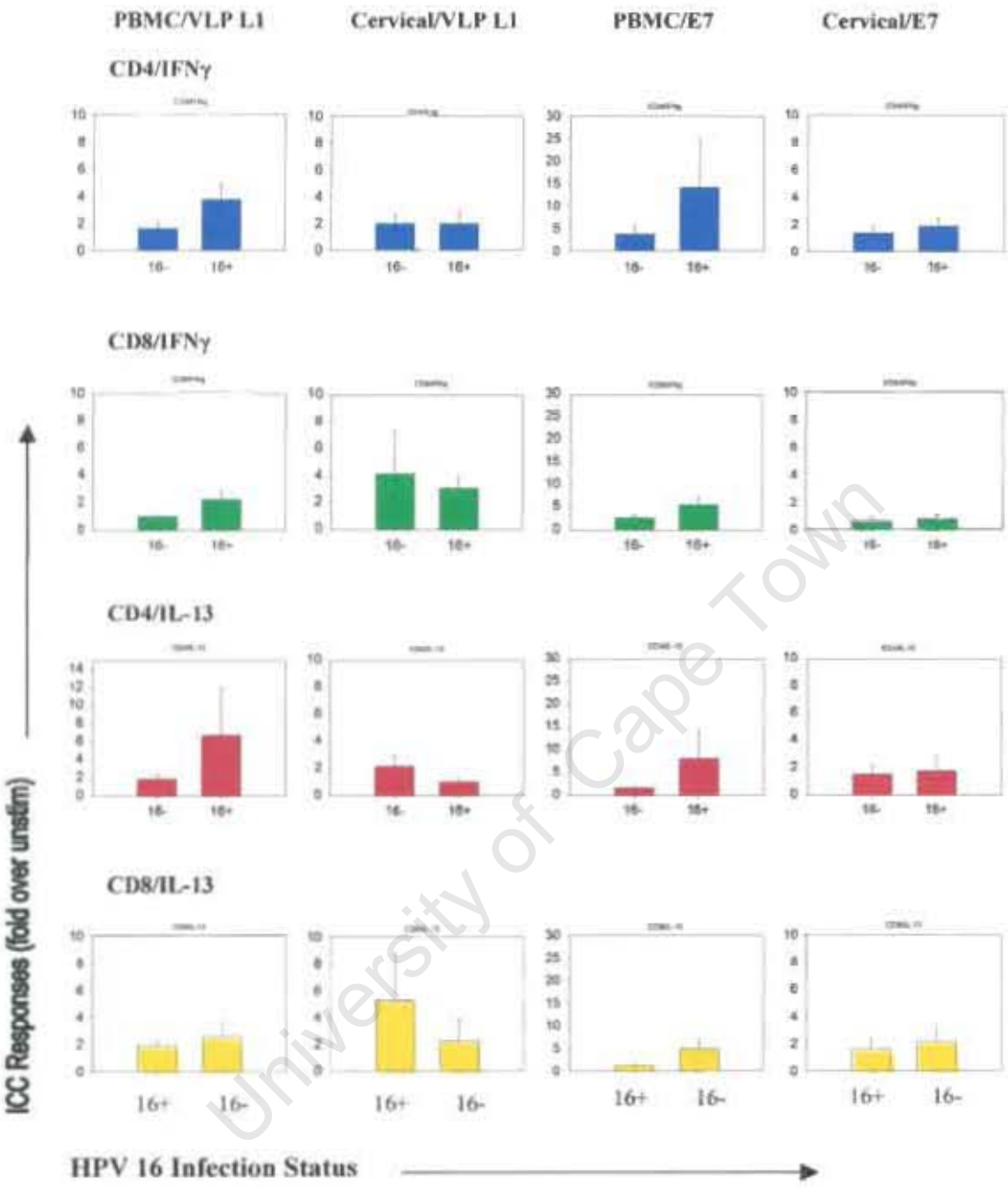


Figure 3.10 Mean ICC responses to HPV16 specific antigens VLP L1 (panel 1 and 2) or E7 (panel 3 and 4) elicited by PBMC (panel 1 and 3) and cervical (panel 2 and 4) T cells of HPV16 infected women, compared to those of HPV16 uninfected women, irrespective of their grade of disease at the cervix. Each bar on the graph represents the mean value (with standard error of the mean bars) for ICC responses (fold above background).

The ICC responses to HPV16 antigen from either HPV16 infected women or HPV16 DNA negative women, are depicted in Figures 3.11 and 3.12 according to the patient's disease status, and the cellular subset eliciting the responses; PBMC or cervical cells. In PBMC cells, the CD4/IFNγ responses to E7 stimulation seemed to increase (with disease grade) in HPV16 negative women but decrease in HPV 16 infected women. Most of the PBMC responses had such large error bars (SEM) that they were not able to indicate true trends occurring in the data. There was one significant positive correlation in increasing CD8/IL-13 responses with disease grade following E7 stimulation elicited by PBMC from the HPV16 infected women (regression value, r=0.752). The PBMC from women with further progressed CIN produced much higher amounts of IL-13 cytokine than women with early stage or no CIN disease at the cervix.

In the graphs correlating the CIN status of the patients (either HPV16+ or 16- women separately) with the average T cell response elicited by their cervical T cells, the trends observed showed decreasing levels of IL-13 cytokine production correlated to an increase in disease progression in the CD8 T cell subset of HPV16 infected samples (Figure 3.12). The correlation between CIN status and magnitude of CD8+IL-13+ responses to HPV16 E7 antigen in the HPV16 infected patients was statistically strong (r=0.57), with the CIN negative patients producing large Th2 responses and the CIN3 patients producing the smallest Th2 responses. These trends were only observed in the HPV16 infected patient groups.

Statistical comparisons of the cervical T cell responses between patients who were suffering from different stages of CIN but who were all infected with HPV16, revealed that HPV16 infected patients suffering from CIN 3 responded to HPV16 VLP L1 with significantly higher levels of CD4+IFNγ+ T cells than the HPV16

infected but CIN negative patients (0.050 ± 0.05 and 1.948 ± 0.891 respectively; mean ± SEM) (p=0.034). The HPV16 negative women with no CIN disease had significantly higher levels of CD4+IL-13+ T cells than the HPV16 negative women suffering from CIN3 at the cervix (4.108 ± 1.753 and 0.405 ± 0.237 respectively; p=0.014).

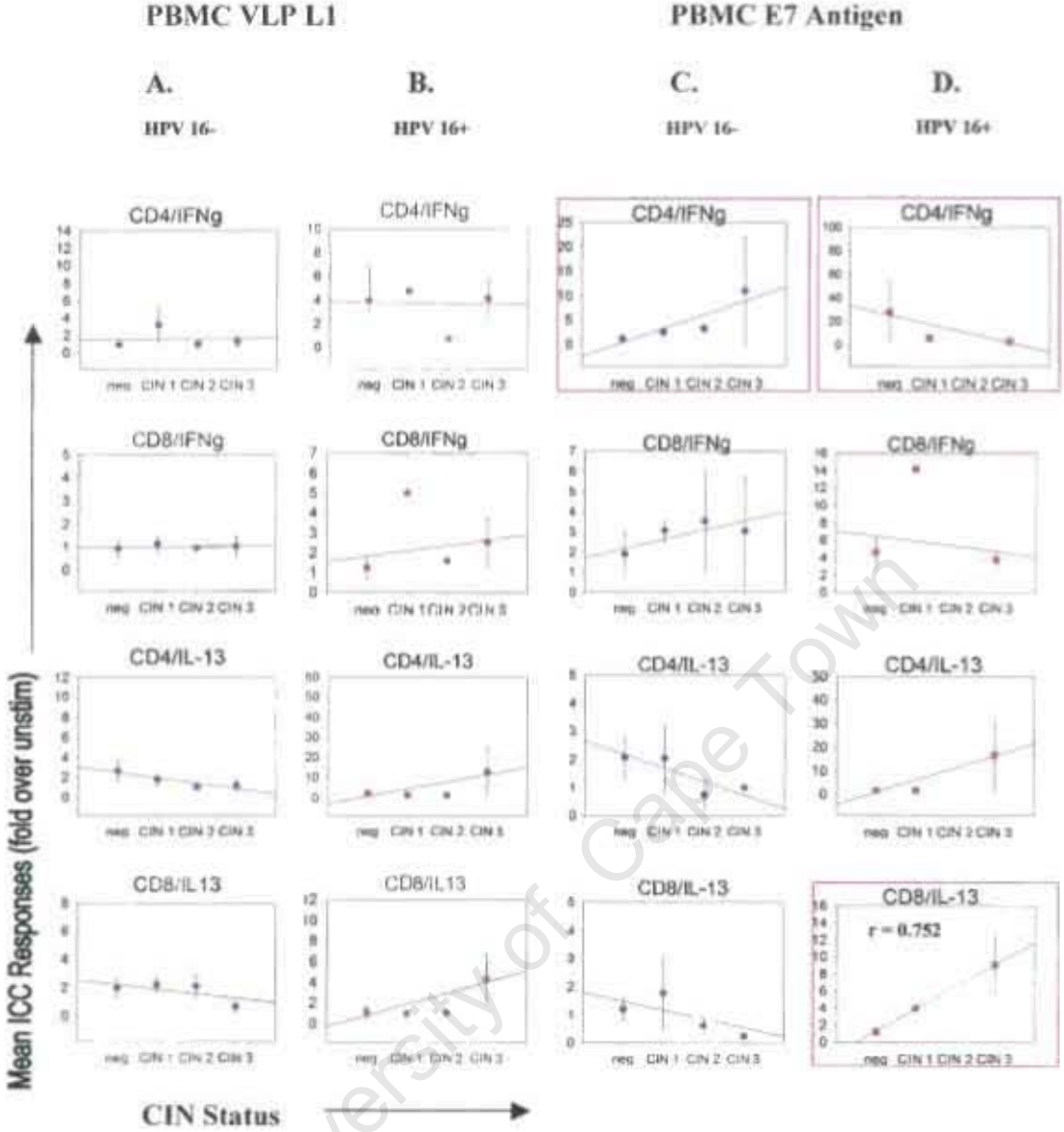


Figure 3.11. Correlation of mean PBMC T cell responses with varying grades of CIN from women either infected with HPV16 (panels 2 and 4; red plots) or not infected with HPV 16 (panels 1 and 3; blue plots) at the cervix. T cells were isolated, stimulated with HPV16 specific antigens VLP L1 (panels 1 and 2) and E7 (panels 3 and 4). Cells were then stained with fluorochrome conjugated antibodies to CD3, CD8, IFN-γ and IL-13. Each point on the graph represents the mean value for ICC responses (fold above background) with standard error bars for each group of women; CIN negative, CIN1, CIN 2 and CIN 3. Lines indicate regression and R values have been shown for those with strong support (R–0.6). Red blocks around graphs are to highlight responses with the strongest correlation with disease severity.

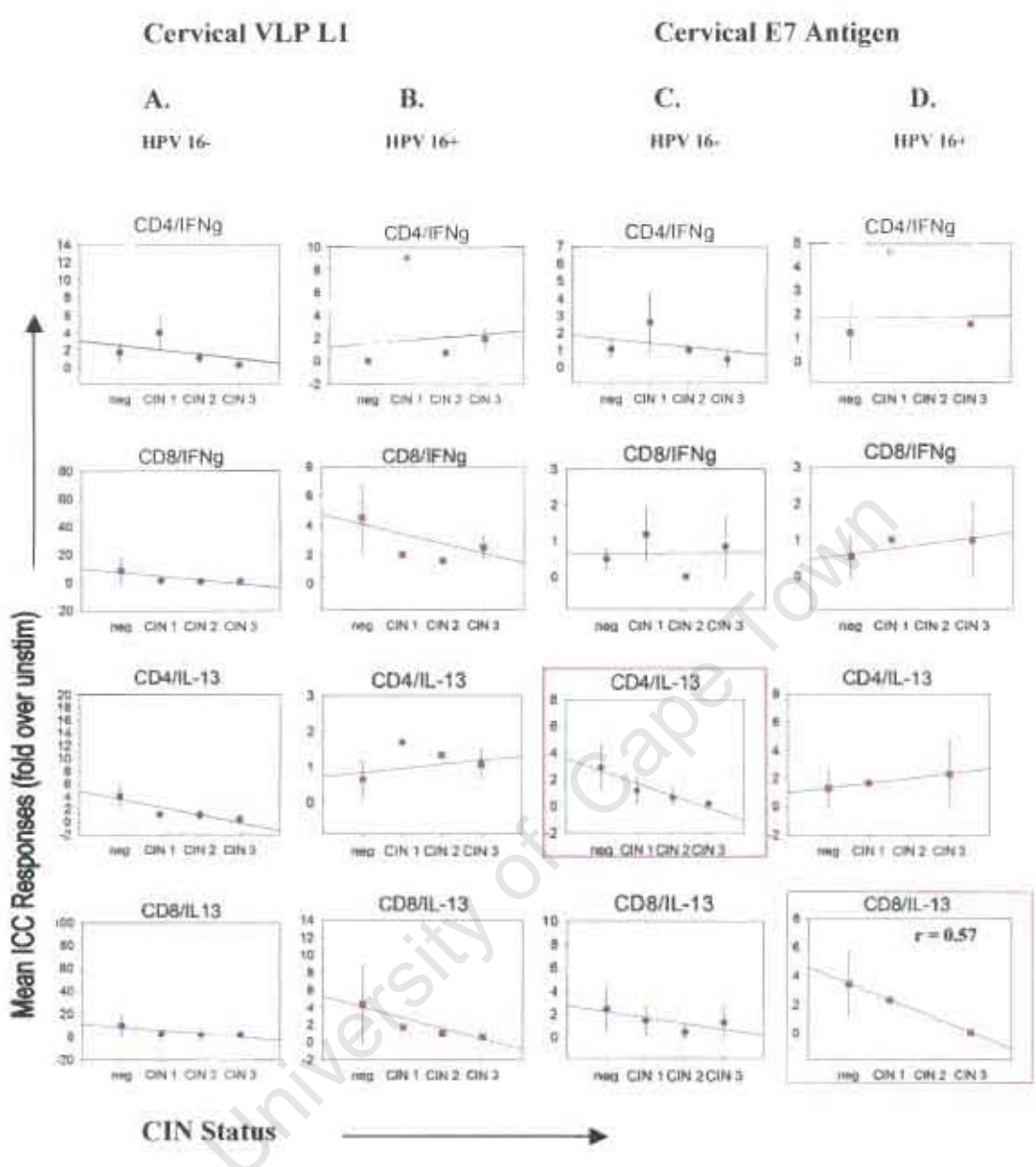


Figure 3.12 Correlation of mean cervical T cell responses with varying grades of CIN from women either infected with HPV16 (panels 2 and 4; red plots) or not infected with HPV 16 (panels 1 and 3; blue plots) at the cervix. T cells were isolated, stimulated with HPV16 specific antigens VLP L1 (panels 1 and 2) and E7 (panels 3 and 4). Cells were then stained with fluorochrome conjugated antibodies to CD3, CD8, IFN-γ and IL-13. Each point on the graph represents the mean value for ICC responses (fold above background) with standard error bars for each group of women; CIN negative, CIN1, CIN 2 and CIN 3. Lines indicate regression and R values have been shown for those with strong support (R~0.6). Red blocks around graphs are to highlight responses with the strongest correlation with disease severity.

3.3.4 Correlation between HPV Viral Load and cytokine response at the cervix and systemically

The viral load results determined by Digene Hybrid Capture® II, were compared to the actual ICC T cell responses elicited by PBMC and cervical cells from all of the study patients (Figure 3.13). This was done to determine whether there was any correlation between the relative quantity of viral DNA at the cervix, and the ability of the T cells to respond to HPV specific antigens.

Interestingly, it was noted that the extent to which a virus has been undergoing replication seemed to have an impact on the magnitude of the CD4+IFNγ+ responses in both PBMC and cervical T cells. In the regression scatter plots comparing the impact of viral load on the number of T cell responses (Figure 3.13), there seemed to be a trend in both cervical and PBMC CD4 T cell responses to VLP L1, of an increase in IFNγ production with increasing viral load titres. These observations are supported by regression values of r=0.57 for PBMC and r=0.51 for cervical T cells. It was also observed in stimulation using HPV16 E7 antigen, that increased viral load titres seemed to be correlated to decreasing numbers of cervical CD4 T cells producing ICC (either IFNγ or IL-13), although this was not significant.

It is interesting to note that when the outlier point which has exceptionally higher viral load that the other samples is removed, the regression values for PMBC and cervical CD4 T cell responses to VLP L1 are reduced to an extent that there is no longer a significant correlation between high viral load and the extent to which T cells respond to viral antigen.

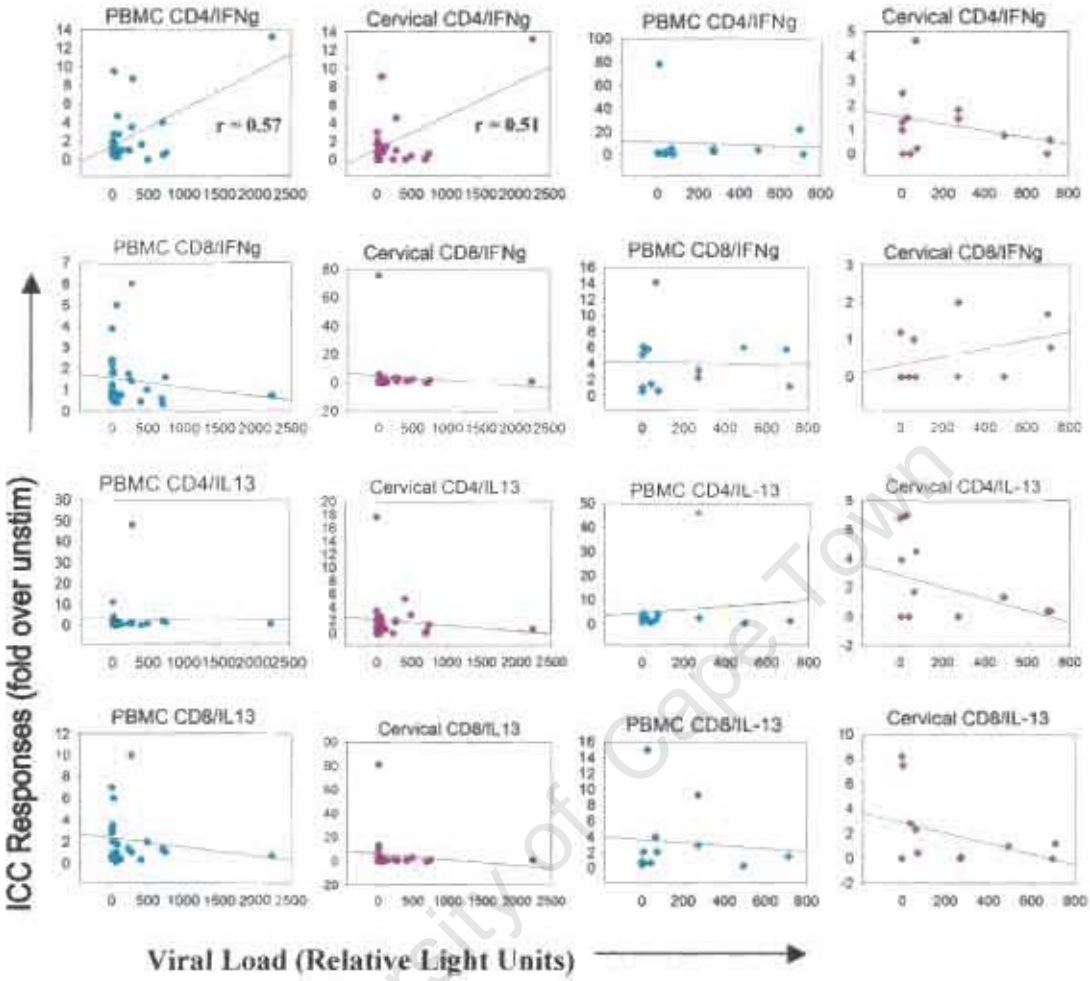


Figure 3.13 Correlation between viral load titres and T cell responses elicited by either PBMC (panel 1 and 3) or cervical cells (panel 2 and 4) in response to HPV 16 VLP L1 (panel 1 and 2) and E7 antigens (panel 3 and 4). Responses were determined by the production of ICC and are shown as fold above background. They were detected using fluorochrome conjugated antibodies to the markers of interest and analysed using a BD FACS Caliber Flow Cytometer with BD CellQuest software. The relative viral load in each cervical sample was determined using the Digene Hybrid Capture® II HPV Test.

3.3.5 Th1 versus Th2 responses in the blood versus at the cervix to HPV antigens L1 and E7

In order to interpret the data in another way, it was necessary to determine a positive cut off point, above which the observed responses could be assumed to be positive responses to the antigen, thereby allowing us to approximate the number of donors who elicited responses to HPV16 specific antigens. Usually in immunological studies (of for example, Human Immunodeficiency Virus [HIV] or Tuberculosis [TB]) negative controls can be obtained from people who have not yet been infected by the virus (Trigona et al., 2003). The patient's unexposed status can usually be confirmed by a lack of viral particles in the patient and no antibody response to viral antigen in their serum. In the case of HPV, this is not possible. Firstly there are many different types of HPV which can cause infection (and which might be able to induce immune responses which could be cross reactive against other HPV types; Höpfl et al., 2000) and secondly not all patients are able to produce antibodies in response to the virus, therefore the lack of antibodies can not be taken as a sign that the patient has never been infected with HPV (Carter et al., 2000; Kirnbauer et al., 1994; Le Cann et al., 1995). Therefore, in studies analysing responses to HPV infection, obtaining a true negative control is a complicated task. For the purposes of this study, since it was not possible to obtain a true negative control, an empirical cut-off of two fold above background (unstimulated) was considered a positive response. Therefore when stating that a patient had a positive response to a specific antigen, this study makes reference to the fact that the patient's antigen specific stimulated T cells elicited an ICC response greater than or equal to 2 times the percentage of ICC producing T cells that were detected in their unstimulated sample population.

In response to the HPV16 VLP L1, PBMC appeared to induce more Th2 type responses. At the cervix, the T cells induced more often Th2 (IL-13) cytokines in response to E7 antigen but in the blood, T cells produced many more Th1 cytokine (IFN-γ) responses when stimulated with E7 antigen (Table 3.2 shown in graphical format in Figure 3.14).

In all analyses, the CIN 1 group elicited the highest percentages of responses against either antigen. In the cervical T cell stimulations, the total percentages of responding T cells (Th1 plus Th2) were lowest in the group of women suffering with CIN 3 and were highest in the women with grade 1 CIN lesions at the cervix.

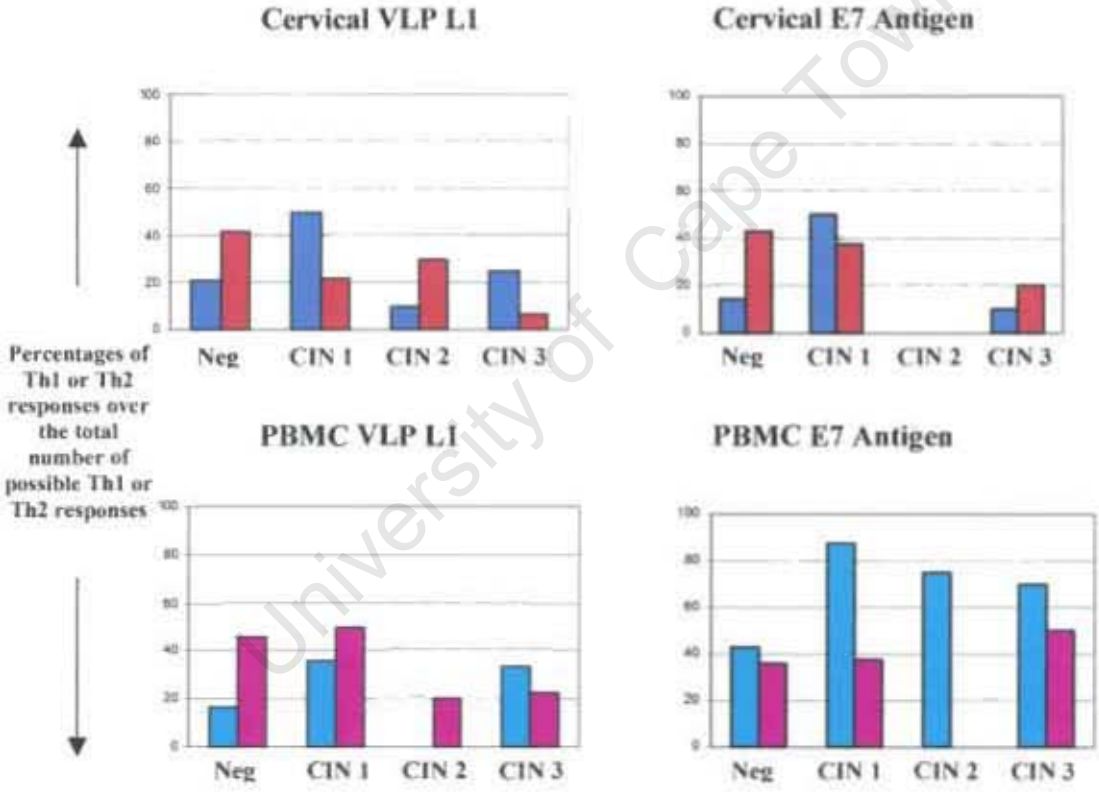


Figure 3.14 Graphs to show distribution of Th1 (dark blue and light blue bars) and Th2 (red and pink bars) responses to VLP L1 or E7 HPV specific antigens, in cervical and PBMC T cells from patients suffering with varying grades of cervical disease (CIN neg, 1, 2 and 3). The magnitude of the bars indicates the percentage of Th1 or Th2 positive responses, out of the total possible number of Th1 or Th2 responses possible (i.e. for each patient in the group two Th1 and two Th2 responses were possible; CD4 and CD8, IFNγ or IL-13).

Table 3.2. Characteristics of donor groups separated by their CIN status and the ability of each group to elicit either Th1 or Th2 responses against HPV Specific Antigens through their cervical and peripheral blood T cells.

CIN Status	No. Patients	Antibody Positive	HPV16+	Stimulation Condition	Responses in Cervical T Cells[b]				Responses in Peripheral Blood T Cells[b]			
					CD4+ IFNγ+	CD4+ IL-13+	CD8+ IFNγ+	CD8+ IL-13+	CD4+ IFNγ+	CD4+ IL-13+	CD8+ IFNg+	CD8+ IL-13+
Neg	12	58.3% (7/12)	25% (3/12)	VLP L1	17% (2/12)	50% (6/12)	25% (3/12)	30% (4/12)	17% (2/12)	60% (6/10)	17% (2/12)	42% (5/12)
				E7[a]	29% (2/7)	43% (3/7)	0% (0/7)	43% (3/7)	43% (3/7)	43% (3/7)	43% (3/7)	29% (2/7)
				PMA/I	100% (9/9)	33% (3/9)	100% (10/10)	50% (5/10)	90% (9/10)	80% (8/10)	100% (10/10)	70% (7/10)
CIN1	7	42.9% (3/7)	14% (1/7)	VLP L1	43% (3/7)	14% (1/7)	57% (4/7)	29% (2/7)	43% (3/7)	43% (3/7)	29% (2/7)	57% (4/7)
				E7	75% (3/4)	25% (1/4)	25% (1/4)	50% (2/4)	75% (3/4)	25% (1/4)	100% (4/4)	50% (2/4)
				PMA/I	100% (6/6)	50% (3/6)	100% (6/6)	67% (4/6)	100% (6/6)	83% (5/6)	100% (6/6)	83% (5/6)
CIN2	5	0% (0/5)	20% (1/5)	VLP L1	20% (1/5)	20% (1/5)	0% (0/5)	40% (2/5)	0% (0/5)	0%(0/5)	0% (0/5)	40% (2/5)
				E7	0% (0/2)	0% (0/4)	50% (0/2)	0% (0/2)	100% (2/2)	0% (0/2)	50% (1/2)	0% (0/2)
				PMA/I	100% (4/4)	50% (2/4)	100% (2/2)	50% (1/2)	100%(4/4)	75% (3/4)	100% (4/4)	100% (3/3)
CIN3	9	33.3% (3/9)	44.4% (4/9)	VLP L1	13% (1/8)	0% (0/8)	38% (3/8)	13% (1/8)	44% (4/9)	22% (2/9)	22% (2/9)	22% (2/9)
				E7	0% (0/5)	20% (1/5)	20% (1/5)	20% (1/5)	60% (3/5)	40% (2/5)	80% (4/5)	60% (3/5)
				PMA/I	100% (6/6)	67% (4/6)	100% (5/5)	60% (3/5)	100% (6/6)	100% (6/6)	100% (6/6)	67% (4/6)

[a] E7 responses were not measured in all donors but only in 7/12 CIN negative donors, 4/7 CIN 1 donors, 2/5 CIN 2 donors and 5/9 CIN 3 donors.
[b] Positive responses were determined as 2 fold above the unstimulated background responses

3.3.7 The cervical cytokine microenvironment of HPV infected women

Through the use of the BD Cytometric Bead Array Kit, it was possible to determine the cytokine microenvironment at the cervix of patients from whom cervical cytobrush samples were collected. In this study, the CBA beads were applied to a sample of the cytobrush collection and transport media, into which the cytobrush had been inserted following rotation in the cervical os. Therefore, any cytokines that the cytobrush might have collected from the cervical tissues would have been resuspended in the cervical specimen supernatant. The kit used for detection of inflammatory cytokines at the cervix can determine the concentrations of IL-12p70, TNF, IL-10, IL-6, IL-1β and IL-8 (the functions of which are listed in Table 3.3).

The resultant concentrations of each cytokine revealed very low (perhaps negligible) levels of IL-12p70, TNFα and IL-10 in all of the patient samples. The concentration was less than detectable in most cases, and never exceeded 9pg/ml. In comparison, for IL-6, IL-1β and IL-8 there were considerably higher amounts of cytokine, the lowest concentration detected was >10pg/ml. Therefore, analysis of the data has been focused on the latter three cytokines. The data depicted in box and whisker plots in Figure 3.15 allows comparison of the results of cytokine concentrations for each of the CIN grade groups (Neg, CIN 1, CIN 2, CIN 3). It is clear to see that there are significantly higher levels of IL-8 cytokine than any of the other cytokines at the cervices of the all of patients. CIN 1 patients had higher average levels of IL-8 cytokine than the other patient groups (2711.5 pg/ml ± 737.3; mean ± SEM), but this result was only statistically significant when compared to the levels of IL-8 at the cervix of CIN 3 patients (636.9 pg/ml ± 130.5) (p=0.0394). The CIN negative patients also seemed to display higher levels of the IL-8 cytokine than CIN 2 and CIN 3 patients, but this was not significant. IL-6 was also present at a

significantly higher concentration in CIN 1 patients (707 pg/ml ± 205.5) than in women with CIN 3 disease (266 pg/ml ± 96.8), (p=0.05). Interestingly the mean levels of IL-6 present at the cervix of all women were much lower than those for IL-8. In fact, IL-8 was present at significantly higher levels than both IL-6 and IL-1β throughout all women when grouped according to their grade of disease ($p<0.026$). Although IL-1β cytokine was present at the cervices of all patients at relatively high detectable levels, it remained at a fairly constant concentration through all of the patient samples and did not seem to vary significantly with the grade of CIN lesion of the patient.

Table 3.3 Description of the functions of the cytokines detected by the Inflammation CBA Bead Kit[a]

Cytokine Detected	Local Effects of Cytokine at Cervix
IL-12p70	Most potent stimulator and differentiator of T cells to mature Th1 or CTL
TNF	Inflammation activator and costimulator of T cells
IL-10	Inhibition of inflammatory cells; promotion of anti-inflammatory responses
IL-6	Growth of mature B cells and costimulator of anti-inflmmatory T cells
IL-1β	Mediator of inflammatory response; also activates and costimulates the T cells
IL-8	Inflammatory action on endothelial cells

[a]Information summarized from Abbas, 1994

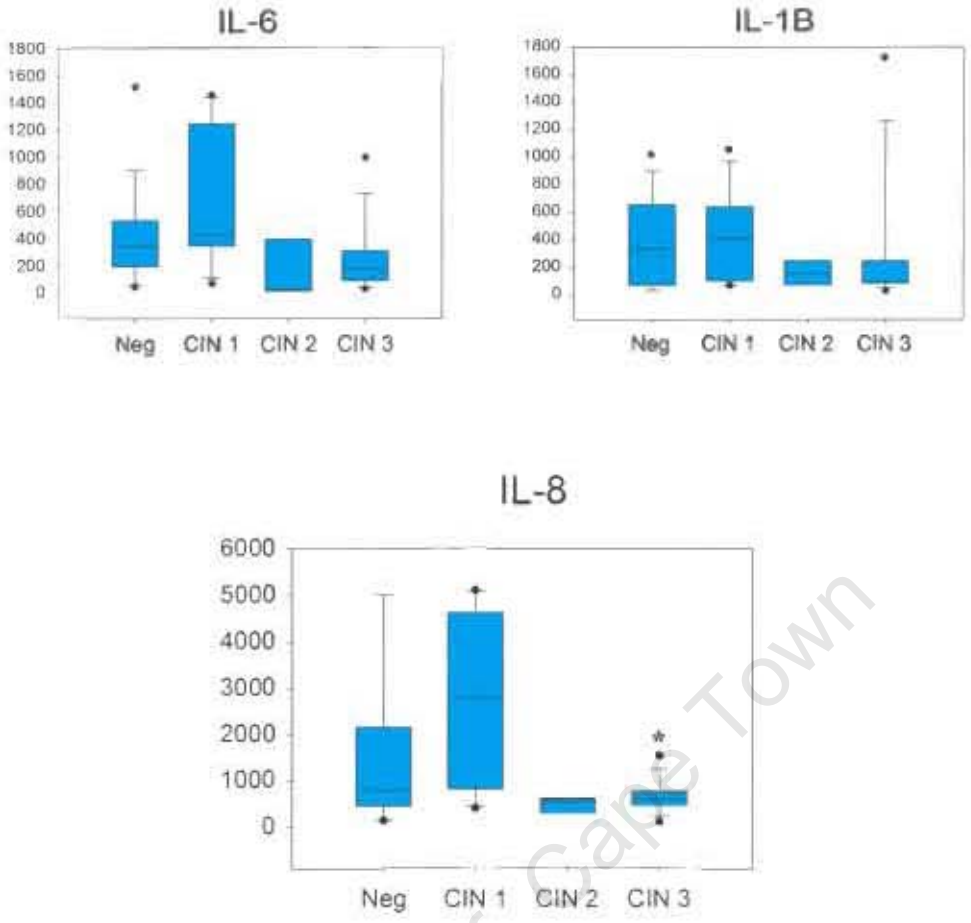


Figure 3.15 Box and Whisker plots showing the presence of pro- and anti-inflammatory cytokines (IL-8, IL-6 and IL-1β) at the cervix of CIN negative, CIN 1, CIN2 and CIN 3 women. Concentrations of the cytokines IL-6, IL-1β and IL-8 in each patient sample (pg/ml), were determined through the use of a BD CBA bead kit and a BD FACS Caliber flow cytometer with BD CBA and BD CellQuest software. * indicates result is statistically significant.

3.4 Discussion

Since HPV infections do not disseminate, the major aim of this study was to investigate HPV-16 L1 and E7 immune responses at the cervix in women with cervical HPV infections and/or HPV-associated cervical disease and then to compare these responses to those detected in peripheral blood. This was done by determining the phenotype of T cells responding (CD4 versus CD8) and their ability to produce cytokines [Th1 (IFNγ): Th2 (IL-13)] in response to HPV specific stimulus. After recruiting 100 women into the study from the Groote Schuur Hospital Outpatients Colposcopy Clinic, only 33 women with cervical disease ranging from negative to CIN3 were suitable for study. This study has shown that the cytobrush method of obtaining cervical lymphocytes combined with intracellular cytokine analysis and flow cytometry in a non-invasive and potentially useful approach to studying immune responses in the genital tract.

Although various HPV-specific T cell responses emerged as being significant, the most notable responses were women with CIN 1 consistently having the strongest CD4 IFN-γ (but not necessarily CD8 T cell) responses at the cervix to HPV-16 antigens compared to women with no cervical neoplasia or those with more severe disease (CIN 2/3). This was observed particularly if one focused on women with active HPV-16 infection but also if one looked at the group as a whole (irrespective of the type of HPV causing infection).

There was a significant trend towards decreasing Th2 responses (IL-13 production) with increasing disease severity, in cervical CD4 and CD8 T cells to both HPV antigens (L1 and E7) if one looked at the group as a whole (and didn't stratify according to active infection with HPV-16). Conversely, this trend was reversed with

increasing Th2 responses with increasing disease severity in the women with active HPV-16 infection (although the correlation coefficients for both increasing and decreasing Th2 responses were not particularly strong).

When PBMC responses from women with HPV-16 DNA at the cervix were compared with those that were infected with other HPV types, the HPV-16 DNA+ women generally produced a Th1 dominant response (more IFN-γ and less IL-13) which changed to a Th2 dominant response with increasing disease severity (particularly for E7 antigen). In contrast, the HPV-16 negative women (infected with other HPV types) showed a complete reversal of this profile with increasing IFN-γ responses and decreasing IL-13 responses with increasing disease grade. The only cervical immune response that correlated with disease grade in this study was that both CD4 and CD8 T cell IL-13 production decreased with increasing disease severity but this was observed in both women infected with HPV-16 and those infected with other HPV types. Although evidence of HPV-16 specificity is lacking, the results do imply that Th2 dominant responses are associated with a "healthier" disease state and IL-13 responses (possibly driving a protective antibody-mediated response) diminish with increasing disease grade. Surprisingly, the magnitude of Th1 responses elicited by cervical T cells was generally lower than those produced by T cells from peripheral blood.

3.4.1 Does age correlate with a decrease in T cells in the cervical epithelium?

During menopause, the epithelial cells of the transformation zone have been shown to migrate upwards from the ecto-cervical area into the endo-cervical region through the cervical os (Crompton, 1976; Cartier, 1984). Therefore, one would expect lower numbers of cervical T cells to be recovered from the more mature cervices of older

women, since there is less of the transformation zone visible to the eye and within reach of the cervical cytobrush. Most studies therefore use age exclusion to ensure that the cervices of the patients being sampled are able to yield sufficient numbers of cervical T cells (Nakagawa et al., 2000; de Gruil et al., 1997). In this study a comparison of age versus cervical T cell did not give a significant correlation, but the reason for that might be because we had already excluded post-menopausal women above the age of 40.

3.4.2 Lack of HPV16 Prevalence in Study Population

HPV DNA was associated with 91% of CIN in this study, which is slightly higher than the prevalence of HPV in CIN observed by other recent studies (Kay et al, 2003; Chan et al., 2003; Molano et al., 2003). HPV16 was found to be the most common type of HPV infecting the cervix of patients in this study. Yet the results from previous studies have indicated that the prevalence of HPV16 in women suffering from CIN is in the region of 50% (Bosch et al., 1995; Kay et al., 2003). The 27% HPV16 prevalence observed in this study was far lower than what we had expected. This could be due to the fact that the specimens used for determining the HPV type infecting the cervix was different in the previous studies. Cervical biopsy specimens of CIN diseased women were obtained and the HPV DNA extracted and typed whereas in this study we extracted and typed HPV DNA from cervical cytobrush specimens. A biopsy yields a much higher quantity of infected tissue (usually from deeper layers as well) than a cytobrush, therefore there may have been a much higher quantity of HPV16 DNA available in the biopsy specimens for analysing and therefore higher levels of HPV16 positive patients.

In this study 39% of women were infected with 2 or more types of HPV. This is considerably high compared to other studies that have shown a multiple infection rate of 12% (Kay et al., 2003). According to a follow up study of cytologically normal women infected with HPV at the cervix, it was shown that multiple HPV types were unlikely to put women at higher risk of lesion progression since clearance of multiple HPV types occurred at the same rate as clearance of single HPV types (Molano et al., 2003). Perhaps the numbers of HPV infections are increased in this study due to a decreased immune response in patients who might be immuno-compromised due to co-infection with human immunodeficiency virus (HIV). HIV is currently highly prevalent in the local South African population, but we did not have ethical approval to determine the HIV status of the patients. When interpreting the above data, however, it should be recognized that the small study size is not conducive to discussing HPV prevalence in the larger female population.

3.4.3 Type of cytokine microenvironment at the cervix

The Cytometric Bead Array (CBA) kit allowed some insight into the cytokine microenvironment present at the cervix when the cytobrush specimens were taken. It was observed that the cytokine present at the highest concentrations at the cervix was IL-8 followed by IL-6 and then IL-1β. Interestingly, the women suffering from grade 1 CIN had the highest amounts of IL-8 cytokine present in their cervical specimens compared with women with higher disease grades (CIN2 or 3) and those without disease. Since IL-8 is an inflammatory chemokine, this could imply that the immune cells at the cervix of women with CIN 1 lesions are eliciting large inflammatory responses to induce lesion regression. This is supported by data which has shown that 85% of CIN 1 lesions actually regress back to CIN negative status (Iatrakis et al.,

2004). Yet recent studies have shown a role for IL-8 in cancer progression, and have shown that cancer cells produced higher levels of IL-8, which correlated with tumour progression (Yuan et al., 2005). In view of these developments this could suggest that it is the actively proliferating tumour cells that are inducing higher levels of IL-8 and perhaps IL-8 is not indicative of a strong immune response to the cervical lesion.

The concentration of IL-8 was significantly higher at the cervix of all patients, in comparison to IL-6 and IL-1β, perhaps because all of the women in the study are either presently suffering or were previously suffering with CIN, therefore all of the women are likely to have inflamed cervical epitheliums due to the original disease. This hypothesis could not be tested, however, because no healthy HPV-infected women were included in the study.

The concentrations of IL-12p70, TNF and IL-10 were lower than results shown in previous studies, which show a peak of IL-12 cytokine at the cervices of the CIN 1 patients (Giannini at al., 1998). The supernatant in which the cytobrush was transported and washed vigorously is probably not ideal for measuring cervical cytokines concentrations (although it did contain the entire mucolytic component of the cytobrush sample and all cervical components other than cells). Future studies aiming at measuring cytokines from the cervix would have to investigate alternative collection methods and compare these with the approach used above. It would probably be more suitable for the samples of the cytokines present at the cervix to be collected by cervical lavage (where the top of the vagina and the cervix are flushed repeatedly with PBS). This might have improved the detection of the IL-10, IL-12p70 and TNF cytokines.

3.4.4 Individual ICC Responses of four interesting patients

This study analysed a total of 33 women's cervical and PBMC responses to HPV specific antigens. Four donors who appeared to have the strongest and most interesting immune responses were isolated and their results described in detail. Two patients were from the CIN negative women; the patient with an active HPV 16 infection at the cervix elicited a strong PBMC CD4/IFNγ response to both HPV16 VLP L1 and E7 antigens. Yet the responses from the patient's cervical T cells were marked by a strong production of Th2 cytokine (IL-13). This suggests that perhaps following clearance of a lesion at the cervix this patient is attempting to regulate and balance the cervical environment, by inducing Th2 responses in the cervical T cells (reflected in the presence of serum antibodies to HPV16 VLP), yet in the PBMC population, there is still an immunological memory against the HPV antigens since there is still HPV16 infection at the cervix which needs to be cleared.

The second CIN negative patients was not infected with HPV 16 but still had antibodies, which suggests that perhaps this patient had successfully cleared a previous HPV16 infection. In this patient, both PBMC and cervical T cell responses were skewed towards a Th2 profile, which suggests that after successful clearance of HPV infection and regression of CIN, the patient is left with antibody immunity to prevent future HPV16 infections.

The third patient was from the CIN 1 group of women. This patient was actively infected with HPV16, but was seronegative at the time of specimen collection. Interestingly, this patient had a high response in the cervical CD4/IFNγ population to both the HPV16 specific antigens, which suggests that this CIN 1 patient is currently attempting to induce lesion regression and viral clearance at the cervix through inflammatory methods.

Finally, the fourth patient was of interest because the cervical T cell responses of this patient were no higher than the background unstimulated population. In the PBMC T cells of this patient there was high production of Th2 IL-13 cytokines. This patient was infected with HPV16 at the cervix, but their antibody status was equivocal, and could not be reliably determined. Therefore this patient might be an example of the type of immune response (or lack thereof), which facilitates progression of CIN lesions to CIN 3 and can not induce clearance of the HPV infection.

3.4.5 Women with CIN 1 consistently showed the strongest responses to HPV antigens

Women with CIN 1 consistently showed stronger CD4 IFN-γ responses at the cervix but also in peripheral blood to HPV-16 antigens in all women with HPV infection as well as in women with active HPV-16 infection. This was not a function of increased HPV viral load, CD3 cervical count or any of the other criteria measured in this study. This significantly elevated recall response to HPV-16 antigens in women with CIN 1 was also mirrored in direct measurement of inflammatory cytokines (IL-8 and IL-6) in the cervical washing. It is clear from recent publications that progression from persistent HPV-infection to CIN1 occurs with much higher frequency than progression of CIN1 to CIN2/3 (Schlecht et al., 2003; Brenner and Syrjänen, 2003). This indicates that the majority of CIN1 lesions spontaneously regress following an effective immune response. Consistent with previous evidence from wart infiltrating lymphocytes that show CD4 T cell production of IFN-γ correlates with lesion regression (Nicholls et al., 2001; Stanley, 2001), this study also

found significantly higher levels of cervical CD4-mediated IFN-γ production in CIN 1 than in either CIN2/3 or women with no lesions.

3.4.6 Trends in T helper responses between patients with varying grades of cervical disease

The type of T helper response predominating in the environment where the host immune cells encounter a foreign organism, can influence the type of response that the host immune defence system chooses to fight the pathogen – i.e. anti-inflammatory or inflammatory (Openshaw et al., 1995; Counsins et al., 2002). Therefore it is of interest to determine whether the cervical or blood T cells are predisposed to induce a Th1 or Th2 environment (or type of response) when stimulated with HPV antigens following HPV infection.

The trends in the data indicate that the numbers of Th1 responses at the cervix were far fewer than that in the blood and this was found to be significant for CD4+ T cells producing IFNγ in particular. It seemed that cervical T cells from patients suffering with late stage cervical dysplasia (CIN 3) were significantly less responsive to HPV16 specific VLP L1 antigens than their PBMCs. Since there was no significant difference between the magnitudes of the T cell responses elicited by PBMC and cervical cells isolated from women with no lesion, CIN 1 or CIN 2, this could imply that these patients had equally effective cervical and PBMC responses. This suggests that patients with weak cervical CD4 T cell IFNγ responses are unable to control their HPV infection and therefore are likely to progress to CIN 3. This supports the data found in previous studies of a correlation between Th1 cytokines at the cervix and subsequent lesion regression (Al-Saleh et al., 1998; Luxton et al., 1997). Although an alternative (and equally likely) explanation would be that

women with CIN 3 have higher frequencies of HPV-responsive cells in their PBMC than women with lower grade disease and this increased frequency results in significantly elevated PBMC responses compared with those at the cervix. The reasons for comparatively depressed responses in CIN3 women at the cervix compared to PBMC are unknown but very interesting. HPV causes localized infections that do not induce an inflammatory response and hence, it is generally believed that the systemic immune system remains ignorant of the infection. The finding here then that peripheral blood responses are greater in magnitude than cervical HPV responses is difficult to reconcile with this hypothesis of peripheral immunological ignorance. The female genital tract is however recognized as a necessary "immuno-privileged" or tolerogenic environment to ensure reproductive success and this could largely account for the observed weak cervical responses measured in this study.

The differing cervical and PBMC T cells responses found in this study are important to bear in mind when interpreting the data from other studies only analyzing PBMC responses to HPV specific antigens. Since it is clear from this study that PBMC responses to HPV antigens do not necessarily imply that there are T cell responses to HPV at the cervix of the patient, were active infection is occurring.

The data from this study highlight a potential correlation of decreased Th2 responses of both cervical and peripheral blood T cells with increased severity of cervical disease. Yet the difference between the PBMC and cervical populations was that the cervical cells isolated from women with no CIN had the highest Th2 responses, whereas in the PBMC, it was the women with the most severe CIN 3 disease who had on average the largest Th2 responses to HPV16 VLP. These results imply that a CD4/IL-13 response to HPV16 VLP L1 at the cervix might be optimal

for prevention of lesion formation, whereas a CD4/IL-13 response detected in the peripheral blood T cells of patients could be a risk factor for disease progression to late stage cervical dysplasia. Another important factor is that perhaps responses in CIN negative women are due to an effective immunological memory, which in previous studies has been shown to last for 20 months (Nakagawa et al., 2002).

3.4.7 Impact of HPV-16 infection on local and systemic T cell responses

As would be expected, patients actively infected with HPV16 at the cervix were found generally to exhibit higher PBMC responses to the HPV16 specific antigens. This was not reflected in the cervical T cell populations, which is interesting to note since the cervical T cells are located at the site of active HPV replication. Therefore one would expect the cervical T cells to be more responsive to HPV-16-specific antigens since they are the immune cells closest to the cervical infection to receive priming of the HPV16 antigens through presentation by the infected keratinocytes. Because it is well recognized that epithelial cells do not have the necessary co-stimulatory molecules to enhance the antigen presenting synapse (by virtue of the fact that they are not antigen-presenting cells) and that T cell responses inadequately primed by poor costimulation are often termed tolerant or anergic, the results from this study must be interpreted with this in mind (Maljezyk et al.,1997).

Interestingly, women infected with HPV types other than HPV-16 were also able to elicit responses to the HPV16 specific antigens which imply that the T cell primed to other HPV types may be able to cross react with HPV16 VLP and E7. This phenomenon has been observed in previous studies (Kadish et al., 2002). This information is valuable for future vaccine considerations, since it suggests that vaccination against HPV16 could cross protect the cervix from other types of HPV

infection. More likely, however, is that the T cell responses detected in women with HPV infections other than HPV-16 may reflect that they had been infected with HPV-16 at some time point prior to this study (supported by detection of HPV-16 specific antibodies in 39.4% of women) but had subsequently cleared this infection and had readily detectable HPV-16-specific memory T cell responses in the absence of active infection. There was, however, no significant difference observed between the T cell responses of patients who were HPV-16 seropositive versus those that were seronegative.

In the comparisons of responses from HPV16 infected women and those infected with other HPV types according to the severity of their cervical disease, there was a strong positive correlation between IL-13 production from CD8 T cells in PBMC and severity of the cervical lesion. These responses were induced by the E7 protein stimulations only, which suggests that perhaps due to the nature of a non-productive HPV infection (where E6 and E7 are produced abundantly in comparison to L1 and L2), the further the patient's CIN has progressed, the more E7 would be found in the region of the CIN (Middleton et al., 2003). This also supports evidence of Th2 responses allowing lesion progression (Scott et al., 1999; de Gruil et al., 1999).

The results also showed that the CD4 T cells from the cervical specimens of the HPV16 infected CIN 3 patients produced significantly higher levels of IFNγ than the CD4 cells of the CIN negative group. Either this suggests that a CD4 IFNγ response at the cervix is not a correlate of protection for patients exposed to HPV or it implies that CIN3 women induce a Th1 type immune response at their cervices in order to defend the host against both the pathogenic invasion and neoplastic changes. This latter idea is supported by the results from women with non-HPV16 type infections that showed significantly lower levels of IL-13 cytokine production in the

CIN 3 group than the CIN negative group. This could be due to a skewing of the immune profile at the highly dysplastic cervix to a Th1 inflammatory response, in an attempt to clear the HPV infection (too little too late). Since many studies have shown the importance of the Th1 inflammatory profile in clearance of HPV and regression of lesions, it seems that the latter explanation is more likely. This is also supported by the finding that CIN negative women have a more balanced Th1:Th2 ratio at the cervix than CIN 1 patients (who all had high levels of Th1 cytokines at the cervix; Scott et al., 1999).

3.4.8 Effect of HPV viral load on T cell responses at the cervix

Detection of the quantity of HPV DNA in cervical specimens by Digene Hybrid Capture can be used as a useful correlate of HPV viral load (Prétet, 2004). The limitations to this assay are that the viral load is not absolute but more a relative guide. The main disadvantage of this approach is that there is no control for the number of cells collected by the cytobrush. Therefore if there were a higher number of cells collected for one patient, the resultant viral load could be confounded if this result were compared directly to the same result from a patient with low cervical cell count

No correlation between grade of cervical disease and HPV viral load was found in this study. This was important to note, because if viral load was associated with CIN status this might skew the T cell responses being investigated. A significant correlation between high viral loads and high levels of IFNγ cytokine production by CD4 T cells was observed following stimulation of the T cells with HPV16 VLP L1. This result is important since the significance held true for CD4 IFNγ production in both the cervical and the PBMC specimens. These results suggest

that high levels of viral DNA at the cervix results in CD4+ T cells undergoing constant priming by the capsid proteins of the viral particles as the virus replicates and produces much viral progeny. This shows that in response to an active HPV16 infection, the immune system elicits primarily Th1 CD4 responses both locally and systemically and that this is directly associated with the amount of antigen present (viral load).

3.4.9 Summary of the cellular immune environment in response to HPV infection at the cervix of women with varying grades of CIN

Even though there were many interesting trends in both cervical and systemic T cell responses to HPV antigens that associated with grade of cervical dysplasia, this study did not have the statistical power to significantly confirm these trends and this was primarily due to the fact that so few of the 100 women enrolled had sufficient T cell numbers in their cervical specimens for in depth analysis.

Therefore for future studies, the following three points should be taken into consideration. Firstly, it is recommended that the study recruits the largest number of women possible. If only approximately one third of the women recruited are eligible for study, then at least 300 women would have to be evaluated to give 100 eligible study participants.

Secondly, this study found that PBMC and cervical T cell responses to a given HPV antigen are often significantly different in matched samples from the same woman. This shows that future studies can not only study PBMC responses to HPV antigens as being representive of the cervical immune response to HPV infection. It is necessary to study both the cervical and the PBMC responses in order to obtain a

more accurate picture of the complete immune response elicited by a patient to HPV infection.

Thirdly, it was also noted that the CIN 3 patients elicited significantly higher Th1 cervical responses to HPV antigens, whereas the CIN negative patients produced significantly higher Th2 responses to HPV antigens. In terms of correlates of protection, this implies that Th1 type responses are not protective but are associated with increasing disease severity. In contrast, responses from the CIN negative patient populations appear to be associated most strongly with Th2 cytokine production, which could be the result of the immune system switching from Th1 responses (which might have induced previous lesion regression) to Th2 responses to HPV antigens, since there is no longer a lesion and therefore no longer a need for inflammatory action in response to HPV antigens.

Finally, the most significant points of interest which emerged from analysis of the data collected in this study were that patients with early cervical dysplasia (CIN 1) elicited the highest and largest overall responses to HPV antigens, whereas patients with late stage cervical lesions (CIN 3) elicited the fewest and lowest responses to HPV16 specific antigens.

CHAPTER FOUR:

HPV-16 SPECIFIC CD8 T CELL MEDIATED CYTOTOXICITY IN WOMEN WITH CIN: PERFORIN AND CD107A AS MARKERS OF CYTOLYTIC POTENTIAL AND FUNCTION

4.1 Introduction

Host immune responses to viral pathogens rely on a combination of different mechanisms to elicit protection. One of the most important mechanisms is antigen specific cytotoxicity elicited by the CD8 cytotoxic T lymphocytes (CTLs) (Barry and Bleackley, 2002). These T cells are vital in response to viral infection since they are able to specifically kill the cells which are infected with virus, and therefore interrupt the viral lifecycle and abort viral reproduction. Cytolytic activity in CD8 T cells is induced when the T cell is activated by antigen that it recognizes as foreign to the host. CD8+ T cell receptors interact with the MHC I antigen presenting molecules which are displayed ubiquitously on all cells (Abbas et al., 1994). If a CTL recognizes that the peptides displayed by MHC I on antigen presenting cells are foreign it will induce cytokine production and cytotolytic activity in order to induce apoptosis of the infected cell. Direct cytotoxicity involves recruitment of cytotoxic granules to the membrane location of the activated T cell receptor (Peters et al., 1991). These lytic granules contain lytic proteins perforin and granzymes. The membrane of cytotoxic granules (enclosing these cytotoxic proteins) contain lysosomal associated membrane glycoproteins (LAMPs; also known as CD107), which are not usually found on the outer surface membrane of the T cells (Peters et al., 1991). CD107 or

LAMP is further divided into CD107a (LAMP-1) and CD107b (LAMP-2). On stimulation of the CTL, these granules fuse to the cell surface membrane and secrete their contents via exocytosis into the synapse between the T cell and the target cell. Perforin molecules polymerize and form ion channels through the surface membrane of the target cell (Abbas et al., 1994). This permeabilisation of the infected cell's outer membrane, allow various granzyme proteins into the cell and these trigger a cascade of nuclear destruction and subsequent cellular apoptosis by cleaving a family of caspase proteins (Barry and Bleackley, 2002).

Since the functional capability of CD8 T cells is an important factor in the strength of an immune response again viral pathogen, many studies of HPV infection have used assays to directly measure the amount of target cell lysis by primed CTL (measured by Chromium release). Previous studies of cytotoxic response to HPV infection in women suffering from CIN have revealed conflicting results, although it appears that there are significantly more cytotoxic responses to HPV antigens in disease free women, than in patients suffering with CIN (Nimako et al., 1997; Nakagawa et al., 1996, 1997). CTL responses from HPV16+ patients seem to be associated with clearance of the infection and have been shown to last for up to 20 months post clearance (Nakagawa, 2000, 2002). These results indicate the importance of a CD8 T cell cytolytic response in the defense against HPV infection and associated CIN. Therefore in order to more comprehensively understand the abilities of the immune system in defense of HPV infections, it was decided that this study would include the development of an assay to assess the cytolytic ability of T cells. The assay was developed on PBMC with the intention for future use on cervical T cell specimens.

Traditionally the method used for testing the cytolytic functional ability of T cells was the chromium release assay (Nakagawa et al., 2000). This is a complicated assay and the process involves many steps. Firstly, it requires 1-3 week stimulation and culturing of the patient's T cells. Secondly, it in necessary to prepare target cells which are infected with modified viral genomes to induce production of HPV specific antigens (e.g. VLP L1, E6 or E7) and which are labeled with radioactive chromium. Thirdly, these target cells are added to the cultured effector CD8 T cells and the amount of chromium which is released during the assay is measured as a reflection of the ability of the CD8 T cells to elicit cytotoxic functions against HPV infection (Nimako et al., 1997). Apart from being time consuming and involving potent radioactivity, a further disadvantage is the need for *in vitro* bulking up of responsive CD8 T cells in order to obtain an activity level which is detectable. This step is necessary since the assay is not sensitive enough to detect responses directly *ex vivo*.

Another method of studying CTL activity involves the use of flow cytometry in order to measure the various cytotoxic markers inside T cells (such as perforin, granzyme A and B, or LAMP) (Appay et al., 2000; Sandberg et al., 2001; Betts et al., 2003). Perforin is stored inside CTLs in granules and following activation of the T cell by antigen presented by an antigen presenting cell, these granules are released into the synapse between the two cells in order to cause damage to the infected cell's surface membrane, and thereby cause the cell to undergo apoptosis. Because perforin is expressed by CTL and is one of the main cytolytic molecules involved in lysing infected target cells, it could potentially be a useful surrogate marker for either cytolytic potential or function. There are two main complications when using perforin as a marker of cytotoxic activity: firstly, constitutive levels of perforin vary widely between different donors and are often very low (Lichtenheld et al., 1988; Appay et

al., 2000; Sandberg et al., 2001) and secondly, perforin is released when CTLs degranulate to facilitate direct cytotoxicity. One must therefore measure perforin both before and after stimulation in order to determine how much perforin was released and from this infer how cytolytically active the cell was. Perforin is instead a very useful marker of cytolytic potential (how much cytotoxicity can be generated by a particular CD8 cell) rather than cytolytic function.

A recently published paper has established a new technique for the *ex vivo* study of cytotoxic function in T cells using extracellular surface expression of LAMP or CD107 as a marker (Betts et al., 2003). CD107 are found in the membranes of cytotoxic granules, therefore when cytolytic granules move to the cell surface during CTL degranulation in order to release their cytotoxic contents, the CD107 molecules are transiently exposed on the outer surface of the cell (Peters et al., 1991). The transient exposure of the CD107 molecule is sufficient to allow antibody conjugation and staining of the CD107a molecule. In this way it is possible to determine precisely how much cytotoxic activity was mediated by each T cell following stimulation.

In this chapter, the use of CD107a staining as a marker of cytotoxic activity of T cells has been investigated on PBMC with the aim that the assay could at a later stage be applied to cervical cells. Both CD107a and CD107b can be used as markers of cytolytic function by T cells but Betts et al. (2003) demonstrated that CD107a staining consistently gave the most reliable results. Development of the CD107a Cytotoxicity Assay involved (i) comparing markers of degranulation (perforin versus CD107a) and determining which marker provides the most efficient and accurate representation of the degree to which degranulation has occurred, (ii) establishing the optimal positive control for CD107a expression, and (iii) determining the optimal period of stimulation with whole protein antigens for CD107a expression. The

CD107a Cytotoxicity Assay was used to determine the cytotoxic ability of HPV16 L1 and E7 responsive T cells isolated from the peripheral blood of women with HPV-associated CIN.

4.2 Materials and Methods

4.2.1 Isolation of PBMC

Donor PBMC used for the development of the cytotoxicity assay were obtained from the Western Province Blood Transfusion Service (WPBTS), but once the assay was optimised, it was performed on PBMC isolated from heparinized whole blood samples from 23 consenting women who were attending the Groote Schuur Colposcopy Clinic. Essentially all PBMC were isolated using the Hypaque-Ficoll (Sigma) density centrifugation method described in Chapter 3 (Section 3.2.2.2). This method relies on the weights of the constituents of the blood sample in order to differentiate and separate the mononuclear cells (lymphocytes and monocytes) from the more dense red blood cells and granulotcytes.

LeucoSep® tubes were used as described in detail in Chapter 3. Isolated PBMC were stored at a concentration of 20 x 10^6/ml per cryovial in 10% Dimethylsulphoxide (DMSO) Fetal Calf Serum (FCS) freezing medium at –80°C. In order to ensure the temperature of the freezing medium was decreased at a constant rate, thereby preventing excessive rupturing of the cellular membrane by the formation of ice crystals, a Mr Frosty container (Sigma) was used, which uses isopropanol to regulate the freezing temperature to decrease at an average of 1°C per minute.

When it was necessary to thaw PBMC, the cryovial containing the frozen cells was thawed in a 37°C waterbath. Then 1ml of 10%FCS RPMI was carefully added dropwise whilst agitating the sample. This step was performed cautiously since the difference in osmotic potential between the two types of media (freezing and

culturing) could result in cell death if it was adjusted too rapidly. Cells were then washed twice with 10%FCS RPMI for 10min at 200 x g. Cells were usually adjusted to a concentration of 2 x 10^6/ml.

4.2.2 Analysis of Perforin as a marker of cytotoxic activity

4.2.2.1 Stimulation of PBMC to determine constitutive and post-stimulation levels of perforin

PBMC from eight anonymous donors from Western Province Blood Transfusion Services (WPBTS) were thawed (as described in section 4.2.1), adjusted to 2x10^6cells/ml and transferred in 200µl aliquots in duplicate into a 96-well round-bottom plate for incubation either unstimulated or with PMA/Ionomycin (PMA 25ng/ml; Ionomycin 1µg/ml). Cells were incubated at 37°C, 5%CO_2 for 1 hr (in the presence of BFA, 10µg/ml) or 4hr (with last 3hr in presence of BFA).

4.2.2.2 Intracellular Staining for Perforin

Following stimulation, it is necessary to stain the cells with antibodies to the various phenotypic and functional markers. Cell surface markers (e.g. CD3 and CD8) can easily be stained since these are displayed on the surface of the cell. For intracellular markers, such as perforin, the staining process involves an additional step – permeabilisation of the cellular membrane. This is necessary since it creates openings through which the antibodies to intracellular molecules are able to enter the cell and bind to their respective targets. It is also important when performing intracellular staining to first 'fix' the cells, to prevent the target intracellular molecules from being released through the same permeabilised membrane openings.

Stimulated cells were transferred to individual BD Falcon FACS tubes and fixed and permeabilised simultaneously in the dark for 10min in 500µl BD Cytofix/CytoPerm solution (BD Pharmingen). The cells were pelleted through centrifugation at 200 x g in a Labofuge 400R centrifuge (Heraeus Instruments) and washed with 1ml 0.1% Saponin PBS (containing 0.01% Sodium Azide). Pellets were resuspended in the dead volume of saponin (approx. 50-100µl) and antibodies were added to the tubes as follows; anti-CD3-APC, anti-CD8-FITC and anti-Perforin-PE. Staining was performed at 4°C, protected from light for 30min. Thereafter the cells were washed with 1ml 0.1% Saponin, fixed in 400µl BD CellFix reagent (BD Pharmingen) and stored at 4°C to be analysed on the Becton Dickenson FACS Caliber flow cytometer using the BD Cellquest Aquistion software.

4.2.3 Staining of PBMC for CD107a expression

4.2.3.1 Intracellular staining for CD107a

Freshly thawed PBMC from three independent donors were adjusted to $1x10^6$cells/ml and viability was confirmed to be above 90% using trypan staining and counting on a haemocytometer. Cells were stimulated in 96-well round-bottom plates (in 200µl) as described above with either no antigen (unstimulated) or with SEB (10µg/ml) in the presence of Monensin (10µg/ml). Monenesin is a Na^+/H^+ ionophore which prevents the secretion of intracellular molecules but does not block CTL degranulation. It also neutralizes the pH of the intracellular endosomes and lysosomes which is important since acidic conditions within granule compartments may quench the fluorescence from the tagged intracellular antibody to CD107 (Mollenhauer et al., 1990). Anti-CD107a-CyChrome was added at the beginning of the incubation period as Betts et al. (2003) had previously demonstrated that CD107a

surface expression is transient and its incorporation right from the beginning of stimulation substantially increased the level of sensitivity of the assay. Once stimulation was initiated the cells were incubated at 37°C, 5%CO_2. After 4 hours, unstimulated and stimulated cells were transferred to BD Falcon FACS tubes and stained for cell surface antigens using anti-CD3-APC, anti-CD8-FITC. CD107a would have been stained during the incubation period. Cells were analysed on a BD FACS Caliber Flow Cytometer within 24hr of staining and data analysed using BD Cell Quest software.

4.2.3.2 Kinetics of cell surface CD107a versus intracellular Perforin expression following stimulation

In order to compare the kinetics of perforin release versus CTL degranulation (indicated by cell surface CD107a expression), PBMC were stimulated with SEB and levels of intracellular perforin and exposed labeled CD107a molecules were compared. Again freshly thawed PBMC from three independent donors were adjusted to $1x10^6$cells/ml and viability was confirmed to be above 90%. Cells were stimulated in 96-well round-bottom plates (in 200µl) as described above with either no antigen (unstimulated) or with SEB (10µg/ml) in the presence of Monensin (10µg/ml). Anti-CD107a-CyChrome was added at the beginning of the incubation period as described by Betts et al. (2003). Once stimulation was initiated the cells were incubated at 37°C, 5%CO_2. At 30 minutes, 2hr, and 4hr, stimulated cells were transferred to BD Falcon FACS tubes and immediately incubated in the dark for 10min in the presence of 500µl BD CytofixCytoperm reagent. Thereafter they were stained following the protocols described in section 4.2.2.2, using anti-CD3-APC, anti-CD8-FITC and anti-Perforin-PE. Cells were analysed on a BD FACS Caliber

Flow Cytometer within 24hr of fixing and data analysed using BD Cell Quest software.

4.2.3.3 Comparison of PHA, PMA/Ionomycin and SEB-stimulation for induction of CD107a expression

To further optimize the CD107a cytotoxicity assay, various stimulants were investigated for use as a positive control. The stimulants compared were (i) a mitogenic agent, phytohaemagglutinin (PHA) which non-specifically interacts with the T cell receptor (TCR) through cross linking thereby mediating T cell activation and subsequent degranulation (Baran et al., 2001), (ii) a pharmacological agent; phorbyl myristate ester (PMA) which activates a Ca^{+} dependant protein kinase C to phosphorylate the CD4 co-receptor and induce signaling cascades through the TCR with the assistance of a calcium ionophore (Ionomycin) [Imboden and Stobo, 1985]; and (iii) a superantigen, *Staphylococcus* enterotoxin B (SEB), which is a potent T cell stimulator (Herman et al., 1991). It was necessary to compare the stimulants, since each mediates T cell activation through a different method and this study aimed to find the stimulation agent which generated the highest levels of degranulation of the T cell. PMA/I has been shown to be the most potent stimulator of T cells (Baran et al., 2001), yet it also results in down regulation of the CD4 receptor molecule (Petersen et al., 1992). This down regulation does not happen following stimulation of T cells with PHA (Baran et al., 2001). Superantigens, such as SEB, are antigens from bacteria or viruses, but unlike the HPV16 specific antigens being used in this study, superantigens are capable of inducing massive T cell responses in most T cells since they stimulate the T cell through a different and more generic interaction with the T cell receptor than the specific antigens which can only induce responses in T cells

which have been primed to recognise a specific element of the antigen (Herman et al., 1991).

Freshly thawed PBMC ($2x10^6$/ml) were transferred in 200µl aliquots into 96-well round-bottom plates and incubated at 37°C, 5% CO_2 for 5hr in the presence of (i) no antigen (unstimulated), (ii) PHA (5µg/ml), (iii) PMA (25ng/ml)/Ionomycin (1µg/ml), or (iv) SEB (1µg/ml). Antibody to the CD107a molecule (anti-CD107a-CChr) was added to each well for the full incubation period. Additionally, the microtubule inhibitor Brefeldin A (BFA; 10µg/ml) was added to each well to block the endoplasmic reticulum and golgi transport apparatus, thereby preventing the secretion of intracellular cytokines. In this instance, BFA was used instead of the secretion inhibitor monensin since it had subsequently come to my attention that studies have shown that monensin can induce IL-1 expression in monocytes (Yewdell and Bennink, 1989). Since IL-1 acts as a mediator of immune responses and costimulator of T cells, the resultant T cell responses might be skewed by the unnatural production of the IL-1 cytokine (Abbas et al., 1994).

After 5 hours, cells were transferred to BD Falcon FACS tubes and stained following the protocols described in section 4.2.2.2. Briefly, the cells were fixed, permeabilised and washed with 0.1% Saponin. Pellets were resuspended in the dead volume (approximately 50-100µl) of saponin and antibodies to the cell surface markers CD3 and CD8 (anti-CD3-APC and anti-CD8-FITC) were added to each tube and incubated at 4°C in the dark for 30 min. Cells were then washed and finally fixed in 400µl BD CellFix solution. Stained cells were acquired using a BD FACS Caliber Flow cytometer and analyzed using BD CellQuest software.

4.2.4 HPV-16 L1 and E7 specific cytotoxicity (CD107a) Assay on PBMC from women with HPV-associated CIN

4.2.4.1 Colposcopy Clinic Study Population

Twenty three women from the larger cohort of 100 women who had been referred to the Groote Schuur Colposcopy Clinic following diagnosis by cytology of CIN disease at the cervix were recruited into this study. The details of the 23 women recruited into this part of the study are described in Table 4.1. In accordance with the Research Ethics Committee of the University of Cape Town all participants were informed regarding the study and their written consent was obtained. From each patient, a 10 ml anti-coagulated (Lithium Heparin) peripheral blood sample was taken for PBMC isolation (as described previously) and a Digene cervical cytobrush was taken for HPV typing.

4.2.4.2 HPV Typing using Roche Reverse Line Blots

It was of interest to determine for each patient the types of HPV infecting their cervical tissues and this was done using Roche Reverse Line Blot strips. The experiments were performed by Candice Sampson from the Medical Virology Department, UCT. The methods used for HPV typing cervical specimens using Roche Reverse Line Blot technology were exactly the same as described in detail in Chapter 3, Section 3.2.3.

4.2.4.3 CD107a Cytotoxicity Assay following stimulation with HPV-16 L1 and E7

The final cytotoxicity assay that was used on the PBMC from patients of the Colposcopy clinic combined the methods used for the 5hr positive control stimulation and the 21hr antigen specific stimulation (sections 4.2.3.3 and 4.2.3.4). PBMC

($2x10^6$cell/ml) from the 23 patients were aliquoted into 96-well round-bottom plates and subjected to the following stimulation conditions: (i) no antigen (unstimulated), PMA/Ionomycin (25ng/ml, 1μg/ml; 5 hours; positive control), HPV16 VLP (10μg/ml; 21 hours; kindly provided by Mr E. van der Walt) and HPV16 E7 antigen (9μg/ml; 21 hours; kindly provided by Dr I. Becker). Antibodies against costimulatory molecules (anti-CD28/49d, 1mg/ml) were added to each well along with anti-CD107a-CChr. BFA (10μg/ml, Sigma) was added to each well for the last 5hr of stimulation in order to inhibit release of the intracellular cytokines. Once incubation at 37°C, 5%CO_2 was complete, the cells were transferred to 5 ml BD Falcon FACS tubes and stained using the protocols described in section 4.2.2.2, with antibodies to CD3, CD8 and IFNγ. Following fixation of the cells, they were immediately acquired and analysed on the BD FACS Caliber flow cytometer using BD CellQuest software.

4.2.5 Statistical analysis

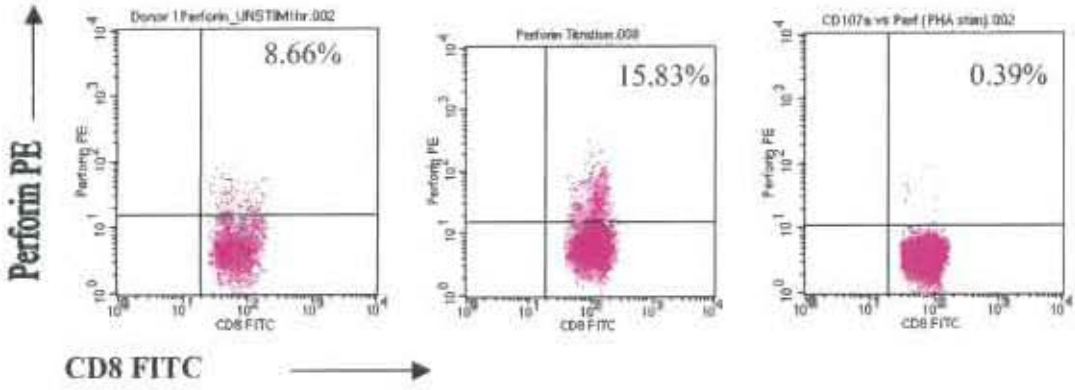
Where indicated, results were analysed for statistical significance using either the Mann-Whitney U Test for unpaired non-parametric data or the Wilcoxon Rank Test for paired non-parametric data (Statistica ®).

4.2 Results

4.2.1 Heterogeneity in the level of intracellular Perforin expression in CTLs from different donors

In order to investigate donor variability in the intracellular levels of perforin in CD8+ CTL, perforin expression in 8 different PBMC donors (from WP Blood Transfusion Services) was assessed. In these donors, perforin expression in CD8 T cells ranged from 0.1-26.2% (Figure 4.1 panel A and B). When the same donor was assessed in independent experiments, the level of perforin positive T cells varied widely (ranging from 0.1% - 2.77%; Fig 4.1C). This indicates that not only is there substantial inter-donor variability in perforin expression, but there is also significant inter-assay variability. This is an important indicator that perforin might not be an accurate and reliable marker of CTL activity or functional capability. The high levels of inter-assay variability is particularly concerning as it implies that either (i) the assay itself is error prone, (ii) the assay is technically poorly standardized; or (iii) that depending on thawing and general culturing conditions, constitutive levels of perforin may really vary.

A.



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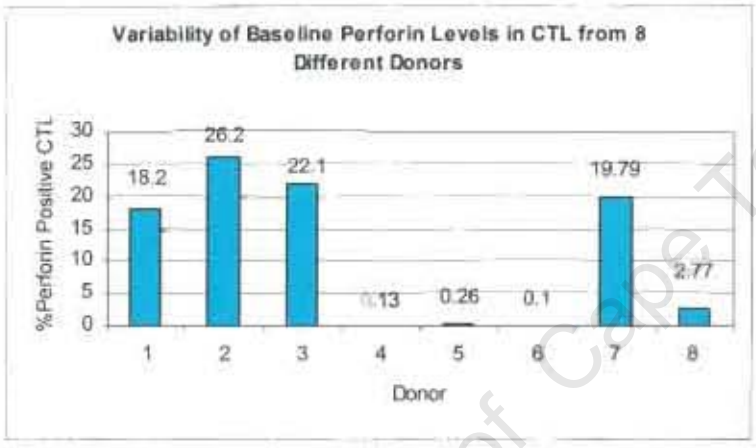


C.

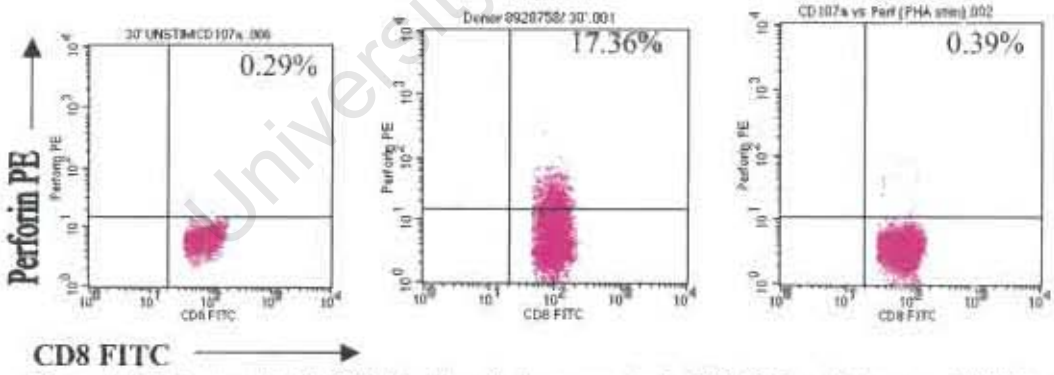


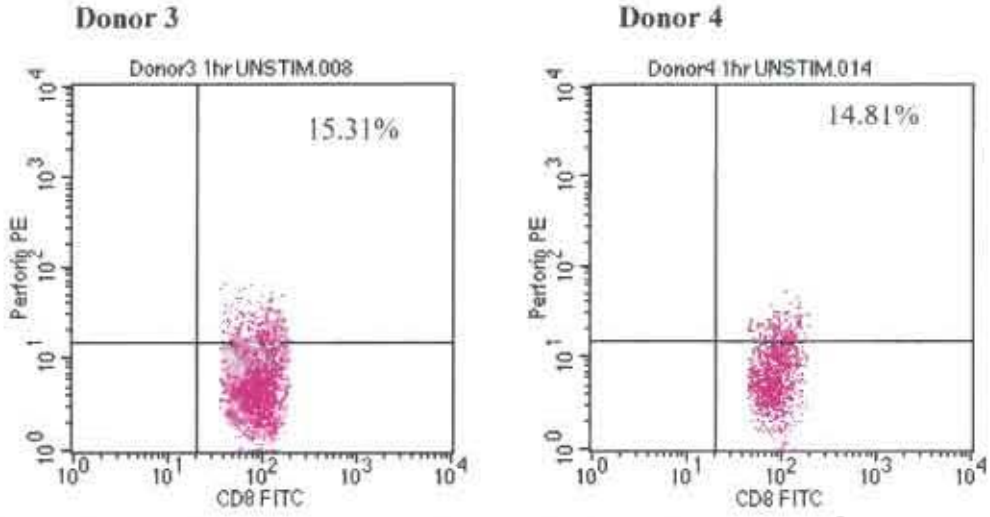
Figure 4.1 Heterogeneity in CD8 T cell perforin expression in PBMC from 8 donors. PBMC were incubated without antigen in the presence of Brefaldin A (BFA) for 30 min, then fixed, permeabilised and stained with antibodies to CD3, CD8 and Perforin molecules. (A) Facs plots depicting variable perforin levels between three different donors. (B) Graph depicting the heterogeneity of perforin levels in 8 independent donors. (C) Facs plots depicting fluctuating percentages of perforin positive CD8 T cells in same donor between three separate experiments. All events shown in all plots are gated for CD3+CD8+ cells only. Values in upper right quadrant indicate percentage perforin positive CD8 T cells.

4.2.2 Impact of T cell activation and degranulation on intracellular Perforin expression

In order to investigate whether activation of T cells with PMA/ionomycin or SEB causes higher levels of degranulation and release of perforin than in the unstimulated cells, intracellular perforin levels were assessed before and following 5 hours of stimulation with PMA (or SEB) (Figure 4.2). The data shows that the baseline levels of perforin positive CTL for donors 1-3 were 18.2%, 26.2% and 22.1% respectively (22.17 ± 4.0 %; mean ± SD) before stimulation. Following stimulation, release of perforin resulted in an average reduction of 56% in the perforin positive CTL population (decreases of 58%, 63% and 45% for each donor respectively).

CTLs from different donors have been shown to exhibit large variability in their base levels of perforin (section 4.2.1). This is an important factor when using perforin release as a marker of cytotoxic function, since the amount of perforin released by CTL in response to antigen specific stimulus might differ significantly between donors but might not be indicative of a significant difference between each donor's cytotoxic ability.

A. Perforin Levels in Unstimulated CTL:



B. Perforin Levels after PMA/Ionomycin stimulation:

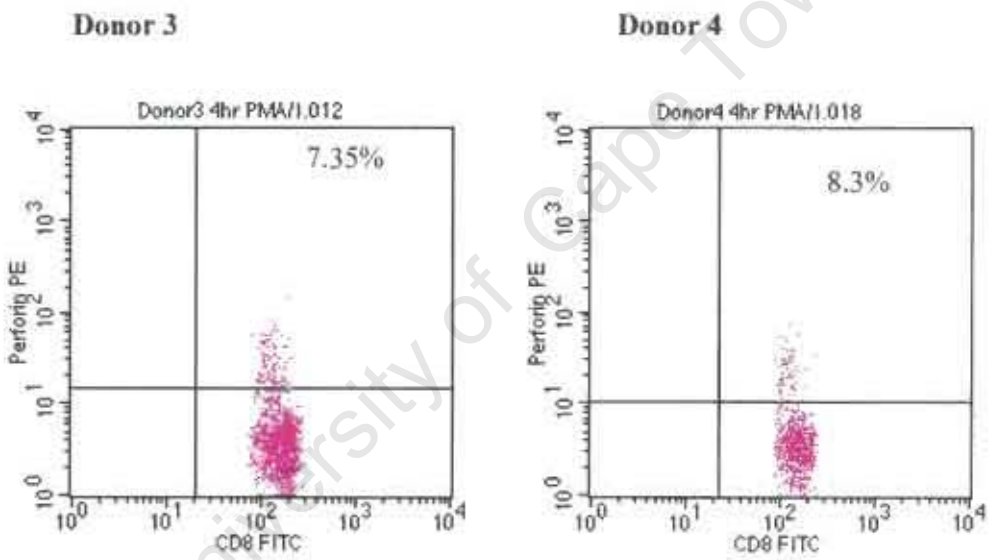


Figure 4.2. Comparison of perforin expression in CD8+ CTL from two different donors (A) before stimulation and (B) following 5hr stimulation with PMA/I. PBMC were either left unstimulated or incubated in the presence of BFA for 5hr with PMA/I stimulation. Then cells were simultaneously fixed and permeabilised before being stained with antibodies to CD3, CD8 and perforin. Events shown are gated for CD3+CD8+ T cells and values in upper right quadrant indicate percentage of perforin positive CTL.

4.2.3 CD107a as a Reliable Marker of Cytotoxic Activity

There are several complications when using perforin as a marker of cytotoxic activity and it was recently suggested that CD107a might be a more representative marker of cytolytic function (Betts et al., 2003). CD107a molecules are found on the membrane of vacuoles containing cytotoxic granules (Peters et al., 1991), therefore when the vacuole containing cytolytic granules fuses with the T cell membrane in order to release its cytotoxic contents (degranulates), the CD107a molecules are transiently exposed on the outer surface of the cell. This transient exposure of the CD107a molecule is sufficient to allow antibody conjugation and staining of the CD107a molecule and in this way it is possible to determine how much cytotoxic activity is elicited by each T cell following stimulation.

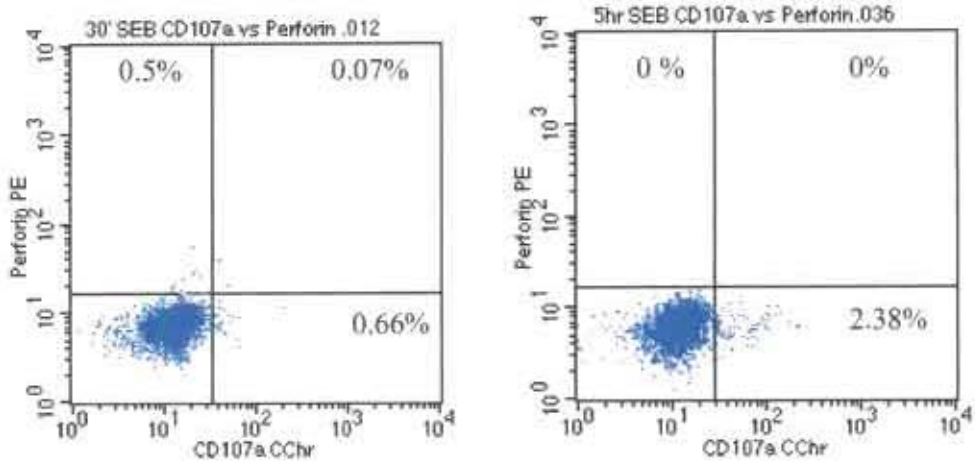
4.2.3.1 Association between CD107a expression and perforin release supports the use of CD107a as an effective marker of cytotoxic activity

A comparison of CD107a expression and perforin release during stimulation was performed (Figure 4.3). The results for three donors show a progressive increase in CD107a expression correlated with a decrease in perforin levels in the cells. This provides evidence to support the theory that CD107a markers are exposed as the cells degranulate and release perforin in response to stimulus. CD107a expression for Donor 1 increased from 0.24% to 0.94% (3.9-fold increase at 30 minutes versus 4 hours), Donor 2 increased from 0.18% to 1.59% (8.8-fold increase); and Donor 3 increased from 0.91% to 1.74% (1.9-fold increase). In comparison the percentages of cells positive for perforin stain were initially 0.13%, 026% and 0.1% respectively in

the unstimulated population at the 30min time point. Following stimulation with SEB the percentages of perforin positive cells in all donor samples had decreased to 0%.

From this data it is evident that there is an association between increasing CD107a expression and loss of intracellular perforin. Therefore it was concluded that CD107a expression could reliably be used as a marker of cytotoxic function.

A.



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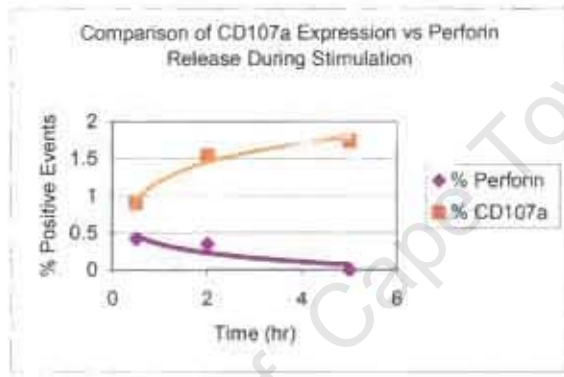


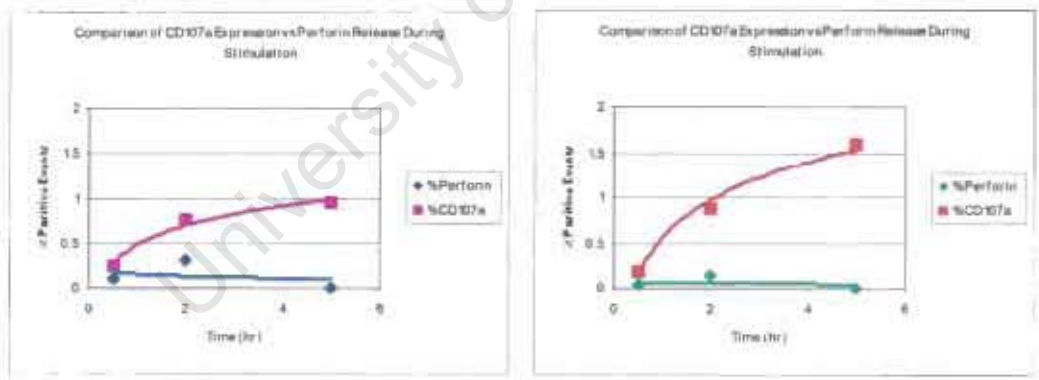


Figure 4.3 Comparison of CD107a expression vs Perforin release in 3 donors, at 3 time points during stimulation with SEB; 30 min, 2hr and 4hr. Cells were stained immediately following completion of the stimulation period and analysed on the flow cytometer within 24hr. (A) Facs plots depicting percentages of CD107a and/or perforin positive CD3+CD8+ T cells at 30 min and 4hr time points on same donor. (B) Graphs illustrating the association between perforin release and subsequent CD107a expression on surface of the cellular membrane. In each plot the top trendline shows the percentage of CD107a positive cells increasing whilst the bottom trendline depicts the % perforin positive cells decreasing.

4.2.3.2 Inter-assay reproducibility of CD107a as a marker of degranulation

Due to previous results depicting high inter-assay variability in percentages of perforin positive cells before stimulation (Figure 4.3), the levels of CD107a expression were also evaluated to determine whether they varied similarly between three independent experiments which utilised aliquots of the same donor's PBMC (Fig 4.4). The results also revealed some degree of inter-assay variability with a maximum difference of 5% between the highest and lowest percentages for each stimulation condition (20.81 – 15.52% PHA stimulated; 3.84 - 1.74% SEB stimulated).

Interesting to note, there is a considerable difference in the CD107a expression depending on which positive control agent was used to stimulate the PBMC. SEB stimulation yielded the lowest percentage CD107a expression (3.84%) whilst PHA stimulation exceeded this value by over 5 fold (20.81% CD107a positive T cells).

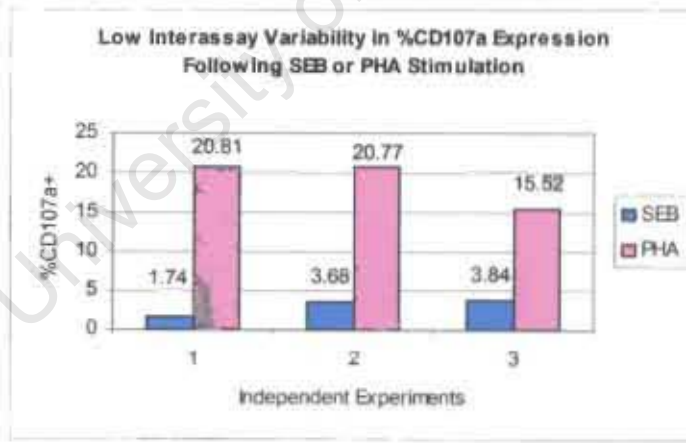


Figure 4.4. Interassay variability when using CD107a as a marker of cytotoxic activity of CD8+ T cells. The results shown are from one donor used in three independent experiments. Each bar represents percentage of CD107a positive events in the CD8+CD3+ population following 5hr stimulation with either SEB (blue bars) or PHA (pink bars), where CD107a antibody was present for full incubation period. Cells were fixed, permeabilised and stained following stimulation and were read on the BD FACS Caliber flow cytometer within 24hr of staining.

4.2.4 Comparison of PHA, SEB and PMA/Ionomycin as agents to induce maximal CD107a expression on activated T cells

Once CD107a was confirmed to be a useful marker for cytotoxic functioning in cells, it was necessary to optimise by comparing various agents for use as a positive control. Some common positive controls (reagents which are capable of stimulating all PBMCs) which have been utilized in many of the experiments in this project, are PHA, PMA/Ionomycin and SEB. The ability of PHA, PMA/Ionomycin and SEB to activate the cytotoxic functions of the CD8 T cells was compared in 2 independent experiments shown in Fig 4.6. It was found initially that PHA was a much better CD107a expression inducer than SEB (9.41 % vs. 5.57 % respectively; Figure 4.5 A). In the second experiment, PHA and PMA/I activation were compared and PMA/Ionomycin was found to be a better stimulant than PHA, yielding percentages of CD107a positive events of 7.22 % vs. 3.67% respectively (Figure 4.5 B). Therefore it was decided that the positive control for use in the cytotoxicity experiments would be PMA/Ionomycin.

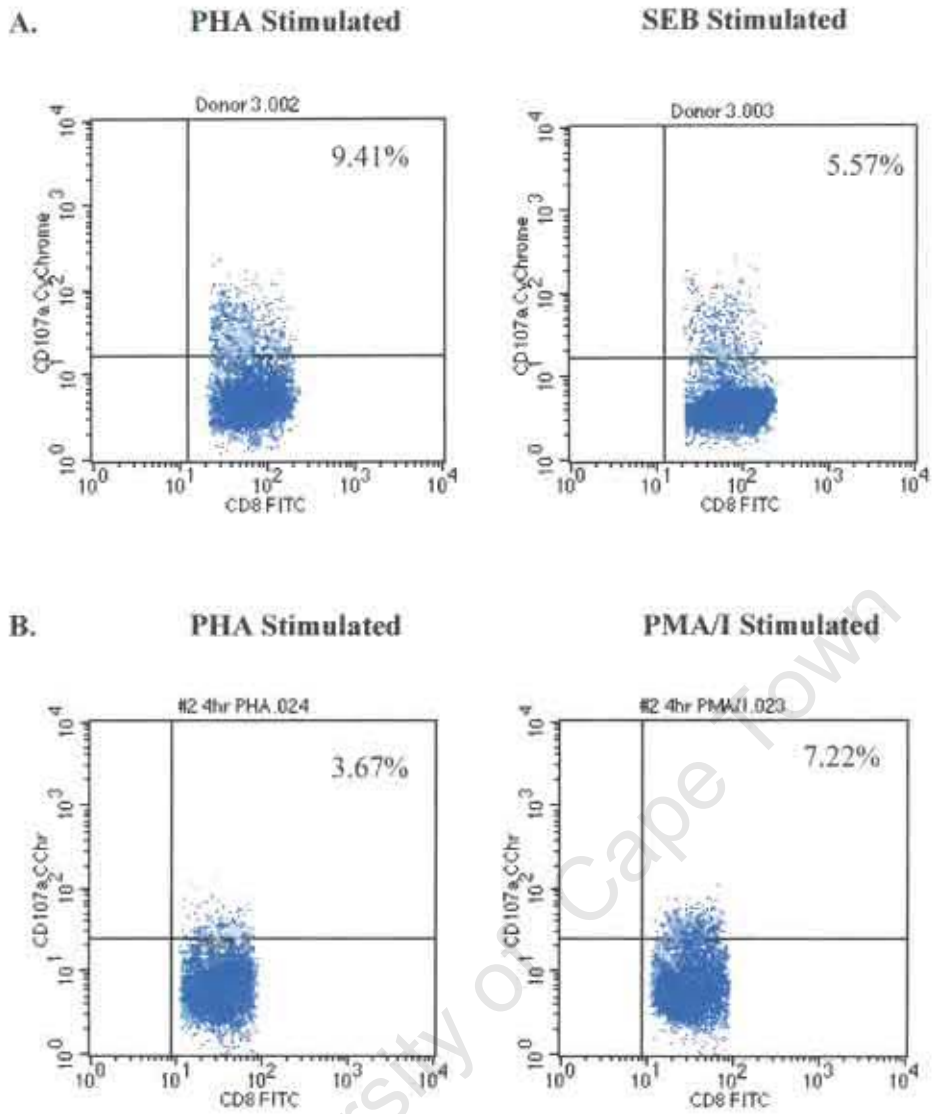


Figure 4.5. Comparison of PMA/Ionomycin, PHA and SEB to induced cytotoxic activities (CD107a expression) of CTL. We compared a total of 3 donors using PHA/SEB stimulation and two donors with PMA/PHA stimulation. PBMC were stimulated for 5 hr in the presence of co-stimulatory antibodies, anti-CD107a-CChr and the respective positive control (A) SEB or PHA and (B) PMA/I or PHA. Following stimulation cells were stained with antibodies to the CD3 and CD8 markers, fixed and then aquired and analysed on the BD FACS Caliber flow cytometer using BD CellQuest software.

4.2.5 CD107a expression following stimulation with HPV-16 L1 and E7 Antigen by PBMC from patients with cervical disease

4.2.5.1 Description of women with HPV-associated cervical disease enrolled in this study

This study recruited 23 women who were presenting with abnormal cytology at the cervix and who had been referred to the Groote Schuur Outpatients Colposcopy Cclinic run by Dr Lynnette Denny (Dept. Obstetrics and Gynaecology, Groote Schuur Hospital). Of the 23 patients, 12/23 (52.2%) were actively infected with HPV-16 (indicated by the presence of HPV-16 DNA at the cervix) while 11/23 (47.8%) were infected with types other than HPV-16. Table 4.1 gives a full description of the 23 women investigated in this chapter together with their CIN status and HPV typing status. Thirteen of twenty three (56.5%) of the women had histologically confirmed CIN or cancer with 6/23 (26.1%) presenting with CIN 1, 4/23 (17.4%) presenting with CIN 2, 2/23 (8.7%) presenting with CIN 3, and 1/23 (4.3%) presenting with adeno carcinoma. The remaining 10/23 (43.5%) of the women were found to be negative for disease at the cervix upon presentation at the Colposcopy clinic. Twelve of twenty three (52.2%) of the women were actively infected with HPV-16, 9/23 (39.1%) were infected with other HPV-types and 2/23 (8.7%) had no detectable HPV DNA at the cervix. HPV-16 was the most frequently detected HPV type.

Table 4.1 Description of women recruited into this study showing severity of cervical disease and type of HPV infecting the cervix

HPV-16 Status	Donor	CIN Status	HPV types
HPV-16 DNA+	JP042	1	16
	JP043	1	16, 33
	JP047	neg	16, 69, 83
	JP085	neg	16, 58, 61
	JP086	3	16
	JP087	neg	16
	JP088	neg	16
	JP092	1	16
	JP093	neg	16
	JP095	2	16
	JP098	2	16, 6, 26, 31
	JP100	2	16, 52, 66
	N=12	5/12 neg 3/12 CIN1 3/12 CIN2 1/12 CIN3	
HPV-16 DNA-	JP038	3	33, 56
	JP044	neg	-
	JP045	neg	35
	JP052	2	18, 58
	JP084	1	56
	JP089	1	35, 62
	JP090	neg	33, 61
	JP091	1	51, 53, 59, 68
	JP094	neg	31, 58
	JP096	Adenocarcinoma	18
	JP097	neg	-
	N=11	5/11 neg 3/11 CIN1 1/11 CIN2 1/11 CIN3 1/11 Adenocarcinoma	

4.2.5.2 HPV-16 L1 and E7-specific cytotoxicity using CD107a as a marker of CD8 degranulation

PBMC collected from 23 women attending the Colposcopy Clinic (Table 4.1) were stimulated with either HPV-16 L1 or E7 antigens for 21 hours and the percentage cells expressing CD107a (cytolytic marker) and IFN-γ was measured. The level of both IFN-γ and CD107a expression in response to PMA stimulation was highly variable. A mean of 1.48% (± 1.76 SD) CD8 T cells expressed CD107

following PMA stimulation. Of the PBMC which degranulated (i.e. were CD107a+) following PMA/I stimulation, the majority also induced production of IFN-γ (87.4 ± 19.6 %; mean ± SD) as illustrated in Figure 4.6 and 4.7 below. In comparison, only a minority of IFN-γ producing CD8 T cells also express CD107a following stimulation [14.67% (± SD 9.07%) of the total IFN-γ producing cells were also CD107a positive]. This data indicates that cells which are strongly stimulated to degranulate, will very likely also produce IFN-γ in response to the stimulus. But cells that produce IFN-γ in response to the stimulus do not necessarily also exhibit cytolytic degranulation.

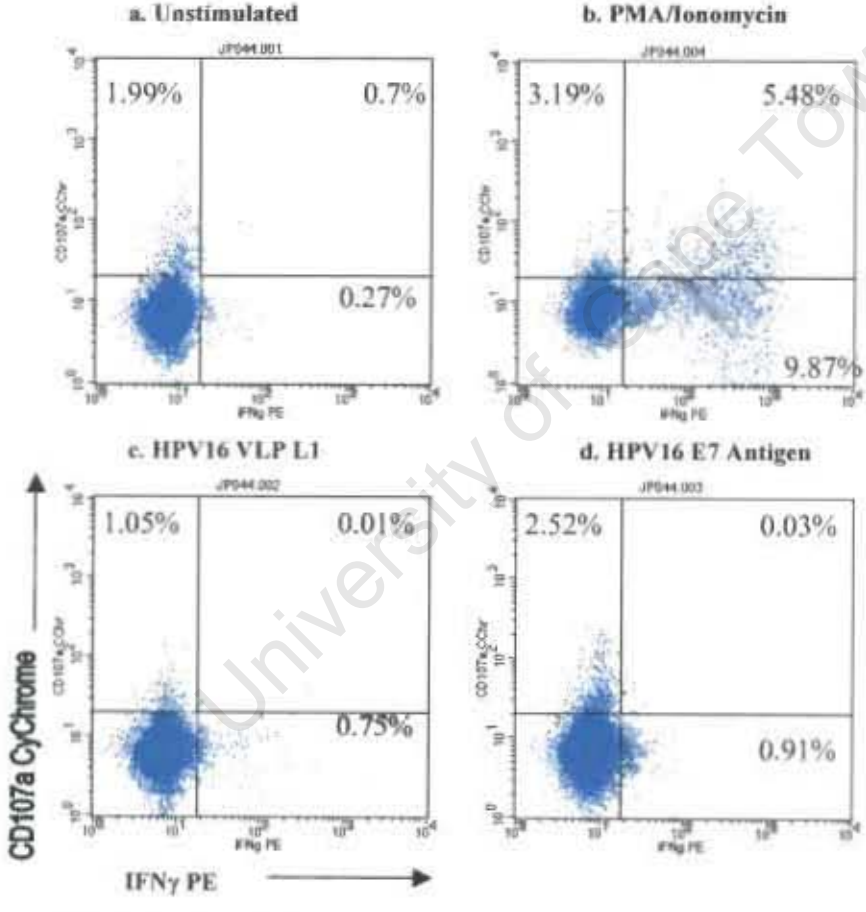
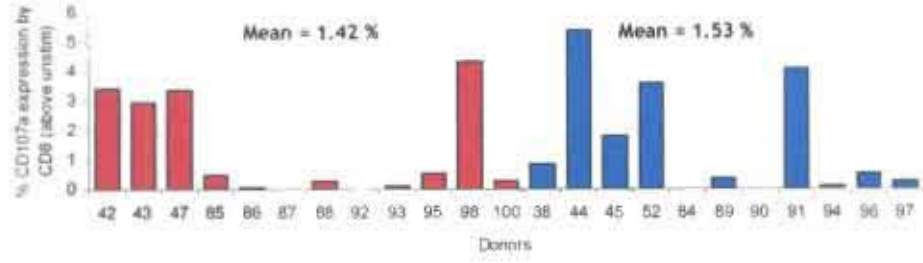


Figure 4.6 Representative flow cytometry plots of donor JP044 after (a) incubation without antigen (b) stimulation with PMA/I (c) stimulation with HPV16 VLP L1 and (d) stimulation with HPV16 E7 antigen. Cells were stimulated for 5hr with CD107a-CChr antibody, co-stimulatory antibodies and BFA, before being permeabilised and stained with anti-CD3-APC, anti-CD8-FITC and anti-IFNγ-PE. All events shown are gated to select for CD3+CD8+ CTL only.

A.



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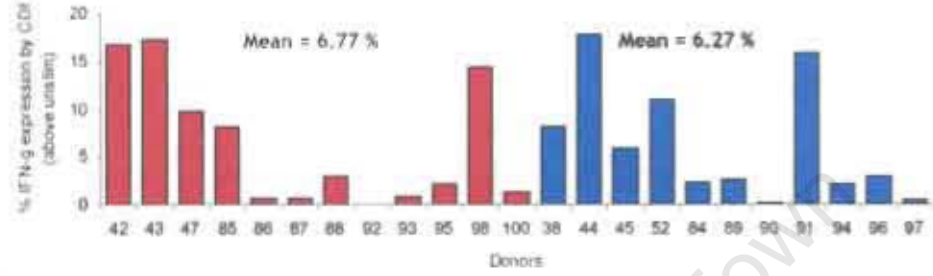

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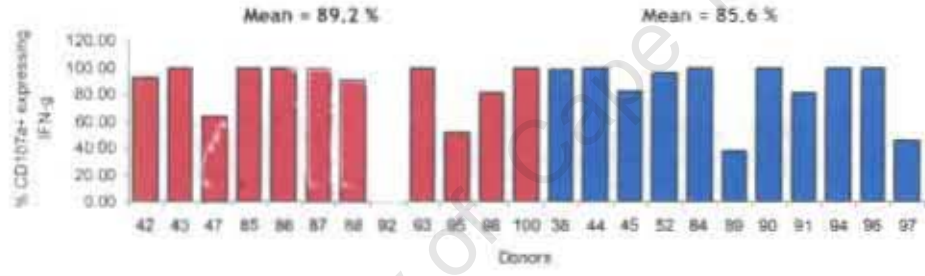

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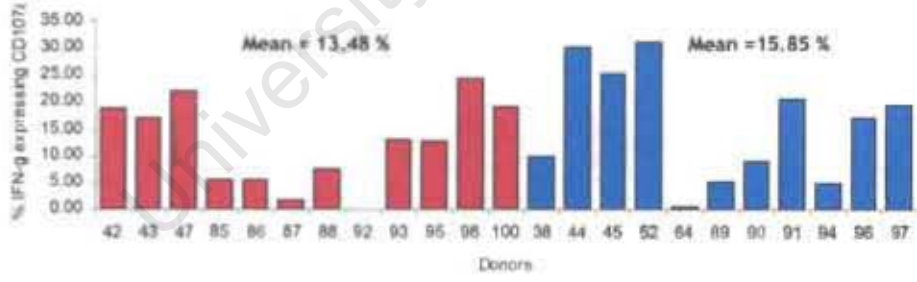


Figure 4.7 CD107a expression and IFN-γ production in PBMC following mitogenic stimulation for 5hr with PMA/I. (A) CD107a expression following stimulation with PMA/ionomycin. Red bars indicate responses in HPV-16 DNA + women while blue bars indicate responses in HPV-16 negative women. (B) IFN-g production to PMA/ionomycin stimulation. (C) CD107a+ cells also expressing IFN-g. (D) IFN-g producing cells also expressing CD107a. CD107a antibodies were added to wells for entire stimulation period. Following stimulation cells were fixed, permeabilised and stained for CD3, CD8 and IFN-γ markers. Acquistion and analysis was then performed on a BD FACS Caliber flow cytometer using BD CellQuest software. Each bar in the graph represents for one donor, the (A) total %CD107a positive per donor, (B) total %IFNγ positive per donor, (C) %CD107a cells also positive for IFN-γ production and (D) % IFN-γ positive cells also expressing CD107a.

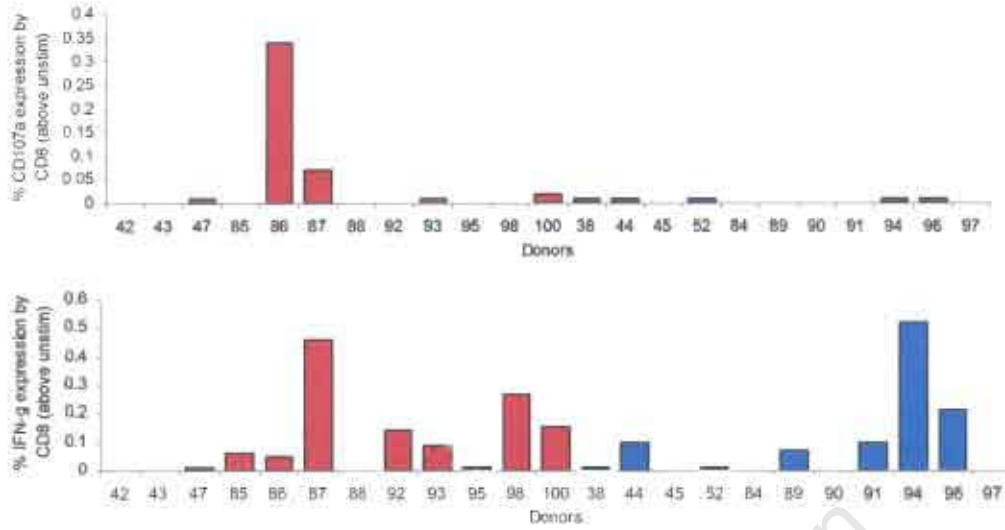
In comparison with the strong IFN-γ association to cells expressing CD107a shown above after PMA/I stimulation, the data from responses to stimulation with the HPV specific antigens was much lower (Figure 4.8). This is understandable since one expects far fewer responding cells in the latter stimulation because antigen specific T cells are rare events and therefore far lower percentages of cells will be capable of responding to the stimulating agent.

In response to HPV16 VLP L1 stimulation, an average of 0.1% (± 0.14% SD) of CD8 T cells produced IFN-γ and 0.02% (± 0.07% SD) degranulated (i.e. expressed CD107a during stimulation) (Figure 4.8; these results are expressed as percentage CD107a expression to VLP above background unstimulated). Of the PBMC that did degranulate, 75% (± 42.49% SD) were also producing IFN-γ and of the IFN-γ producing PBMC only 20.9% (± 39.41% SD) were positive for CD107a (Figure 4.9).

Stimulation of PBMC with HPV16 E7 antigen induced cytotoxic activity (i.e. CD107a expression during stimulation) in an average of 0.16% (± 0.2% SD) and IFN-γ production in 0.13% (± 0.14% SD) of CD8 T cells (Figure 4.8; again results are expressed as percentage CD107a expression to E7 above background unstimulated). As can be seen in the graphs below (Figure 4.8) the E7 antigen appears to elicit many more cytotoxic responses from T cells than the VLP L1 antigen. The average percentage of cells positive for both markers was reduced in the E7 stimulated populations in comparison with the VLP L1 stimulated results. Only 13.67% (± 32.13% SD) of the PBMC which degranulated were also producing IFN-γ and the percentage of IFN-γ producing cells that were also expressing CD107a on their surface during stimulation was 8.8% (± 23.71% SD) (Figure 4.9). These values all have large standard deviations, which indicate that there was a high level of

variability in the magnitude of responses between the various PBMC donors. It is also interesting to note when comparing the antigen specific responses to the mitogen (PMA/I) responses, that the average percentages of CD107a+ cells which were also producing IFN-γ cytokine were slightly reduced in the CD8 T cells stimulated with VLP (75% versus 87% in PMA/I) but were greatly reduced in CD8 T cells stimulated with HPV 16 E7 antigen (13.7%) (Figure 4.9). The average percentages of IFN-γ producing cells which had also degranulated in response to the stimulus, were less variable; 14.7% in PMA/I stimulated, 20.9% in VLP L1 stimulated and 8.8% in E7 stimulated PBMC. These results appear to indicate that stimulation with VLP L1 induces a comprehensive response (of degranulation and cytokine production) in a higher number of the CD8 T cells than the positive control PMA/I stimulation.

[A] Responses to HPV16 L1



[B] Responses to HPV16 E7

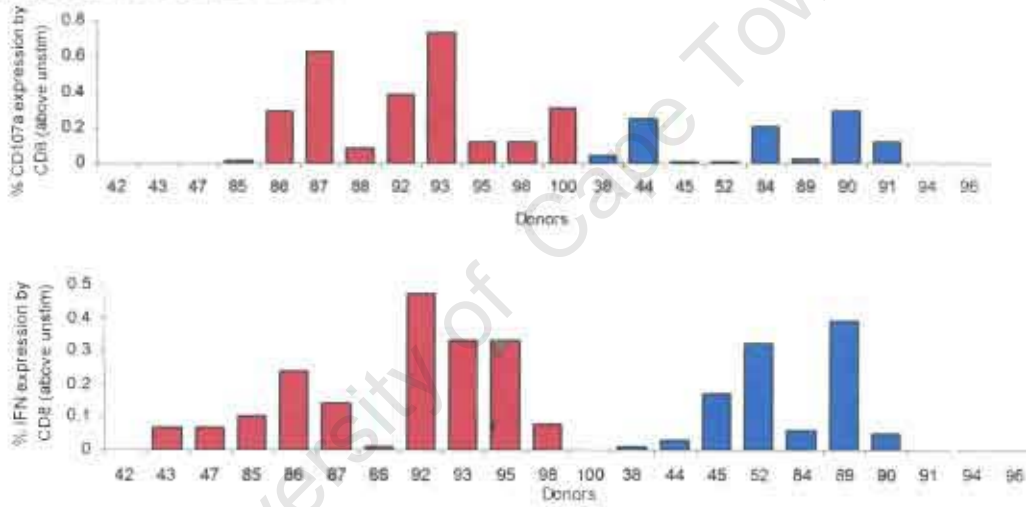


Figure 4.8. Individual responses elicited by CD8 T cells of 23 donors following stimulation with HPV specific antigens. [A] Responses to VLP-16. [B] Responses to E7. Red bars indicate active infection with HPV-16 at the cervix while blue bars represent no HPV-16 DNA at the cervix. PBMC were stimulated for 21hr with either HPV16 VLP L1 or HPV16 E7, in the presence of costimulatory molecules, anti-CD107a-CChr antibody and Brefaldin A for the last 5hr of stimulation. Cells were then fixed, permeabilised and stained for CD3, CD8 and IFN-γ. Acquisition and analysis of stained and fixed PBMC was performed on a BD FACS Caliber flow cytometer using BD CellQuest software. Each bar on the graph represents total percentage of positive events for one donor (Green bars, %CD107a expression; Red bars, %IFN-γ production). All events were gated for CD3+CD8+ CTL only. The values given have been adjusted for background (i.e. the percentage positive from the unstimulated sample has been subtracted from the test sample).

A. B.

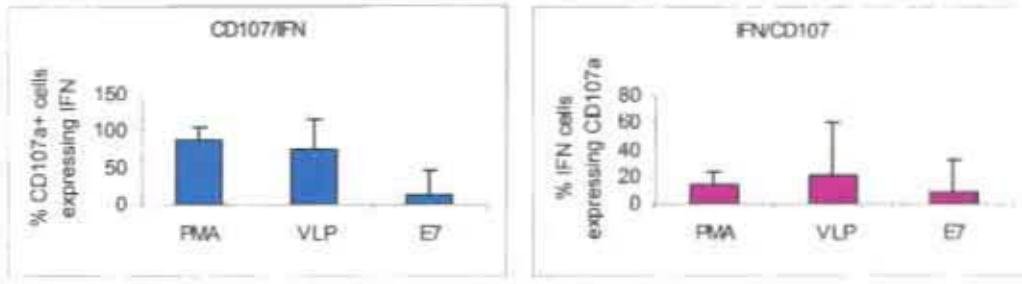


Figure 4.9 Graphs to indicate the average percentages (and standard error of the mean) of stimulated PBMC co-expressing both the IFN-γ and CD107a markers. PBMC were stimulated for 21 hr (with Brefaldin A for the last 5 hr) in the presence of costimulatory molecules, anti-CD107a-CChr and either PMA/I (1st bar), HPV16 VLP L1 (2nd bar) or HPV16 E7 antigen (3rd bar). Following stimulation cells were fixed, permeabilised and stained for CD3, CD8 and IFN-γ. The graphs shown give a comparison of either (A) %CD107a+ cells also producing IFN-γ or (B) %IFN-γ+ cells also degranulating and expressing CD107a after stimulation with each respective condition. The values given have been adjusted for background (i.e. the percentage positive from the unstimulated sample has been subtracted from the test sample).

The small sample size of this study limits the statistical significance of a comparison between the characteristics of the responses from HPV16 infected versus women with no HPV-16 DNA at the cervix. But there are some interesting trends in the data which may be noted. Graphical representation of the data showing average percentage responses to the VLP L1 antigen reveals that IFN-γ responses in the HPV16 infected group (0.1 ± 0.04 %; mean ± SEM) are similar to responses in the group with no HPV-16 DNA at the cervix (0.09 ± 0.05 %; Figure 4.10). Yet CD107a expression in response to VLP L1 appears to only occur in the HPV16+ group (0.04 ± 0.03 %) since the average for the HPV16- group was 0%. These percentages are exceptionally low and significance of positive values as low as 0.04% is doubtful. The same situation seems to occur in the PBMC stimulated with HPV16 E7 antigen; 0.15% (± 0.04; SEM) of HPV16+ women on average produced IFN-γ in response to E7, which is comparable to 0.1% (± 0.05) in the 16- group. The average percentage of CD107a expression was higher following E7 stimulation than after VLP L1 in both

the HPV-16 infected women (0.23 ± 0.07%) and the non-HPV-16+ group (0.1 ± 0.04%). There appears to be a noticeable difference between the percentages of responding cells from women who have been exposed to the HPV16 virus and women who have other non-HPV-16 type infections (0.23% versus 0.1%, respectively, 2.3-fold difference).

In the event of no true negative control, to facilitate analysis of the data, responses were deemed to be positive if they were 3 fold above the background. In the VLP L1 stimulated populations, only 1/12 HPV-16 DNA+ patient responded with CD107a cytotoxicity (8.3%) while 0/11 women with non-HPV-16 type infections responded. In the E7 stimulated samples, 4/12 (33.3%) HPV-16 DNA+ patients had cytotoxic responses to HPV16 E7 antigen while only 1/11 (9.1%) women with non-HPV-16 infections had responses >3-fold above background.

Also interesting to note is that only one patient had cytotoxic responses to both of the HPV 16 antigens, VLP L1 and E7. This patient was infected with HPV 16 at the cervix and suffering from severe grade CIN 3. The four other cytotoxic responses were elicited to E7 antigen only, in women who were not suffering from CIN at the cervix.

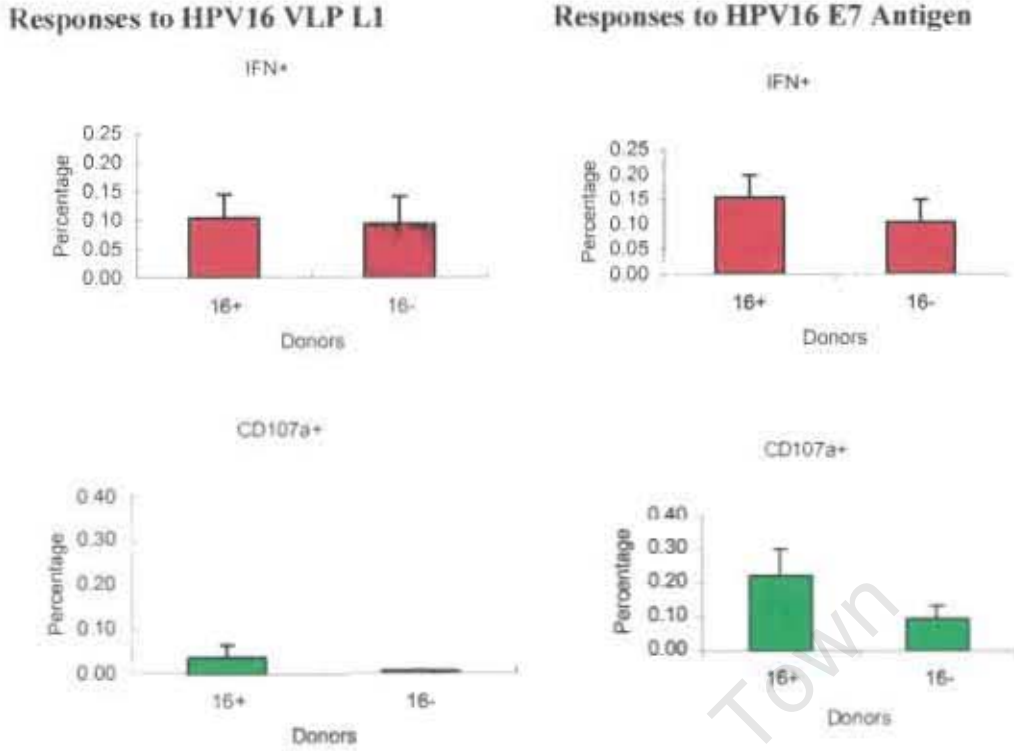


Figure 4.10. Comparison between the cytokine (IFN-γ) and cytotoxic (CD107a) responses elicited by the CD8 T cells of HPV16 infected (HPV16+) versus HPV16 negative donors, following stimulation with HPV16 specific antigens. PBMC were stimulated for 21 hr in the presence of either VLP L1 or E7 antigen, costimulatory molecules and with Brefaldin A added for the last 5hr. Antibodies to CD107a were added for entire stimulation period and after stimulation cells were fixed, permeabilised and stained with antibodies to CD3, CD8 and IFN-γ. Stained and fixed cells were analysed on the BC FACS Caliber flow cytometer using BD CellQuest software. Each bar of the graph depicts the mean and the standard error of the percentages of positive events recorded for either CD107a expression (green graphs) or IFN-γ production (red graphs). The values given have been adjusted for background (i.e. the percentage positive from the unstimulated sample has been subtracted from the test sample).

4.3 Discussion

The ability of T cells to elicit cytotoxic acitivity in response to viral pathogens is a highly important part of the host's immune system. Viral pathogens use the cells they have infected both for producing viral offspring and also to hide from the immune system. In order to engage in effective defense of the host it is necessary for the immune cells to be able to specifically identify and kill cells which are infected with viral pathogens. Therefore cytotoxic functional abilities of T cells are always of interest to immunologists studying immune responses to viral pathogens.

Many studies analyzing the T cell immune response to HPV have utilised Chromium release CTL assays (Nakagawa, 2000). These allow determination of the cytotoxic ability of CTL through the use of target HPV infected cells that release radioactive chromium (Cr^{51}) when lysed. Therefore when the HPV specific CTL recognize the HPV antigen presented by the infected target cells, they will attempt to lyse the target cell and if they are successful it will result in release of radioactive chromium into the culture medium which can then be analysed using a γ-radiation counter. This technique is laborious requiring an 1 – 3 week *in vitro* stimulation period and preparation of HPV antigen expressing, antigen presenting cells (APC) which must then be labeled with radioactive chromiun before the actual CTL assay commences (Nakagawa et al., 1999; Nimako, 1997).

In this chapter, the development of a CTL assay which will allow direct *ex vivo* determination of the cytotoxic ability of HPV specific T cells, using the technique of flow cytometry and fluorochrome conjugated antibodies to cytotoxic markers has been described. The aim of this chapter was to develop this assay and use it in conjunction with the ICC assay on cervical T cell samples. Previous studies have

utilised fluorochrome-conjugated antibodies to label cytotoxic markers of interest (such as perforin and granzyme B) for analysis through fluorescent microscopy (Bontkes et al., 1997). This allowed determination of whether cells in cervical biopsies had the potential to elicit a 'kill' response, by analysis of whether cytotoxic molecules were present in the cells. But this does not indicate whether those cells actually induce a cytotoxic response to antigens. Recently papers have been published which describe the use of flow cytometry to analyse the functional cytotoxic ability of PBMC in response to antigen (Appay et al., 2000; Sandberg et al., 2001; Betts et al., 2003). This technique uses fluorochrome conjugated antibodies to various cytotoxic markers to stain PBMC, following stimulation with antigen. The labeled cells were analysed using flow cytometry to determine whether a cytotoxic response had been initiated.

The markers of interest when studying cytotoxic responses in T cells are located within cytotoxic granules (Peters et al., 1991). Following stimulation of a T cell receptor and induction of cytotoxic signals, these granules are recruited to the location of the activated T cell receptor, where they fuse with the cell surface membrane and exocytose their cytolytic contents (including granzyme B and perforin) into the immunological synapse between the T cell and the infected cell. During this process, lysosomal associated membrane glycoproteins (LAMPs, also known as CD107a/b) which are found only in the lipid bilayer of the cytotoxic granules, become transiently exposed on the outer surface of the T cell. Thereafter the LAMPs are endocytosed and return to their original location in the lipid bilayer of the cytotoxic granules. Perforin, Granzyme B and CD107a are the markers of interest when analyzing cytotoxic activity in T cells.

For the purposes of this study, the efficiency of CD107a and perforin as markers of cytotoxicity were compared. The results show that immediate disadvantages of using perforin as a marker of cytotoxicity are (i) the high variability in the levels of intracellular perforin between different donors (as much as 0.1 – 26.2%) and (ii) the inter-assay variability in the intracellular levels of perforin in one individual donor (assayed in 3 independent experiments) (0.1 – 2.6%). These results indicate that the reproducibility of the cytotoxicity results when using perforin as a marker could be compromised. Finally, since cytolytic activity involves perforin release, the final percentage perforin levels in the cells have to be compared to the original levels in order to determine the relative amount of activity which occurred during stimulation. This is an important factor when using perforin release as a marker of cytotoxic function, since the amount of perforin released by CTL in response to antigen specific stimulus might differ significantly between donors but might not be indicative of a significant difference between each donor's cytotoxic ability.

The advantages of labeling CD107a while it is transiently expressed during the process of degranulation are (i) it is a direct marker of cytotoxic potential in the cell and (ii) the labeled CD107a molecules accumulate inside the T cell allowing subsequent analysis of cytotoxic activity in individual cells with the flow cytometer. Comparison of the data from three independent experiments, where PBMC from the same donor were stimulated identically, shows that CD107a is a relatively reliable marker capable of producing reproducible results. CD107a was also shown to be an accurate marker of the degranulation process, since the percentage of CD107a positive cells increased as the percentage of perforin positive cells decreased. Therefore it was concluded that CD107a could be reliably used as an effective and accurate marker of the cytolytic activity occurring in CD8 T cells.

The first stage of developing any assay is to determine the optimal positive control – in other words the protocol control test that can be included in every experiment to verify that the assay itself had worked and the results should be reliable. This assay involves the stimulation of PBMC with an HPV specific antigen and then the staining of the cells to determine whether any of the T cells were able to mount a cytotoxic immune response. Therefore the positive control in this situation needed to be an antigen that would non-specifically cause CD8 T cell stimulation and therefore reveal whether the donors T cells were capable of reacting to any stimulus at all. Previous experiments have utilised various positive controls including: mitogenic agents (PHA), pharmacologic agents (PMA/I) and superantigens (SEB). Comparison of these agents revealed PMA/I to be the optimal positive control for determining inherent cytotoxic ability in CD8 T cells. Interestingly the cells stimulated with SEB, which should induce massive CTL responses (Herman et al., 1991), actually only expressed 5 fold less CD107a than the PHA stimulated cells. A possible explanation for this vast difference in degranulation capabilities is that SEB induces less intracellular calcium during the T cell stimulation process than other antigens, which results in an inability of the CTL to perform cytoloysis (Fuller and Braciale, 1998).

When developing assays for use with specific antigens, to which only 'rare event' responses are expected, it is necessary to consider other factors; for example the optimal length of stimulation period in order to generate a detectable response. In this case, the optimal stimulation for the HPV16 antigens (VLP L1 and E7) was 21hr, therefore it was necessary to establish at which point in the stimulation period the antibodies to CD107a should be added to the culture medium and whether the CyChrome fluorochrome would be sufficiently stable to withstand 21hr of incubation at 37°C, 5%CO_2 without becoming bleached and losing fluorescence intensity. The

results revealed that the anti-CD107a-CChr antibody was stable enough to be used for the duration of the 21hr stimulation period, and it was preferable to add the antibody for the entire 21hr stimulation period (as opposed to just for the last 5hr as was done for the positive control), which suggests that cytotoxicity is not a cumulative event (such as cytokine production) but that the cell responds immediately to the T cell receptor stimulus with release of most of its cytotoxic granules.

The optimised cytotoxicity assay was successful in assessing the cytotoxic function of CD8 T cells when stimulated with either the positive control or the HPV16 specific antigens. The benefit of this flow cytometry based assay for cytotoxic activity is that unlike previous assays which can only quantify the amount of cytolytic activity which has occurred by determining target cell lysis, this assay facilitates the analysis of the functional characteristics of the cells which are performing the cytolytic activity. Therefore with this cytotoxicity assay it is possible to determine which cells are capable of 'killing' the infected cells and what other functional characteristics these cells possess (e.g. cytokine production).

Due to the small size of the study population in this pilot study of cytotoxic responses, it is not possible to draw any statistically significant conclusions. Yet, this assay has still revealed some interesting points. It was noticed that HPV16 E7 was a much more effective antigen than VLP L1, inducing higher levels of CD107a expression (i.e. degranulation) across a larger number of patient samples. The production of IFN-γ cytokine and degranulation process seemed to be mutually exclusive events in the PBMC responding to E7, and observing double positive responses (IFN-γ+CD107a+) to E7 antigen was rare. This was unexpected since in all the positive control stimulated PBMC samples, most of the CD107a+ cells were also producing IFN-γ, which would seem to be the natural process of immune response

events in T cells. Perhaps the unusual nature of the T cell response to E7 is due to the fact that HPV infected patients the infection might cause a disturbance in the function of the T cells and results in impaired immune responses to the HPV antigens (Nickoloff et al., 1994, 1995). This observation may however be an artifact of the low numbers of positive events following stimulation with HPV antigen. So perhaps if responses to HPV specific antigens occurred more often or if a greater number of PBMC could be analyzed to improve the significance of each positive event, this would result in a greater number of double positive cells (IFN-γ+CD107a+) in the HPV antigen stimulated populations.

The majority of cytotoxic responses were elicited by women infected with HPV16 at the cervix which supports previous studies that have shown that patients with CTL responses were likely to be suffering from persistant HPV infections (de Gruil et al., 1996). Also, the majority of the cytotoxic responses were elicited by CIN negative women (in whom cervical lesions are likely to have regressed since these women were referred to the clinic with CIN but on arrival at the clinic were found to be disease free). This concurs with previous studies, which have shown that T cells from healthy women elicited more effective CTL responses to HPV antigens than T cells from CIN diseased women (Nakagawa et al., 1997).

Despite extensive setup and validation of the assay on PBMC samples, the low frequency of CD107a expression to HPV antigens and the low yields of cervical T cells from patient cytobrush samples made it impossible to perform this assay on cervical samples as originally intended.

In conclusion, this chapter has described the development and optimization of a cytotoxicity assay which may prove invaluable in the analysis of CTL responses to antigen specific stimuli. It has been shown how CD107a is a reliable and direct

marker of the degree of degranulation in CD8 T cells, with reproducible results across independently performed experiments. Since CTL defense in the face of viral pathogens is a crucial part of the immune response, this is a highly important area in immune response research. This assay has described a simple, direct *ex vivo* method for accurately quantifying the magnitude of potential CTL activity and assessing various other phenotypic or functional characteristics on each individual cell of the experimental population.

CHAPTER 5:

DISCUSSION

5.1 Overall Objectives of this Project

First and foremost, the main aim of this project was to investigate the type of cellular immune response elicited by cervical T cells in women with HPV-associated cervical disease (CIN 1-3) in response to HPV infection and to compare these cervical T cell responses with those from peripheral blood. Many published reports of CMI responses during HPV infection have focused on responses in the peripheral blood of infected or diseased women. It is well recognized that HPV types that infect the genital mucosa (such as HPV-16) do not cause systemic infection but rather, viral replication is localized, highly tissue specific (only infecting basal keritinocytes) and tightly controlled. The value of studies of systemic T cell responses to such a localized infection is questionable without a representative comparison from T cells isolated from the site of pathology, the genital mucosa.

Secondly, the fact that almost half (47-58%) of CIN2/3 lesions and 85% of CIN1 lesions are likely to regress completely (Chan et al., 2003; Schlecht et al., 2003; Brenner and Syrjänen, 2003; Iatrakis et al., 2004) highlights the value in determining (through this cross-sectional study) the type of immune responses which were elicited by T cells from women with CIN. Therefore it was of interest in this project to compare the ICC responses from CIN negative women to those suffering with cervical dysplasia, to determine whether there were any significant differences between their responses and whether those significant responses were potential correlates of protection to HPV infection and associated cervical disease.

This project also aimed to develop techniques in order to (i) improve the efficiency of the current protocols for processing cervical cytobrush samples and (ii) to analyse the functional cytotoxic ability of T cells using a newly described marker of degranulation in combination with the technique of flow cytometry.

5.2 Development of Methods to Process Cervical Samples

Since this study was interested in the actual immune response at the site of mucosal HPV infection, it was necessary to isolate cervical T cells for further analysis and process them in such a way that these cells would be both competent for use in direct *ex vivo* functional studies and sufficient in number for the results to be statistically meaningful. The published protocols on the processing of cervical cytobrush samples needed to be refined in several areas (Musey et al., 1997).

Of primary importance, it was necessary to determine whether a cervical cytobrush sample had yielded enough CD3+ T cells in order to continue with the stimulation and analysis of the sample. This is because, when T cells undergo *ex vivo* stimulation with various virus specific antigens, the frequency of positive events that occur to those specific antigens is low (Waldrop et al., 1997), therefore the number of events evaluated by flow cytometry need to be as large as possible so that the strength of numbers might lend significance to the few positive events above the background unstimulated population. From these experiments it was determined that a CD3+ Screen could be utilised to determine the validity of the cellular sample and whether it should be analysed or discarded. It was established that a CD3+ event number >500 in the CD3+ screen would serve as an optimal cut off, ensuring sufficient T cell yield in the sample such that in the final analysis the number of T cells would be sufficient to support differences between the antigen specific stimulation and a background of

0.85% to 1.7%. The differences between antigen stimulated responses and unstimulated responses which were observed in this study were, however, rarely large enough, with the frustrating result that many of the individual low positive responses could not be considered significant.

5.3 Analysis of HPV-specific cervical and peripheral blood T cell Responses by intracellular cytokine staining and flow cytometry

This project aimed to use flow cytometry to analyse the type of cytokine (Th1 versus Th2) responses elicited by the CD4 and the CD8 T cells. There are many benefits of the use of flow cytometry to determine the T cell responses to HPV specific antigens in comparison with techniques previously used such as proliferation (Luxton et al., 2003) or Chromium release assays (Nakagawa et al., 2001). Firstly, other assays require a 1-3 week *in vitro* culturing period in order to induce expansion of the T cells and facilitate subsequent detection of the positive responses. Flow cytometry has higher sensitivity to detect positive responses, therefore it allows the direct *ex vivo* analysis of T cells and eliminates the need for a potentially problematic and misrepresentative culturing period.

Secondly, flow cytometry has the added advantage of allowing detection of multiple parameters individually on each single cell of a cellular population. Therefore, through this method it is possible to determine the exact numbers of responding T cells, whether they belong to the CD4 or CD8 subset, and whether the responding cells are capable of producing one or more cytokines of interest. Therefore flow cytometry facilitates a comprehensive and accurate analysis of the responding populations of T cells following stimulation with specific antigens.

Due to the sample size of this study, where only one third of the 100 recruited women had sufficient cervical T cell numbers to be fully analysed, it was found that statistical power of the results were limited. This being so, analysis of the data was continued and subsequently many interesting trends were observed.

Interestingly, it was found that often the types of responses eilicted by matched PBMC and cervical T cells samples from a single patient, to stimulation with the same HPV specific antigen, were highly different. The differences between cervical T cell responses, and those elicited by the PBMC were most significant in the CIN 3 women, whose responses were significantly reduced at the cervix. This could imply that patients with weak cervical CD4 T cells IFNγ responses are unable to control their HPV infection and therefore are likely to progress to CIN 3. It is recommended that an important consideration for future studies, especially when interpreting results from PBMC responses, would be that the detection of responses to a specific antigen in a patient's PBMC specimen does not necessarily imply that there are T cell responses at the cervix of that same patient.

The most consistently high and significant responses were elicited by the PBMC and cervical cells of women with CIN 1. These women had the strongest CD4 IFN-γ responses at the cervix to HPV-16 antigens compared to women with no cervical neoplasia or those with more severe disease (CIN 2/3). This is consistent with previous evidence from wart infiltrating lymphocytes studies (Nicholls et al., 2001; Stanley, 2001), which show that CD4 T cell production of IFN-γ correlates with lesion regression. These results also support those reported in other studies where a correlation was found between Th1 cytokines at the cervix, and subsequent lesion regression (Al-Saleh et al., 1998; Luxton et al., 1997). The strong inflammatory Th1 responses detected were also reflected by the detection of inflammatory cytokines in

cervical washing, which revealed significantly higher inflammation at the cervix of the women with CIN 1 (IL-8) than any of the other patient groups. This implies that the immune cells at the cervix of women with CIN 1 lesions might be eliciting large inflammatory responses to induce lesion regression and HPV clearance. This supports the results of previous studies, which have shown that the vast majority (85%) of CIN 1 lesions spontaneously regress (Iatrakis et al., 2004) as opposed to only half (47-58%) of CIN2 and CIN3 lesions (Schlecht et al., 2003). Finally, a significant correlation was observed between high viral loads and high levels of IFNγ cytokine production by CD4 T cells following stimulation of the T cells with HPV16 VLP L1 demonstrating increased immune responsiveness driven by increased antigen load. This shows that in response to an active HPV16 infection, the immune system elicits primarily Th1 CD4 responses both locally and systemically.

As expected, patients actively infected with HPV16 at the cervix were found generally to exhibit higher PBMC responses to the HPV16 specific antigens, although women infected with HPV types other than HPV-16 were also able to elicit responses to the HPV16 specific antigens. In the absence of no true negative control group (a group of women without previous history of HPV infection; both HPV DNA and antibody negative), the magnitude of responses in women with active HPV-16 infection compared with women infected with non-HPV-16 responses is difficult to interpret as is the potential cross-recognition of HPV-16 antigens by T cells elicited during non-HPV-16 infections.

In the comparisons of responses from HPV16 infected women and those infected with other HPV types according to the severity of their cervical disease, there was a strong positive correlation between IL-13 production from PBMC CD8 T cells and severity of the cervical lesion in women infected with HPV16, when cells were

exposed to HPV16 E7 antigen. This data indicates that in women with active HPV16 infections at the cervix, the immune response to the HPV antigens is predominantly anti-inflammatory (Th2) and this supports the hypothesis that an inadequate inflammatory immune response could result in progression of the cervical lesion.

Yet it has also emerged from this study that CIN 3 patients appear to elicit Th1 IFNγ responses (possibly as a last attempt to rid the host of the infection), which could be interpreted as a contradiction to the hypothesis that lesion progression is associated to a Th2 type of cytokine profile. However, overall the CIN 3 Th1 type responses were noted to be smaller in magnitude and number than the responses elicited by other patient groups. In comparison, the CIN 1 patients elicited the highest number and largest responses to HPV specific antigens and it is the data from these patients that suggest that a Th1 inflammatory (CD4/IFNγ) response at the cervix of HPV infected women is the strongest correlate of protection against HPV infection and subsequent cervical disease.

5.4 Markers of HPV-16 specific Cytolytic Activity by peripheral blood T cells: CD107a versus perforin

One of the most important immune response mechanisms is the antigen specific kill mechanism elicited by the CD8 cytotoxic T lymphocytes (CTLs). In HPV infected women, these responses were found to be highly associated with the regression of CIN (Nakagawa et al., 1996; 1997). CTL responses from HPV16+ patients have been shown to last for up to 20 months post clearance (Nakagawa, 2000, 2002). These results indicate the importance of a CD8 T cell cytolytic response in the defense against HPV infection and associated CIN.

There are various methods of detecting the cytotoxic function in T cells. Most HPV immune response studies have utilised the chromium release CTL assay. But due to a lack of sensitivity, this method requires a 1-3 week culturing of the T cells prior to the assay, in order to amplify responding cells in order to facilitate detection of the positive responses. It also involves generating a line of HPV antigen expressing antigen presenting cells to serve as targets for the HPV specific T cells. It is through the lysis of the target cells by the CD8 T cell population that the relative amount of cytotoxic activity is determined. This highlights another disadvantage of this method, it does not measure the actual cytolytic activity of CD8 T cells, rather it measures the death of target cells, and therefore indirectly the relative amount of cytotoxic activity. A recently published method used the flow cytometer to determine directly on antigen stimulated CD8 T cells how much degranulation had occurred in response to the stimulus (Betts et al., 2003). It reported that a fluorochrome conjugated antibody to the CD107a molecule could be used as a marker of degranulation, since CD107a is located only on cytolytic granules, and is transiently exposed on the surface of a degranulating cell during exocytosis of the cytotoxic granules contents (e.g. perforin and granzyme) (Betts et al., 2003).

Therefore, this study sought to develop an assay that could analyse the cytotoxic ability of T cells in response to HPV16 specific antigens, through the use of the flow cytometer. Initially cytotoxic T cell responses to HPV-16 L1 and E7 antigens were investigated in PBMC from twenty three women with HPV-associated cervical disease (CIN), with the intention to develop the assay for use on cervical cytobrush samples. Although the study population was small and statistical power limited, I observed that higher levels of CD107a expression (i.e. degranulation) were elicited much more frequently to HPV16 E7 than VLP L1. Yet in comparison to the

PMA/I stimulated PBMC where all CD107a+ CTL were also producing IFNγ in response to the mitogenic stimulation, PBMC which were stimulated with HPV16 specific E7 antigen showed a completely different profile with the production of IFN-γ cytokine and degranulation process seeming to be mutually exclusive events (i.e. to observe double positive responses (IFN-γ+CD107a+) to E7 antigen was rare). Whether this is an artifact of the very low frequencies of these events or indicative that cytokine production and the cytotoxic ability of the T cells are induced by different and independent T cells, remains to be determined.

In this study, the majority of cytotoxic responses were elicited by CIN negative women. This supports previous studies, which have shown that T cells from healthy women elicited more effective CTL responses to HPV antigens than T cells from CIN diseased women (Nakagawa et al., 1997), and also confirms that this assay could be a reliable method for detecting HPV specific CTL activity in HPV infected women.

Despite extensive set up and validation on PBMC, due to the low frequencies of CD107a expression in response to HPV antigens and the low yields of cervical T cells from cytobrush samples, it was impossible to perform the developed CD107a assay on cervical cytobrush specimens.

5.5 Future Considerations for the analysis of HPV-specific cervical T cell responses

Through the experiments performed in this thesis, some fundamental considerations have been established, which will significantly assist future studies. Firstly, it is crucial for a sample to have sufficient numbers of T cells for further analysis. It is beneficial in the early stages of the study to determine a CD3 screen

and appropriate cut off value in order to prevent the loss of time, money, valuable resources and intensive labour on the stimulation, staining and subsequent attempt at analysis of samples with insufficient T cell yields.

Secondly, due to the heterogenous nature of cervical cytobrush specimens, which yield such variable quantities of T cells, and of which so many samples had to be discarded due to insufficient T cell populations, the following options are highly recommended. When commencing applications to undertake a project which analyses cells through collection using cervical cytobrush, the applicant should request ethical permission for as large a study population as is physically possible to process. Yet even if this is taken into consideration, an inclusion rate of 33% (33/100) as described in this study is very low. Since screening and processing of the 66% of samples, which were ultimately not suitable for inclusion into the study, also required significant personnel and financial resources, enrolling more women to increase suitable cervical specimen inclusion might not be the most viable option.

Perhaps a more suitable and efficient option would be to increase the numbers of responding T cells using *in vitro* culturing and repeated stimulation with either mitogenic stimuli, or HPV specific antigens (Luxton et al., 2003). This would greatly improve the numbers of responsive T cells in the total population and thereby prevent the need for a CD3 screen, allowing the inclusion of many more cervical T cell samples into the study population. The later option will be investigated in future studies of HPV-specific T cell responses from cervical cytobrush specimens.

Appendix A

Table A.1 Raw Data from ICC Responses of cervical T cells stimulated with PMA/I, HPV16 VLP L1 and E7[a]

Donor	CIN	Stimulation Condition	% CD4+ IFN+	% Above Unstim	Abs Fold (Stim/Unstim)	% CD8+IFN+	% Above Unstim	Abs Fold	% CD4+1L-13+	% Above Unstim	Abs Fold	% CD8+IL-13+	% Above Unstim	Abs Fold
JP001	neg	UNSTIM	0			15.63			0			26.32		
		PMA/I	4.76	4.76	#DIV/0!	40.55	24.92	2.59	0	0	#DIV/0!	5.27	-21.05	0.20
		VLP L1	0	0.14	#DIV/0!	16.28	0.14	1.04	5.27	0.14	#DIV/0!	13.88	0.14	0.53
JP010	neg	UNSTIM	0.17			0.24			0.44			0.06		
		PMA/I	35.55	35.38	209.12	72.73	72.49	303.04	20.83	20.39	47.34	9.1	9.04	151.67
		VLP L1	0.13	0	0.76	0.29	0.05	1.21	0.88	0.44	2	0.17	0.11	2.83
JP018	neg	UNSTIM	0			1.01			0			0.87		
		PMA/I	5.55	5.55	#DIV/0!	32.65	31.64	32.33	0	0	#DIV/0!	5.93	5.06	6.82
		VLP L1	0	0	#DIV/0!	0	0	0	1.8	1.8	#DIV/0!	2.12	1.25	2.44
JP028	neg	UNSTIM	0.28			0.57			1.97			1.15		
		PMA/I	7.58	7.3	27.07	77.73	77.16	136.37	0.38	-1.59	0.19	1.3	0.15	1.13
		VLP L1	0.5	0	1.79	1.47	0.9	2.58	0.33	-1.64	0.17	1.13	-0.02	0.98
JP034	neg	UNSTIM	0.51			0.5			0.51			0.52		
		PMA/I	17.29	16.78	33.90	87.34	86.84	174.68	0	-0.51	0.00	1.98	1.46	3.81
		VLP L1	4.66	4.15	9.14	1.31	0.81	2.62	0	-0.51	0.00	0.37	-0.15	0.71
JP035	neg	UNSTIM	1.03			1.06			0.93			1.05		
		PMA/I	26.39	25.36	25.62	67.42	66.36	63.60	0	-0.93	0	1.5	0.45	1.43
		VLP L1	0	-1.03	0	3.7	2.64	3.49	0	0	0	1.07	0.02	1.02
		E7	1	-0.03	0.97	0	-1.06	0	1.16	0	0	0	-1.05	0
JP041	neg	UNSTIM	0.23			0.27			0.05			0.09		
		PMA/I	1.89	1.66	8.22	4.69	4.42	17.37	0.05	0	1	0	-0.09	0
		VLP L1	0.7	0.47	3.04	4.76	4.49	17.63	3.77	3.72	75.4	7.27	7.18	80.78
		E7	0.58	0.35	2.52	0.32	0.05	1.19	0.34	0.29	6.8	0.74	0.65	8.22
JP045	neg	UNSTIM	0.45			0.3			0.08			0.6		
		PMA/I	7.73	7.28	17.18	23.99	23.69	79.97	0.42	0.34	5.25	0.69	0.09	1.15
		VLP L1	0.42	0	0.93	0.36	0.06	1.2	0	-0.08	0.00	0.09	0	0.15
		E7	0.1	0	0.22	0	0	0	0.36	0.28	4.50	0.29	0	0.48

Donor	CIN	Stimulation Condition	% CD4+ IFN+	% Above Unstim	Abs Fold (Stim/Unstim)	% CD8+IFN+	% Above Unstim	Abs Fold	% CD4+1L-13+	% Above Unstim	Abs Fold	% CD8+IL-13+	% Above Unstim	Abs Fold
JP047	neg	UNSTIM	0.67			1.09			0.4			0.35		
		PMA/I	3.68	3.01	5.49	21.61	20.52	19.83	3.4	3	8.5	4.77	4.42	13.63
		VLP L1	0.1	0	0.15	0.29	0	0.27	2.41	2.01	6.03	4.55	4.2	13.00
		E7	0	0	0	0	0	0	1.55	1.15	3.88	2.61	2.26	7.46
JP058	neg	UNSTIM	1.63			2.08			0.81			2.08		
		PMA/I	17.2	15.57	10.55	86.67	84.59	41.67	1.08	0.27	1.33	20	17.92	9.62
		VLP L1	0	-1.63	0.00	0	-2.08	0.00	0	-0.81	0.00	0	-2.08	0.00
		E7	0	-1.63	0.00	0	-2.08	0.00	0	-0.81	0.00	5.88	3.8	2.83
JP061	neg	UNSTIM	0.25			0.24			0.87			0.16		
		PMA/I	29.59	29.34	118.36	63.63	63.39	265.13	2.24	1.37	2.57	0.49	0.33	3.06
		VLP L1	0.06	0	0.24	0.09	0	0.38	0.06	0	0.07	0	0	0
		E7	0.14	0	0.56	0.19	0	0.79	0.34	0	0.39	0.19	0.03	1.19
JP088	neg	UNSTIM	0.46			0.54			0.91			0.54		
		PMA/I	19.21	18.75	41.76	56.8	56.26	105.19	0	-0.91	0.00	0	-0.54	0.00
		VLP L1	0	-0.46	0.00	4.06	3.52	7.52	1.52	0.61	1.67	0	-0.54	0.00
		E7	1.69	1.23	3.67	0.88	0.34	1.63	0	-0.91	0.00	0	-0.54	0.00
JP007	1	UNSTIM	0.06			0.18			0.05			0.24		
		PMA/I	39.18	39.12	653	88.86	88.68	493.67	0.43	0.38	8.6	2.21	1.97	9.21
		VLP L1	0.1	0.04	1.67	0	-0.18	0.00	0.05	0	1.00	2.11	1.87	8.79
JP013	1	UNSTIM	0.21			0.24			3.1			0.15		
		PMA/I	20.39	20.18	97.10	5.64	5.40	23.50	15.91	12.81	5.13	5.60	5.45	37.33
		VLP L1	2.77	2.56	13.19	0.17	0.00	0.71	2.02	0.00	0.65	0.10	0.00	0.67
JP033	1	UNSTIM	0.19			0.9			0.56			0.9		
		PMA/I	5.48	5.29	28.84	72.41	71.51	80.46	0	-0.56	0.00	0	-0.9	0.00
		VLP L1	0.16	-0.03	0.84	2.85	1.95	3.17	0.39	-0.17	0.70	1.42	0.52	1.58
JP049	1	UNSTIM	0.39			0.06			1			0.18		
		PMA/I	44.25	43.86	113.46	74.93	74.87	1248.83	0.4	-0.6	0.40	0.43	0.25	2.39
		VLP L1	3.55	3.16	9.1	0.12	0.06	2	0.78	-0.22	0.78	0.31	0.13	1.72
		E7	1.81	1.42	4.64	0.06	0	1	1.7	0.7	1.70	0.42	0.24	2.33
JP084	1	UNSTIM	0.65			0.68			0.65			0.68		
		PMA/I	11.71	11.06	18.02	41.18	40.5	60.56	0.71	0.06	1.09	1.79	1.11	2.63
		VLP L1	1.12	0.47	1.72	2.18	1.5	3.21	1.01	0.36	1.55	0	-0.68	0.00
		E7	1.3	0.65	2.00	1.76	1.08	2.59	0.29	-0.36	0.45	0.44	-0.24	0.65

Donor	CIN	Stimulation Condition	% CD4+ IFN+	% Above Unstim	Abs Fold (Stim/Unstim)	% CD8+IFN+	% Above Unstim	Abs Fold	% CD4+1L-13+	% Above Unstim	Abs Fold	% CD8+IL-13+	% Above Unstim	Abs Fold
JP089	1	UNSTIM	0.14			0.24			0.14			0.24		
		PMA/I	15.03	14.89	107.36	61.02	60.78	254.25	1	0.86	7.14	2.26	2.02	9.42
		VLP L1	0.13	-0.01	0.93	0	-0.24	0.00	0.07	-0.07	0.50	0.4	0.16	1.67
		E7	0.82	0.68	5.86	0.23	-0.01	0.96	0.44	0.3	3.14	0.9	0.66	3.75
JP091	1	UNSTIM	0.38			0.34			0.38			0.68		
		PMA/I	20.92	20.54	55.05	69.34	69	203.94	3.35	2.97	8.82	1.09	0.41	1.60
		VLP L1	2.08	1.7	5.47	0.95	0.61	2.79	1.04	0.66	2.74	1.42	0.74	2.09
		E7	0	-0.38	0.00	0	-0.34	0.00	0	-0.38	0.00	0	-0.68	0.00
JP008	2	UNSTIM	0.27			0.46			0.08			0.53		
		PMA/I	69.04	68.77	255.70	78.56	78.10	170.78	60.97	60.89	762.13	76.40	75.87	144.15
		VLP L1	0.51	0.24	1.89	0.43	0.00	0.93	0.04	0.00	0.50	1.89	1.36	3.57
JP011	2	UNSTIM	2.94			0			0.85			0		
		PMA/I	32.89	29.95	11.19	78.95	78.95	#DIV/0!	2.15	1.3	2.53	0	0	#DIV/0!
		VLP L1	10.17	7.23	3.46	0	0	#DIV/0!	0.91	0.06	1.07	0	0	#DIV/0!
JP014	2	UNSTIM	2.02						3.64					
		PMA/I	41.86	39.84	20.7	69.14			13.96	10.32	3.84	6.34		
		VLP L1	4.33	2.31	2.14	0.61			4.23	0.59	1.16	1.33		
JP060	2	UNSTIM	0.53			0.17			0.14			0.17		
		PMA/I	20.41	19.88	38.51	54.93	54.76	323.12	0.15	0.01	1.07	0.53	0.36	3.12
		VLP L1	0.22	0	0.42	0.32	0.15	1.88	0.4	0.26	2.86	0.48	0.31	2.82
		E7	0.4	0	0.75	0	0	0	0.19	0.05	1.36	0.17	0	1
JP062	2	UNSTIM	0.1			1.3			0.1			0.33		
		PMA/I	1.6	1.5	16	12.8	11.5	9.85	0.07	0	0.7	0	0	0
		VLP L1	0	0	0	0	0	0	0	0	0	0	0	0
		E7	0.13	0.03	1.3	0	0	0	0	0	0	0	0	0
JP002	3	UNSTIM												
		PMA/I												
		VLP L1	0	0.14	#DIV/0!	0	0.14	#DIV/0!	0	0.14	#DIV/0!	1.09	0.14	#DIV/0!
JP005	3	UNSTIM	0			11.1			7.51			14.31		
		PMA/I	0	0	#DIV/0!	24.99	13.89	2.25	16.67	9.16	2.22	33.37	19.06	2.33
		VLP L1	0	0.14	#DIV/0!	33.35	0.14	3.00	0	0.14	0.00	20.01	0.14	1.40
JP017	3	UNSTIM	3.14			0			6.97			9.94		
		PMA/I	27.8	24.66	8.85	13.16	13.16	#DIV/0!	0	-6.97	0.00	0.00	-9.94	0.00
		VLP L1	2.55	-0.59	0.81	2.94	2.94	#DIV/0!	4.26	-2.71	0.61	3.02	-6.92	0.30

Donor	CIN	Stimulation Condition	% CD4+ IFN+	% Above Unstim	Abs Fold (Stim/Unstim)	% CD8+IFN+	% Above Unstim	Abs Fold	% CD4+1L-13+	% Above Unstim	Abs Fold	% CD8+IL-13+	% Above Unstim	Abs Fold
JP029	3	UNSTIM	0.06			0.57			0.41			0.26		
		PMA/I	2.15	2.09	35.83	76.26	75.69	133.79	0.37	-0.04	0.90	0	-0.26	0.00
		VLP L1	0.77	0.71	12.83	2.06	1.49	3.61	0.35	-0.06	0.85	0	-0.26	0.00
JP043	3	UNSTIM	0.59			0.25			0.59			0.5		
		PMA/I	23.09	22.5	39.14	57.11	56.86	228.44	2.54	1.95	4.31	1.83	1.33	3.66
		VLP L1	0.62	0.03	1.05	0.28	0.03	1.12	1.04	0.45	1.76	0.28	-0.22	0.56
		E7	1.07	0.48	1.81	0	-0.25	0	0	-0.59	0	0	-0.5	0
JP048	3	UNSTIM	0.81						0.81					
		PMA/I	10.87	10.06	13.42		*Too Low CD8+ Cell Count*		7.97	7.16	9.84		*Too Low CD8+ Cell Count*	
		VLP L1	1.78	0.97	2.20				0.44	-0.37	0.54			
		E7	1.21	0.4	1.49				5.65	4.84	6.98			
JP055	3	UNSTIM	0.25			0.11			0.17			0.22		
		PMA/I	24.74	24.49	98.96	82.49	82.38	749.91	0.85	0.68	5.00	4.75	4.53	21.59
		VLP L1	0.73	0.48	2.92	0.53	0.42	4.82	0.7	0.53	4.12	0.43	0.21	1.95
		E7	0.36	0.11	1.44	0.22	0.11	2.00	0	-0.17	0.00	0.03	-0.19	0.14
66	3	UNSTIM	0.17			3.85			0.5			1.92		
		PMA/I	3.23	3.06	19.00	32.11	28.26	8.34	0.42	-0.08	0.84	1.83	-0.09	0.95
		VLP L1	0	-0.17	0.00	3.85	0	1.00	0	-0.5	0.00	0	-1.92	0.00
		E7	0	-0.17	0.00	6.45	2.6	1.68	0.19	-0.31	0.38	0	-1.92	0.00
68	3	UNSTIM	0.11			0.34			0.11			0.34		
		PMA/I	4.48	4.37	40.73	30.18	29.84	88.76	0.24	0.13	2.18	1.61	1.27	4.74
		VLP L1	0	-0.11	0.00	0.35	0.01	1.03	0.1	0	0.91	2.08	1.74	6.12
		E7	0.1	-0.01	0.91	0	0	0	0	0	0	0.9	0.56	2.65

[a] In some cases donor samples were unable to be stimulated with E7 antigen since it was not available at the time of the experiment.

Table A.2 Raw Data from ICC Responses of Peripheral Blood T cells (PBMC) stimulated with HPV16 VLP L1 and E7[a]

Donor	CIN Status	Stimulation Condition	% CD4+ IFN+	% Above Unstim	Abs Fold	% CD8+IFN+	% Above Unstim	Abs Fold	% CD4+1L-13+	% Above Unstim	Abs Fold	% CD8+IL-13+	% Above Unstim	Abs Fold
JP001	neg	UNSTIM	0.05			0.21			0.45			0.18		
		PMA/I	0.21	0.16	4.2	4.02	3.81	19.14	1.82	1.37	4.04	0.03	-0.15	0.17
		VLP L1	0.08	0.03	1.6	0.09	-0.12	0.43	0.03	-0.42	0.07	0.06	-0.12	0.33
JP010	neg	UNSTIM	0.17			0.24			0.44			0.06		
		PMA/I	6.48	6.31	38.12	11.23	10.99	46.79	1.64	1.20	3.73	0.25	0.19	4.17
		VLP L1	0.13	-0.04	0.76	0.29	0.05	1.21	0.88	0.44	2.00	0.17	0.11	2.83
JP018	neg	UNSTIM	0.57			2.6			0.49			0.67		
		PMA/I	5.94	5.37	10.42	23.57	20.97	9.07	0.65	0.16	1.33	3.05	2.38	4.55
		VLP L1	0.64	0.07	1.12	1.93	0	0.74	0.5	0.01	1.02	1.35	0.68	2.01
JP028	neg	UNSTIM	0.06			0.31			0.06			0.07		
		PMA/I	0.87	0.81	14.50	12.22	11.91	39.42	1.40	1.34	23.33	0.61	0.54	8.71
		VLP L1	0.06	0	1.00	0.15	-0.16	0.48	0.24	0.18	4.00	0.14	0.07	2.00
JP034	neg	UNSTIM	0.04			0.16			0.03			0.07		
		PMA/I	3.62	3.58	90.5	29.56	29.4	184.75	0.06	0.03	2	0.05	-0.02	0.71
		VLP L1	0.02	-0.02	0.5	0.07	-0.09	0.4375	0	-0.03	0	0.01	-0.06	0.14
JP035	neg	UNSTIM	0.44			0.61			0.87			0.31		
		PMA/I	0.75	0.31	1.70	9.92	9.31	16.26	21.64	20.77	24.87	0.42	0.11	1.35
		VLP L1	0	-0.44	0.00	0.18	-0.43	0.30	0.00	-0.87	0.00	0.00	-0.31	0.00
		E7	0.56	0.12	1.27	0.35	-0.26	0.57	2.26	1.39	2.60	0.18	-0.13	0.58
JP041	neg	UNSTIM	0.03			0.07			0.03			0.02		
		PMA/I	4.93	4.9	164.33	12.07	12.00	172.43	0.41	0.38	13.67	0.24	0.22	12.00
		VLP L1	0.04	0.01	1.33	0.27	0.20	3.86	0.33	0.30	11.00	0.14	0.12	7.00
		E7	0.06	0.03	2.00	0.36	0.29	5.14	0.02	-0.01	0.67	0.01	-0.01	0.50
JP045	neg	UNSTIM	0.06			0.10			3.13			0.04		
		PMA/I	1.83	1.77	30.50	2.11	2.01	21.10	2.17	-0.96	0.69	0.02	-0.02	0.50
		VLP L1	0.02	-0.04	0.33	0.04	-0.06	0.40	3.37	0.24	1.08	0.07	0.03	1.75
		E7	0.03	-0.03	0.50	0.06	-0.04	0.60	1.46	-1.67	0.47	0.02	-0.02	0.50
JP047	neg	UNSTIM	0.02			0.16			0.02			0.07		
		PMA/I	2.66	2.64	133.00	6.44	6.28	40.25	0.21	0.19	10.50	0.47	0.40	6.71
		VLP L1	0.19	0.17	9.50	0.14	-0.02	0.88	0.02	0.00	1.00	0.03	-0.04	0.43
		E7	1.57	1.55	78.50	0.97	0.81	6.06	0.08	0.06	4.00	0.15	0.08	2.14
JP058	neg	UNSTIM	0.07			0.06			0.17			0.04		
		PMA/I	2	1.93	28.57	5.27	5.21	87.83	1.08	0.91	6.35	0.37	0.33	9.25
		VLP L1	0.02	-0.05	0.29	0.03	-0.03	0.50	0.09	-0.08	0.53	0.04	0.00	1.00
		E7	0.1	0.03	1.43	0.09	0.03	1.50	0.07	-0.10	0.41	0.03	-0.01	0.75
JP061	neg	UNSTIM	0.04			0.34			0.08			0.07		
		PMA/I	3.86	3.82	96.50	15.88	15.54	46.71	1.29	1.21	16.13	0.14	0.07	2.00
		VLP L1	0.02	-0.02	0.50	0.12	-0.22	0.35	0.16	0.08	2.00	0.09	0.02	1.29
		E7	0.03	-0.01	0.75	0.41	0.07	1.21	0.08	0.00	1.00	0.11	0.04	1.57

Donor	CIN Status	Stimulation Condition	% CD4+ IFN+	% Above Unstim	Abs Fold	% CD8+IFN+	% Above Unstim	Abs Fold	% CD4+IL-13+	% Above Unstim	Abs Fold	% CD8+IL-13+	% Above Unstim	Abs Fold
JP088	neg	UNSTIM	0.02			0.03			0.01			0.01		
		PMA/I	6.4	6.38	320.00	16.19	16.16	539.67	0.06	0.05	6.00	0.02	0.01	2.00
		VLP L1	0.04	0.02	2.00	0.07	0.04	2.33	0.05	0.04	5.00	0.02	0.01	2.00
		E7	0.08	0.06	4.00	0.19	0.16	6.33	0.01	0	1.00	0.01	0	1.00
JP007	1	UNSTIM	0.15			0.15			1.06			0.35		
		PMA/I	22.48	22.33	149.87	15.53	15.38	103.53	0.61	-0.45	0.58	0.10	-0.25	0.29
		VLP L1	0.29	0.14	1.93	0.36	0.21	2.40	0.21	-0.85	0.20	1.10	0.75	3.14
JP013	1	UNSTIM	0.21			0.24			3.10			0.15		
		PMA/I	22.33	22.12	106.33	17.42	17.18	72.58	1.01	-2.09	0.33	0.12	-0.03	0.80
		VLP L1	2.77	2.56	13.19	0.17	-0.07	0.71	2.02	-1.08	0.65	0.10	-0.05	0.67
JP033	1	UNSTIM	0.03			0.09			0.02			0.02		
		PMA/I	4.37	4.34	145.67	11.94	11.85	132.67	0.48	0.46	24.00	0.17	0.15	8.50
		VLP L1	0.08	0.05	2.67	0.16	0.07	1.78	0.02	0.00	1.00	0.06	0.04	3.00
JP049	1	UNSTIM	0.06			0.05			0.35			0.01		
		PMA/I	4.65	4.59	77.50	12.69	12.64	253.80	0.86	0.51	2.46	0.10	0.09	10.00
		VLP L1	0.28	0.22	4.67	0.25	0.20	5.00	0.47	0.12	1.34	0.01	0.00	1.00
		E7	0.35	0.29	5.83	0.71	0.66	14.20	0.59	0.24	1.69	0.04	0.03	4.00
JP084	1	UNSTIM	0.2			0.41			0.13			0.13		
		PMA/I	8.75	8.55	43.75	33.4	32.99	81.46	2.41	2.28	18.54	1.45	1.32	11.15
		VLP L1	0.2	0	1.00	0.4	-0.01	0.98	0.55	0.42	4.23	0.46	0.33	3.54
		E7	0.47	0.27	2.35	0.89	0.48	2.17	0.58	0.45	4.46	0.56	0.43	4.31
JP089	1	UNSTIM	0.01			0.01			0.01			0.02		
		PMA/I	1.45	1.44	145.00	5.55	5.54	555.00	0.05	0.04	5.00	0.06	0.04	3.00
		VLP L1	0	-0.01	0.00	0	-0.01	0.00	0.02	0.01	2.00	0.02	0	1.00
		E7	0.04	0.03	4.00	0.04	0.03	4.00	0.01	0	1.00	0.01	-0.01	0.50
JP091	1	UNSTIM	0.05			0.01			0.03			0.02		
		PMA/I	12.59	12.54	251.80	34.22	34.21	3422.00	0.38	0.35	12.67	0.05	0.03	2.50
		VLP L1	0.02	-0.03	0.40	0.01	0	1.00	0.08	0.05	2.67	0.04	0.02	2.00
		E7	0.06	0.01	1.20	0.03	0.02	3.00	0.02	-0.01	0.67	0.01	-0.01	0.50
JP008	2	UNSTIM	0.27			0.46			0.08			0.53		
		PMA/I	5.83	5.56	21.59	19.73	19.27	42.89	0.22	0.14	2.75	0.08	-0.45	0.15
		VLP L1	0.51	0.24	1.89	0.43	-0.03	0.93	0.04	-0.04	0.50	1.89	1.36	3.57
JP011	2	UNSTIM	0.07			0.12			0.09			0.18		
		PMA/I	8.98	8.91	128.29	20.18	20.06	168.17	0.56	0.47	6.22	0.17	-0.01	0.94
		VLP L1	0.05	-0.02	0.71	0.19	0.07	1.58	0.12	0.03	1.33	0.19	0.01	1.06
JP014	2	UNSTIM	2.13			0.29			3.16					
		PMA/I	11.73	9.6	5.51	27.2	26.91	93.8	3.21	0.05	1.02	0.77		
		VLP L1	1.59	0	0.75	0.34	0.05	1.17	3.79	0.63	1.2	0.22		

Donor	CIN Status	Stimulation Condition	% *CD4+ IFN+*	% Above Unstim	Abs Fold	% *CD8+IFN+*	% Above Unstim	Abs Fold	% *CD4+1L-13+*	% Above Unstim	Abs Fold	% *CD8+IL-13+*	% Above Unstim	Abs Fold
JP060	2	UNSTIM	0.01			0.01			0.08			0.03		
		PMA/I	3.38	3.37	338	5.89	5.88	589	1.18	1.1	14.75	5.35	5.32	178.33
		VLP L1	0	-0.01	0	0.01	0	1	0.07	-0.01	0.875	0.06	0.03	2.00
		E7	0.04	0.03	4	0.06	0.05	6	0.02	-0.06	0.25	0.01	-0.02	0.33
JP062	2	UNSTIM	0.04			0.2			0.04			0.14		
		PMA/I	3	2.96	75	10.25	10.05	51.25	0.92	0.88	23	0.85	0.71	6.07
		VLP L1	0.05	0.01	1.25	0.15	-0.05	0.75	0.06	0.02	1.5	0.11	-0.03	0.79
		E7	0.1	0.06	2.5	0.21	0.01	1.05	0.05	0.01	1.25	0.13	-0.01	0.93
JP002	3	UNSTIM	0.06			0.06			0.09			0		
		PMA/I	2.46	2.4	41	11.62	11.56	193.67	0.56	0.47	6.22	0.02	0.02	#DIV/0!
		VLP L1	0.03	-0.03	0.5	0.13	0.07	2.17	0.06	-0.03	0.67	0.03	0.03	#DIV/0!
JP005	3	UNSTIM	0.04			0.19			0.07			0.85		
		PMA/I	24.72	24.68	618	50.42	50.23	265.37	8.66	8.59	123.71	22.28	21.43	26.21
		VLP L1	0.04	0	1	0.33	0.14	1.74	0.05	-0.02	0.71	1.17	0.32	1.38
JP017	3	UNSTIM	0.28			1.23			2.14			1.18		
		PMA/I	7.19	6.91	25.68	10.94	9.71	8.89	8.56	6.42	4	0.5	0	0.42
		VLP L1	0.76	0.48	2.71	0.96	0	0.78	3.34	1.2	1.56	0.48	0	0.41
JP029	3	UNSTIM	0.04			0.32			0.04			0.27		
		PMA/I	1.07	1.03	26.75	20.03	19.71	62.59	0.12	0.08	3.00	0.43	0.16	1.59
		VLP L1	0.04	0	1	0.24	-0.08	0.75	0.02	-0.02	0.50	0.11	-0.16	0.41
JP043	3	UNSTIM	0.02			0.12			0.01			0.03		
		PMA/I	5.64	5.62	282	11.62	11.5	96.83	2.00	1.99	200.00	0.06	0.03	2.00
		VLP L1	0.07	0.05	3.5	0.17	0.05	1.42	0.48	0.47	48.00	0.30	0.27	10.00
		E7	0.1	0.08	5	0.27	0.15	2.25	0.46	0.45	46.00	0.28	0.25	9.33
JP048	3	UNSTIM	0.08			0.06			0.09			0.01		
		PMA/I	2.79	2.71	34.88	7.78	7.72	129.67	1.08	0.99	12.00	0.32	0.31	32
		VLP L1	0.12	0.04	1.50	0.11	0.05	1.83	0.08	-0.01	0.89	0.06	0.05	6
		E7	0.12	0.04	1.50	0.35	0.29	5.83	0.16	0.07	1.78	0.15	0.14	15
JP055	3	UNSTIM	0.03			0.05			0.03			0.01		
		PMA/I	8.55	8.52	285	55.86	55.81	1117.2	2.3	2.27	76.67	1.5	1.49	150
		VLP L1	0.26	0.23	8.67	0.3	0.25	6	0.03	0	1.00	0.01	0	1
		E7	0.08	0.05	2.67	0.16	0.11	3.2	0.07	0.04	2.33	0.03	0.02	3
JP066	3	UNSTIM	0.01			0.16			0			0		
		PMA/I	0.47	0.46	47	2.89	2.73	18.06	0.17	0.17	#DIV/0!	0.23	0.23	#DIV/0!
		VLP L1	0.04	0.03	4	0.09	-0.07	0.56	0	0	#DIV/0!	0.02	0.02	#DIV/0!
		E7	0.22	0.21	22	0.92	0.76	5.75	0.02	0.02	#DIV/0!	0.07	0.07	#DIV/0!
JP068	3	UNSTIM	0.08			0.03			0.01			0.04		
		PMA/I	0.55	0.47	6.875	0.31	0.28	10.33	0.09	0.08	9	0.07	0.03	1.75
		VLP L1	0.01	-0.07	0.125	0	-0.03	0.00	0.03	0.02	3	0.01	-0.03	0.25
		E7	0	-0.08	0	0.01	-0.02	0.33	0.01	0	1	0.01	-0.03	0.25

APPENDIX B : SUBSTRATES AND SOLUTIONS

Cell Stimulation Solutions

PBS – phosphate buffered saline pH 7.2

Ingredient	Final Concentration	Amount Added
KCl	2.7mM	0.20g
KH_2PO_4	1.2mM	0.20g
NaCl	138mM final	8.0g
$Na_2HPO_4.7H_2O$	8.1mM	2.16g
Water		900ml

Adjust pH to 7.2
Make up Water to 1*l*
Autoclave and store at 4°C.

RPMI

1640 Medium with Glutamax-1 and 25mM HEPES
(Invitrogen Corporation, UK)

To all RPMI, the following were added:
Penicillin (1 x 106iu/ml) 1ml to 100ml RPMI
Streptomycin (0.5 µg/ml) 1ml to 100ml RPMI
Amphotericin B 1ml to 100ml RPMI
(supplied by Invitrogen Corporation UK)

10% FCS RPMI

Fetal Calf Serum (Fetal Bovine Serum) was heat inactivated at 56°C and then added to RPMI (10ml to 100ml RPMI).

10% AB RPMI

Heat inactivated serum collected from AB positive blood donors, was heat inactivated and filtered before addition to RPMI (10ml per 100ml RPMI)

Staining Solutions

Staining Solution

5% FCS
0.01% Azide
made up to volume with PBS.

0.1% Saponin Permeabilization Solution

0.2g saponin
0.01% azide
200ml PBS

1% Azide Stock Solution

0.5g Sodium Azide (NaN_3)
50ml PBS

Agarose Gel Solutions

1.8% Agarose Gel (w/v)

1.8g Agarose
1 x TAE (make up to 100ml)

Tris Acetate Buffer (TAE) (50x)

242g Tris
57.1 ml Glacial acetic acid
100ml 0.5M EDTA

Loading Buffer

Ingredient	Final Concentration	/10ml
Xylene cyanol	0.25% (w/v)	25mg
Sucrose	40% (w/v)	4.0g
EDTA (0.5M,pH 8.0)	20mM	0.4ml
Water		To 10 ml

Roche Reverse Line Blot Solutions

SSPE (20x)

For *1l* : *74g* NaCl
27.6g $Na_6H_2PO_4H_2O$
7.4g EDTA

Hybridisation Solution

1x (4x SSPE, 0.5% Sodium Dodecyl Sulphate)
for 1*l* : 20x SSPE 200ml
20% SDS 25ml
dH_2O 775ml

Wash Solution

1x (1x SSPE; 0.1% SDS)
for 1*l* : 20x SSPE 50ml
20% SDS 5ml
dH_2O 945ml

SA-HRP(streptavidin conjugated Horse radish peroxidase)

Conjugate Solution
For 100ml : 1x wash solution 100ml
Amplicor SA-HRP 0.3ml

Citrate Buffer

For 1*l* :	20x Citrate Solution	50ml
	dH_2O	950ml

ELISA Solutions

Blocking Solution

0.5% PVA
make up to required volume in PBS pH 7.4

OPD Substrate Solution

3 OPD tablets (2mg/tablet)
9 ml RO H_2O
3.75µl H_2O_2

Stop Solution

0.5M H_2SO_4

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