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HPV Mediated Antagonism of the IL-18 Proinflammatory Pathway in Head and Neck Cancer

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Abstract

In this thesis, I examined the effect of human papillomavirus (HPV) on the proinflammatory IL-18 cytokine pathway in head and neck cancers. I investigated the expression and methylation of genes associated with this pathway using The Cancer Genome Atlas (TCGA) data. In HPV+ cancers, *IL18*, *CASP1*, and *AIM2* were downregulated, while *IL18BP* was upregulated compared to HPV- cancers and adjacent non-cancerous tissues, and *IL18's* promoter was significantly more methylated. I compared HPV+ and HPV- head and neck cancer cell lines for expression of RNA and protein levels of IL-18 and IL-18BP by qPCR, western blot, and ELISA. IL-18 mRNA and protein levels were lower in HPV+ cells, while IL-18BP mRNA was higher. The E7 protein from most HPV types repressed an *IL18* luciferase reporter and this required conserved region 3 of HPV16. Repression of IL-18 mediated inflammation may help HPV evade the immune system and contribute to chronic infection.

Keywords

Human papillomavirus (HPV), epigenetics, gene expression, methylation, head and neck cancer, bioinformatics, The Cancer Genome Atlas, interleukin-18, interleukin-18 binding protein, inflammation, immunology, virology

Summary for Lay Audience

Human papillomaviruses (HPV) are an expanding cause of cancer in the global theater of human disease and are responsible for ~5% of cancers world-wide. HPV is the root cause of practically all cervical cancers, a large number of anogenital cancers, and at the focus of this thesis, a rising number of head and neck cancers. These HPV+ cancers are biologically unique when compared to their HPV- counterparts. They express the viral HPV cancer causing genes early gene 6 (E6) and early gene 7 (E7) integrated into their own genome. These viral genes encode proteins that disrupt our cell's ability to control their replication, repress their ability to die in response to cancerous growth, and signal the immune system that they are infected. This allows HPV infected cells to divide uncontrollably and avoid the immune system. The avoidance of the immune system is important to HPV's ability to cause cancer, as the immune system normally destroys cells within a state of uncontrolled replication. We identified and began to study an additional way by which HPV avoids the immune system, through the suppression of the interleukin-18 (IL-18) inflammatory pathway. IL-18 is an important component of the immune system within our skin, and functions by activating immune cells with anti-viral and anti-cancer properties. As HPV infects skin cells, avoiding the IL-18 proinflammatory response is an important barrier to surpass and may contribute to the ability of this virus to establish and maintain infection. In this thesis, I explore the impact of HPV on expression of IL-18, components of the protein complexes that activate it, and its suppressor IL-18 binding protein (IL-18BP) in head and neck cancers. I found a consistent and targeted downregulation of the IL-18 pathway, which I attributed to HPV E7. Suppression of this inflammatory pathway has significant implications on the establishment and maintenance of infection by HPV and could contribute to the ability of HPV to cause head and neck cancer.

Co-Authorship Statement

The experiments found within this thesis were designed by Dr. Joseph Mymryk, Dr. Steven Gameiro, and Wyatt Anderson, with guidance and mentorship from Tanner Tessier and Martin Prusinkiewicz. All experiments were conducted by Wyatt Anderson with the exception of the primary tumour processing, data collection and normalization analysis that was done by the National Cancer Institute and collected within the Cancer Genome Atlas Program database. The UWO series of cell lines were generated and generously lent to me from the laboratory of Dr. Nichols by Dr. John Barrett. In addition, several plasmids used in this thesis were constructed by Drs. Greg Fonseca and Biljana Todorovic. The following analysis and writing was conducted by Wyatt Anderson, with revisions and edits by Dr. Joseph Mymryk and Dr. Gregory Dekaban.

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List of Abbreviations

AIM2	HIN-200 absent in melanoma protein 2
ASC	Apoptosis-associated speck-like protein containing CARD
bp	Base pairs
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CCNA1	Cyclin A1
CDC	Center for Disease Control
CpG- 5’	Cytosine-phosphate-Guanine
CR1	Conserved region 1
CR2	Conserved region 2
CR3	Conserved region 3
CXCL14	Cystine-X-Cystine Motif Chemokine Ligand 14
DAMP	Danger associated molecular pattern
DMEM	Dulbecco’s Modified Eagle Medium
DMEM/F12	Dulbecco’s Modified Eagle Medium/Hams nutrient mixture F12
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
dsDNA	Double stranded DNA
E1	Early protein 1

E2	Early protein 2
E4	Early protein 4
E5	Early protein 5
E6	Early protein 6
E6-AP	Early protein 6 adaptor protein
E7	Early protein 7
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
HBV	Hepatitis B
HCV	Hepatitis C
HLA	Human leukocyte antigen
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HRP	Horseradish peroxidase
HTLV-1	Human adult T-cell leukemia
ICE	Interlukein-1β converting enzyme

IFNγ	Interferon gamma
IL-1β	Interleukin-1 beta
IL-18	Interleukin-18
IL-18BP	Interleukin-18 binding protein
IL-18R	IL-18 receptor
IL-33	Interleukin-33
IL-36	Interleukin-36
IL-37	Interleukin-37
IU	International units
KSHV	Kaposi's sarcoma associated herpesvirus
L1	Late protein 1
L2	Late protein 2
LCR	Long control region
mRNA	Messenger ribonucleic acid
MHC	Major histocompatibility complex
NF-κB	Nuclear factor kappa light-chain-enhancer of activated B cells
NHGRI	National Human Genome Research Institute
NCI	National Cancer Institute
NK-cell	Natural killer cell
NLRP	Nod-like receptor protein

PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRR	Pathogen recognition receptor
PV	*Papillomaviridae*
pRb	Retinoblastoma protein
PYCARD	PYD and CARD domain containing
PYD	Pyrin domain
qPCR	Quantitative polymerase chain reaction
RCF	Relative centrifugal force
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSEM	RNA-seq by expectation maximization
TCGA	The Cancer Genome Atlas
TNF	Tumor necrosis factor
TGF-B	Tissue growth factor beta
VEGF-D	Vascular endothelial cell growth factor-D
VRL	Victoria Research Labs
WHO	World Health Organization

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Prelude

In regard to viruses, wherever life can be found viruses can be found too. From the atmosphere to the depths of the sea, viruses are as abundant as they are diverse and infect all kinds of life. Structurally, viruses are bundled packages of either DNA or RNA, tightly packed within a series of repeating and interlocking proteins called the viral capsid. This capsid too can be enveloped within a host derived phospholipid membrane. Viruses are obligate intracellular parasites. This means that they require the biochemical machinery of the cells they infect to replicate themselves and transcribe their genetic material into mRNA and translate those into proteins (Krupovic M., *et al* 2019). Due to their lack of endogenous metabolism and ability to replicate themselves, it is a topic of debate whether viruses are actually living entities or just bundled packages of genetic material (Wessner D. R. 2010).

Considering the state of the world in 2020 and the current global pandemic of the SARS-CoV2 Coronavirus, the importance of understanding and studying viruses has never been clearer. Upon infection of a host, viruses can cause a variety of diseases and disorders by disrupting biological homeostasis and activating or suppressing the immune system (Woolhouse M. *et al* 2012). This hijacking of host endogenous functions by viruses to promote replication and infection can have a wide variety of negative effects, producing symptoms that may cause both immediate and long-lasting harm (Speck S. H. and Ganem D. 2011). These symptoms encompass a wide variety of illnesses, including excessive inflammation, the breakdown of infected tissue, and the abnormal replication of host cells. A subset of viral infections can induce oncogenesis in their infected hosts and are aptly named tumour viruses (Table 1.1).

According to the World Health Organization (WHO), roughly 12-20% of the global cancer burden is derived from viral infections (Mersi E. A. *et al* 2015). This is typically attributed to the virus's ability to alter host gene function and protein expression. Specifically, in order to create an environment ideal for viral infection and cellular replication, these viruses repress host inflammation and anti-tumour genes, while promoting cellular proliferation. One such example of these viruses is the Human Papilloma Virus (HPV). The focus of this thesis is to identify and investigate interactions of HPV with its host cells that contribute to head and neck cancer. Specifically, I am examining the effects of the HPV oncogenes on the IL-18 pathway, and how this interaction contributes to the etiology of HPV-derived head and neck cancer.

Chapter 1

1 HPV

1.1 Introduction to HPV

HPV is the etiological agent of what are colloquially known as warts or papillomas. According to the Center for Disease Control (CDC), HPV is one of the most common sexually transmitted diseases on earth. It is estimated that nearly 80% of sexually active adults will contract this virus at some point within their lifetime (CDC, 2017). This is not a new phenomenon however, as HPV and the subsequent cutaneous warts they can induce have been recorded since the times of ancient Rome (Celcus A. C. 47AD). The etiological agent of these warts had eluded researchers for centuries, being thought to have been caused by anything from close contact with farm animals, to religious punishment for masturbatory behaviors (Burns D. A. 1992). It was not until the experiments of Dr. Giuseppe Ciuffo in 1907 that it was hypothesized to be an infectious agent, most likely a virus, that was inducing these cutaneous lesions. He explored this theory experimentally by taking the lysate of a wart that had been passed through a Berkfeld filter, a filter fine enough to remove both bacterial and fungal pathogens and inoculating his own hand with the lysate. Subsequently, at the site of inoculation warts developed within the following weeks (Ciuffo G. 1907). The viral cause was later supported in the year 1949 where the crystal structure of virus like particles were observed in processed wart tissue samples (Strauss *et al* 1949). As time passed and these lesions were investigated and researched, they were eventually designated as a group of viruses called the papova viruses, and this was later changed to the papillomaviruses of *Papillomaviridae* (PV) family (Strauss *et al* 1949, Melnick J. L. 1962, Bravo I. C. and Félez-Sánchez M. 2015).

This research and identification of the PV viruses cumulated in one of the most important scientific discoveries of the century within both the fields of virology and oncology. In 1983, Dr. Harald zur Hausen identified HPV-16 and 18 viral genomes within the genome of carcinomas of cervical cancer patients, as well as cervical cell lines. He was able to

Table 1.1: Currently described oncogenic tumour viruses associated with human cancers

This table encompasses the viruses in which have been confirmed to cause human cancers. Virally derived cancers account for approximately 12-20% of cancers worldwide through the alteration of host endogenous gene expression and function.

Adapted from Brubaker J. *American Society for Microbiology* 2019

Virus Family	**Human Tumours**
Hepatitis B (HBV)	Hepatocellular carcinomas. The integration of HBV into the host genome and an overexpression HBV surface antigen in the endoplasmic reticulum causing hepatocellular damage through reactive oxygen species (ROS) and decrease apoptosis initiated by p53, Fas, Tumor Necrosis Factor (TNF) and Tissue Growth Factor Beta (TGF-B). The hepatitis family of viruses cause roughly 5% of worldwide cancers
Hepatitis C (HCV)	Hepatocellular carcinomas. Unlike HBV, HCV is unable to integrate into hepatocytes genome. Instead they remain latent within hepatocytes causing irreversible liver fibrosis, genetic, and epigenetic changes, of anti-tumour genes like p53, PIKCA, and beta-catenin
Epstein-Barr Virus (EBV)	Burkitt's Lymphoma, Hodgkin's Disease, nasopharyngeal carcinoma, stomach adenocarcinoma. EBV dysregulates the expression of the *myc* proto-oncogene causing alteration in cell cycle, apoptosis, and cellular transformation. Causes roughly 1% of the world-wide cancer burden.
Merkel Cell Polyomavirus	Merkel cell carcinoma. This is a skin cancer of neuroendocrine origin associated with infection by this polyomavirus and is most commonly found within sun-exposed individuals and the immunocompromised.
Kaposi's Sarcoma Associated Herpesviruses (KSHV)	Kaposi's sarcoma, primary effusion lymphomas, and multicentric Castleman's disease. This is the most common cancer in untreated HIV patients. KSHV in immunocompromised patients results in dysregulation of cell proliferation and metabolism in endothelial cells, resulting in roughly 1% of the worldwide cancer burden.
Human Adult T-cell Leukemia Virus Type-1 (HTLV-1)	T-cell leukemia. HTLV-1 is a retrovirus that infects T cells, and infection leads to adult-T-cell leukemia in roughly 2-5% of infected individuals.
Human Papilloma Viruses (HPV)	Cervical/Anogenital/Head and Neck Squamous cell carcinomas. Carcinomas of the infected mucosa through integration of oncogenic viral genes into the host genome, dysregulating apoptosis through degradation of p53 and the pRb family of cell cycle regulators. Causes roughly 5% of the worldwide cancer burden.

draw the connection between the virus and its ability to cause these abnormal growths of epithelial cells into papillomas and the eventual development of cancer (zur Hausen H., *et al* 1983). This groundbreaking research established that HPV infections are not only medically significant, but that human viruses can induce oncogeneses in their host cells. This earned Dr. zur Hausen the Nobel prize in medicine in 2008.

Following the revelation of HPV's association with cervical cancer, the need for a vaccination against this infection became of interest for the health of the general population. This push for an effective and safe HPV vaccine eventually came to fruition in 2006, as a prophylactic vaccine entitled Gardasil became commercially available from Merck & Co. pharmaceuticals. This vaccine is based on the major capsid protein of HPVs viral protein shell, which can self-assemble into a virus-like particle capable of initiating an adaptive immune response. This promotes the development of neutralizing antibodies, allowing for the sequestration and removal of viral particles upon infectious exposure, resulting in a lasting immunological response (Johnson A. L. 2007).

1.1.1 HPV Biology

HPV is a member of the *Papillomaviridae* family of viruses. These viruses are notorious for their ability to cause both benign and malignant growths of epithelial cells across the animal kingdom, with PV species infecting not only mammals, but birds, fish, and reptiles as well (Van Doorslaer K *et al.* 2018). These viruses are highly species specific. As such, HPV can only productively infect the epithelia of humans. As it currently stands, there are well over 200 different HPV types.

HPV is a non-enveloped, double-stranded circular DNA tumour virus with a genome of approximately 8000 nucleotides, with 8 described genes. These includes HPV early genes E1, E2, E4, E5, E6, and E7, as well as late genes L1 and L2 (Figure 1.1). This genome is packaged within an icosahedral capsid consisting of 72 protein pentamers consisting of HPVs major and minor capsid proteins, L1 and L2 respectively (Figure 1.1). This results in a 55nm diameter viral infectious particle (DiGiuseppe S. *et al* 2017). Despite sharing these common traits across the many different types of HPV, these viruses are roughly divided into five separate categories based on a variety of factors, including the tissues they

infect, the type of lesion they produce, their ability to immortalize cells, and histological presentation. These categories of HPVs include the Alpha-viruses, Beta-viruses, Gamma-viruses, Mu-viruses, and Nu-viruses (de Villers *et al* 2004). The topic of this thesis will encompass the activities and oncogenic properties of the HPV Alpha-viruses.

The alpha-HPVs infect the mucosal tissues of humans and induce abnormal, continuous replication of epithelial cells (Gheit T. 2019). The alpha-HPVs are of interest and scientific importance as they contain what are considered the high-risk and low risk HPVs. The high-risk HPVs infect only the mucosal epithelia of the cervix, the head and neck, and the anogenital track and initially present as a papilloma of the mucosa. These papilloma start out as benign lesions, but should the host fail to clear the viral infection, the lesion may progress to malignancy (Shanmugasundaram S. and You J. 2017). The currently identified high-risk HPVs are HPV-16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, and 82 (Munoz N. *et al* 2003, Burd E. M. 2003, Sunderström *et al* 2015). The low risk alpha-HPVs are mucosal HPVs that typically cause benign lesions and warts, although these infrequently progress to carcinomas. The low risk alpha-HPVs encompass HPV 6, 11, 42, 43, 70, and 90 (Burd E. M. 2003, Sunderström *et al* 2015)

1.1.2 HPV Viral Entry

The infectious cycle of HPV is a complex dance of gene expression, where the translation of their 8 genes is carefully orchestrated in a manner that allows the virus to remain undetected, yet still accomplish its goals of infection and progeny production. This process begins when the virus is introduced to the epithelia through contact with virus present in shed infected epithelia. Here, the virus will descend through the stratified epithelia infect the lowest defined layer, the basal epithelium. The virus passes through these upper cornified layers of the epithelium through micro abrasions, which are small cuts and tears within both mucosal and cutaneous epithelium that occur through the regular wear and tear of daily life (Figure 1.2)(Stanley M. *et al* 2010). Once within the basal layer of the epithelium, HPV virions now enter the cell. The L1 virion protein binds membrane-bound cellular receptors the heparin sulphate proteoglycans (Schiller J. T. *et al* 2010, Raff A. B. *et al* 2013). This induces the endocytosis of the virion.

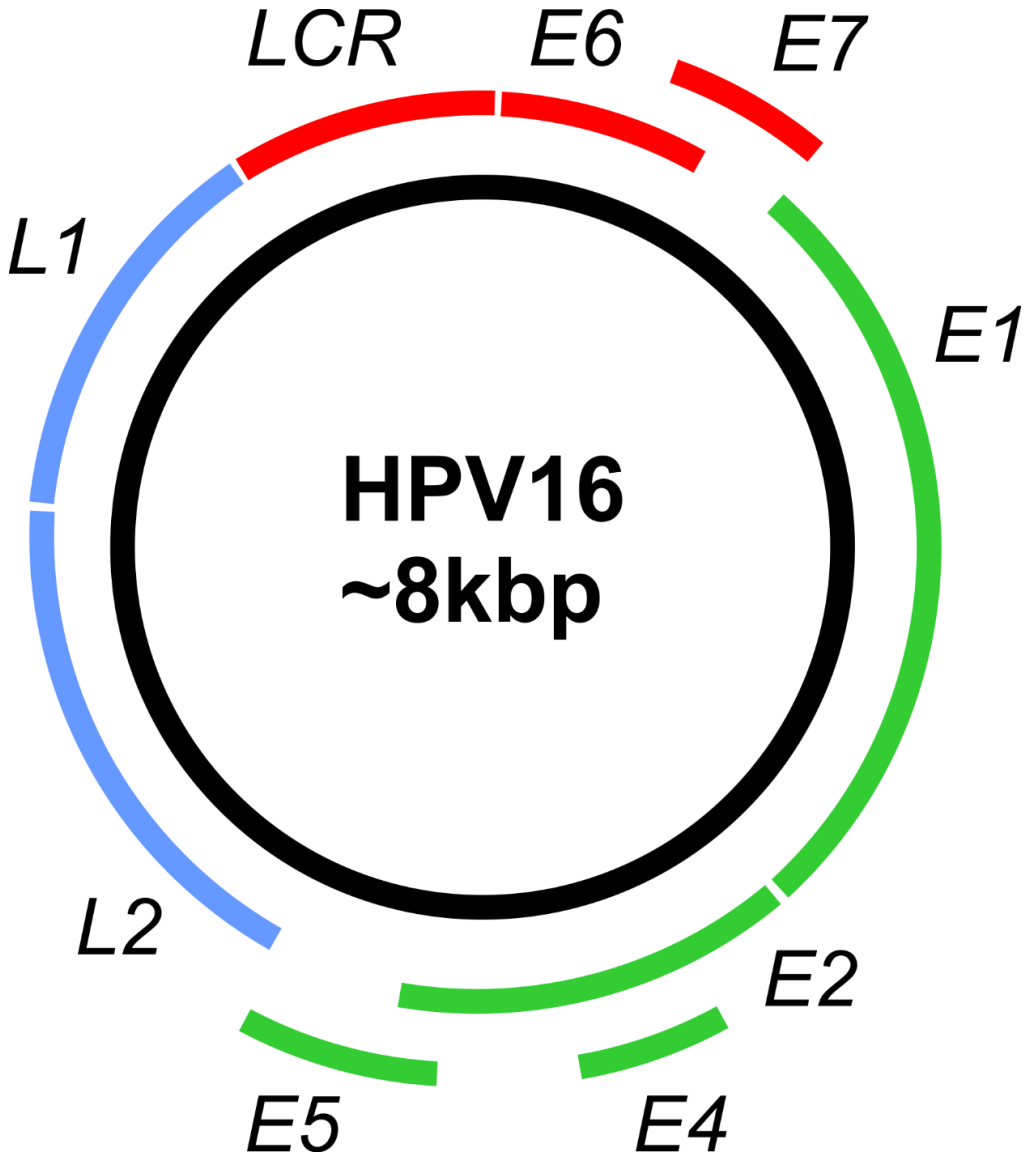


Figure 1.1: The genomic organization of HPV-16's genes

HPV-16 has a roughly 8000bp long double-stranded DNA genome which contains 8 genes. The genome is divided into three regions. The early region, where the E1-E7 genes reside, are expressed during the initial infection. The late region, where the capsid proteins L1 and L2 reside, are only expressed in terminally differentiated keratinocytes. The third region is the regulatory region, called the long control region (LCR), which contains the viral origin of replication and transcriptional control signals for early and late gene expression.

Upon maturation of the endosome and transport via the trans-Golgi network, the viral particle arrives at the nucleus through the endoplasmic reticulum. The L2 protein facilitates the disassembly of the viral capsid and the release of the viral DNA from the endosome to the nucleus upon cellular replication and disassembly of the nuclear membrane. The HPV genome is subsequently maintained extra-chromosomally, as an episome (DiGiussipe S. *et al* 2014/2016, Aydin I. *et al* 2014).

1.1.3 E1 and E2

Once the virus has established itself in the nucleus, its next requirement is to replicate its own genome and to begin expressing the viral early genes. The first viral proteins expressed upon infection are the early genes E1 and E2, which direct and facilitate the replication and expression of the rest of HPVs genes. E1 is the only protein within the HPV genome with a conserved enzymatic activity and its main function is to initiate and regulate the replication of the HPV genome (Bream G. L. *et al* 1993). E1 initially binds to the HPVs genome origin of replication within the LCR, and acts as an ATPase, as well as a DNA helicase. Once bound to the viral DNA, E1 forms a hexamer and initiates elongation of viral DNA using the cellular DNA replication apparatus. This binding efficiency is weak, however, and is amplified with the assistance of E2 (Castro-Muñoz L. J. *et al* 2019).

E2 is a regulatory protein that modulates the expression and replication of HPVs genes. E2 is both a transcriptional repressor, or a transcriptional activator, depending on where in the genome it associates and what it is bound to (Spalholz B. A. *et al* 1985). The E1-DNA complex has a weak affinity to the origin of replication on the HPV genome, an affinity that is greatly increased when E2 is present. This protein complex acts to regulate the recruitment of DNA replication machinery as well as translational machinery. This E1-E2 complex allows for a controlled replication of not only the HPV genome, but also a controlled, restrained expression of HPVs oncogenes depending on the differentiation status of the host cell (Hedge R. S. 2002). Typically, within an HPV infection, there are fewer than 100 copies of the genome and low expression of HPVs other early proteins, E6 and E7, within the basal layer of the epithelium. This repression can be attributed to the inhibitory effects of E2 (Maglennon G. A. *et al.* 2012). Despite the low level of

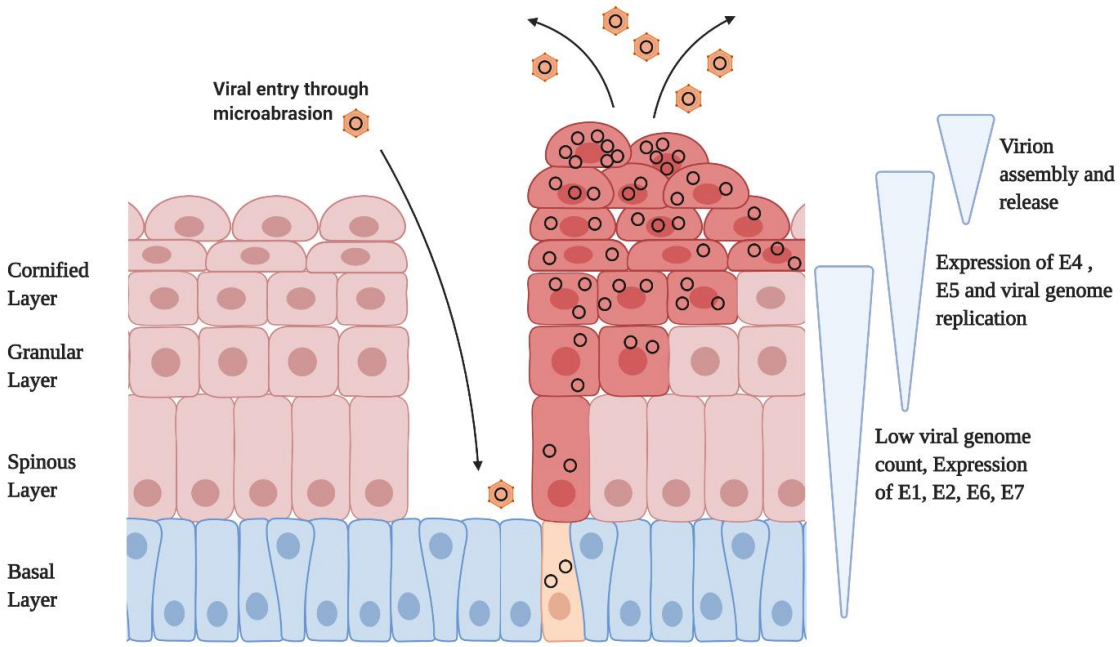


Figure 1.2: The lifecycle of HPV

The HPV virion enters the host through micro-abrasions to infect the cells comprising the basal layer of the epithelium. Here, the viral genome is maintained at a low frequency with low expression of viral early genes. The basal cells then replicate, leaving one infected daughter cell in the basal layer, as one infected daughter cell rises through the epithelium. Typically, healthy cells differentiate and stop replicating as they move upwards through the skin. These differentiated cells continue to replicate through a dysregulation of the cell cycle mediated by the viral early proteins. As the cells rise further through the epithelium layer, the late genes begin to be expressed coincident with terminal differentiation and the viral genome is amplified. Here at the cornified layer of the epithelium, dead, virus containing cells are shed into the environment continuing the cycle of viral dissemination and infection. (Figure adapted from C. A. Moody and L. A. Laimins 2010)

expression of its early genes, HPV is able to replicate its genome using both virus-derived proteins and host-derived proteins, and is able to maintain a low antigenic presence within the basal layer of the epithelium that helps evade the immune system, typically for months or even years (Peh W. L. *et al* 2002).

1.1.4 E4 and E5

Two of HPVs least understood proteins, E4 and E5 are expressed during the differentiation and replication of keratinocytes. HPV E4 is synthesized as a fusion protein with a portion of E1, represented as E1^E4, as a result of RNA splicing, and sequence overlap with the open reading frame (ORF) of E1 (Wang *et al* 2011). Despite being in the series of early proteins it is suspected that the function of E4 falls within the later stages of viral propagation as it is nearly undetectable in early viral infection (Doorbar *et al* 2013). While its function is currently undescribed, it is suspected to function as a component of viral release through the binding of keratin (Brown *et al* 2006).

HPV E5 has been gaining interest in the scientific community in recent years. While E5's functional properties are yet to be fully understood, it is now considered one of HPVs oncogenes. Although its function is not necessary for HPV infection and oncogenesis, E5 has been shown to augment the transformative properties of E6 and E7 (Maufort J. P. *et al* 2010). E5 expression delays differentiation of the infected epithelial cell, assisting in the maintenance of cell cycle progression (Muller M. *et al* 2015). E5 lacks any currently described enzymatic activity, and it accumulates within the Golgi apparatus, the endoplasmic reticulum, and the plasma membrane. E5 appears to be a transmembrane protein and contains three close proximity hydrophobic regions with α-helical structures similar to other transmembrane proteins (Conrad M. *et al* 1993, Krawczyk E. *et al* 2011). Through its association with these membranes, E5 is suspected to function as a viroporin. It functions by promoting cell cycle progression through interaction with the epidermal growth factor receptors pathway at the membrane and through blocking acidification of the endocytic vacuole (Pim D. *et al* 1992, Schrevel M. *et al* 2011).

1.1.5 HPV Transforming Protein E6

Despite there being currently three described HPV oncogenes, only two of them are consistently retained within HPV induced cancers (Münger K. *et al* 2004). These proteins are HPVs transforming E6 and the E7 oncoproteins. E6 protein is roughly 150 amino acids (aa) long, containing two zinc binding domains within its structure. These domains are crucial for HPVs survival and ability to transform cells (Martinez-Zapien D. *et al* 2016). This is due to its ability to inhibit pro-apoptotic and anti-tumour protein, p53 (Pol A. B. V. and Klingelhutz A. J. 2013). The function of p53 is one of the largest hurdles any tumour virus or cancer has to overcome, due to its ability to induce apoptosis within cells where there is irreparable DNA damage/mutation, oncogene expression, cellular stress like hypoxia found within tumour environments, and most importantly, inappropriate DNA and cellular replication (Zilfou J. T. and Lowe S. W. 2009).

To avoid the function of this pro-apoptosis gene, E6 binds and degrades p53. It does this through associating with and co-opting the function of the cellular E6-associated protein (E6-AP). E6 and E6-AP form a heterodimer, which binds p53 to form a heterotrimer (Scheffner M. *et al* 1993). E6 and E6-AP function as a ubiquitin-protein ligase and they ubiquitinate p53, tagging it for proteolytic degradation through the proteasome (Figure 1.3) (Garnett T. O. and Duerksen-Hughes 2006). E6 has also been reported to prevent p53 independent apoptosis through the binding and inhibition of several other proapoptotic proteins, including Bak, c-Myc, and TNF receptor 1 (Thomas M. and Banks L. 1998, Gross-Mesilaty S. *et al* 1998, Filippova M. *et al* 2002).

1.1.6 The HPV E7 Oncogene

E7 is a roughly 100aa long protein and is a highly conserved cancer-causing gene across the different types of HPV. As the function of E7 is predominantly nuclear, it is found mostly in the nucleus of infected cells (Smotkin D. and Wettstein F. O. 1987). The E7 100aa are divided into a series of conserved regions (CR), CR1, CR2, and CR3. CR1 of E7 spans the first 15aa of the protein, CR2 encompasses residues 16-38, and CR3 has the lion's share domains with significant sequence homology to the human adenovirus (hAdV) early

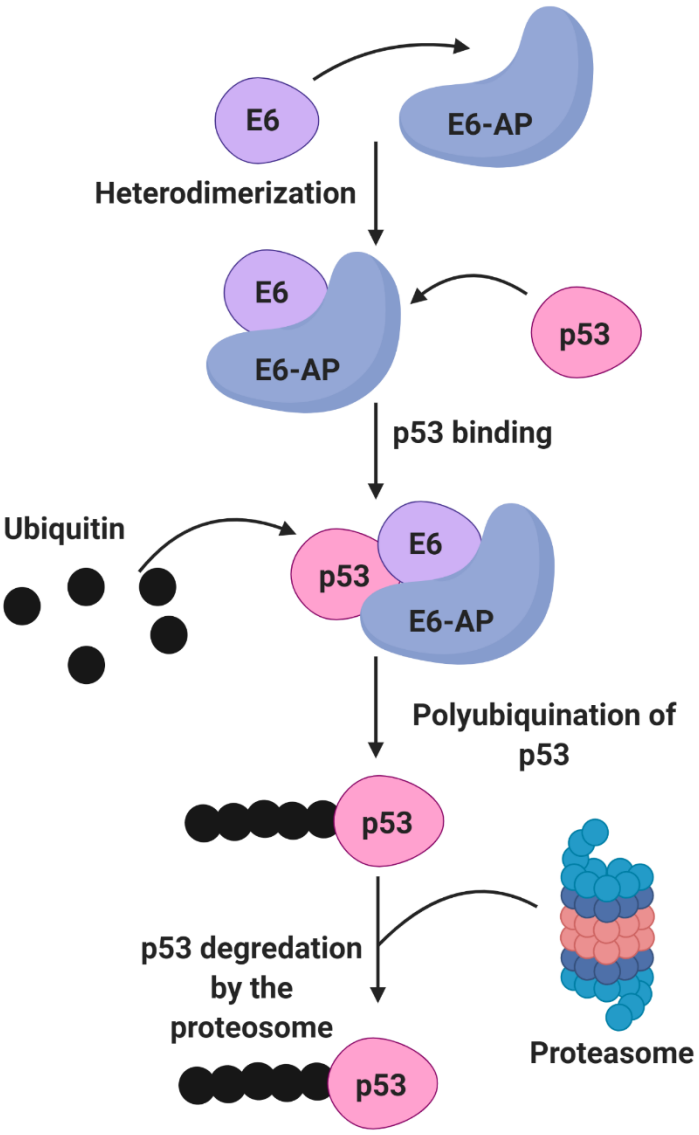


Figure 1.3: The activity of HPV-16 E6

The activity of E6 as represented through a stepwise diagram of the assembly of the ubiquitin ligase complex. E6 initially binds the E6-AP resulting in a heterodimer that complexes with p53. Ubiquitin is then ligated to p53, marking it for proteolytic destruction by the proteasome.

protein 1A (E1A) (Phelps W. C. *et al* 1998). E7 CR2 contains a LxCxE motif (where x represents any amino acid) which is responsible for E7s high affinity interaction with the retinoblastoma family of tumour suppressors (pRb, p107 and p130) (Barbosa M. S. *et al* 1990, Münger K. *et al* 1989). E7s carboxyl terminal CR3 contains a zinc-binding domain, comprised of two pairs of cysteines separated by roughly 30aa. These two domains are essential for protein stability and function and have been implicated in E7s ability to regulate transcription (Todorovic B. *et al* 2011, Songcock W. K. *et al* 2017).

E7 is utilized to keep the cells within a state of constant cell cycling through the dysregulation of the host cell cycle G1 checkpoints. E7 does this through the exploitation of the E2F transcription factors and inhibition of their negative regulators pRb, p107 and p130 (Chan Y. K. and Gack M. U. 2016). The pRb family of proteins function as negative regulatory proteins that sequester the E2F transcription factors, preventing them from inducing transcription of their target genes. E2F transcription factors are the regulators of expression for genes involved in controlling cell cycle progression from the G1 phase of cellular growth to the S-phase of DNA replication (Johnson D. G. and Schneider-Broussard R. 1998). This is where the synergistic effects of E6 are important, as the dysregulation of the cell cycle is one signal that potently activates p53. High-risk HPV are especially efficient in this deregulation of the pRb family of proteins by destabilization via ubiquitination and subsequent proteasome degradation instead of just binding and inhibiting pRb through steric hindrance (Figure 1.4) (Todorovic B. *et al* 2012, Oh K. J. *et al* 2010).

E7 also has pro-HPV replication activity within these epithelial cells through altering the methylation of gene promoters. E7 CR3 has been shown to facilitate the binding and activation of host cell DNA methyltransferase 1 (DMNT1) (Burgers W. A. *et al* 2007, Songcock W. K. *et al* 2017). While the exact mechanism in which binding effects gene methylation is poorly understood, it has been implicated in the altered methylation of several gene promoters that interfere with the function and infection of HPV. These include genes encoding cell cycle regulators and proinflammatory cytokines. For example, knockdown of E7 using siRNA in HPV positive cells significantly reduced promoter methylation of Cyclin A1's promoter (CCNA1), an important regulator of the G1-S-phase

cell cycle checkpoint (Chalerpet K. *et al.* 2015). The proinflammatory chemokine CXCL14, which promotes immune cell migration (Cicchini L. *et al* 2016), and Human Leukocyte Antigen E (HLA-E/MHC-I) a regulator of NK cell and CD8+ cell interaction (Cicchini L. *et al* 2017) have both been shown to have hypermethylated promoters in HPV derived cervical cancers.

1.1.7 L1 and L2

Once the host cell reaches the upper layers of the epithelium, terminal differentiation has occurred, and this initiates the production of HPV late proteins. These consist of the L1 and L2. The major capsid protein L1, once translated, spontaneously self-assembles without the need of chaperone or scaffold proteins into a facsimile of the final virion (Schiller and Lowy 2012). The purification of recombinant L1 protein is the basis for current HPV vaccines, as even *in vitro* the purified L1 protein can self-assemble into virus-like particles (VLP) (Buck C. B. *et al.* 2013). The minor capsid protein L2, however, is vital to produce mature virions. L2 associates with HPVs genome and the L1 major capsid proteins, facilitating encapsulation of the viral genome. These two proteins working in tandem are required for the final production of mature virions, ready to be released and infect anew (Wang J. W., Roden R. B. S. 2013).

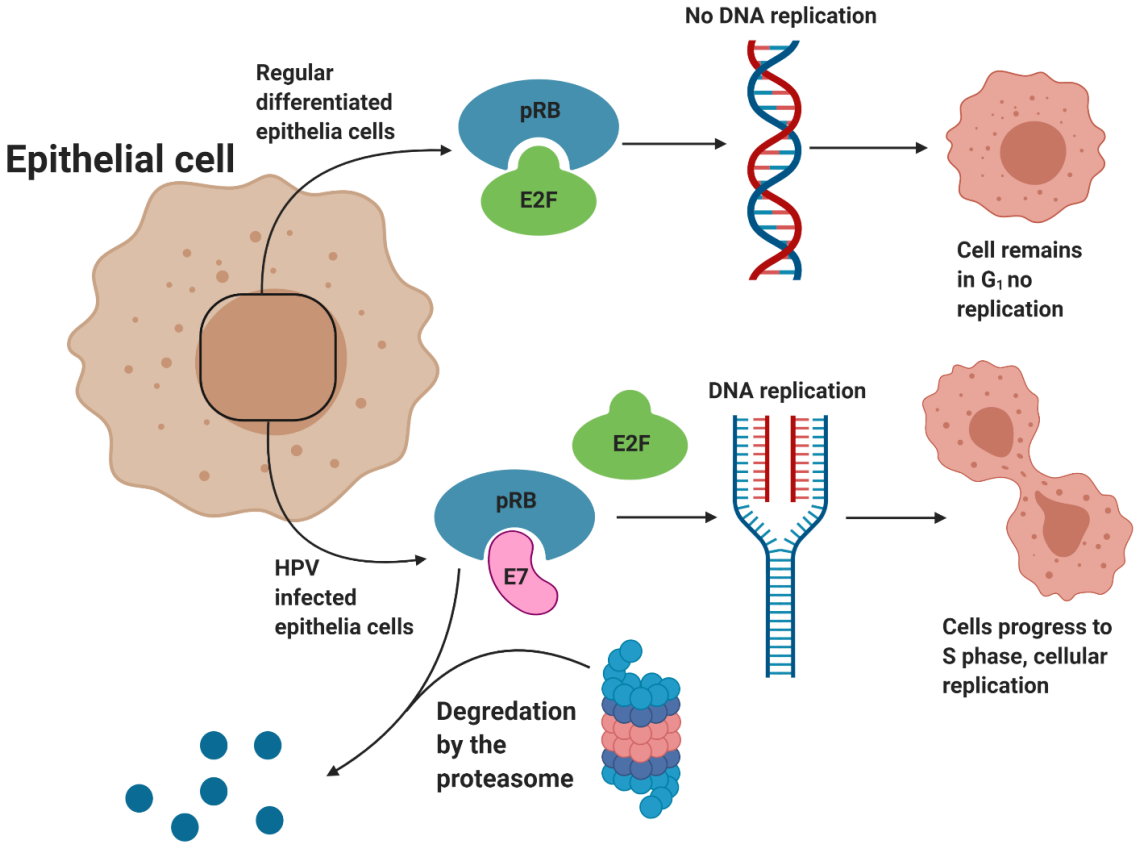


Figure 1.4: The activity of HPV-16 E7 on the cell cycle

E7 functions through binding the pRb family of proteins, blocking them from binding and inhibiting their target the E2F transcription factors. In high-risk HPV like HPV-16, this repression is further mediated through the proteolytic destruction of pRb through the proteasome.

1.2 HPV and Cancer

The transformation of HPV infected cells is not the typical end result of infection. In most cases, HPV infections persist for a few months before successful resolution of the infection by the immune system of the host. These infections may be asymptomatic or present with the development of papilloma and warts. HPV-derived cancers are far more infrequent, and are the product of long-term, chronic infections by high-risk HPVs in the mucosa, either from chronic reinfection of the tissue or from the host's inability to clear HPV infection (Shanmugasundaram S. and You J. 2017). These long-term infections may also be asymptomatic, resulting in an insidious infection with few warning signs to the cancer that may lie in the future. Chronic infections by high-risk HPV make up approximately 10-20% of cases of HPV infection (Moreno-Acosta P. *et al* 2020). There are several alleles within the human body that are associated with enhanced susceptibility to these chronic infections, like the phenotype of the infected HLA series of proteins, being of the male sex, and patient ethnicity (Bernal-Silva S. *et al* 2013, Wank R. and Thomssen C. 1991).

These chronic infections can result in a tumultuous downwards spiral of the HPV infected cells, where the continuous expression of viral oncogenes wreak havoc on the host cell. As the cells are forced to continuously replicate, the host genome will begin to acquire DNA damage and accumulate potentially oncogenic mutations. As HPV E6 inhibits apoptosis in response to the DNA damage response, the host cell is unable to die in response to the accumulation of mutation and damage (Garnett T. O. *et al.* 2006). As mutations begin to accumulate in the host cell's genome, genetically unstable regions of host DNA, called common fragile sites, will reach their breaking point and undergo double-stranded DNA breaks (McBride A. A. 2017). It is in these common fragile sites that the HPV viral oncogenes can be found to be integrated into the host genome, a hallmark of HPV derived cancer. With expression of HPVs oncogenes from integrated viral genomes (Jeon S. and Lambert P. F. 1995), and the loss of E2 expression and its repressive effects on E6 and E7 expression (Collins S. I. *et al* 2009), these oncogenes are expressed at much higher levels, driving malignant progression.

In the past decades, HPV has been rising in prevalence as an etiological agent of a variety of cancers. It is currently estimated that HPV is responsible for roughly 5% of human

cancers world-wide and is particularly impactful on populations within developing countries. HPV is perhaps best known for its role in cervical cancers. It has been identified to be nearly universally present within cervical cancers, with the high-risk subtypes HPV-16 and HPV-18 making up to 70% of these cases. According to the Global Cancer Observatory database, a summation of cancer data from 185 countries, there were approximately 600,000 cases of HPV derived cervical cancers and 311,000 deaths annually (Arbyn M., *et al* 2019). It is not only the cervix where HPV sows the seeds of cancer. HPV is also the etiological agent of a large subset of anogenital cancers, as well as a growing subset of cancers of the head and neck (Bansal A. *et al* 2016).

1.2.1 Head and Neck Squamous Cell Carcinomas (HNSCC)

Head and neck squamous cell carcinomas (HNSCC) are cancers that arise within the epithelium within the mucosa of the mouth and throat. They can be divided into 4 subsites, comprised of the oral cavity, hypopharynx, oropharynx, and the larynx (Boyle P and Levin B. 2008, Jemal A. *et al* 2007). Each year there are roughly 600,000 cancer diagnoses of the head and neck, and nearly 300,000 corresponding deaths, making it the sixth most prominent causes of cancer world-wide, and one of the deadliest (Alsahafi E. *et al* 2019). The prognosis of head and neck cancers is typically quite poor, with a five-year survival rate of 40-50% depending on stage of disease, HPV status, and biological subsite (Leemans C. R. *et al* 2011). Cancers of the head and neck are a heterogenous disease, and can be divided into two biochemically distinct subclasses, HPV+ tumours and HPV- tumours.

Typically, cancers of the head and neck are derived from habitually consumed carcinogens like tobacco and excessive drinking, environmental factors like air quality and workplace gaseous hazards, and infection with high-risk HPVs (Shaw R. and Beasley N. 2016). Treatment of cancers of the head and neck ranges from surgical removal, radiation therapy, and/or chemotherapy. Due to the location of these cancers and the current state of medical intervention, there is a sharp reduction in the quality of life of head and neck cancer patients, as treatment can cause discomfort, disfigurement, and loss of function of important components of their head and throat. This ranges from cosmetic damage to the face, to the inhibition of speech and inability to swallow due to damage to the jaw and larynx (Dropkin M. 1989). Thankfully, due to awareness campaigns and initiatives to

reduce smoking, the incidence of HPV- HNSCC has been on the decline (Fakhry C. *et al* 2018).

This decrease has not, however, caused an overall decline of all cancers of the head and neck, as HPV derived HNSCCs have been rising at epidemic rates. As the etiological agent of these cancers begins to change, so too has the demographics of the patients. Patients with HPV+ cancers tend to be younger and healthier, with an overall better prognosis when compared to traditional HPV- HNSCC patients who are older with mutagen derived cancers. In the work of Dr. Adam Burr, he has shown that patient survival with HPV+ head and neck cancers has a median time of 23.8 months, nearly twice the median amount of months as those with HPV- cancers at 11.1 months (Ang K. K. *et al* 2010, Burr A. R., *et al* 2018). While the prognosis is still quite poor, patients with HPV-associated head and neck cancer respond better to radiotherapy and have improved survival (Maxwell J. H. *et al* 2016). While the reasoning behind this improved prognosis and survival is still being investigated, it is understood that these differences in survival are most likely attributed to the younger age of the HPV+ patients, improved health in non-smoking HPV+ cancers, as well as the unique tumour microenvironment associated with HPV+ cancer.

1.2.2 The Cancer Genome Atlas (TCGA)

One of the most powerful research tools in the sphere of bioinformatics are databases. One such database that has been the foundation for countless cancer research projects is the Cancer Genome Atlas (TCGA). This online database represents a massive collection of molecular and clinical data acquired from cancer patient samples all over the world. Cancer patients consented to the use of their tumour and treatment data for scientific research allowing the National Cancer Institute (NCI) and National Human Genome Research Institute (NHGRI) to coalesce the data all within a single database. This resulted in nearly 2.5 petabytes of data, encompassing the information of over 11,000 cancer patients with 33 different types of cancer, all within a single source. This includes genomic, epigenetic, proteomic, transcriptomic, survival data, and clinical data. Importantly, with respect to the analyses undertaken in this thesis, the TCGA includes DNA methylation data and quantitative RNA-seq data (Li B. and Dewey C. N. 2011).

Within this database, there is a subsection of data encompassing a large cohort of head and neck cancer patients. In addition to the analysis of tumour samples, in some cases the TCGA repeated the same analysis they conducted on tumours on non-cancerous adjacent tissues, which serves as a useful control. In addition to this, the HPV status of these head and neck tumours is reported within the database. This results in three distinct subsections of head and neck samples, HPV+, HPV-, and normal adjacent non-cancerous tissues. Since the data was generated via a uniform pipeline and normalized, it allows for quantitative comparison between the three tissue types.

1.3 The Epithelium

To look at the immunogenic environment that HPV must overcome to establish and maintain an infection and identify potential immunological components that can contribute to cancer etiology, I will briefly describe the immune environment of the skin. As described above, high-risk HPV infects keratinocytes of the mucosal epithelia. The epithelium is an important physical and chemical barrier to microbes, including both commensal and pathogenic, and has a robust immunogenic landscape (Duckney P. *et al* 2013). The epithelium is the largest organ within the human body, and typically our first line of defense against pathogens and inflammatory agents in our environment. Due to its status as the first line of defense, our epithelium must be able to both respond quickly to immunological insult through the innate immune system and appropriately activate the adaptive immune system.

The epithelium functions as a physiological barrier to infection, a source of chemical defense, and a place for immune cells to reside. In addition to the integrity of the mucosal epithelium itself, an additional barrier to entry is a difficult to traverse layer of mucus, which is filled with a variety of antimicrobial peptides and molecules, including lysozymes, peroxidases, compliment components, defensins, and neutralizing antibodies like immunoglobulin A (Schleimer R. P. *et al* 2007). Epithelial cells themselves recognize pathogen-associated molecular patterns (PAMPs), which trigger innate immune responses, such as upregulation of inflammatory mediators like type I interferons, and enhanced antigen presentation in the context of Major Histocompatibility Complex Class I (MHC-I)

(Hobbs C. G. L. *et al* 2006). An important cellular mediator of innate immune system involved in barrier maintenance are Natural Killer cells (NK cells). Upon activation, NK cells have anti-bacterial, anti-viral, and anti-tumour effects vital to the maintenance of epithelial health through the secretion of interferon gamma (IFN-γ) and killing of bacterial/virally infected cells (Fuchs A. and Colonna M. 2011).

The adaptive immune system is a potent and highly precise division of the immune system and is pathogen specific. In response to inflammatory stimuli within the epithelium, the recruitment of adaptive immune cells is mediated through the secretion of proinflammatory cytokines and chemokines to induce T-cell differentiation and recruitment of CD4+ helper T-cells (Th1 cells) and cognate cytotoxic CD8+ T-cells. In the epithelium, these cells are vital for MHC-I recognition and the clearance of intracellular pathogens (Sinigaglia F. and D'Ambrosio D. 2000). One cytokine of particular importance in epithelial barrier function, which spans both the activation of the innate and adaptive immune systems in response to viral infection, is Interleukin-18 (IL-18).

1.3.1 An Introduction to IL-18

IL-18 is an interferon inducing factor. It is constitutively produced by epithelial cells and macrophages (Pirhonen *et al* 1999, Sugawara S. *et al.* 2001). IL-18 is a member of the IL-1 superfamily of cytokines. This family of proinflammatory cytokines include the proto-typical IL-1, as well as IL-18, IL-33, and recently IL-36 and IL-37 (Dinarello C. *et al.* 2010). These cytokines are all related through their biological origin, the structure of their receptor, and the pathways they activate (Dinarello C. 2009). IL-18, similarly to IL-1, requires cleavage from its initial pro-form to be rendered biologically active, most commonly through the activity of the protease caspase-1.

Once IL-18 is activated, it is then subsequently secreted to initiate a proinflammatory response from several types of immune cells. The method in which IL-18 is secreted is a topic in which is poorly understood, as the protein lacks a secretory signal sequence (Gardella S. *et al* 2000). It is currently understood that the secretion of IL-18 is increased through the expression of vascular endothelial cell growth factor-D (VEGF-D). As VEGF-D contains a metalloprotease domain, knockdown of the ADAM33 metallopeptidase was

shown to inhibit the secretion of IL-18, indicating importance in the secretion process (Kim K. E. *et al* 2009, Kim K. E. 2007)

Upon secretion, IL-18 activity is suppressed through sequestration by the soluble IL-18 binding protein (IL-18BP). The balance between IL-18 and IL-18BP determines biological response. Once sufficient IL-18 has been secreted to overcome repression by IL-18BP sequestration, it binds to receptors on both innate and adaptive immune cells, including NK cells and T-helper cells respectively (Tomingaga K. *et al* 2000). IL-18 stimulates the production of interferon gamma (IFN-γ) and immune cell activation, resulting in a swift and powerful inflammatory response with a wide range of antiviral, antibacterial, and anti-tumour effects (Okumara *et al* 1998).

1.3.2 IL-18 Activation Through the Inflammasome

As IL-18 is constitutively produced within epithelial cells, the means of activation and sequestration are the main methods of modulating its activity. IL-18 is initially produced as an intracellular 24kDa pro-IL-18 protein, which is biologically inert (Mejias N. H. *et al* 2018). Pro-IL-18 is rendered biologically active through cleavage by caspase-1, which is itself activated by the activity of one of several different types of inflammasomes. Inflammasomes are multiprotein complexes that are constructed of either the NOD-like receptor proteins (NLRPs) or the HIN-200 absent in melanoma protein 2 (AIM2) (Walle L. V. and Lamkanfi M. 2011)

The NRLP inflammasomes are a complex of several proteins with NLRP1, NLRP3, and NLRC4 being vital for its activity, with the NLRP3 inflammasome being directly responsible for caspase-1 activation (Kanneganti T. D. *et al* 2006). In addition to its function as a mediator of apoptosis and pyropotosis, it is also a potent activator of the immune system in response to PAMPs and danger associated molecular patterns (DAMP). PAMPs, as their name suggests, are foreign biological materials that cells can recognize due to their exogenous nature and origin, like the lipopolysaccharide components of the bacterial cell wall, viral DNA within the cytoplasm, double stranded RNA and viral 5’triphosphate RNA (Thompson M. R. *et al* 2011). These PAMPS are recognized through either cytoplasmic or membrane-bound pattern recognition receptors (PRR). Upon the

activation and priming of PRRs in response to PAMPS, NLRP3 transcription is upregulated, transcribed, and assembled (Latz E. *et al* 2013). This also occurs as a response to cellular stressors indicative of either tissue or DNA damage (Kelley N., *et al* 2019). The inflammasome then mediates inflammation through the cleavage of several biological mediators of inflammation, including the activation of caspase-1 (Cerreti D. P. *et al* 1992).

AIM2 is specifically important in regards to the activation of the immune system upon infection with dsDNA viruses like HPV (Riva G. *et al* 2019). AIM2 is a PRR with a HIN domain that senses the presence of dsDNA in the cytoplasm. Normally, in regular somatic, non-dividing cellular environments, host DNA is sequestered within the nucleus. Thus, surveillance and detection of cytoplasmic DNA is indicative of viral infection or severe nuclear damage (Man S. M. *et al* 2015). Unlike other PRRs, which mediate the transcription of NLRP inflammasome constituents, AIM2 binds dsDNA through their HIN domain and subsequently binds with an adaptor protein ASC using their respective PYD protein domains (Hara H. *et al* 2013). These ASCs contain a caspase recruitment domain (CARD) that recruits pro-caspase-1. Using these protein structures as a scaffold, this AIM2-ASC-Caspase-1 complex triggers the cleavage and activation of Caspase-1, developing a catalytically active inflammasome protein complex (Wang B. and Yin Q. 2017).

Caspase-1, previously known as interleukin-1β converting enzyme (ICE), is a member of intracellular cysteine proteases, which are able to cleave a narrow range of proteins in response to its own activation (Nicholson D. W. 1999). As mentioned above, Caspase-1 is initially translated as procaspase-1 and activated through its association with large macromolecule scaffolds within the inflammasome through CARD-CARD (caspase recruitment domains) and PYD-PYD (pyrin domain) protein interaction. After activation, Caspase-1 subsequently cleaves and activates pro-IL-1β and pro-IL-18, to produce the biologically active cytokine. (Franchi L. *et al* 2009).

1.3.3 IL-18 Binding Protein

Before secreted IL-18 can reach its receptor, it must overcome sequestration by IL-18 Binding Protein (IL-18BP). This secreted protein has an exceptionally high affinity for

biologically active IL-18, binding it such that IL-18 is unable to interact with its receptor (Kim S. H. *et al* 2000). IL-18BP is considered a decoy receptor and IL-18 becomes biologically inert when sequestered by IL-18BP (Novick D. *et al* 1999).

IL-18BP, like IL-18, is also constitutively produced and secreted. In healthy humans without inflammatory stimulus, IL-18BP is found at a nearly 20-fold higher concentration in human serum compared to active IL-18 (Novick D. *et al* 2001). This is an important method for keeping Th1-mediated inflammation in check, as even low levels of IL-18 activation have been associated with autoimmune and inflammatory disorders. Thus, the constitutive production of IL-18BP surpassing that of IL-18 is important for maintaining this low inflammatory level in homeostasis in the skin (Novick D. *et al* 2009, 2011).

IL-18BP is also important for terminating the IL-18 inflammatory response post-activation. Indeed, IL-18-mediated inflammation needs to be silenced after triggering the desired response, otherwise this risks excessive and damaging inflammation (Hurgin V. *et al* 2002). IL-18BP transcription and production is significantly increased in response to IFN-γ in non-leukocyte cells, including epithelial cells. As IFN-γ is the key resulting product of IL-18-mediated activation of NK and T-cells, the activation of IL-18BP expression spells the end of the IL-18 inflammatory response (Mühl H. *et al* 2000). This essentially creates a negative feedback loop, where IL-18's suppressor is produced in response to IL-18 activation, allowing IL-18 to have a potent and fast stimulus of inflammation that is short in duration.

1.3.4 IL-18 Activity

Once IL-18 has been activated, it is then subsequently secreted from the inflammasome reactive epithelial cells in the skin. IL-18 then interacts with several different immune cells through the IL-18 receptor (IL-18R). The IL-18R is a heterodimer of two membrane bound proteins, IL-18Rα and β. They share similar homology to the IL-1β receptor, but the IL-18R recognizes a molecular pattern within the second domain of IL-18 that is unique to the cytokine (Tsutsumi N. *et al* 2014). This initiates the intracellular signaling through the MyD88 pathway, eventually resulting in the activation of Nuclear Factor κB (NFκB) and the mitogen-activated pathways of protein kinases (Weinstock J. V. *et al* 2003). NFκB is

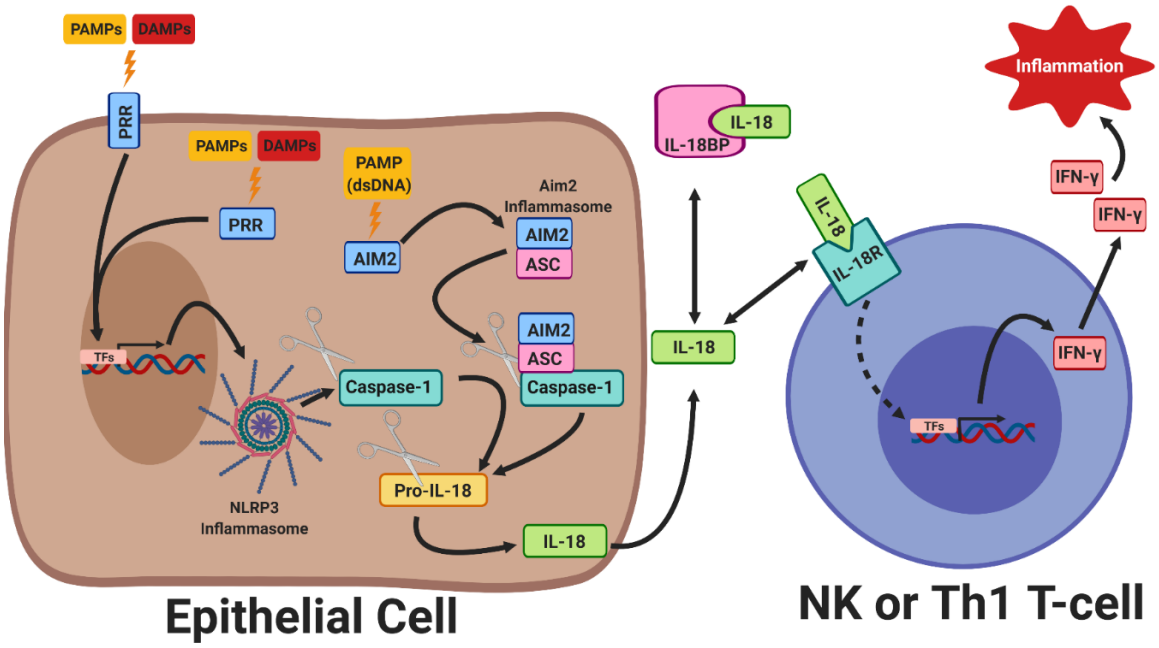


Figure 1.5: A summation of the IL-18 mediated proinflammatory pathway

In the presence of foreign PAMPs or endogenous DAMPs, membrane bound and intracellular PRRs recognize their cognate molecular patterns. Upon recognition, the stimulation of specific transcription factors induces the expression and subsequent formation of the protein mega-complex called the NLRP3 inflammasome, or the AIM2 PRR directly binds to its associated proteins to form the AIM2 inflammasome. The inflammasomes provide the scaffolding for the cleavage of pro-Caspase-1 into its active form. Caspase-1 in turn cleaves pro-IL-18 into its active form in preparation for secretion. Upon secretion, IL-18 must overcome the inhibitory threshold of IL-18BP, which serves as a decoy receptor, to achieve a concentration sufficient for the activation of NK cells and Th1 cells. Upon stimulation of the IL-18R, the NFκB transcription factors induce the production and release of IFN-γ, which contributes to subsequent anti-viral functions.

a transcription factor that regulates the expression of genes in response to an inflammatory stimulus. In NK and T-cells, NFκB activation induces the production of several proinflammatory cytokines, and primarily among these is IFN-γ (Pfeffer L. M. 2011). IL-18 has the distinctive ability to activate components of both the innate and adaptive immune system. Specifically, IL-18's activity is costimulatory with IL-12 in the activation and development of Th1 cells, NK cells, and B-cells (Harandi A. M. *et al* 2001). This is largely due to IL-12's ability to upregulate the production and presentation of the IL-18R on these target immune cells. This enhances their sensitivity to the signaling of active IL-18, assisting in the strong and swift induction of INF-γ (Yoshimoto T. *et al* 1998, Nakanishi K. 2018).

1.3.5 IL18 and Disease

As previously mentioned, IL-18 has a complex relationship with a variety of diseases, including autoimmune diseases, viral infection, and cancer. IL-18 has been implicated as a marker for several inflammatory diseases, including systemic lupus erythematosus and Wegener's granulomatosis. In these diseases, the basal level of active IL-18 in the serum of patients is high enough to overcome the barrier of IL-18BP inhibition, resulting in a constitutive low level of inflammation dangerous to the circulatory system (Novick D. *et al* 2009, Novick D. *et al* 2011).

IL-18 has also been proven to be an important cytokine for inhibiting viruses that infect the epithelium. Indeed, several viruses that specifically infect the epithelium encode their own homolog of IL-18BP in an effort to neutralize the IL-18-mediated inflammatory response and the recruitment of cytotoxic T cells and NK cells. These viruses include *Molluscum contagiosum*, a common infection of the skin (Xiang Y. *et al* 2001), as well as one of the most devastating family of epithelial viruses known to man, the variola viruses, including the smallpox virus (Xiang Y *et al* 2007). HPV, another epithelial tropic virus, does not encode a homolog for IL-18BP, but instead upregulates the expression of host cell encoded IL-18BP. In a paper by the MacDonald laboratory of Leeds University, keratinocytes expressing HPV E6 and E7 displayed a pronounced increase in IL-18BP production and secretion as a means of IL-18 sequestration. This paper directly identified

the suppression of IL-18-mediated inflammation as a new immune evasion mechanism of HPV (Richards K. H. *et al* 2014).

Cancer also exhibits sensitivity to IL-18-mediated inflammation. In many cases, cancers alter the activity of the cytokine by increasing the expression of IL-18BP and/or decreasing the expression of IL-18. For example, IL-18BP has been found at elevated levels within human ovarian epithelial cancers and has been hypothesized to represent an immunotherapeutic target. (Carbotti G. *et al.* 2013). IL-18 has been suggested to exert its anti-tumour effects through the activation of NK cells and cytotoxic T cells. These two immune cell types have potent anti-tumour activity through the recognition and clearance of cells showing signs of stress or uncontrolled replication. The activation of these cells is important in the modulation of cancer treatment and destruction of precancerous cells, as well as in cancer immunotherapy (Hu W. *et al* 2019, Farhood B. *et al* 2018). However, IL-18's relationship with cancer is significantly more complicated. The expression of IL-18 at high concentrations in cancer over a longer period was significantly correlated with poorer prognosis in a variety of cancers. This has been linked to an increase in myeloid-derived suppressor cell differentiation and activity, which suppresses inflammation (Nakamura K. *et al.* 2018, Tangkijvanich P. *et al* 2007).

1.4 Overarching Hypothesis and Objectives

HPV infects the epithelium and avoids the immune system in order to establish and sustain an infection. This results in chronic long-term infections that may last from months to even years (Ramanakumar A. V. *et al* 2016). It is through these chronic infections that HPV can drive the neoplastic transformation of cells in which they infect, resulting in cancers of the mucosa, including those of the head and neck. I hypothesized that HPV's suppression of the proinflammatory activity of IL-18 not only contributes to viral evasion of the immune system within the epithelium, but also contributes to maintenance of chronic HPV infections that lead to HPV derived cancers.

In pursuit of my thesis's overarching hypothesis, I undertook several different lines of investigation. I explored the RNA and DNA methylation data within the TCGA, explored the endogenous properties of HNSCC cell lines derived from both HPV+ and HPV-

cancers, and performed biochemical assays exploring the effects of HPV's major oncoproteins on IL-18 expression. These objectives can be summarized as:

1. Utilize the RNA-seq and DNA methylation data within the TCGA to compare the mRNA expression levels of IL-18 and other genes associated with this inflammatory pathway in HPV+, HPV-, and normal non-cancerous tissues within the head and neck.
2. Compare the mRNA and protein expression levels of IL-18 and IL-18BP between HPV+ and HPV- HNSCC cell lines to determine if the IL-18 reduction seen within the TCGA is also observed within HNSCC cell lines.
3. Determine if expression of the E7 oncogene, which is consistently expressed in HPV+ cancers, is sufficient to repress transcription of the IL-18 gene.

Chapter 2

2 Methods

2.1 TCGA RNA Expression and Analysis

Tumour and tissue samples from the head and neck cohort (HNSC) from the TCGA were collected by researchers and health practitioners located across the globe (Cancer Genome Atlas Network, 2015). These primary tumor samples were subjected to molecular characterization, including mRNA-sequencing using the Illumina platform. The RNA sequencing data was analyzed via a unified bioinformatic pipeline, and these results can be freely downloaded from the Broad Genome Data Analysis Center Firehouse server (https://gdac.broadinstitute.org/). The analysis pipeline used for all TCGA cohorts results in level 3 RNA-seq by Expectation Maximization (RSEM) data that has been normalized through RSEM normalization and encapsulates the mRNA content of over 22,000 expressed genes, allowing for comparisons across different tumor types and adjacent normal control tissues collected at the time of tumour resection (Cancer Genome Atlas Network, 2015). For this study, I performed a comparative analysis of eight genes in the IL-18 pathway, including *IL-18, IL-33, IL-1β, IL-18BP*, *CASP1*, *NLRP3*, *AIM2 and PYCARD*. The normalized mRNA expression values for each TCGA HNSC sample were exported into Microsoft Excel for further organization and exported into additional statistical software (details described below). In Excel, HPV status was manually designated and derived from a previously published dataset (Bratman S. V. et al 2016). Tumours derived from secondary metastatic lesions, as well as tumours with an unknown HPV status were excluded from this study. This resulted in a total of 515 different tumours available for analysis, with 73 HPV+ tumours, 442 HPV- tumours, and 43 samples from normal adjacent tissues from this cohort. Statistical tests were conducted using GraphPad Prism version 8.4 using a two-tailed non-parametric statistical T-tests, the Mann-Whitney U test.

2.2 DNA Methylation Comparison

In addition to the mRNA-sequencing data, this study also used genome-wide DNA methylation data obtained from the same TCGA HNSC cohort of cancer samples (Cancer

Genome Atlas Network, 2015). This data was collected using the Illumina Infinium Human Methylation450 Beadchip array, which encompasses over 450,000 methylation probes, corresponding to CpG sites across the human genome. Like the RNA-sequencing data, the normalized methylation values are publicly available from the Broad Genome Data Analysis Center Firehouse server (https://gdac.broadinstitute.org/). There are 31 CpG DNA probes within the IL-18BP upstream promoter region and 24 CpG probes within the upstream promoter region of IL-18. These probes were identified using the online tool Wanderer (Díez-Villanueva A. *et al.* 2015), which provides a digital representation of the genome where CpG probes are identified within a given stretch of genomic DNA.

This methylation data is represented as β-Values, representing the fraction of CpG methylated at that specific locus, and was exported into Microsoft Excel. These values were manually curated for HPV status as described in section 2.1, and then used to quantitatively compare the difference in methylation of the genomic DNA prior to the beginning of IL-18 and IL-18BP gene respectively. This resulted in a total of 515 different samples available for analysis, with 73 HPV+ tumours, 440 HPV-, and 23 samples from normal adjacent tissues within these patients. Statistical tests were conducted using GraphPad Prism version 8.4 using a two-tailed non-parametric statistical T-tests, the Mann-WhitneyU test.

2.3 Generation of Plasmids

Plasmids were generated using Polymerase Chain Reactions (PCR) to amplify the gene of interest from a previously constructed template. PCR amplification of HPVs E7 was conducted using Phusion Polymerase (ThermoFisher) with forward and reverse DNA oligonucleotide primers (Integrated DNA Technologies, IDT) matching the beginning and ending sequence of the gene of interest (Table 2.3). This PCR was completed using 10µM of each primer, 10ng of template plasmid DNA, and 5xPhusion buffer (ThermoFisher) to a final volume of 20µl. Reactions underwent 30 cycles of amplification, with an annealing temperature of 60°C for 15 seconds, extension at 72°C for 20 seconds, and a release temperature of 98°C for 20 seconds within a thermocycler (BioRad, C1000 Touch Thermocycler). Ten microliters of this PCR reaction was loaded onto a 1% Agarose Gel (FroggaBio) in gel running buffer (0.5x TBE) with 0.01% ethidium bromide and subjected

to electrophoresis to confirm PCR amplification by the presence of a band of the predicted size. Forty microliters of the PCR reaction were purified using the EZ-10 Spin Column DNA Gel Extraction Minipreps Kit (BioBasic) according to the manufacturer's protocol. This purified PCR product and 1µg of the zsGreen circular plasmid (pLVX-3xTy1-T2A-zsGreen supplied by Dr. Greg Fonseca) was then digested with XhoI and EcoRI-HF (NEB) within CutSmart buffer (NEB) at a final volume of 20µl for 1 hour at 37°C, with 1 unit of each enzyme. The digested plasmid was then loaded and run within a 2% agarose gel (FroggaBio) with ethidium bromide as described above for confirmation of linearization, and the band of interest was then cut for further purification using the EZ-10 Spin Column DNA Gel Extraction Minipreps Kit (BioBasic). Both the digested plasmid and the digested PCR product were then purified again in preparation for ligation using Quick Ligase Enzyme (NEB) and Quick Ligase x2 Buffer for 10 minutes at room temperature.

To construct our IL-18 promoter luciferase vector, we amplified a fragment of DNA containing the promoter region of IL-18 purchased from IDT. This construct corresponds to the 1.5kb region of DNA immediately prior to the IL-18 gene. PCR amplification was performed using a Q5 Polymerase (NEB) with 10µM of each primer (Forward and reverse E7 primer for cloning, Table 2.4), 10ng of template plasmid DNA, and 5xPhusion buffer to a final volume of 50µl. This reaction underwent 15 cycles of amplification using an annealing temperature of 67°C for 30 seconds, extension temperature of 72°C for 20 seconds, and a release temperature of 98°C. Ten microliters of this PCR reaction was loaded into a 1% Agarose Gel (FroggaBio) with ethidium bromide for electrophoresis to confirm PCR amplification. Forty microliters of the PCR reaction were purified using the EZ-10 Spin Column DNA Gel Extraction Minipreps Kit (BioBasic) according to the manufacturer's protocol. This purified PCR product and 1µg of pGL3-Basic circular plasmid obtained from the laboratory of Dr. Fred Dick in the Victoria Research Labs (VRL) at Victoria Hospital., was then digested with XhoI-HF and KpnI-HF within CutSmart buffer (NEB) at a final volume of 20µl for 1 hour at 37°C. The digested plasmid was then loaded and run within a 1% agarose gel with ethidium bromide for confirmation of linearization, and the band of interest was then cut for further purification. Both the digested plasmid and the digested PCR product were then purified again in preparation for

ligation. Ligation was conducted using Quick Ligase Enzyme (NEB) and Quick Ligase x2 Buffer for 10 minutes at room temperature.

Plasmids 7-24 (Table 2.1) were developed by previous PhD student Dr. Biljana Todorovic and are described in her previous publication (Todorovic B. *et al* 2011). These plasmids were digested to extract the gene fragments of interest and then ligated into a plasmid vector containing an N-terminal GFP and a myc tag (Table 2.2)

All plasmids were transformed into the bacterium *Escherichia coli* (strain DH5α) and subsequently plated onto lysogeny broth (LB, 10g/L tryptone, 5g/L yeast extract, 86mM NaCl) plates with ampicillin selection (50µg/ml, BioShop). The resulting colonies were screened for proper transformation and ligation of the insert through colony PCR and PureLink Quick Plasmid Miniprep kits. Briefly, colonies were picked under sterile conditions using a p200 pipette tip. The tip was dipped within a prepared Phusion polymerase reaction. This PCR was completed using 10µM of each primer to the respective insert (Table 2.4), the picked colony of bacteria as the source of template plasmid DNA, and 5xPhusion buffer to a final volume of 50µl. This reaction underwent 30 cycles of amplification, with an annealing temperature of 60°C for 15 seconds, extension temperature of 72°C for 20 seconds, and a release temperature of 98°C. The entire PCR product was then loaded into a 1% agarose gel with ethidium bromide and electrophoresed to confirm PCR amplification of the insert. The p200 pipette tip used to pick the colony was also submerged in 5ml of liquid LB + ampicillin solution and incubated for 16 hours (overnight) at 37°C to make a small preparation of the plasmid DNA. The small-scale plasmid preparations of the plasmids in which the colony PCR was positive were done using the manufacturer's instructions for the PureLink Quick Plasmid Miniprep kits (Geneaid). These plasmids were sent for sequencing at the BioBasic facility in Burlington, Ontario where the samples underwent Sanger sequencing for sequence confirmation.

2.4 Cell Culture and Transfection

HT1080 fibrosarcoma cells were used for testing expression from recently cloned expression vector plasmids and for co-transfections. The HNSCC cell lines PE/CA, Cal27, Cal33, PIC6B, SCC2, SCC9, SCC47, SCC61, SCC152, UWO8, UWO23, UWO37, FaDu,

and 93-VU (Table 2.3) were used as a source of mRNA for qPCR assays of gene expression and protein for western blotting. Cal33 cells were also grown and transfected with expression vectors for HPV oncogenes and luciferase assays. These cell lines were generously shared with me from the laboratory of Dr. Anthony Nichols (Western University), with Dr. John Barret generating the UWO series of HNSCC HPV+ cell lines.

HT1080 cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) supplemented with 100µg/mL streptomycin and 100 international units (IU) per ml of penicillin. All cells were cultivated in T75 adherent tissue culture flasks (Sarstedt) within an incubator at 37°C with 5% CO_2. Cells were harvested and split through washing with phosphate buffered saline (PBS; Wisent) then incubated in 1ml of a 0.25% trypsin/EDTA solution (Wisent) to release the cells from the adherent flasks.

For HT1080 transfections, cells were seeded and cultivated within 10cm adherent cell culture plates (Sarstedt) and grown until ~80% confluency. Medium was replaced with fresh DMEM with 10% FBS, and cells were then transfected with 8µg of zsGreen-HPV16 E7 using the transfection reagent X-tremeGene DNA Transfection Reagent (Roche). These transfections were completed by mixing a ratio of 2µl of transfection reagent with 1µg total of plasmid DNA in 1ml of serum-free cell culture media. Cells were incubated with the transfection reagent for 24h and expression was confirmed by fluorescent microscopy.

Cal33 head and neck cancer cells were selected for the luciferase assays as they expressed high levels of IL-18 mRNA and are HPV- (Table 2.3). These cells were cultivated in Dulbecco's modified eagle medium/Hams nutrient mixture F12 (DMEM/F12) with 10% FBS with 100µg/mL streptomycin and 100IU of penicillin. in T75 adherent tissue culture flasks (Sarstedt) within an incubator at 37°C with 5% CO_2. Cells were harvested and split through washing with PBS (Wisent) then incubated in 1ml of a 0.25% trypsin/EDTA solution (Wisent) to release the cells from the adherent flasks. Cal33 were seeded within 6-well adherent cell culture plates and grown until ~80% confluency. Medium was replaced with fresh DMEM/F12 with 10% FBS and cells were transfected with a total of 5µg of DNA using Lipofectamine 3000 adherent cell culture transfection reagent

(ThermoFisher). This was done with 7.5µl of transfection reagent and 2µl of p3000 reagent per 1µg of DNA. Cells were transfected with 5µg of zsGreen-HPV16 E7 for the purposes of qPCR, western blot, and ELISA. Cells were transfected with 2.5µg of pGL3-Basic-IL-18 promoter with 2.5µg of one of the pCANmyc-EGFP E7 constructs for luciferase assays (Table 2.2). Cells were incubated with the transfection reagent and DNA for 10 hours, then the transfection media was replaced with fresh DMEM/F12 for an additional 38 hours of incubation. Transfection and expression were confirmed through the use of fluorescent microscopy. The microscope used was the Nikon Eclipse Ti with the fluorescent light supplied through the Nikon Intensilight C-HGFI Unit. Images were captured using a Retiga EXi FAST Cooled mono 12-Bit camera and then processed through the Volocity 6.1.1 Image software

2.5 Quantitative Reverse Transcription PCR

HNSCC cell lines were harvested upon reaching 80% confluency. Media was removed by aspiration and cells were washed with 1ml of cold PBS. One milliliter of cell lysis buffer (sourced from NEB) with protease inhibitor was applied to each plate and cells were harvested using a cell scraper. The cells were incubated with lysis buffer at 4°C for 10 minutes. RNA was collected using the Monarch Total RNA Miniprep Kit (NEB) with PureLink DNase (ThermoFisher). cDNA was obtained by reverse transcription of 1µg of RNA utilizing the SuperScript VILO cDNA Synthesis kit (ThermoFisher). The quantification of IL-18 and IL-18BP RNA was completed using the TaqMan Gene Expression Assay (ThermoFisher) according to the manufacturer's protocol with 100ng of cDNA. Analysis of expression was performed through the quantification of the -ΔΔCT values calculated by RT-qPCR and standard curves. Standard curves were established through the serial dilution of a plasmid containing the DNA sequence matching the mature mRNA sequence of IL-18, IL-18BP, E7, and GAPDH. 100ng, 10ng, 1ng, 0.1ng, and 0.01ng of DNA standards were analyzed using the TaqMan Gene Expression Assay (ThermoFisher) according to the manufacturers protocol. The -ΔΔCT values were then subsequently plotted to establish a standard curve. The plasmid has a known size in base pairs, and together with the slope of the standard curve I was able to ascribe a DNA copy number to the given CT values. As qPCR probes are dependent on the quantity of DNA in

the sample, we are able to apply this standard curve to the given CT values of our cellular mRNA made cDNA.

$$\text{mRNA content} = \frac{(\text{Slope} * \text{avg}\Delta\Delta\text{CT} + \text{yintercept}) * 6.022 * 10^{23}\text{molecules/mole}}{(\text{Plasmid base pairs} * \frac{660\text{g}}{\text{mole}}) * 1 * 10^{9}\ \text{ng/g}}$$

2.6 Western Blot

Protein samples were prepared through the lysis of cells with RIPA buffer at 4°C for 15 minutes. Cells were then gently centrifuged for 10 mins at an RCF of 500xg at 4°C to pellet cell debris, and the supernatant was removed. Twenty microliters of the cell lysate were then mixed with LDS loading buffer ([2xLDS buffer, 1,4-Dithiothreitol (DTT)] Novex). Samples were initially loaded into a NuPage 10% Bis-Tris polyacrylamide gel (Invitrogen). These samples were resolved by electrophoresis at 200V in MES running buffer (50mM MES, 50mM Tris, 3.47mM SDS, 1.03 EDTA, BioShop). The proteins within the gel were then transferred into a 0.45µm pore polyvinylidene fluoride membrane (PVDF; FroggaBio) using the XCell SureLock docking system (Invitrogen) using our own custom transfer buffer (25mM Bicine, 25mM Bis-Tris, 1.03 EDTA, 20mM chlorobutanol, 10% methanol. Bicine from BioBasic, remaining reagents from BioShop). Before protein transfer, the PVDF membrane was "activated" by a brief soak in methanol. Protein transfer from the gel to the membrane was done at 18V for 1.1 hours. After transfer, the PVDF was briefly soaked in TBS-T (Tris buffered saline [20mM Tris, 136mM NaCl] with 0.1% Tween-20: Tween-20 from Sigma, the rest from BioShop). The membrane was subsequently blocked with skim milk blocking buffer (5% w/v Skim Milk Powder in TBS-T) for an hour at room temperature. The membrane was then incubated overnight in blocking solution containing the primary antibody (Table 2.1). The membranes were then washed thrice for 20 mins at room temperature with TBS-T. The membrane was then gently shaken for an hour with a secondary antibody (Table 2.1), a species-specific monoclonal antibody conjugated with horseradish peroxidase (HRP) and diluted in blocking buffer. After three further 20-minute washes with TBS-T at room temperature, the membrane was then treated with Immobilon Luminata Crescendo Western HRP Substrate or Immobilon Luminata Forte Western HRP

Substrate (Millipore) for two minutes. Images were obtained using the ChemiDoc XRS Gel Imaging system and software (BioRad).

2.7 Bradford Assay

The concentration of protein in cell extracts was determined using the Bradford assay dye reagent (BioRad). Serial dilutions of bovine serum albumin (BSA, Sigma) were used to create protein standards containing 1, 2, 5, 10, and 40µg/ml of BSA in double distilled water (ddH_2O). One microliter of these standards was added ddH_2O to a final volume of 800µl and mixed with 200µl of the 5x Protein Assay Dye Reagent (BioRad). Two hundred microliters of this final solution were added to the wells of clear flat bottomed 96-well plates (ThermoFisher) in technical duplicates. Using a Multiscan Ascent plate-reader (ThermoFisher) at 450nM, the absorbance values were measured and plotted to develop the standard curve utilized in protein concentration calculations.

2.8 Sandwich Enzyme Linked Immunosorbent Assay (ELISA)

HNSCC cell lines were cultivated in 10cm cell culture dishes until ~80% confluency. Cell culture media was replaced with fresh media, which was collected 24 hours later. Media was centrifuged at an RCF of 500xg for 10 minutes at 4°C to pellet cellular debris, which was discarded. Cells were collected from the plates with a scraper and counted using a hemocytometer. Two hundred microliters of the cell culture media were incubated in duplicate with IL-18 specific antibody coated wells upon a plate shaker at 200rpm at 4°C overnight (Abcam). These plates then had the media aspirated and underwent 3 rounds of washing with PBS. The plates then underwent a secondary incubation period shaking at 500rpm at room temperature with the supplied secondary antibody conjugated to HRP. The wells were then again washed 3 times with PBS and were then activated through the addition of a TMB development substrate and shaking at 500rpm in complete darkness for 10 minutes. The plates OD values were then collected using the Multiscan Ascent plate-reader (ThermoFisher) at 450nm.

Lyophilized recombinant IL-18 (Abcam) was used as a protein standard. A series of dilutions of IL-18 were created at 8000pg/ml, 4000pg/ml, 2000pg/ml, 1000pg/ml,

500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, and a sample containing no recombinant IL-18. These serial dilutions were subjected to analysis by ELISA as described above to generate a standard curve.

2.9 Luciferase assay

Three hundred thousand Cal33 cells were seeded within 6-well plates. After 24 hours, they were transfected with the IL-18 luciferase reporter vector and either a control vector or a vector expressing an HPV oncoprotein using lipofectamine 3000. The cells were then incubated for 10 hours before the transfection media was removed by aspiration. Cells were washed using 1ml of room temperature PBS and collected using a cell scraper. Cells were pelleted by low speed centrifugation and the pellet was incubated in 200 µl of 1x Cell Lysis Buffer (Promega) at 4°C for 10 minutes. Cell lysates were centrifuged at an RCF of 200xg at 4°C for 10 minutes to pellet cell debris and the supernatant was collected. 50µl of cell lysate was mixed with 100ul of luciferase assay substrate (Promega) immediately before analysis using the Lumat LB 9507 luminometer (Bertold Instruments) to measure luminescence. Measurements were taken immediately after mixing with a 10 second measurement time. Twenty microliters of cell lysate were kept for confirmation of HPV-16 E7 expression by Western Blot as described above. Relative light units (RLU) values normalized based on protein levels as determined by the Bradford Assay.

Table 2.1: List of antibodies used within this thesis

Antibody	Animal of Origin	Usage	Dilution factor	Company
Anti- HPV-16E7	Mouse polyclonal	WB, primary conjugated HRP	1:500	SantaCruz
Anti-IL-18	Mouse polyclonal	WB, primary antibody	1:1000	AbCam
Anti-IL-18BP	Rabbit polyclonal	WB, primary antibody	1:1000	AbCam
Anti-Tubulin	Mouse polyclonal	WB, primary antibody	1:2000	Sigma
Anti-Mouse antibody (IgG)	Rat monoclonal	WB, secondary for IL-18 and tubulin, conjugated to HRP	1:20000	Jackson Laboratories
Anti-Rabbit antibody (IgG)	Goat polyclonal	WB, secondary for IL-18BP, conjugated to HRP	1:20000	Jackson Laboratories
Anti-c-Myc	Mouse monoclonal	WB, primary antibody	1:2000	Invitrogen

Table 2.2: List of plasmids used within this thesis

#	Name	Backbone	Description/insert
1	pCANmyc-EGFP-hIL18	pCANmyc-EGFP	Plasmid containing an exon-exon junction within the transcribed IL-18 mRNA sequence for qPCR standard curve production
2	pCANmyc-EGFP-hIL18BP	pCANmyc-EGFP	Plasmid containing an exon-exon junction within the IL-18BP genomic sequence for qPCR standard curve production
3	pGEM-GAPDH	pGEM	Plasmid containing an exon-exon junction within the GAPDH genomic sequence for qPCR standard curve production
4	pCANmyc-EGFP	pCANmyc-EGFP	Plasmid containing EGFP behind a multiple cloning site
5	pGL3-Basic	pGL3-Basic	Plasmid containing a promoterless luciferase gene
6	pGL3-Basic-IL18-promoter	pGL3-Basic	Plasmid containing luciferase vector with the 1.5kb IL-18 promoter region
7	HPV16-E7	pCANmyc-EGFP	HPV-16 E7 ligated to EGFP, used for the amplification, and cloning of E7, transfection of E7, and co-transfection of E7 and luciferase vector
8	HPV6-E7	pCANmyc-EGFP	HPV-6 E7 with N-terminal myc-tag for co-transfection of E7 and luciferase vector
9	HPV11-E7	pCANmyc-EGFP	HPV-11 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector
10	HPV18-E7	pCANmyc-EGFP	HPV-18 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector
11	HPV31-E7	pCANmyc-EGFP	HPV-31 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector
12	HPV35-E7	pCANmyc-EGFP	HPV-35 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector
13	HPV39-E7	pCANmyc-EGFP	HPV-39 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector

14	HPV45-E7	pCANmyc-EGFP	HPV-45 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector
15	HPV52-E7	pCANmyc-EGFP	HPV-52 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector
16	HPV55-E7	pCANmyc-EGFP	HPV-55 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector
17	HPV58-E7	pCANmyc-EGFP	HPV-58 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector
18	HPV59-E7	pCANmyc-EGFP	HPV-59 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector
19	HPV67-E7	pCANmyc-EGFP	HPV-67 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector
20	HPV74-E7	pCANmyc-EGFP	HPV-74 E7 with N-terminal myc tag for co-transfection of E7 and luciferase vector
21	HPV16-E7 CR1-2 aa1-39	pCANmyc-EGFP	HPV-16 E7's conserved regions 1 and 2 aa1-39 with N-terminal EGFP
22	HPV16-E7 CR3 aa39-98	pCANmyc-EGFP	HPV-16 E7's conserved region 3 aa39-98 with N-terminal EGFP
23	HPV16-E7 CR3 Binding Domain 1 aa39-58	pCANmyc-EGFP	HPV-16 E7's conserved region 3 with its first binding domain aa39-58 with N-terminal EGFP
24	HPV16-E7 CR3 Binding Domain 2 aa58-99	pCANmyc-EGFP	HPV-16 E7's conserved region 3 with its second zinc-binding domain aa59-98 with N-terminal EGFP
25	pEGFP-C2	pEGFP-C2	GFP vector for transfection efficiency
26	pLVX-3xTy1-T2A-zsGreen	pLVX-3xTy1-T2A-zsGreen	Empty vector with zsGreen fluorescent protein behind a multiple cloning site with a T2A self-cleavage site
27	pLVX-3xTy1-T2A-HPV16-E7	pLVX-3xTy1-T2A	HPV-16 E7 ligated to zsGreen through a T2A self-cleavage site for transfections and luciferase assays

Table 2.3: List of cell culture cell lines utilized in this thesis
All cell lines are HNSCC cell lines and were cultivated in DMEM/F12 with 10%FBS and 1% Pen/Strep with the exception of HT1080 cell lines which are fibrosarcoma and were cultivated in DMEM with 10% FBS and 1% Pen/Strep

Cell Line	Cell Line HPV Status
HT1080	HPV-
CAL33	HPV-
CAL27	HPV-
SCC2	HPV+
SCC9	HPV-
SCC40	HPV-
SCC47	HPV+
SCC61	HPV-
SCC152	HPV+
PIC6B	HPV-
PE/CA	HPV-
93-VU	HPV+
FaDu	HPV-
UWO8	HPV+
UWO23	HPV+
UWO37	HPV+

Table 2.4: List of oligonucleotide ssDNA primers used within this thesis

Primer Name	Primer Sequence 5'-3'	Primer Usage
GAPDH Forward primer	ATGACAACTTTGGTATCGTGGAAGG	qPCR
GAPDH Reverse primer	GAAATGAGCTTGACAAAGTGGTCGT	qPCR
IL-18 Forward Primer	CCTGGAATCAGATTACTTTGGC	qPCR
IL-18 Reverse Primer	GGGTGCATTATCTCTACCAGTCAGA	qPCR
IL-18BP Forward Primer	CCAGTGTTCCCAGCAGCTAA	qPCR
IL-18BP Reverse Primer	CTGCAGGCCACACAGGATAA	qPCR
HPV-16 E7 Forward Primer	ACAGCTCAGAGGAGGAGGAT	qPCR
HPV-16 E7 Reverse Primer	ACCGAAGCGTAGAGTCACAC	qPCR
HPV-16 E7 Forward Primer	TATACTCGAGATGCATAGGAGATACA	Cloning
HPV-16 E7 Reverse Primer	TATACTTAAGTGGTTTCTGAGAACAGATG	Cloning
IL-18 Promoter region forward primer	TATGGTACCCTACTTGATCCCACTTCGTGCTTTC	Cloning
IL-18 Promoter region reverse Primer	TATACTCGAGCTCCAGCAAGCTGGGGAGAG	Cloning
pGL3-Basic Forward Primer	CAAAACGAAACAAAACAAACTAGC	Sequencing
pGL3-Basic Luciferase gene Reverse Primer	GCGGTTCCATCTTCCAGCGG	Sequencing
CMV promoter Forward primer	GGCGTGTACGGTGGGAGGTC	Sequencing

Chapter 3

3 Results

3.1 Investigation of the Impact of HPV on the IL-18 Pathway Using the TCGA Head and Neck Cancer Cohort

The data obtained by the TCGA represents a powerful opportunity to undertake a bioinformatics-based analysis of the impact of HPV on the IL-18 pathway in head and neck cancer. Specifically, this allows us to utilize at a wide array of processed and analyzed samples of real head and neck tumours from across the globe. In the head and neck cohort of the TCGA, the HPV status was also previously determined (Bratman S. V. *et al* 2016, Cancer Genome Atlas Network, 2015) resulting in a pool of 73 HPV+ cancers, 442 HPV- cancers, and 43 samples of normal, non-cancerous adjacent tissues extracted along with patient tumours. These normal adjacent tumours are tissues samples excised from patients in addition to their primary tumour and represent non-cancerous tissue from the same tumour location. This allows us to compare our HPV+ cancers to the non-cancerous tissues that surround it as a normal control.

To explore the expression of IL-18 and its associated pathway constituents, I used both the transcriptomic database as well as the database of genomic methylation data from the head and neck cohort of the TCGA. The transcriptomic data contains the normalized mRNA content of the tumours and normal adjacent tissues, which allows for the quantitative analysis of changes in mRNA expression between individual tumour samples and normal adjacent tissues. The genomic methylation database contains β-values, which represent proportional DNA methylation of CpG sites located throughout the human genome, allowing for quantitative analysis of changes in methylation of genes between HPV+, HPV-, and normal adjacent tissues.

3.1.1 The Impact of HPV status on the Expression of *IL18* and Related Proinflammatory Cytokines in Primary Human Head and Neck Cancers

We initially began our investigation by examining expression of IL-18's inhibitory protein IL-18BP and IL-18 itself. Previously, *In vitro* cell culture analysis conducted by the

MacDonald laboratory at Leeds University has shown an increase in the production of IL-18BP by human foreskin keratinocytes in response to introduction of HPV's oncogenes (Richard K. H. *et al* 2013). To see if this increase in IL-18BP the MacDonald lab discovered was mirrored in real cancers, I turned to the TCGA for answers. These findings are similarly present in the TCGA HNSCC cohort, as there is a significant increase in the expression of the *IL18BP* gene's mRNA in HPV+ cancers when compared to HPV- cancers and normal adjacent tissue (Figure 3.1A).

Next, I examined the expression of *IL18* mRNA to see if there were any additional alterations to the IL-18 pathway in these HPV+ cancers. Here I found that it was significantly reduced in HPV+ cancers when compared to HPV- cancers and normal adjacent tissues (Figure 3.1B). IL-18 is one of three proinflammatory cytokines within the IL-1 family of cytokines, so we also examined expression of the other constituents of the IL-1 family, IL-1β and IL-33. The mRNA levels of *IL1β*, while reduced in HPV+ cancers when compared to HPV- cancers, were not significantly different when compared to normal adjacent tissues (Figure 3.1C). The mRNA levels of *IL33* were significantly reduced in both HPV+ and HPV- cancers when compared to normal adjacent tissue, but the two cancer types had comparable levels of cytokine transcription (Figure 3.1D). Thus, of the three members of the IL-1 family of proinflammatory cytokines, only *IL18* mRNA expression is significantly reduced in HPV+ cancers compared to the expression of normal adjacent tissues or HPV- cancers, suggesting a targeted reduction in IL-18's activity by HPV (Figure 3.1).

The head and neck can be divided into different subsites, including the oropharynx, the alveolar ridge, oral cavity, larynx, and hypopharynx. It is well established that patient outcome and survival is improved in HPV+ HNSCC within the oropharynx when compared to HPV- oropharyngeal cancer (Ang K. K. *et al* 2010, Burr A. R., *et al* 2018). We compared the expression of *IL18* in the 73 HPV+ cancers across the anatomical subsites to see if the expression of *IL18* could be correlated to the varied changes in patient outcome in HPV+ cancers of the oropharynx compared to the other head and neck subsites. One sample was excluded from the HPV+ cancers, as the anatomical head and neck subsite was not clarified. There was no significant difference in *IL18* expression between the

anatomical subsites of HPV+ head and neck cancers, suggesting that HPV represses IL-18 across all anatomical subsites, though due to low sample number there is a noticeable lack of statistical power when comparing the different head and neck subsites (Figure 3.2A).

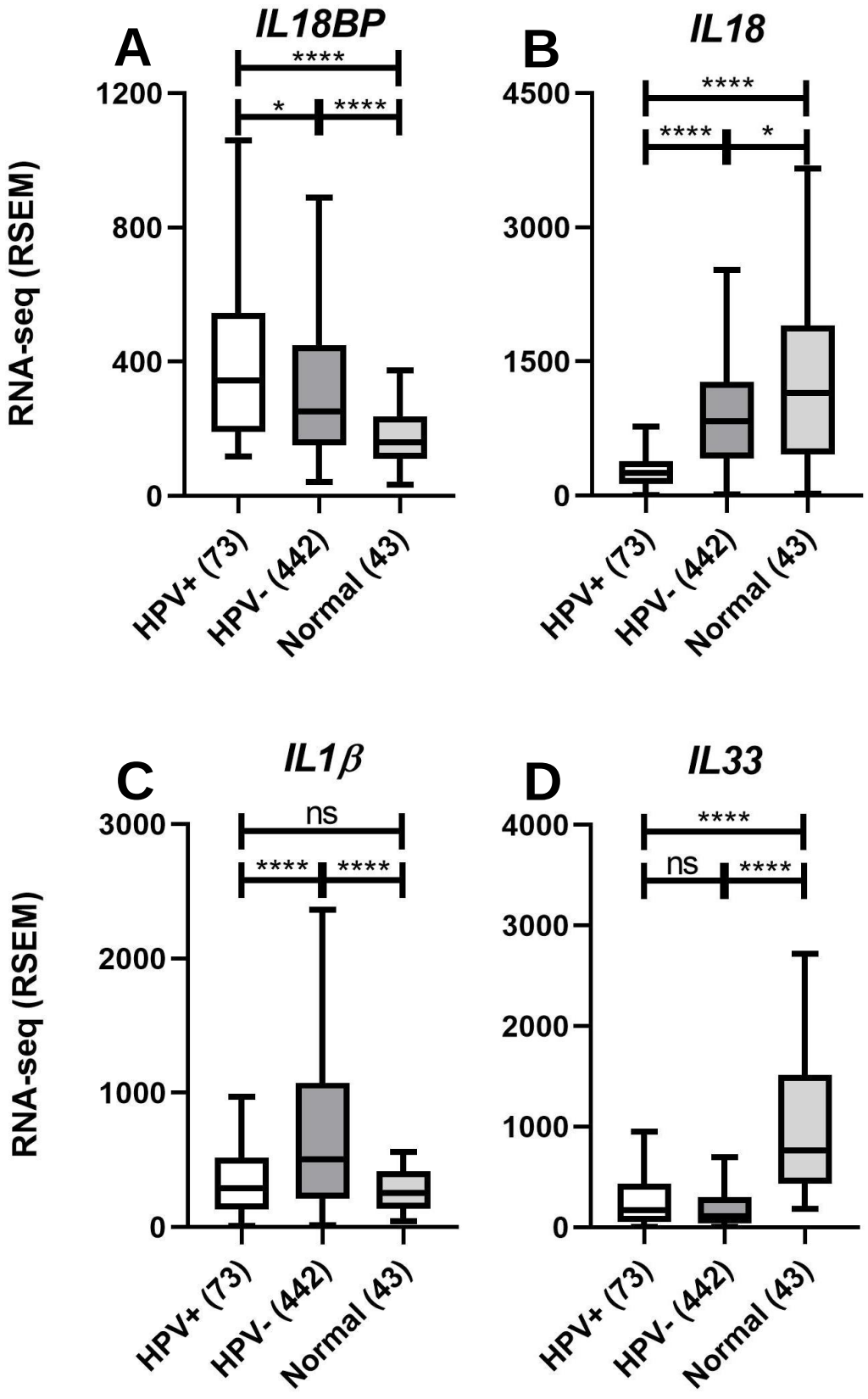
In the sphere of HPV+ cancers, HPV-16 is the most prevalent HPV type found within HPV+ head and neck cancers as well as cervical cancers (D'Souza G. *et al* 2010). As different types of HPV are identified in the head and neck cohort of the TCGA, I next examined the expression of *IL18* between samples grouped by the different types of HPV to examine if the repression of *IL18* is found across HPV types to explore if the repression of IL-18 could contribute to the heightened success of HPV-16 in establishing long-term oncogenic infections compared to the rarer HPV types. There was no difference in *IL18* expression between the different HPV types suggesting the repression of *IL18* is a conserved function among the high-risk HPV within the head and neck cancers (Figure 3.2B).

3.1.2 Impact of HPV on The Expression of the Inflammasome Components *AIM2, PYCARD, NLRP3,* and *CASP-1* in Primary Human Head and Neck Cancers

As previously mentioned, for IL-18 to be rendered biologically active it must be cleaved by active capsase-1. The activation of caspase-1 is the result of stimulated PRRs and the formation of a complex of proteins called the inflammasome. Two distinct types of inflammasomes induce caspase-1 activation. These include the AIM2 inflammasome in response to detection of dsDNA in the cytoplasm, or the NLRP3 inflammasome in response to various cellular stresses like microbial infection, reactive oxygen species, cellular dysfunction, and cellular damage (Kelley N. *et al* 2019). We looked at the impact of HPV on expression of the genes encoding the constituents of these inflammasomes, including *NLRP3*, the *AIM2* encoded PRR and the adaptor protein ASC (encoded by the *PYCARD* gene), which allows for caspase-1 binding to both AIM2 and NLRP3. With regards to the AIM2 inflammasome, there is no significant difference in the expression of *AIM2* mRNA between the HPV+ and HPV- cancers, although expression was elevated in both cancers when compared to normal adjacent tissue (Figure 3.3A). Expression of both *PYCARD* and *CASP1* mRNAs were both significantly reduced in HPV+ cancers when compared to HPV-

cancers, although *PYCARD* mRNA levels were not different compared to the normal adjacent tissue (Figure 3.3B/C). There is no apparent change to in levels of *NLRP3* mRNA as there was no significant difference between HPV+, HPV- and the normal adjacent tissue (Figure 3.3D). Taken together, these data suggest that another potential means of IL-18 suppression by HPV could be mediated through the reduced expression of two vital components of IL-18 activation, caspase-1 and its adaptor protein ASC, which are both needed for the AIM2 and NLRP3 inflammasome (Figure 3.3A-C).

Figure 3.1: Expression of mRNAs encoding IL-18, IL-18BP and related cytokines in tumor samples from the TCGA head and neck cancer cohort. The mRNA expression levels of *IL18BP* **(A)**, *IL18* **(B)**, *IL33* **(C)**, and *IL1β* **(D)** were compared between HPV+, HPV- tumours, and non-cancerous adjacent tissues from TCGA HNSCC patient data. (**A)** There was significantly more *IL-18BP* mRNA in HPV+ cancers when compared to HPV- cancers and normal adjacent tissues. **(B)** There was significantly less *IL18* mRNA in HPV+ cancers compared to HPV- cancers and normal adjacent tissues. **(C)** There was significantly less *IL1β* mRNA in HPV+ cancers compared to HPV- cancers but there was no significant difference between HPV+ cancers and normal adjacent tissue. **(D)** There was significantly less *IL33* mRNA in HPV+ cancers then compared to normal adjacent tissues but there was no significant difference between HPV+ and HPV- cancers. Statistical analysis was conducted through a Non-parametric Mann-WhitneyU test ($p<0.0001$=****, $p<0.001$=***, $p<0.05$=*, $p>0.05$=ns).


A
IL18BP
****
*
****
1200
800
400
0
RNA-seq (RSEM)
HPV+ (73)
HPV- (442)
Normal (43)
B
IL18
****
****
*
4500
3000
1500
0
HPV+ (73)
HPV- (442)
Normal (43)
C
IL1β
ns
****
****
3000
2000
1000
0
RNA-seq (RSEM)
HPV+ (73)
HPV- (442)
Normal (43)
D
IL33
****
ns
****
4000
3000
2000
1000
0
HPV+ (73)
HPV- (442)
Normal (43)

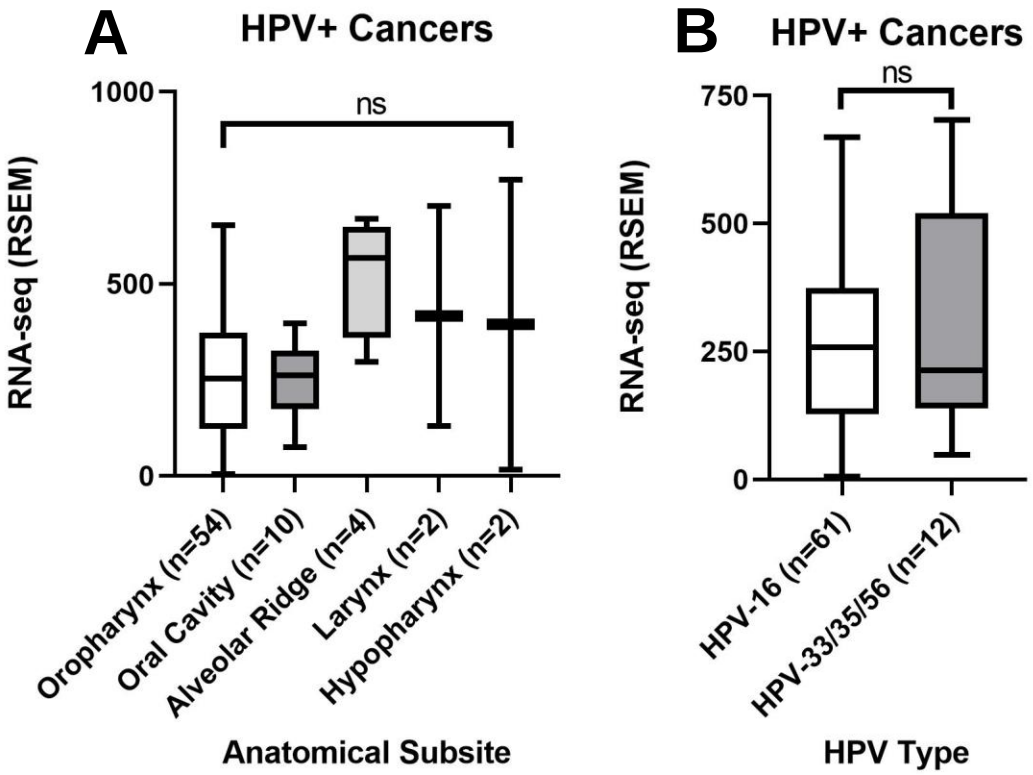
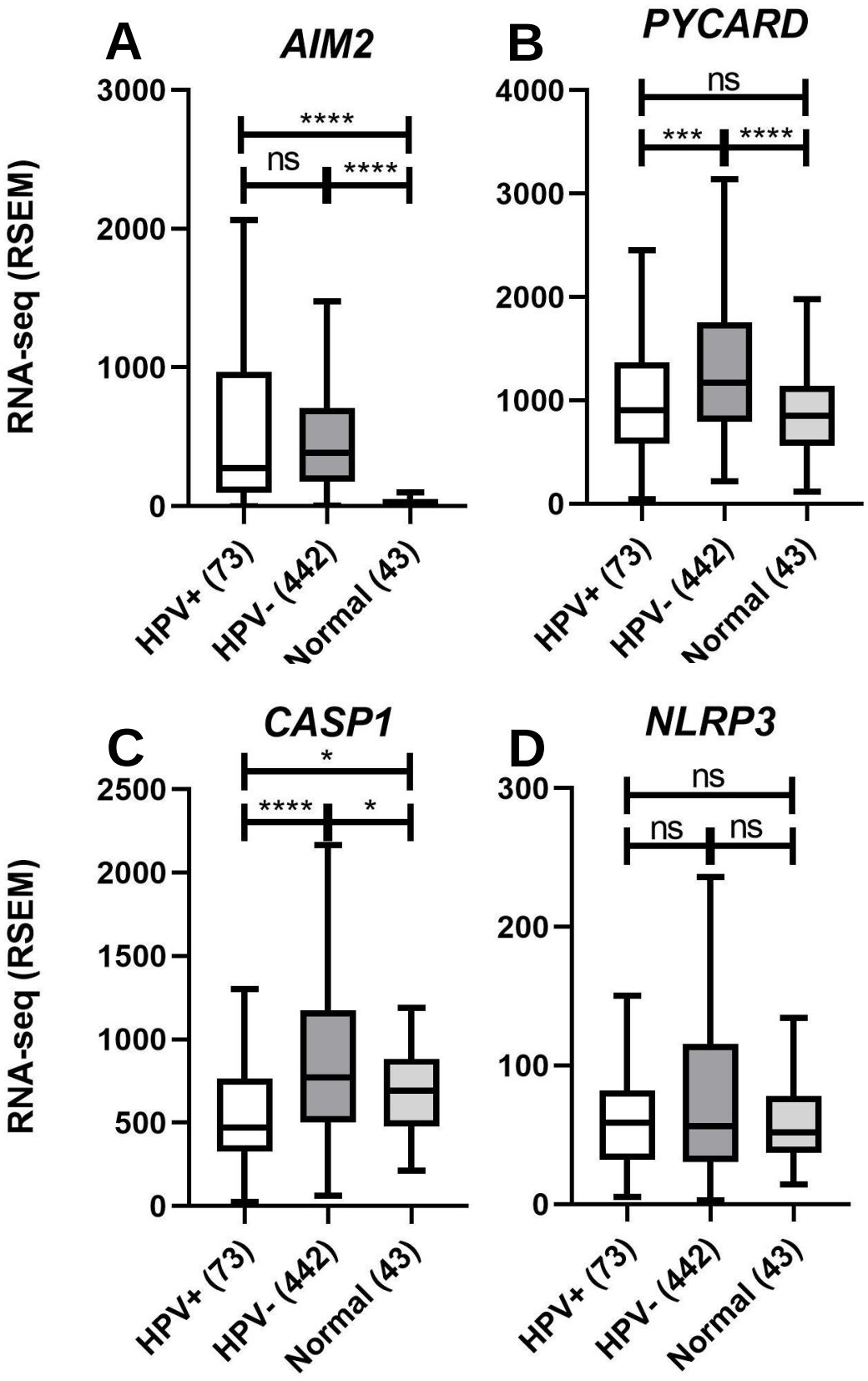


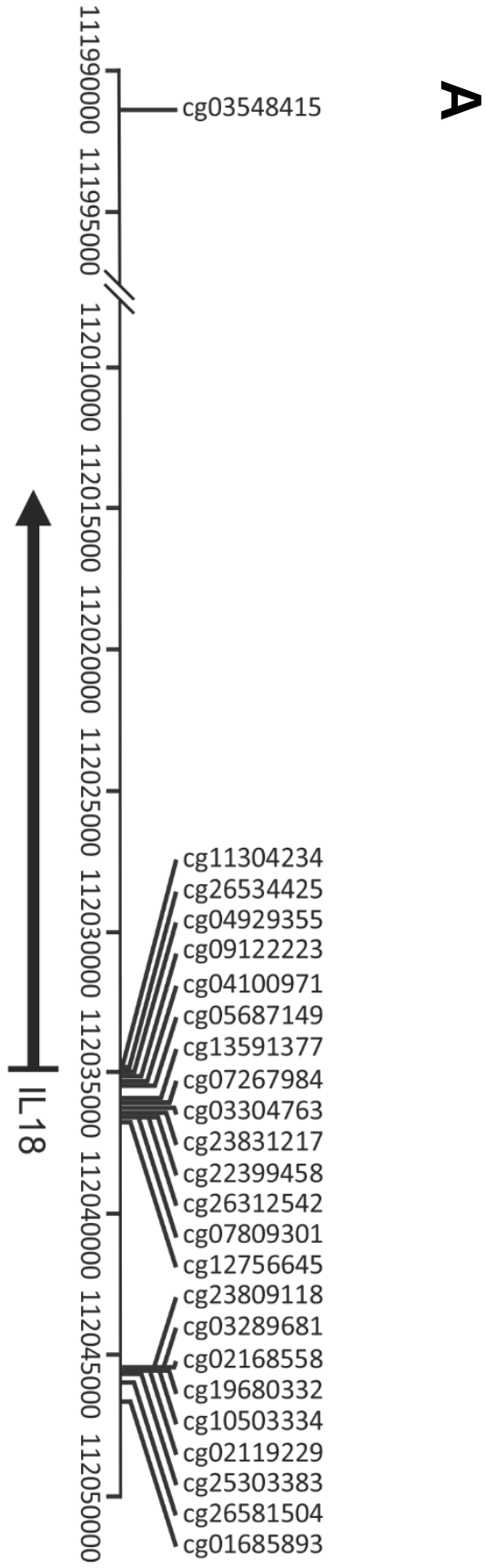
Figure 3.2: Expression of mRNAs encoding IL-18 in HPV+ samples from the TCGA head and neck cancer cohort.
(A) Comparing the expression of *IL18* between the anatomical subsites identified no significant difference between the oropharynx and the other head and neck cancer sites. **(B)** There was also no significant change in expression of *IL18* between HPV-16+ head and neck cancers and those cancers caused by other HPV types. Statistical analysis was conducted through a Non-parametric Mann-WhitneyU test ($p<0.0001$=****, $p<0.001$=***, $p<0.05$=*, $p>0.05$=ns).

Figure 3.3: Impact of HPV on expression of the genes encoding caspase-1 and components of the inflammasomes that activate caspase-1.
The mRNA levels of the components of the inflammasome *CASP1* (A) *PYCARD* (B), *AIM2* (C), and *NLRP3* (D), were calculated for HPV+ tumours, HPV- tumours, and non-cancerous adjacent tissues from patient data within the TCGA head and neck cancer cohort. **(A)** There was significantly less *CASP1* mRNA in HPV+ cancers compared to HPV- cancers and normal adjacent tissues. **(B)** There was significantly less *PYCARD* mRNA in HPV+ cancers compared to HPV- cancers but there was no significant difference between HPV+ cancers and normal adjacent tissues. **(C)** There was no significant difference in *AIM2* mRNA between HPV+ cancers and HPV- cancers but it was significantly higher in both cancer types compared to normal adjacent tissues. **(D)** There was no difference in *NLRP3* expression across HPV+ cancers, HPV- cancers, and normal adjacent tissues. Statistical analysis was conducted through a Non-parametric Mann-WhitneyU test ($p<0.0001$=****, $p<0.001$=***, $p<0.05$=*, $p>0.05$=ns).


A
AIM2
B
PYCARD
C
CASP1
D
NLRP3
RNA-seq (RSEM)
RNA-seq (RSEM)
3000
2000
1000
0
4000
3000
2000
1000
0
2500
2000
1500
1000
500
0
300
200
100
0
****
ns
****
ns
***
****
*
****
*
ns
ns
ns
HPV+ (73)
HPV- (442)
Normal (43)

3.1.3 DNA Methylation Status of CpG Sites Upstream of the *IL18* Gene's Open Reading Frame in Primary Human Head and Neck Cancers

DNA methylation is a well-documented mechanism of control over gene expression, with higher levels of DNA methylation at CpG sites within the genome upstream of the start of a gene being associated with lower transcription and gene expression (Moore L. D. *et al* 2013). HPV E7 has been previously shown to alter transcription of host genes by associating with and activating the DNA methyltransferase protein DNMT1, leading to changes in DNA methylation (Verlaat W. *et al.* 2018, Widschendter A. *et al.* 2004). We utilized the TCGA's DNA methylation data to determine if there was a change in DNA methylation up-stream of the *IL18* locus HPV+ HNSCC. We collected data from 24 DNA probes surrounding the *IL18* coding region on chromosome 11 (Figure 3.4A), which provided β-values representative of proportional methylation for each site in each cancer sample. Methylation of CpG sites in the upstream region of the *IL18* gene in HPV+ cancers exhibited significantly higher levels of methylation compared to their HPV- cancers and normal adjacent tissue counterparts (Figure 3.4B-Y). Increased methylation across these CpG sites may represent a possible mechanism leading to the transcriptional repression in HPV+ HNSCC. The first four probes prior to the *IL18* gene, probes cg11304234, cg26534425, cg04929355, cg09122223, (Figure 3.4 C-F) exhibit the largest increases in the levels of DNA methylation in HPV+ cancers, and are within 1.5kb of the start of *IL18*'s gene. This section of DNA was the selected for usage within luciferase assays presented in section 3.3.


A
111990000
111995000
112010000
112015000
112020000
112025000
112030000
112035000
112040000
112045000
112050000
cg03548415
IL18
cg11304234
cg26534425
cg04929355
cg09122223
cg04100971
cg05687149
cg13591377
cg07267984
cg03304763
cg23831217
cg22399458
cg26312542
cg07809301
cg12756645
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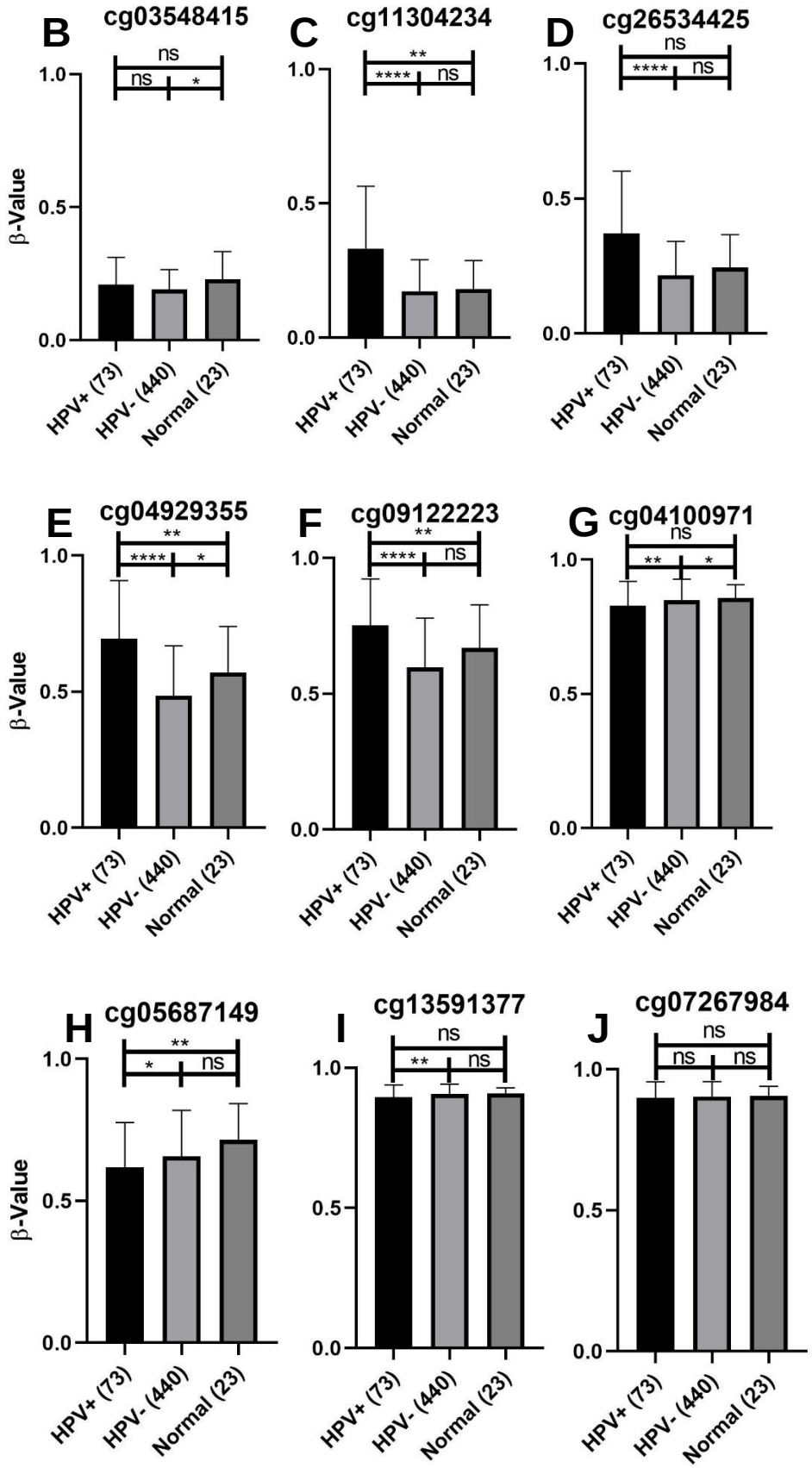
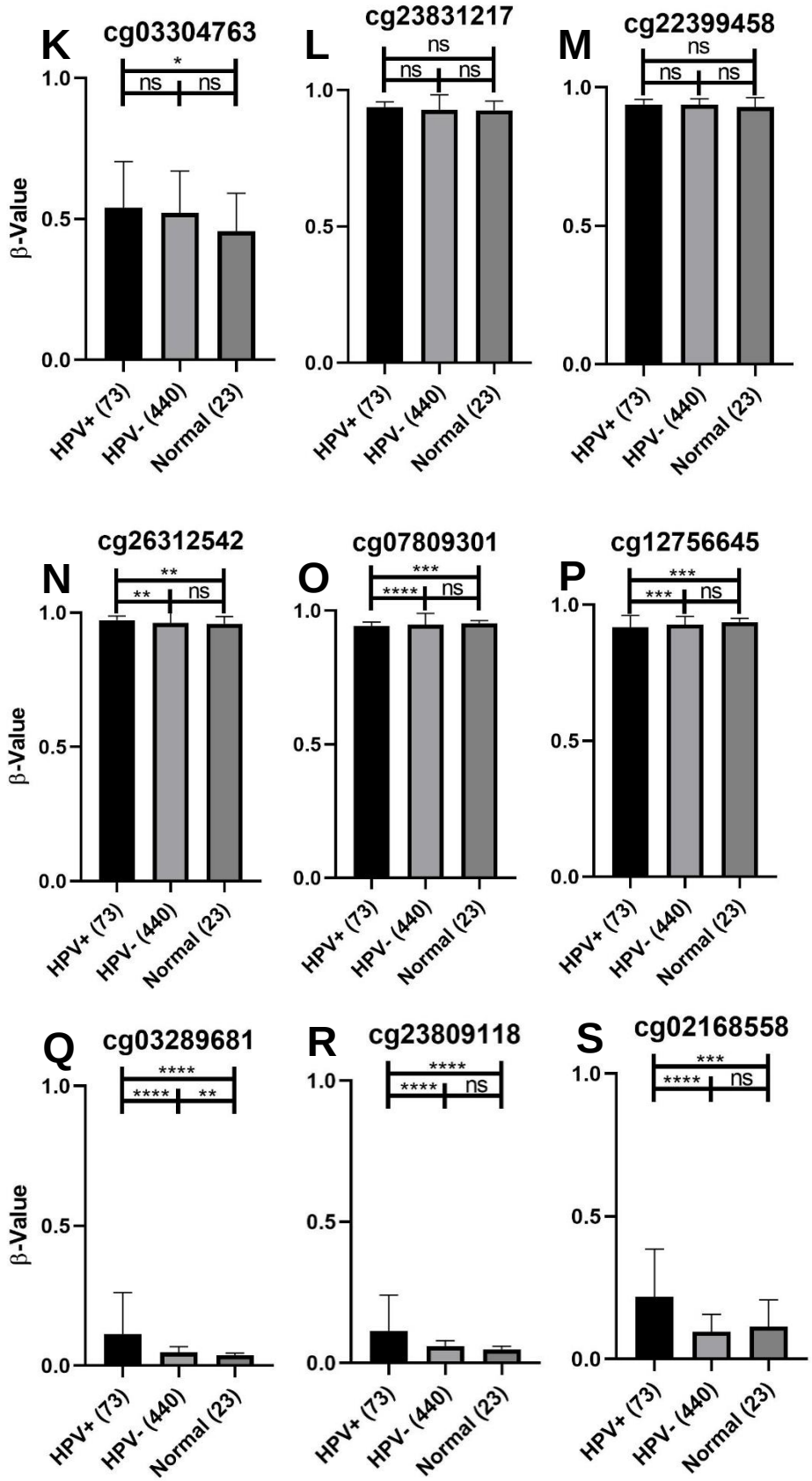
cg25303383
cg26581504
cg01685893


B
cg03548415
C
cg11304234
D
cg26534425
E
cg04929355
F
cg09122223
G
cg04100971
H
cg05687149
I
cg13591377
J
cg07267984
β-Value
1.0
0.5
0.0
ns
*
**
****
HPV+ (73)
HPV- (440)
Normal (23)


K
cg03304763
*
ns
ns
L
cg23831217
ns
ns
ns
M
cg22399458
ns
ns
ns
β-Value
1.0
0.5
0.0
HPV+ (73)
HPV- (440)
Normal (23)
N
cg26312542
**
**
ns
O
cg07809301
***
****
ns
P
cg12756645
***
***
ns
Q
cg03289681
****
****
**
R
cg23809118
****
****
ns
S
cg02168558
***
****
ns

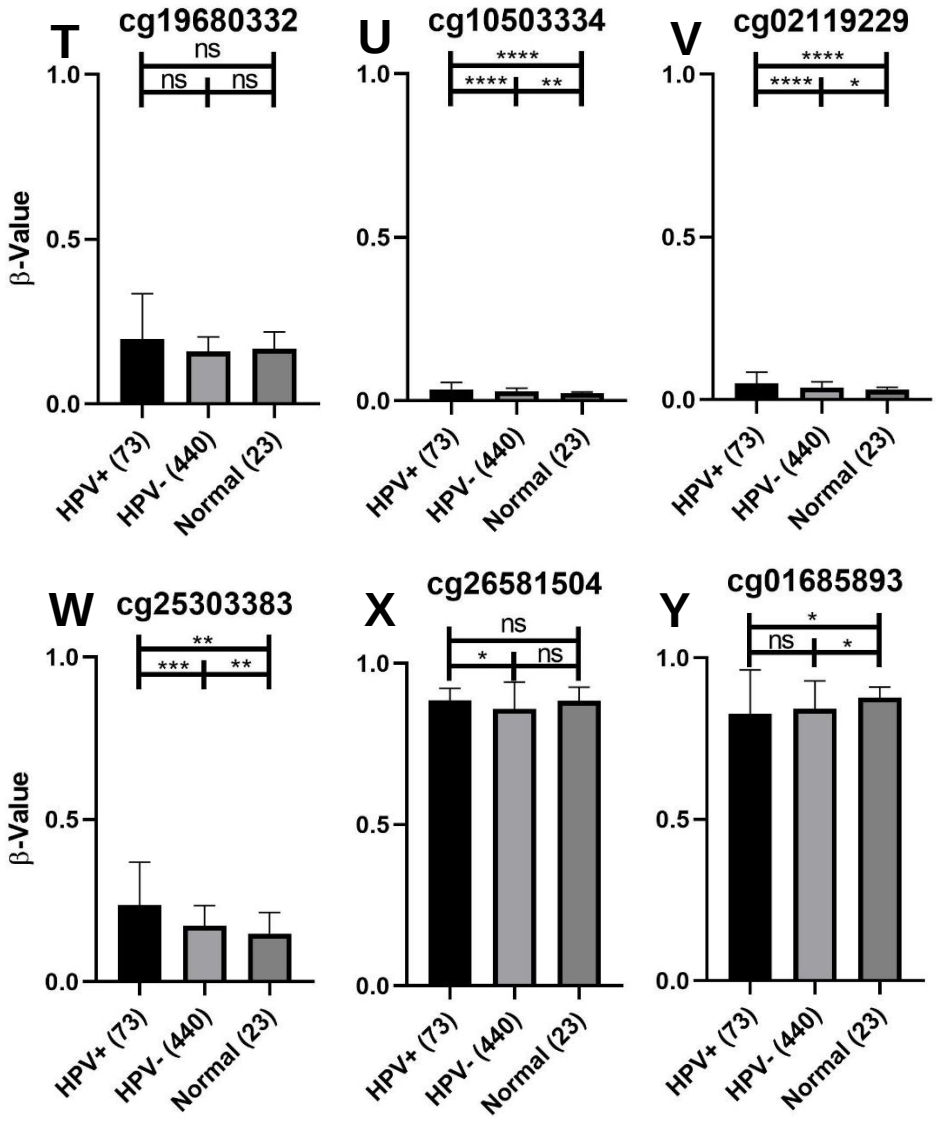


Figure 3.4: Probe location and β-values for CpG methylation within the 5' promoter region of the IL-18 gene

(A) A map of the section of chromosome 11 and the locations of the CpG sites and their corresponding DNA probes with respect to the *IL18* gene. **(B-Y)** The β-values for CpG site methylation for the indicated DNA probes were extracted from the TCGA Illumina BeadChip Array data. These values were calculated for HPV+ tumours, HPV- tumours, and non-cancerous adjacent tissues from patient data within the TCGA head and neck cohort. Statistical analysis was conducted through a Non-parametric Mann-WhitneyU test ($p<0.0001$=****, $p<0.001$=***, $p<0.05$=*, $p>0.05$=ns).

3.2 Examining Expression of IL-18 and IL-18BP in HNSCC Cell Lines

3.2.1 The Effect of HPV Status on IL-18 and IL-18BP Levels in HNSCC Cell Lines

Analysis of the TCGA data showed a significant decrease in IL-18 mRNA and significantly increase IL-18BP mRNA levels in HPV+ primary human head and neck cancers when compared to HPV- cancers and regular tissues. I next tested a panel of established HNSCC cell lines to determine if HPV status was similarly correlated with reduced IL-18 expression and increased IL-18BP expression. These cell lines were generated from excised patient tumours, that through mutation of their biological processes have escaped typical cellular senescence and are able to be grown *in vitro.* These cell lines are all derived from the head and neck, with the HPV+ cell lines containing integrated HPV DNA within their genome. The UWO series of cell lines were generated within the Nichols laboratory by Drs. Anthony Nichols and John Barret and were generously shared with me for my experiments. We hypothesized that if the biological phenomenon of IL-18 repression and IL-18BP upregulation was mirrored in these cell lines, this would allow us to use these cell lines as an experimental system for the investigating the mechanism by which HPV affects expression of IL-18 and its associated pathway components.

I initially began our investigation into IL-18 and IL-18BP expression in HNSCC cell lines using qPCR to examine the levels of *IL18* and *IL18BP* mRNA. I extracted cellular mRNA and converted it to cDNA, which was used for qPCR. I found that HPV+ HNSCC cell lines expressed significantly less *IL18* mRNA than HPV- cell lines (Figure 3.5A). Regarding *IL18BP*, I found a significantly greater production of *IL18BP* mRNA in HPV+ cell lines compared to HPV- cell lines (Figure 3.5B).

3.2.2 The Effect of HPV Status on Expression of IL-18 and IL-18BP Protein and Secretion in HNSCC Cell Lines

To corroborate the mRNA expression level data obtained from our HNSCC cell cultures, I also examined the expression of IL-18 and IL-18BP protein. Cell lysates were collected for western blot analysis, while the cell culture media was collected for analysis by ELISA.

In the western blot analysis, IL-18 was detected in the HPV- cell lines but was barely detectable in most of the HPV+ tumor cell lines, with the noticeable exception of SCC47 (Figure 3.6A). The α-tubulin protein was used as a control to ensure uniform loading of the protein gels. I also used a sandwich ELISA to measure the level of secreted IL-18 in the cell culture media. There were significantly lower concentrations of secreted IL-18 in the HPV+ cell culture media when compared to the cell culture media of the HPV- cell lines (Figure 3.6B). Thus, these cell line studies suggest that HPV represses IL-18 mRNA expression and protein production in most established HNSCC cells, in good agreement with the reduction in mRNA observed using the TCGA data obtained from primary head and neck tumors (Figure 3.1B). Unfortunately, no IL-18BP could be detected by either western blot or ELISA.

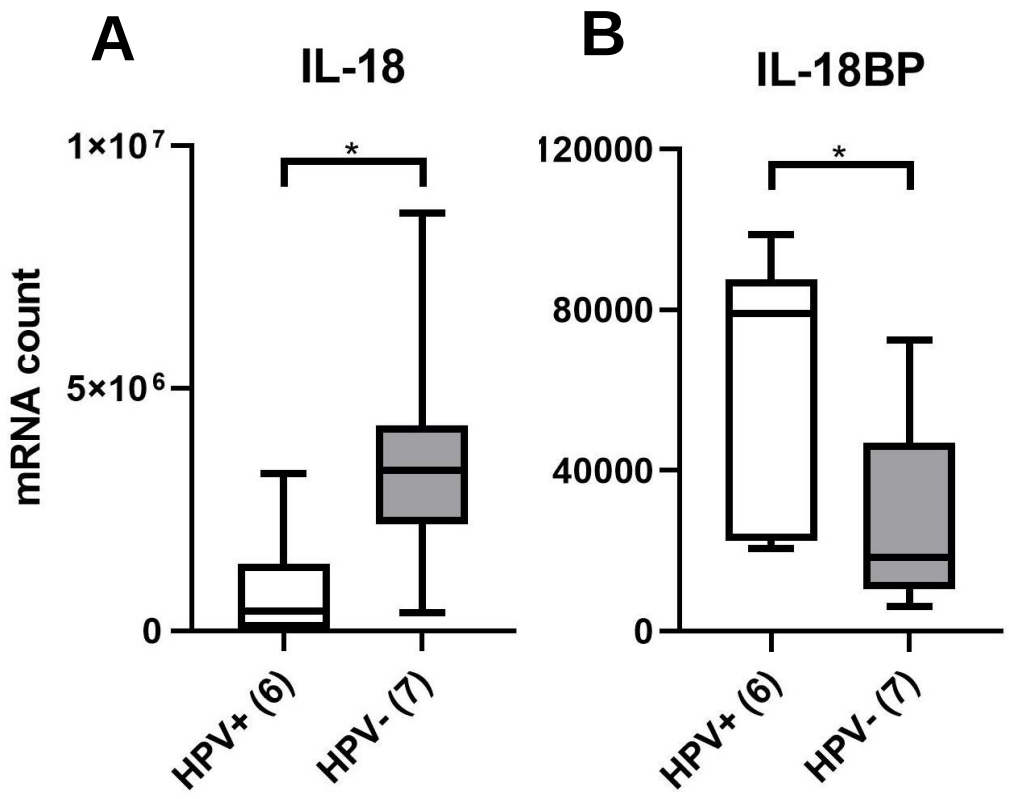
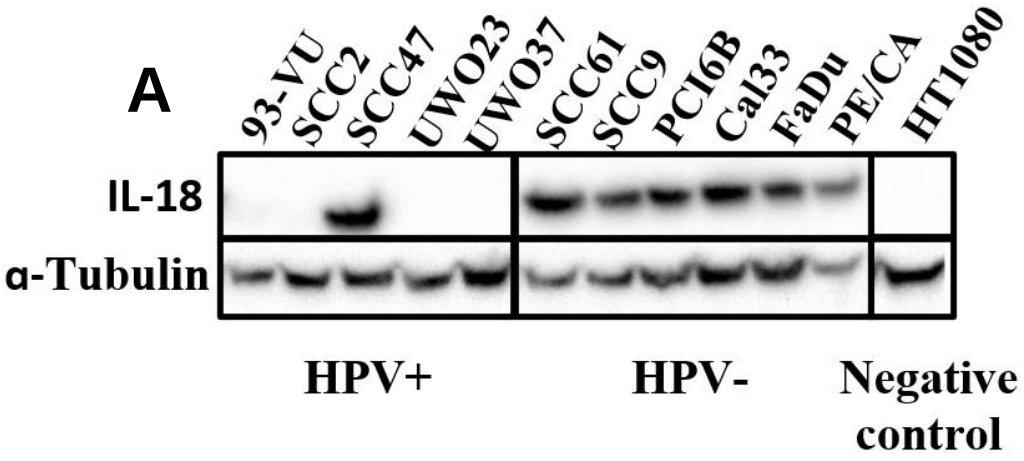
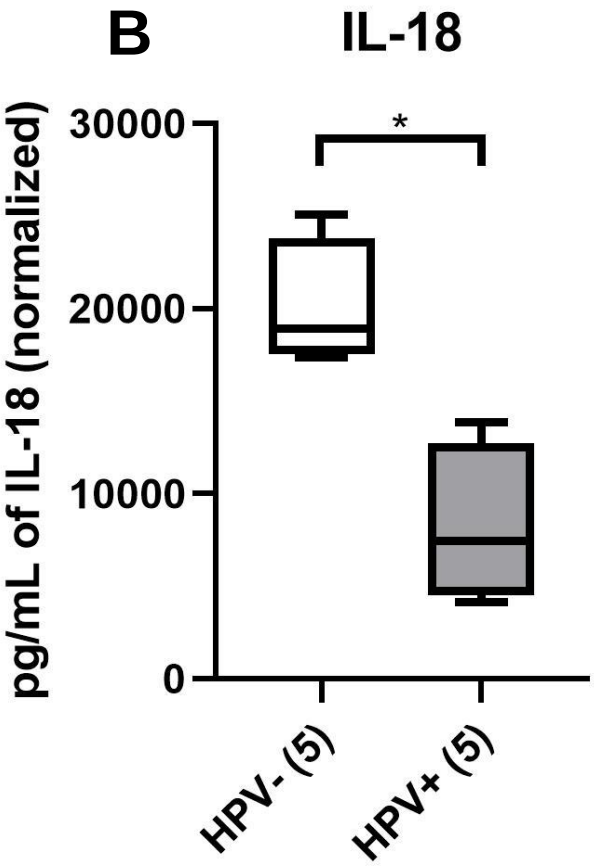


Figure 3.5: mRNA analysis of IL-18 and IL-18BP in established HNSCC cells lines. The mRNA levels of IL-18 (A) and IL-18BP (B) was measured by qPCR in HPV+ (SCC2, SCC47, SCC152, 93-VU, UWO23, UWO37) and HPV– (Cal33, PE/CA, SCC9, SCC40, SCC61, PIC6B, FaDu) HNSCC cell lines to determine the impact of HPV status on expression. **(A)** Significantly lower levels of IL-18 mRNA were detected in HPV+ HNSCC cell lines when compared to the HPV-cell lines ($p<0.05$, $p=0.0309$). **(B)** Significantly higher levels of IL-18BP mRNA were detected in HPV+ HNSCC cell lines when compared to the HPV-cell lines. Statistical analysis was conducted through a Non-parametric Mann-WhitneyU test ($p<0.05$, $p=0.0262$).

Figure 3.6: Analysis of IL-18 protein expression levels in established HNSCC cell lines.

I measured the protein levels of IL-18 (A) and α-tubulin (B) in HNSCC cell lines by western blot and secreted IL-18 by ELISA (C) in HPV+ (SCC2, SCC47, 93-VU, UWO23, UWO37) and HPV– (Cal33, PE/CA, SCC9, SCC61, PIC6B, FaDu) HNSCC cell lines to determine the impact of HPV status on expression. Western blots of the HNSCC cell lysate for **(A)** IL-18 to examine intracellular IL-18 with α-tubulin as a loading control. Intracellular IL-18 was nearly indetectable in HPV+ cells while most HPV- cells have consistently detected IL-18. **(B)** Using an ELISA to detect secreted IL-18 in cell culture media, there was significantly lower levels of IL-18 protein were detected in HPV+ HNSCC cell lines when compared to the HPV-cell lines ($p<0.05$, $p=0.0286$). Western blots and ELISA of IL-18BP protein failed to yield a detectable signal


A
93-VU
SCC2
SCC47
UWO23
UWO37
SCC61
SCC9
PCI6B
Cal33
FaDu
PE/CA
HT1080
IL-18
α-Tubulin
HPV+
HPV-
Negative control


B
IL-18
pg/mL of IL-18 (normalized)
30000
20000
10000
0
*
HPV- (5)
HPV+ (5)

3.3 Determining the Effects of HPV E7 on *IL18* Transcription.

3.3.1 The Effect of HPV16's E7 on the Promoter Region of IL-18 in Cal33 HNSCC Cells

As previously described, HPV E7 has been shown to be involved in alteration to gene expression through interactions with gene promoters (Chalerpet K. *et al.* 2015, Cicchini L. *et al* 2016, Cicchini L. *et al* 2017). To determine if HPV E7 affects the IL-18 promoter, I PCR amplified 1.5kb of the *IL18* genes promoter region from human genomic DNA and cloned it into the pGL3-Basic luciferase expression vector. This region was selected as it contains the *IL18* gene's promoter and sequences that demonstrated significantly more DNA methylation in the TCGA HPV+ samples when compared to HPV- samples and normal adjacent tissues (Figure 3.4 C-F). This region has also previously been shown to function as an active promoter in luciferase assays by another group (Kalina U. *et al* 2000).

To test the efficacy of this newly cloned vector, both pGL3-Basic and IL-18-Promoter-pGL3-Basic were transfected into Cal33 HNSCC cell lines, and after transfection the cell lysate was subjected to a luciferase assay. Cal33 cells were chosen because they are HPV-HNSCC cells that express a relatively high level of endogenous IL-18. This assay confirmed the newly constructed IL-18 reporter displayed significantly greater expression than the parent vector lacking the IL-18 promoter (Figure 3.7). This vector was then subsequently co-transfected with a mammalian expression vector expressing HPV-16 E7 fused with zsGreen fluorescent protein via a T2A self-cleavage site. This allowed me to confirm transfection efficiency and test the function the of HPV-16 E7 protein without potential confounding effects related to fusion to a large fluorescent protein tag. These experiments showed a significant decrease in the expression of luciferase by the IL-18 promoter when it was co-expressed with HPV-16 E7 (Figure 3.8A). I also tested the ability of HPV-16 E7 to impact the IL-18 promoter when tagged with both EGFP and myc tags at the N-terminus. Cal33 HNSCC cells co-transfected with the pCANmycEGFP-HPV16-E7 construct and IL-18 promoter luciferase vector similarly exhibited a reduction in luciferase

expression (Figure 3.8 B). Expression of HPV-16 E7 zsGreen vector was confirmed by western blots (Figure 3.9 A/B).

3.3.2 Mapping the Regions of HPV-16 E7 Involved in Repressing Transcription from the IL-18 Promoter in Cal33 HNSCC Cells

I next tested a series of HPV-16 E7 fragments for their ability to repress luciferase expression from the IL-18 reporter vector. I initially focused on the conserved regions of E7, which are referred to as conserved region 1 (CR1; spanning amino acids 1-15), conserved region 2 (CR2; spanning amino acids 16-28), and conserved region 3 (CR3; spanning amino acids 39-98). These regions were selected to determine which conserved region of E7 was responsible for suppression of the IL-18 promoter. The two fragments of the HPV E7 CR3 were also selected, spanning amino acids 39-58 and amino acids 59-98, as HPV E7 has been described to interact with DNMT1 through CR3 and this interaction could play are role in repression. I co-transfected Cal33 cells with the IL-18 luciferase reporter and either empty vector or the pCANmyc-EGFP constructs expressing the different conserved regions of HPV-16 E7. I observed a significant reduction in the expression of luciferase by the vector expressing the first 19aa of CR3 (pCAN-myc-EGFP-E7aa39-58) when compared to the transfected empty vector, while the other portions of HPV-16 E7 had no significant effect of the expression of luciferase, except for full length HPV-16 E7 (Figure 3.10).

3.3.3 Testing the Effects of Various HPV E7s on Transcription from the Promoter Region of *IL-18* in HNSCC Cal33 Cells

I next examined a panel of different high and low-risk HPV E7 proteins on the expression of the IL-18 reporter in Cal33 HNSCC cells. This panel of 11 HPV E7 genes were expressed from the same pCANmyc-EGFP mammalian expression construct used for HPV-16 E7. I observed near universal suppression of luciferase expression by the E7 proteins of all HPV types, with the noticeable exception of HPV-31 E7 (Figure 3.11). Thus, most, but not all HPV types appear capable of repressing transcription from the IL-18 promoter.

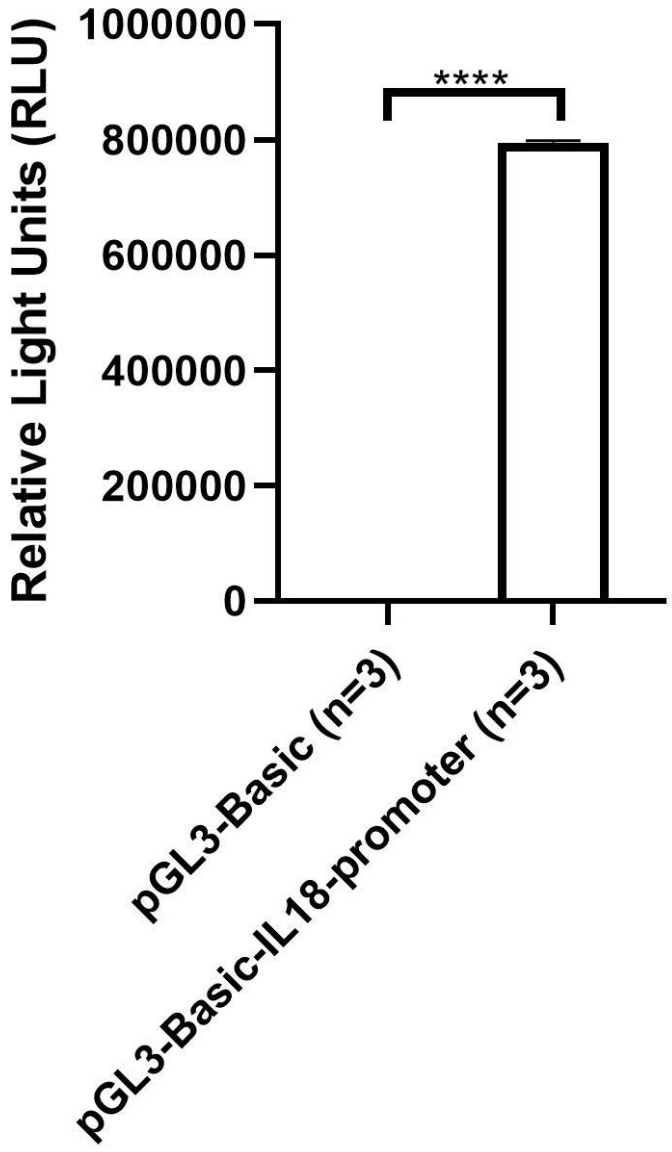


Figure 3.7: A 1.5kBp fragment of the IL-18 promoter is sufficient to drive expression of luciferase transcription when transfected into Cal33 HNSCC cells. Transfections of Cal33 cells were done using Lipofectamine 3000. When compared to the transfection of a promoter-less pGL3-Basic reporter construct, there was a significant increase in the production of luciferase by the vector containing a 1.5kb fragment of the IL-18 promoter. Statistical analysis was conducted through a parametric t-test ($p<0.0001$=****, $p<0.001$=***, $p<0.05$=*, $p>0.05$=ns)

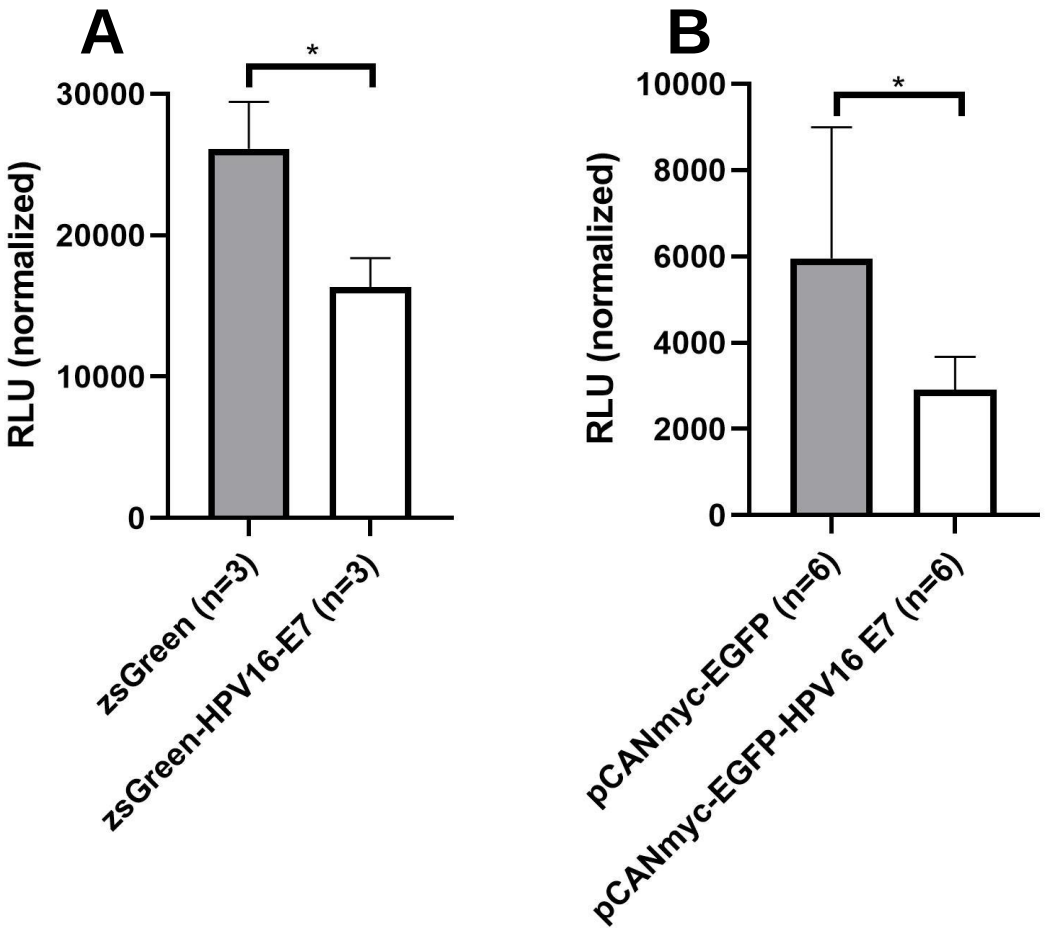


Figure 3.8: Expression of luciferase by the IL-18 reporter is repressed by co-transfection with HPV-16 E7 constructs.
Co-transfections of Cal33 cells were done using lipofectamine 3000 transfection reagent. There was significantly less luciferase expression when the construct was co-transfected with either **(A)** zsGreen HPV-16 E7 expression vector or **(B)** the pCANmyc-EGFP HPV-16 E7 vector. Statistical analysis was conducted through a parametric t-test ($p<0.0001$=****, $p<0.001$=***, $p<0.05$=*, $p>0.05$=ns)

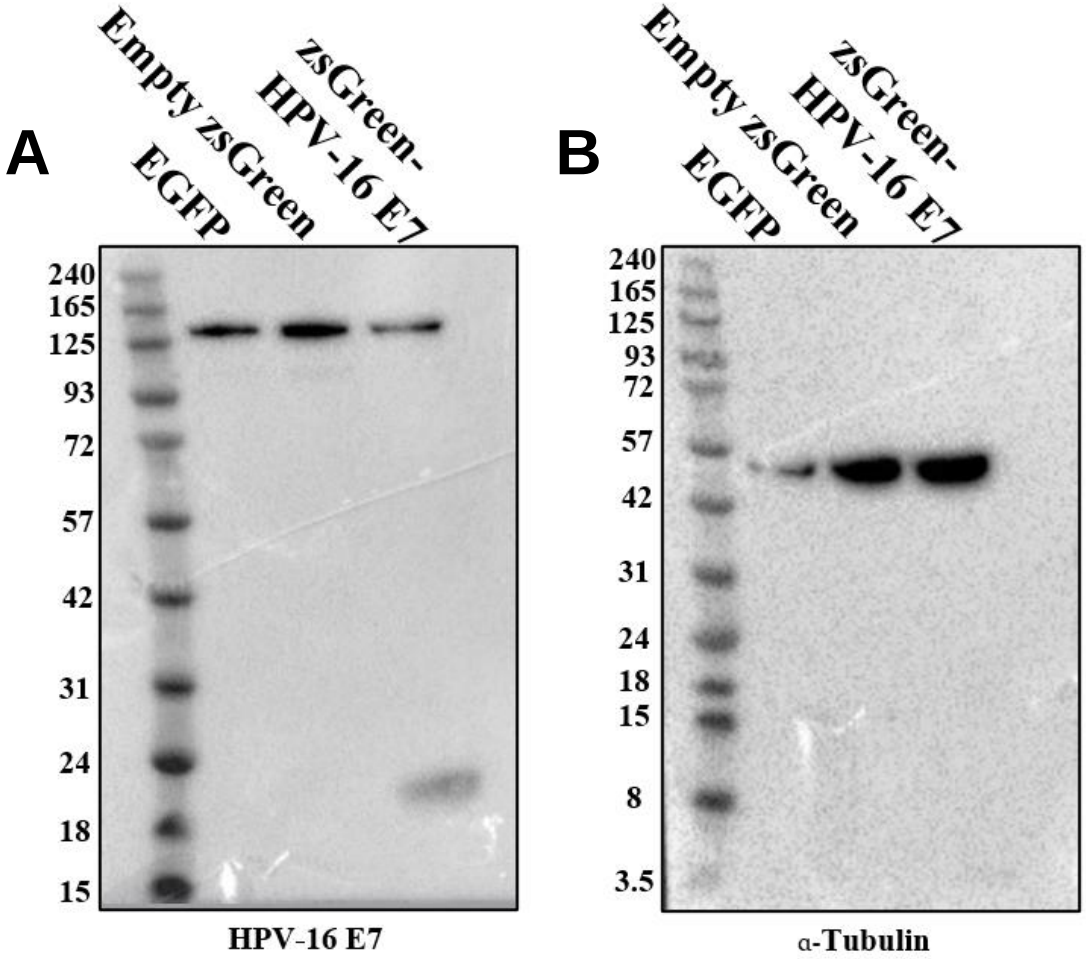


Figure 3.9: Expression of HPV-16 E7 by zsGreen

A western blot was performed using cellular lysate from Cal33 cells transfected with the empty vector zsGreen vector, a transfection control EGFP expressing vector or our zsGreen-HPV-16 E7 mammalian expression vector. **(A)** The blot was probed using anti-E7 antibody and the arrow indicates the band with the size expected for E7 with an additional blot of the cell lysate for our **(B)** loading control of α-tubulin.

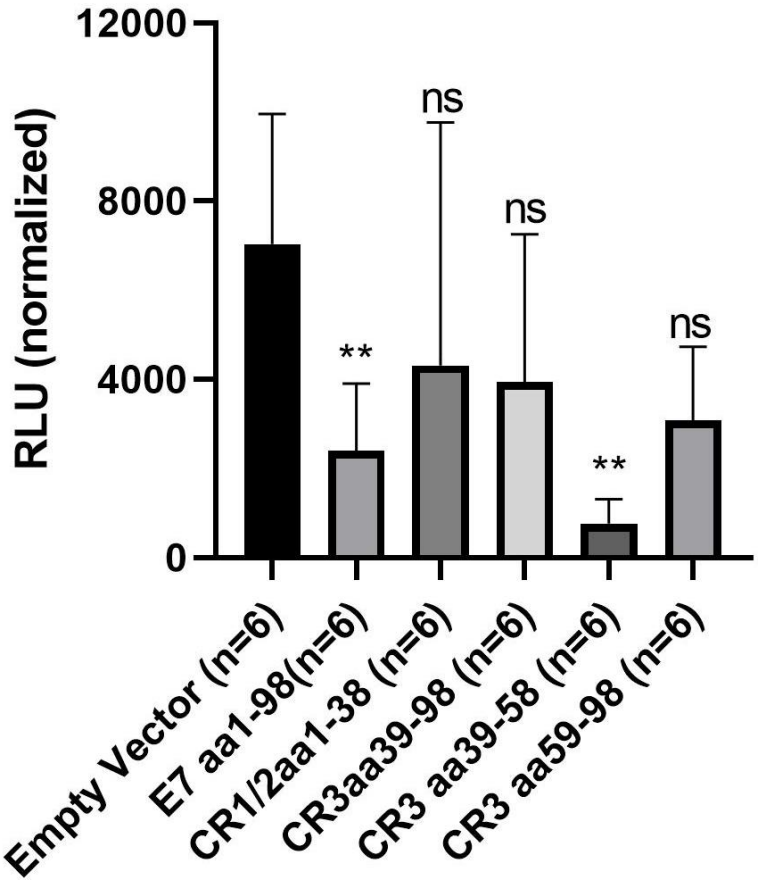


Figure 3.10: Mapping the region of HPV-16 E7 required for repressing expression of the IL-18 promoter luciferase construct luciferase.
Cal33 HNSCC cells were co-transfected with the IL-18 luciferase reporter and vectors expressing the indicated portions of HPV-16 E7 denoted by the amino acids (aa) present. There was significantly less expression of luciferase when the construct was co-transfected with full length E7 or the fragment corresponding to aa39-98. Statistical analysis was conducted through a parametric t-test ($p<0.0001$=****, $p<0.001$=***, $p<0.05$=*, $p>0.05$=ns).

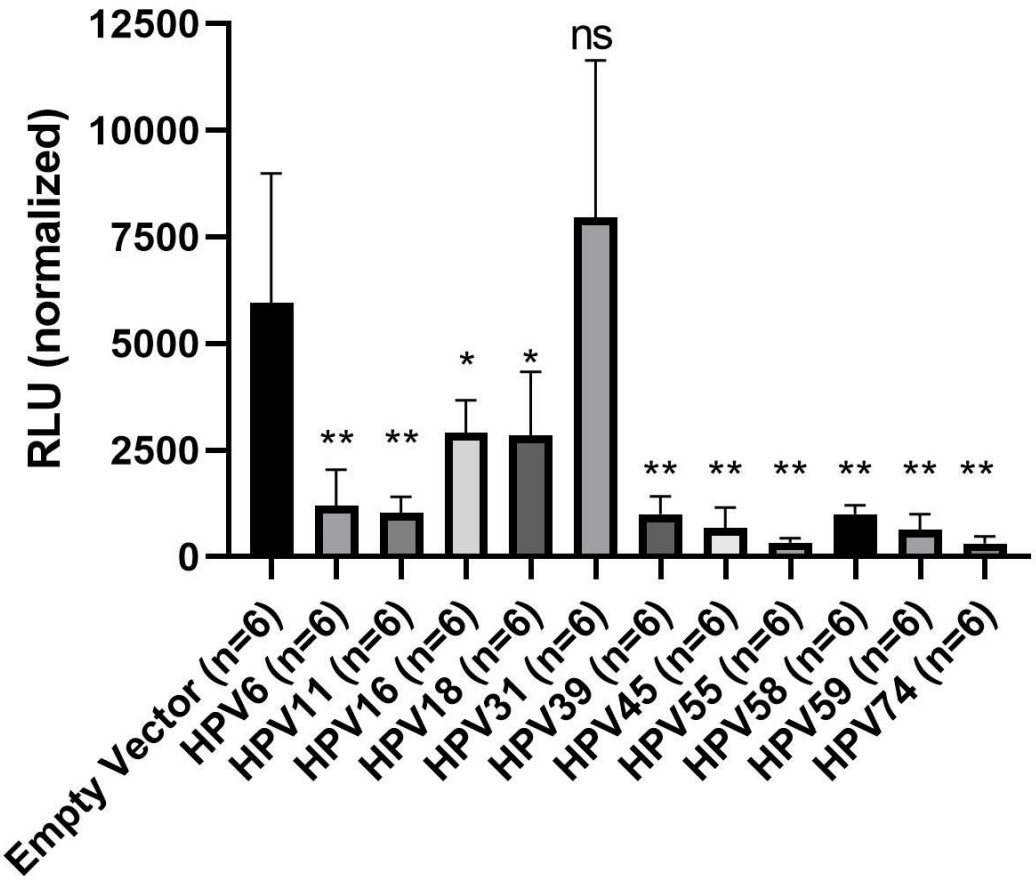


Figure 3.11: The E7 proteins from most HPV types repress expression from the IL-18 luciferase reporter.

Cal33 HNSCC cells were co-transfected with the IL-18 luciferase reporter and vectors expressing the E7 proteins of the indicated HPV types. The E7 proteins from all HPV types except HPV-31 repressed IL-18 expression. Statistical analysis was conducted through a parametric t-test ($p<0.0001$=****, $p<0.001$=***, $p<0.05$=*, $p>0.05$=ns).

Chapter 4

4 Discussion

4.1 General Discussion

4.1.1 The Suppression of the IL-18 Pathway is a Common Element Across HPV+ Head and Neck Cancers in the TCGA Head and Neck Cohort

HPV is a human tumor virus that is responsible for a significant fraction of human cancers (Bansal A. *et al* 2016). In addition to altering host cell growth, we hypothesized that the ability of HPV to suppress local immune responses could contribute to immune evasion and chronic infection. The work presented in this thesis examines the effects of HPV on the immune system, with a specific emphasis on IL-18. IL-18 is a constitutively expressed proinflammatory cytokine in the epithelium (Sugawara S. *et al* 2001). This cytokine is an important component of the barrier function of the mucosa tissues typically infected by HPV. IL-18 has a wide variety of antiviral and anticancer effects in the body through its stimulatory effects on NK cells and T-cells, which leads to the subsequent production of IFNγ. In the context of HPV infections, avoiding these cytotoxic immune cells and apoptosis of infected or oncogenically transformed host cells is important for persistence of viral infection and tumour survival. We hypothesized that the HPV oncogenes expression in HPV-dependent head and neck cancers suppresses the IL-18 proinflammatory pathway, contributing to oncogenesis.

Through analysis of the data from the TCGA, I observed a clear reduction in the level of *IL18* mRNA in HPV+ head and neck cancers compared to their HPV- counterparts and normal non-cancerous adjacent tissues. *IL18* appeared to be specifically targeted, as expression of the other IL-1 superfamily cytokines were not reduced in HPV+ cancers when compared to HPV- cancers and normal adjacent tissues. Likely, IL-18 is the largest barrier to HPV during the infection of the epithelium, as it is the only cytokine within this family that is constitutively produced within these tissues (Lopez-Castejon G. and Brough D. 2017, Seidelin J. B. *et al* 2009). Although constitutively expressed, Il-18 expression is

further elevated in epithelial cells in direct response to the detection of dsDNA outside of the cell nucleus through the AIM2 inflammasome (Yogarajah T. *et al* 2017).

In addition to IL-18 itself, I extended my studies to examine the impact of HPV on the expression of other components of the IL-18 pathway using gene expression data from the TCGA head and neck cohort. In agreement with the findings of the MacDonald lab (Richard K. H. *et al* 2014), I found that *IL18BP* mRNA expression was elevated in HPV+ cancers when compared to HPV- cancers and normal non-cancerous tissues. Expression of *CASP1*, which cleaves and activates pro-IL-18, was also found to be reduced in HPV+ cancers when compared to HPV- cancer and normal adjacent tissues. Interestingly, *CASP1* mRNA expression appears to be elevated in HPV- cancers when compared to normal adjacent tissues, accentuating the difference between HPV+ and HPV- HNSCC. *PYCARD*, which encodes ASC, a key component of the inflammasomes that activates caspase-1, is similarly reduced in HPV+ cancers when compared to HPV- cancers. Taken together, these results suggest HPV exerts a concerted antagonistic effort on expression of the components of the IL-18 inflammatory pathway in head and neck cancers, which may contribute to this disease.

HPV-16 is the most frequent HPV associated with HNSCC (Kobayashi K. *et al* 2018). However, infection with other high-risk HPVs like HPV-33 can also lead to these cancers, and these exhibit a poorer prognosis in comparison to HPV16+ HNSCC (Chatfield-Reed K. *et al* 2020, Bratman S. V. *et al* 2016). A comparison of the relative *IL18* mRNA expression levels across patient samples did not identify any significant difference between HPV-16+ cancers or those caused by other HPV types. This suggests that all HPVs can reduce *IL18* mRNA expression and that the relative activity of this pathway does not contribute to the observed differences in patient prognosis between these two groups of HPV+ HNSCC.

4.1.2 IL-18 and IL-18BP Expression is also Altered in HPV+ HNSCC Cell Lines

After our examination of the expression of IL-18 pathway components in primary head and neck cancer tissues from the TCGA, we examined cultured HNSCC cell lines to see if they

similarly exhibit the same HPV dependent IL-18 repression and IL-18BP upregulation. Not only was I looking for a replication of the TCGA findings, I was also hoping to determine if HNSCC cell lines could serve as potential working models for studying the mechanism by which HPV affects the IL-18 pathway in terms of experimentation and laboratory manipulation. I examined a panel of HPV+ and HPV- HNSCC-derived cell lines for mRNA expression levels of IL-18 pathway components by qPCR, as well as internal and secreted protein levels by western blot and ELISA, respectively. My findings in these cell lines reflected the findings of the TCGA. Specifically, there was a significant reduction in *IL18* mRNA and IL-18 protein in HPV+ cell cultures compared to HPV- cell cultures. In addition, the expression of *IL18BP* mRNA was significantly increased in HPV+ cell lines when compared to HPV- cell lines. However, I was unable to detect IL-18BP protein intracellularly or within the media from cultured cells. My observation that the IL-18 pathway is similarly repressed in HPV+ HNSCC cell lines compared to HPV- lines suggested that the continued expression of HPV oncogenes in these cells is likely consistent or correlates with these effects. These results also suggest that experimental manipulation of HPV- HNSCC cell lines may allow us to investigate the mechanism by with HPV affects this pathway.

4.1.3 HPV Suppression of *IL18* mRNA Expression may be an Epigenetic Effect Mediated by Changes in Promoter Methylation.

IL-18 is constitutively expressed in the epithelium (Sugawara S. *et al* 2001). Actively expressed genes generally exhibit reduced promoter methylation. Importantly, HPV E7 has been shown to repress transcription by other genes by binding and activating DNMT1, a methyltransferase that induces the methylation of CpG sites in genomic DNA (Burgers W. A. 2007). I next determined if HPV was affecting methylation of the *IL18* locus in head and neck cancers using the genome wide methylation data from the TCGA. We looked at data from 24 DNA methylation probes surrounding the *IL18* gene. I found a pattern of significantly elevated DNA methylation in HPV+ cancers throughout the *IL18* gene promoter. These data suggest that increased methylation of the *IL18* promoter by HPV is at least in part responsible for the decreased level of *IL18* mRNA observed in HPV+ HNSCC. Interestingly, HPV+ precancerous lesions and cancers of the cervix develop

distinct patterns of DNA methylation, and this is considered a hallmark of these virally-derived cancers (Verlaat W. *et al.* 2018, Widschendter A. *et al.* 2004) that may similarly extend to HPV+ HNSCC.

4.1.4 HPV E7 is Sufficient to Repress Transcription from the *IL18* Promoter in Cell Culture Models

HPV-dependent head and neck cancers consistently retain expression of both the E6 and E7 oncogenes (Yeo-The N. S. L. *et al* 2018). Given the increases in *IL18* promoter methylation identified using the TCGA data, and the known interaction of HPV-16 E7 with DNMT1 and histone deacetylase 1 (HDAC1), we next tested whether HPV E7 was sufficient to repress transcription from the *IL18* promoter using luciferase reporter assays. We used a luciferase reporter containing a 1500bp of the *IL18* promoter that had previously been shown to be sufficient to direct transcription from the *IL18* gene (Kalina U. *et al* 2000) and also contains the CpG sequences exhibiting the largest changes of methylation between HPV+ and HPV- head and neck cancers. We selected the Cal33 cell line for these experiments, as they are HPV-, express high levels of endogenous IL-18 and are relatively easy to transfect with plasmid DNA. In these experiments, expression of HPV-16 E7 significantly decreased the activity of the *IL18* promoter when compared to our control vectors that did not express E7. This result shows that HPV-16 E7 can suppress transcription from the *IL18* promoter in HPV- cell lines and that this functions via elements contained within the 1500bp located upstream of the *IL18* transcription start site.

Further luciferase assays with vectors expressing isolated conserved regions of HPV-16 E7 mapped the repression function of E7 to a subregion of CR3 spanning residues 39-58. CR3 is responsible for the ability of HPV to bind several proteins involved in gene expression, like DNMT1 and HDAC1, which function by methylating CpG sites, deacetylation of histones, and altered chromatin structure (Delcuve G. P. *et al* 2012, Damelin M. and Bestor T. H. 2007). Although not precisely defined, the interactions of these epigenetic regulators with E7 is usually attributed to the zinc binding domain found near the end of CR3 (Brehm A. *et al* 1999, Longworth M. S. and Laimins L. A. 2004). However, Ski interacting protein (SKIP), a transcriptional co-regulator that aids in overcoming the pRb-mediated cell cycle checkpoints, is known to interact with the 19 amino acids at the beginning of CR3 of

HPV16 E7 (Prathapam T. *et al* 2001). Future investigations into a potential role for SKIP in IL18 dysregulation by HPV should be a high priority.

I also tested a panel of high and low risk HPV E7's for repression of transcription from the *IL18* promoter, and this showed that all the different HPV E7's were able to suppress luciferase activity, with the exception of HPV31 E7. This is also consistent with the data from the TCGA, which showed that head and neck cancers caused by other HPV types besides HPV-16+ cancers expressed similarly reduced levels of *IL18* mRNA. Thus, repression of *IL18* transcription seems to be a highly conserved function across most HPV types, whether high or low risk. Although suppression of the IL-18 pathway may contribute to cancer formation, it is clearly not sufficient, as low risk HPVs also possess this activity.

4.2 Conclusion

The goal of this thesis was to examine the effect of HPV on the IL-18 pathway in head and neck cancers. My investigations using TCGA data from primary human tumors, cultured HNSCC cells, and the IL-18 promoter all indicate that there is a concerted effort by HPV to suppress the activity of IL-18 through the repression of its expression, repression of its activators, and increased expression of IL-18BP, a negative regulator of IL-18. Furthermore, our results indicate that suppression of IL-18 is mediated via the CR3 region of E7 and is a conserved function of both high and low-risk E7. A summation of the effect of HPV on the IL-18 pathway is presented in Figure 4.1A.

My studies identified additional avenues by which HPV can maintain an anti-inflammatory environment to sustain persistent infections that can evade the immune system for months to years within their hosts. Specifically, I have found that HPV can suppress the activity of the proinflammatory IL-18 pathway, which is vital in epithelial defense. This expands our understanding beyond the already known ability of the HPV E6 and E7 oncogenes to increase the expression of IL-18BP (Richards K. H. *et al* 2014). Indeed, our results suggest that the pervasiveness of IL-18 suppression extends far further, to include the effects on expression of inflammasome components and IL-18 itself. The concerted downregulation of IL-18 activators and IL-18 itself confirms the importance of the IL-18 pathway in epithelial barrier function. It further emphasizes how important the reduction of the activity

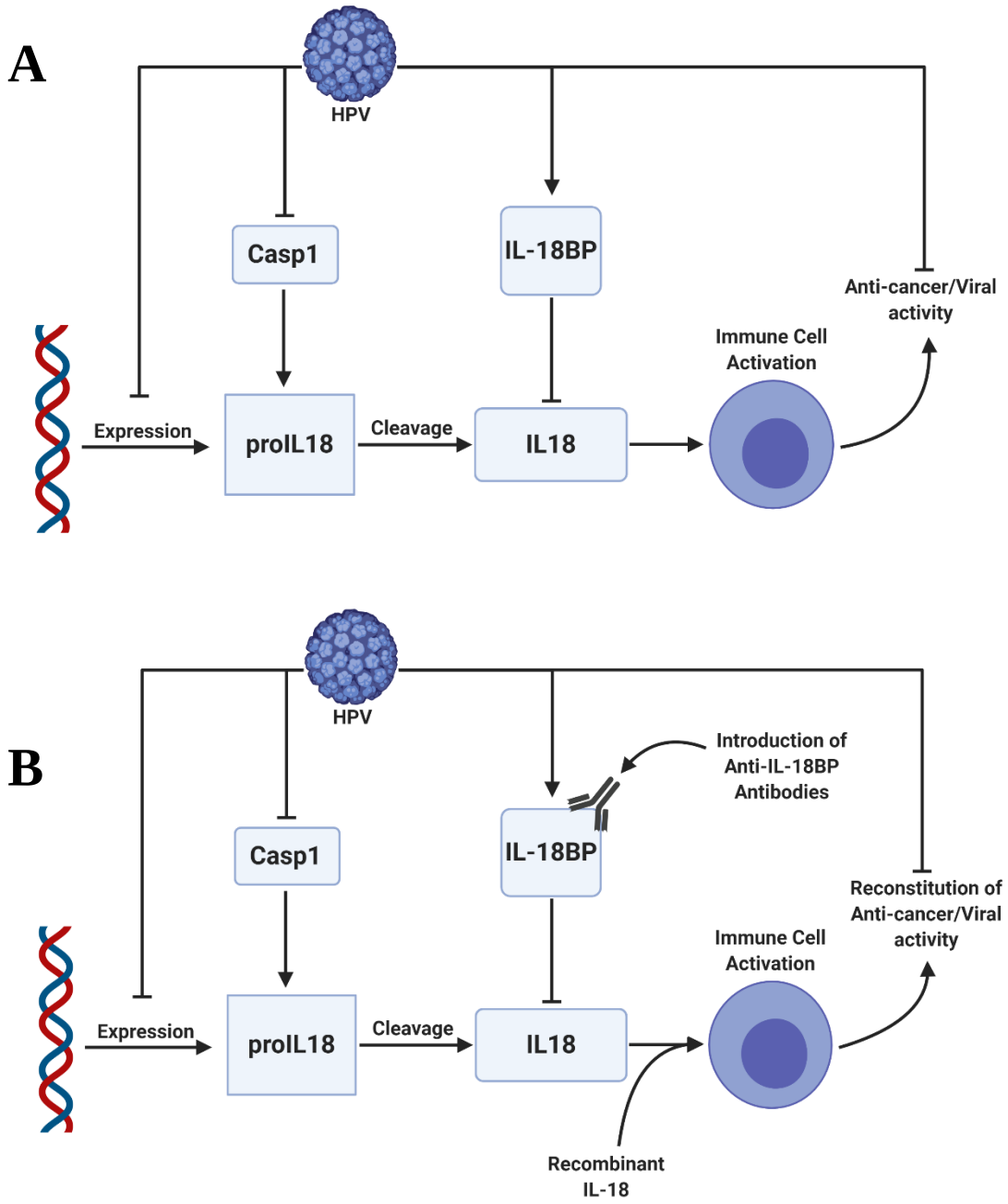


Figure 4.1: A model of the effect of HPV on the IL-18 proinflammatory pathway.

(A) A model of the regulation imposed by HPV on the IL-18 pathway through the repression of IL-18's activator caspase-1 and IL-18 itself, and the upregulation of IL-18BP. **(B)** Points of intervention that could potentially restore immune cell activation dependent on IL-18's antiviral and anticancer activity

of this cytokine is for the HPV replication cycle as well as the oncogenesis of HPV dependent head and neck cancer.

In addition to a further understanding of HPV's interactions with the host immune system, this work suggests that the loss of the IL-18 proinflammatory response could contribute to the virus's ability to induce cancer. The loss of the IL-18 immune response means a loss of the cells ability to activate immune cells in response to the detection of dsDNA in the cytoplasm, a likely indication of HPV infection. As discussed earlier in this thesis, HPV+ cancers are mostly derived from chronic infections with high-risk HPV. These long-term persistent infections are likely aided by the loss immune of cell stimulation. IL-18-mediated activation of NK cells and cytotoxic T-cells not only functions to enhance the destruction of infected cells, but to also destroy cells undergoing uncontrolled replication and showing signs of cellular stress (Yasuda K. *et al* 2019). The loss of these activities could protect HPV infected cells from clearance by the immune system, by contributing to the formation of an anti-inflammatory haven for these precancerous lesions to develop and progress to malignancy.

My studies may have additional impact beyond an enhanced understanding of the biology of HPV infection. Importantly, my work raises the possibility that the restoration of the IL-18 response in these HPV+ cancers could potentiate the benefits of traditional cancer treatments and immunotherapies. Recombinant IL-18 has been purified, is commercially available, and can potentially act to stimulate other immunotherapies in the reactivation of cytotoxic immune cells (Robertson M. J. *et al* 2006, Senju H. *et al* 2018, Zhou T *et al* 2020). For example, IL-18 has been shown to have several protective and stimulatory effects when used in mouse models of gastrointestinal cancer models (Sussman D. A. *et al* 2012, Salcedo R. *et al* 2010), lymphoma models (Ni J. *et al* 2012, Srivastava S. *et al* 2013), melanoma models (Nagai H. *et al* 2002, Cho D. *et al* 2000), and bacterially induced cancer models. These studies show that increased activity in NK cells resulted in reduced tumorigenesis, inhibited tumour growth, and protected against metastatic cancer spread, suggesting that IL-18 could enhance immunotherapy (Srivastava S. *et al* 2010). Alternatively, to combat the HPV-induced overexpression of IL-18's suppressor IL-18BP, the introduction of an antibody-based therapeutic that blocks IL-18BP's binding site could

also allow reactivation of endogenous IL-18 signaling by blocking the sequestration of the IL-18 cytokine (Figure 4.1 B). This would likely have to be used sparingly and in careful dosages, as a chronic overabundance of IL-18 has been associated with inflammatory skin diseases like systemic lupus erythematosus and psoriasis, as well as bowel disease like irritable bowel syndrome (IBS) and Crohn's disease (Kanai T. *et al* 2001, Favilli F. *et al* 2009, Lee J. H. *et al* 2015).

4.3 Future Directions and Experiments

Studies of the effect of HPV on the activity of IL-18 is far from over, and there are several additional experiments that would benefit our understanding of their interactions. This includes using an existing series of point mutants within HPV-16 E7 to identify the exact residues of E7 necessary for repressing transcription from the promoter, examining the ability of non-oncogenic E7s to repress the IL-18 promoter, examining the differences between HPV-16 and HPV-31 E7, and further experimental analysis to try to measure the impact of HPV of the expression of IL-18BP protein. Each of these is briefly described below.

A previous PhD student within the Mymryk lab, Dr. Biljana Todorovic, developed an extensive series of point-mutants within HPV-16's E7, all cloned within the same mycEGFP mammalian expression vector used in my studies (Todorovic B. *et al* 2012). Luciferase assays using these point mutants within CR3 could identify which amino acids are important to the suppression of the IL-18 promoter. Coimmunoprecipitations assays with HDAC1, DMNT1, and SKIP would confirm that wild-type HPV-16 E7 is indeed binding to these transcriptional modulators, and if binding depends on the same regions required for repression of transcription.

Additionally, a comparative analysis of the E7 proteins from HPV-16, HPV-31, and non-oncogenic HPVs, like the Beta-HPV E7s, could help illuminate the mechanisms in which HPV mediates the oncogenesis of their host cells. Examining the non-oncogenic E7's through another series of luciferase experiments could help to understand if the suppression of IL-18 is a function conserved only among cancer causing HPV, or if it is a common activity among most HPVs. Additionally, as HPV-31 did not repress the IL-18 luciferase

vector, I would first like to confirm the expression of E7 from HPV-31 within these cells, as lack of activity could simply reflect a lack of expression. Should the presence of HPV-31 E7 be confirmed, a comparative analysis of the amino acids sequences that differ between HPV-31 and HPV-16 E7 proteins could help identify the important motifs necessary for repression of expression and confirm our point mutation data.

Further experimentation using our cultured cell lines would extend our understanding of the mechanistic basis by which E7 represses IL-18 expression. One such experiment would be through the stable lentivirus-mediated transduction of HPV-16 E7, selected E7 point mutants lacking activity, or an empty control vector into our HPV- cell lines. We would then be able to examine the effects of E7 in a context that more closely resembles a long-term HPV infection. Here, we could conduct an analysis of the endogenous changes in IL-18BP mRNA, promoter methylation, histone acetylation, and IL-18's protein and mRNA through qPCR, methylation analysis, western blots, and ELISAs. The examination of such an engineered cell line through qPCR and western blots could be expanded to include studies of the effect of E7 on the other modulated proteins we identified using the TCGA data, including caspase-1, This would lead to a more comprehensive understanding of the effect of HPV on their expression. This would also provide a more realistic model examining the effect of E7 on histone acetylation and DNA methylation, as they would be interacting with chromosomal DNA instead of transiently transfected promoter templates within a circular plasmid. As E7 interacts with both HDAC1 and DNMT1, they may both functions to exhibit synergistic effects in IL-18 downregulation.

Additionally, as I was unable to detect IL-18BP production by the HNSCC cell lines or induce its expression with IFNγ, I would like to conduct further studies using an *in vitro* model in which IL-18BP can be examined. The MacDonald laboratory (Richards K. H. *et al* 2014) previously reported that they were able to induce the production of IL-18BP with IFNγ within cell cultures of primary foreskin keratinocytes. Future experiments introducing E7 into primary keratinocytes could be more relevant than culture models using tumor cell derived lines for these studies as the activity of E7.

In summation, IL-18 is an important component of barrier maintenance within the epithelium, acting as a first responder cytokine in the activation of both the innate and adaptive immune system. To avoid this response, HPV employs several mechanisms to downregulate this inflammatory pathway. This includes downregulating the expression of *IL18*, the downregulation of its activators *CASP1* and *AIM2*, and the upregulation of the negative regulator *IL18BP*. The downregulation of IL-18 can be attributed to the activity of the HPV oncoprotein E7, specifically via CR3. HPV mediated downregulation of IL-18 transcription is mediated through the first 1.5kbp of the *IL18* promoter. This biological phenomenon warrants further experimental exploration, including the mechanistic studies related to E7 interactions with this promoter, transcriptional regulatory factors, and the important motifs within E7 that mediate this binding. It will also be important to examine this loss of proinflammatory cytokines activity in tumor models, as the downregulation of IL-18 results not only in the loss of its antiviral properties, but also downregulates its anti-cancer properties, which could contribute to the formation and maintenance of an anti-inflammatory environment that allows for HPV infected cells to progress to cancer.

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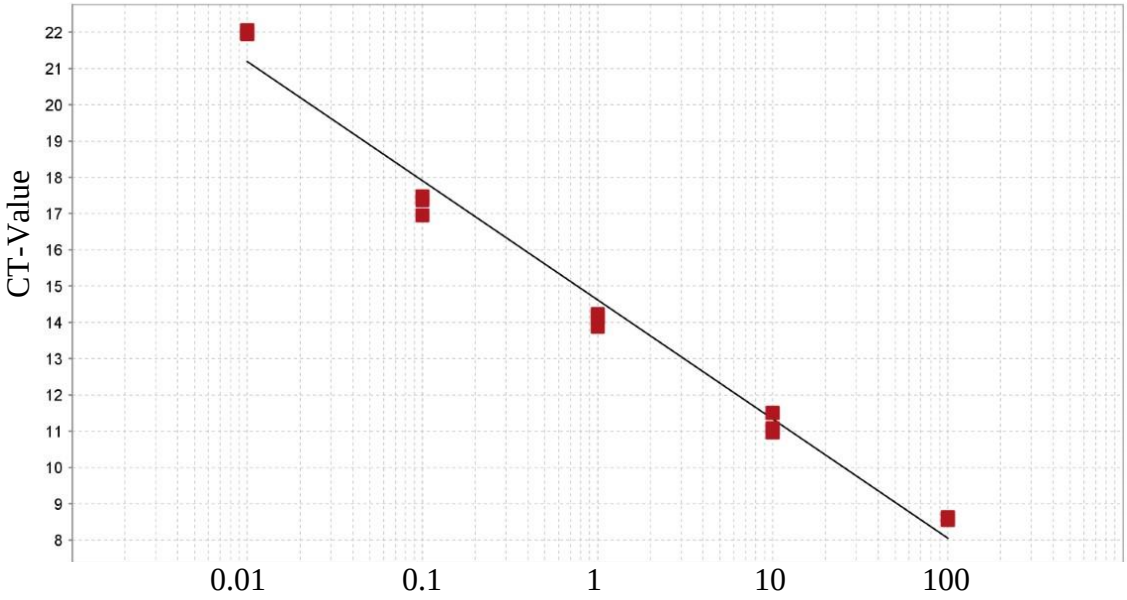
Appendices

Appendix A. Standard curves of GAPDH, IL-18, and IL-18BP used for the calculation of mRNA counts.

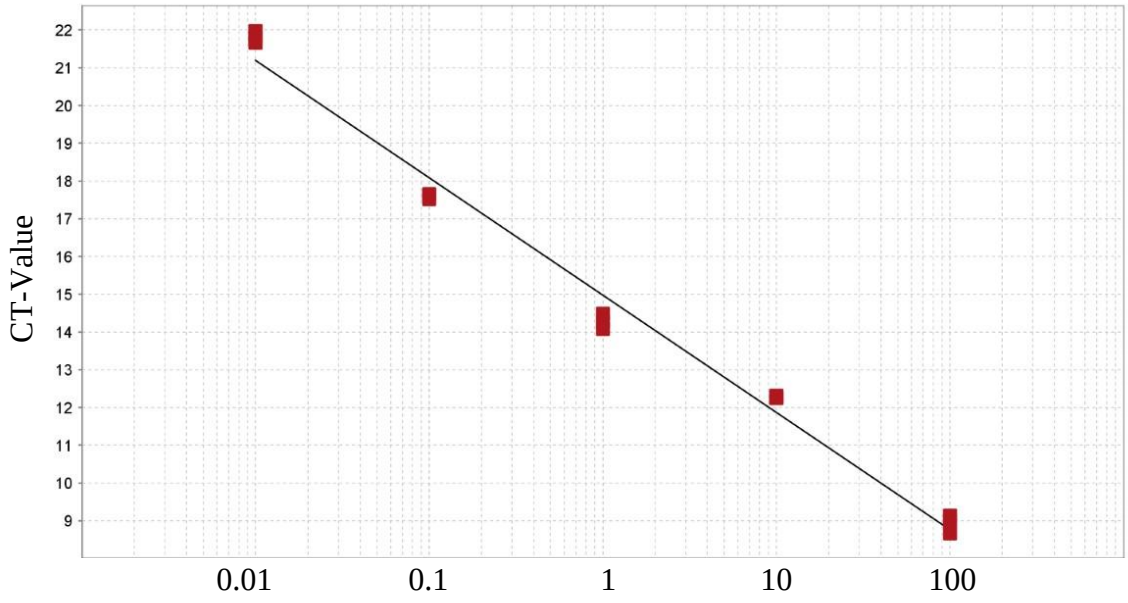
I used plasmid DNA containing the transcribed sequences of **(A)** GAPDH, **(B)** IL-18, and **(C)** IL-18BP within. qPCR of a serial dilution of these plasmids allowed us to develop standard curves in which were utilized in mRNA calculations. To assure proper equation of the line is developed, correlation value of R^2>0.95, and an efficiency of 100%+/-10% must be achieved.

A **GAPDH**



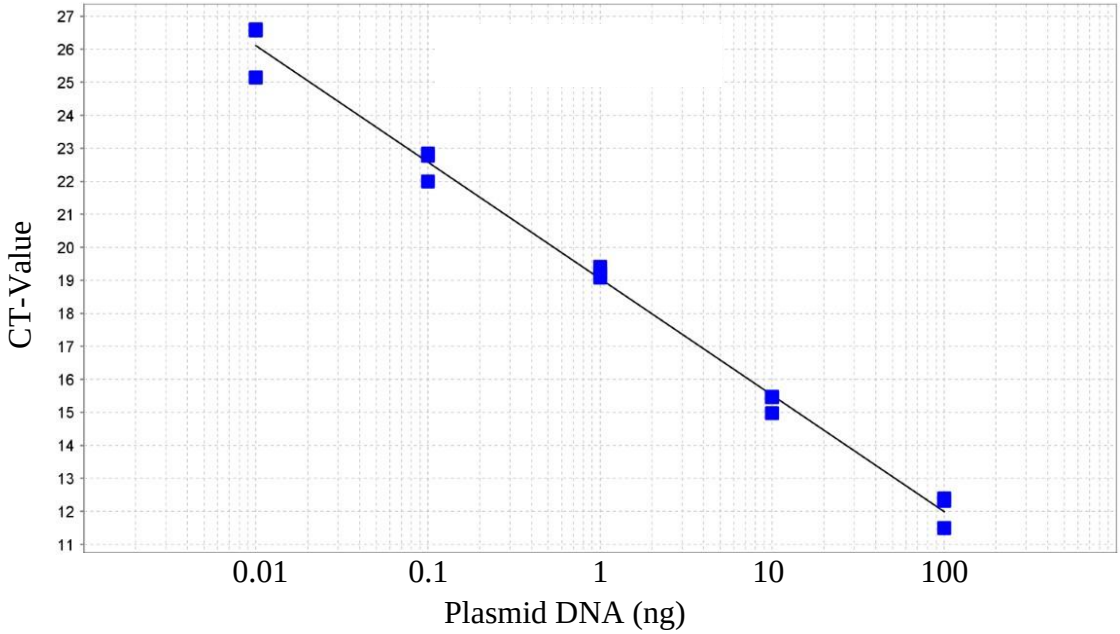
Slope: -3.286, Y-intercept: 14.623, R^2: 0.984, Efficiency: 101.53%

B **IL-18**



Slope: -3.109, Y-intercept: 14.981, R^2: 0.986, Efficiency: 109.728%

C **IL-18BP**



Slope: -3.531, Y-intercept: 19.05, R^2: 0.992, Efficiency: 91.972%

Curriculum Vitae

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International Centre for Genetic Engineering and Biotechnology
Trieste, Italy

Abstract and presentation entitled “The Effect of HPV on the Expression of the Cytokine IL-18 in Head and Neck Squamous Cell Carcinomas.” Selected by committee and presented at the ICGEB DNA tumour virus international conference based specifically within the field of viruses which induce cancers in Trieste, Italy.

Talks on Friday (TOFS) **February 2019**
Lawson Health Research Institute
London, Ontario
Abstract and presentation entitled “The Effect of HPV on the Expression of the Cytokine IL-18 in Head and Neck Cancers.” Selected by TOFS coordinator and presentation at St. Joseph’s Hospital in London, Ontario, Canada.

Poster Presentations

Infection and Immunity Research Forum **November 2019**
Great Hall, Western University
London, Ontario
Abstract and poster entitled “The Effect of HPV on the Expression of the Cytokine IL-18 in Head and Neck Squamous Cell Carcinomas.” Selected by student organized conference for students of Southern Ontario. Abstract and poster presentation presented in London, Ontario, Canada.

Oncology Research Day **June 2018, June 2019**
Best Western/Lamplighter Inn
London, Ontario
Abstract and poster entitled “The Effect of HPV on the Expression of the Cytokine IL-18 in Head and Neck Squamous Cell Carcinomas.” Ontario-wide conference of presentations showcasing the research of students studying human cancers from both scientific and clinical perspectives. Abstract and poster presentation presented in London, Ontario, Canada.

London Health Research Day **April 2019**
London Convention Centre
London, Ontario
Abstract and poster entitled “The Effect of HPV on the Expression of the Cytokine IL-18 in Head and Neck Squamous Cell Carcinomas.” A conference held within London, Ontario with province wide attendance for student and researchers to showcase their work. Presented in London, Ontario, Canada.

Western University Health and Research Conference **October 2019**
Physics and Astronomy Atrium, Western University
London, Ontario
Abstract and poster entitled “The Effect of HPV on the Expression of the Cytokine IL-18 in Head and Neck Squamous Cell Carcinomas.” WUHRC is a conference held within Western University in which Graduate students present their research to undergraduate students interested in graduate studies. Presented in London, Ontario, Canada.

Volunteer Positions

Partners in Experiential Learning Program **September 2019 – February 2020**
Training and supervision of a cooperative education high school student (Nayana Thomas). Daily mentoring, and directing the education and experimentation of her activities within the Mymryk lab.

Sponsor Liaison **February 2018 – November 2019**
Infection and Immunity Research Forum
Contacting, collecting, and coordinating the donations from industry sources for the student run conference IIRF. Worked in event organization and presentation selections

Science Rendezvous Guide **April 2019**
Labour, directions, and greeting for the community science outreach program of Science Rendezvous

Member, Student Outreach Committee **October 2018**
Dept. of Microbiology & Immunology
Representative for the Department of Microbiology and immunology to prospective graduate students

Student Representative, Seminar Committee **October 2018 – August 2019**
Dept. Microbiology & Immunology
Responsible for attending meetings and representing student interest regarding R.G.E. Murray Seminars

Scholarships and Awards

Western Graduate Research Scholarship **September 2018 – May 2020**
$1000 award for Graduate studies

The Western Scholarship of Excellence **September 2014**
$1000 reward for a high entrance average to Western University

Passport to Education **September 2014**
$500 provincial award for a high entrance average to Western University

Publications

Zhang A., Tessier T. M., Galpin K. J. C., King C. R., Gameiro S. F., **Anderson W. W.**, Yousef A. F., Quin W. T., Li S. S. C., and Mymryk J. S. (2018) "The Transcriptional Repressor BS69 is a Conserved Target of the E1A Proteins from Several Human Adenovirus Species". *Viruses* 10(12):662