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Suppression of Innate Immune Response by Primary Human Keratinocytes

Expressing HPV-16 E6 and E7

By

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Submitted in Partial Fulfillment

of the

Requirements for the Degree

Doctor of Philosophy

Supervised by

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Curriculum Vitae

Jennifer Letitia Changery Guess was born in State College, Pennsylvania. She attended the United States Air Force Academy from 1990 to 1994, and graduated with a Bachelor of Science degree and a military officer commission in 1994. She attended the University of Michigan from 1998 to 1999 and graduated with a Masters of Public Health degree in 1999. She came to the University of Rochester in the Summer of 2002 and began graduate studies in Microbiology and Immunology. She pursued her research in immunology aspects of human papillomavirus under the direction of Professor Dennis McCance and received the Master of Science degree from the University of Rochester in 2004.

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My sincerest appreciation and gratitude is extended to my family. First to my parents, who long ago gave me the strength, independence, and love that have enabled me to succeed. Also, to my husband and best friend who has supported me through some of my weakest and strongest times.

Abstract

Human papillomavirus (HPV) types infect the skin and mucosal epithelium. Lesions resulting from HPV infection can linger for months or years suggesting that HPV presence goes unnoticed by the host immune system. If allowed to persist, the high-risk HPV types can result in malignant cellular transformation and eventual progression to invasive carcinoma. The importance of the immune system in clearing HPV infection is substantiated by studies involving immunocompromised individuals. HPV infection could evade the immune response through (1) suppression of immune mediators which direct the migration of immune cells to the site of infection, (2) inhibition of antigen presentation to immune cells, or (3) alteration of the function of immune cells. Histological studies of HPV-positive tissue reveal a lack of immune cells, particularly Langerhans cells (LCs) which function as the major antigen-presenting cells of epithelial tissue. Immature LCs take up residence in the epithelium; however, additional precursor cells can be recruited by cytokines and chemokines secreted by keratinocytes during inflammation. Macrophage inflammatory protein 3 α (MIP-3 α) functions as a potent chemoattractant of Langerhans precursor cells due to their selective expression of the receptor for MIP-3 α .

It has been reported that E6 and E7 of high-risk HPVs interfere with immune mediators, the result of which is thought to be suppression of immune cell recruitment and function. Here we show that expression of HPV-16 E6/E7 in primary human keratinocytes results in the repression of several immune-related genes. Of

particularly interest is the down-regulation of MIP-3 α which results in decreased migration of immature Langerhans precursor-like cells. Interestingly, E6 and E7 from the low-risk HPV types 6 and 11 also inhibited MIP-3 α transcription which implies that the method by which this repression occurs is independent of the well-studied effects of HPV-16 E6 and E7 on p53 and retinoblastoma functions. In this study, the interaction of HPV-16 E6 and E7 with co-activators of nuclear factor- κ B appears to be the mechanism underlying the suppression of MIP-3 α . Overall, the ability of E6/E7-expressing cells to down-regulate mediators important in the recruitment of immune cells suggests that this mechanism may be important in the persistence and progression of HPV infections.

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Chapter 1

Introduction and Background

1.1 Significance

Human papillomaviruses (HPVs) are small DNA viruses which exhibit tissue tropism, infecting only cutaneous or mucosal epithelium. A high percentage of individuals exposed to HPV clear the infection within a short period following initial contact with the virus, but a small fraction will develop lesions resulting from the infection. Most of these low-grade lesions often resolve over time and only a small fraction of initial infections will progress to high-grade lesions or invasive carcinoma. Thus, it appears that the host immune response is imperative in clearing initial or persistent HPV infection. This observation is substantiated by studies highlighting increased prevalence and disease progression in immunosuppressed individuals [reviewed in (93)]. Additionally, reports detailing a disparity in the cytokine response and immune cell population in regressing warts compared to non-regressing warts also suggest a correlation between an apt immune response and clearance of HPV-positive lesions (20).

HPV types are commonly classified as either low-risk or high-risk, based on their potential to cause benign or malignant proliferation of infected cells, respectively. High-risk HPV types have been correlated with 90% of cervical cancers and more than 50% of other anogenital cancers (113). The expression of two early genes of high-risk HPVs, E6 and E7, has been detected in initial infected tissue, high-grade lesions, and cervical carcinomas. Thus, because of their detection in tissues of progressive HPV disease, and their ability to transform cells *in vitro*, E6 and E7 have been characterized as the primary transforming genes of high-risk HPVs. As such, E6

and E7 have been the subjects of intensive research focused on elucidating their roles in promoting advancement of lesions and disease associated with high-risk HPV infection. The role of E6 and E7 in promoting cell cycle progression and inhibiting apoptosis in a cellular environment normally under the control of programmed cell death has been explained through the interactions of these genes with numerous host genes and transcription factors, most notably p53 and retinoblastoma (Rb). However, the ability of the virus to propagate is pointless if the infection is cleared by the host immune response.

Thus, the ability of the virus to evade the immune system would be beneficial to viral persistence and disease progression. The target cell of an HPV infection is the keratinocyte, which responds quickly to infection or injury by initiating the innate immune response. Keratinocytes exposed to pathogens produce pro-inflammatory molecules which function to maintain the immune response and recruit immune cells. The responding immune cells also generate immune mediators, and take up foreign peptide fragments which will be presented to naïve T cells. Upon activation by antigen presentation, T cells are recruited back to the infected tissue and initiate an antigen-specific immune response which will clear the tissue of infected cells. Previous research on immune evasion by HPV has revealed the ability of E6 and E7 to repress the production of cytokines and chemokines, and interfere with the anti-viral response of infected keratinocytes. Additionally, HPV-positive tissue displays decreased expression of immune-related genes as well as decreased numbers of immune cells which suggests that effective clearance of an HPV infection requires the

coordinated effort of the host immune system. In this study, we identify several immune mediators, such as cytokines, chemokines, adhesion molecules, proteases, and receptors, whose expression is down-regulated in keratinocytes expressing HPV-16 E6 and E7. We selected MIP-3 α for further investigation because of its selective role as a potent chemoattractant for Langerhans cells (LCs). Additional experiments were designed to determine the biological significance of decreased MIP-3 α production by E6/E7-expressing keratinocytes, as well as the mechanism underlying the down-regulation of MIP-3 α by E6 and E7.

1.2 Epithelial Immune Response

1.2.1 Innate immune response

The epithelial defense to an infection begins with the innate immune response, a non-specific reaction designed to quickly eliminate invading pathogens. The epithelium itself provides a first line of defense by acting as a physical barrier against pathogens; however, if the barrier is compromised through injury or infection, chemical mediators and cells of the innate immune response become involved. Molecules released from activated epithelial cells and keratinocytes include complement proteins, defensins, cytokines, chemokines, and lipid mediators, while the cellular component includes natural killer (NK) cells, granulocytes, macrophages, and LCs [reviewed in (17)]. Cells of innate immunity respond by secreting additional immune mediators. These pro-inflammatory signals are responsible for a variety of functions, from recruiting more immune cells to altering the function of infected cells

and any resident immune cells. Additionally, cells of the innate immune response initiate the adaptive immune response through uptake of foreign antigen. Both components (chemical mediators and cells) of this first line of defense are described in more detail in the following sections.

Chemokines, cytokines, adhesion molecules and proteases released during the innate immune response are responsible for directing the migration of leukocytes, monocytes, lymphocytes, neutrophils, eosinophils, basophils, NK cells, dendritic cells, and endothelial cells (81). Keratinocytes play an active role in the innate immune response as cellular sources of both cytokines and chemokines in the epithelium. Keratinocytes secrete many cytokines, such as interleukin (IL)-1, -6, -7, -10, -12, -15, -18, -20, which not only influence the migration of immune cells, but also stimulate the further release of cytokines by keratinocytes and responding immune cells (33). The antigen-presenting cells of the epithelium (monocytes/macrophages/LCs) produce tumor necrosis factor (TNF)- α , TNF- β (lymphotoxin- α), IL-1, -6, -12, -15, -18, and -23, which are important for affecting the function of other immune cells. TNF up-regulates adhesion molecules and activates neutrophils, IL-1 activates T lymphocytes through the production of IL-2 and enhances B-cell proliferation, IL-6 and IL-8 activate and recruit T lymphocytes, IL-12 activates proliferation and cytokine production by NK cells, IL-18 assists in expression of cellular adhesion molecules, IL-23 works with IL-12 and -18 in the induction of interferon (IFN)- γ , and IL-15 regulates the function of NK, T, and B cells [reviewed in (10)].

Important in the recruitment of immune cells to the site of inflammation are chemokines, molecules that are chemotactic for neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts, and keratinocytes through the interaction of each chemokine with a specific G-protein-coupled receptor on the responding cells. Chemokines also function as regulators of both T cell differentiation towards T_H1 or T_H2 lineages, and maturation/differentiation of monocytes and immature dendritic cells [reviewed in (10)]. Some chemokines important in the inflammatory response include macrophage inflammatory proteins (MIP)-1 α , -1 β , -3 α , -3 β ; monocyte chemoattractant proteins (MCP)-1, -2, -3, -4; growth-related oncogene (GRO)- α , - β , - γ ; IL-8; monokine induced by interferon- γ ; interferon-inducible T-cell α chemoattractant (I-TAC); interferon- γ inducible protein 10 (IP-10); thymus and activation-regulated chemokine (TARC); and regulated on activation, normal T-cell expressed and secreted (RANTES). Again, keratinocytes are important cellular sources of chemokines in the epithelium such as IL-8, GRO- α / β / γ , IP-10, and MCP-1 (90). Through the production of these chemokines, activated keratinocytes are able to recruit granulocytes, macrophages, and T lymphocytes to the site of inflammation. Granulocytes, which include neutrophils, monocytes, eosinophils, basophils, and mast cells, produce reactive oxygen molecules, TNF- α , IL-12, and chemokines. Along with some granulocytes, macrophages and LCs help the interaction between the innate and adaptive immune responses through the uptake of foreign antigen. These phagocytic cells initiate the response of the second arm of the immune system (the adaptive

immune response) through both the secretion of IL-12 and IFN- γ (by monocytes/macrophages) and the presentation of antigen to T cells (by LCs).

1.2.2 Adaptive immune response

The innate immune response is temporally followed by the adaptive immune response, a defense reaction that takes more time to mount but provides specific and long-term protection against the pathogen. Initiation of the adaptive immune response is the presentation of foreign antigen by antigen-presenting cells to naïve T lymphocytes either within the epidermis or regional lymph nodes. T cells are activated by presentation of foreign antigen peptides on major histocompatibility complex (MHC) molecules. The type of MHC molecule recognized by the T cell receptor determines which type of T cell (cytotoxic or helper) will be activated [reviewed in (17)]. Activation of CD8⁺ cytotoxic T lymphocytes occurs through the presentation of endogenous antigen on infected cells via MHC class I molecules. Endogenous antigen refers to proteins that are processed, within cells bearing class I molecules, into peptide fragments. In the skin, infected keratinocytes have the ability to become antigen-presenting cells with the up-regulation of class I molecules and adhesion molecules by exposure to interferons. Adhesion molecules, such as intracellular cell adhesion molecule (I-CAM), assist in the adhesion of T cells, and class I molecules aid in the foreign antigen being presented to T cells and the subsequent activation of these cells. The activated CD8⁺ cells then kill cells, such as virally-infect or tumor cells, bearing the same antigen peptide. Several chemical

mediators assist in the cytotoxic response of CD8⁺ cells, including IL-2, -4, -5, -6, -7, -10, -11, -12, -15, TNF- α , TNF- β , and IFNs.

In contrast to the activation of CD8⁺ cytotoxic T cells via presentation of endogenous antigen, CD4⁺ helper T cells are activated through the presentation of exogenous antigen on antigen-presenting cells (monocytes, macrophages, dendritic cells, LCs, B cells) via MHC class II molecules. In the case of an epithelial immune response, infected cells of the epithelium recruit antigen-presenting cells to the site of inflammation through the production of cytokines and chemokines. Following uptake of antigen, cells such as LCs migrate to regional lymph nodes where naïve T cells can be sufficiently activated to mount an antigen-specific immune response. Activated CD4⁺ helper T cells follow chemokines back to the site of initial antigen exposure and further differentiate into either T_H1 or T_H2 cells depending on the cytokines produced at the site of inflammation. IL-12 produced by macrophages or NK cells help push helper T cells towards T_H1 differentiation, while IL-4 induces T_H2 differentiation. T_H1 cells then produce IFN- γ , TNF- α , and IL-2 and initiate cell-mediated inflammation, while T_H2 cells assist in the production of antibodies and activation of B cells through the secretion of IL-4, -5, -6, -9, -10, and -13 [reviewed in (92)]. As stated previously, B cells can uptake antigen and present peptides via MHC class II molecules. Antigen processing up-regulates the expression of co-stimulatory molecules CD80/CD86 on the B cells which results in activation of T cells and a corresponding activation of B cells via CD40/CD40 ligand (CD40L) signaling

[reviewed in (17)]. The ensuing activation of B cells leads to the production of antibodies and the development of B cell memory.

The first line of defense against invading pathogens, therefore, involves an interaction between the innate and adaptive immune responses. Immune mediators released by infected cells recruit immune cells to the site of infection where these cells continue the inflammatory response through the sustained production of cytokines, chemokines, adhesion molecules, and proteases. Responding antigen-presenting cells uptake foreign antigen and initiate the adaptive immune response through the presentation of peptide fragments and subsequent activation of naïve T lymphocytes. Activated T cells can kill infected cells by direct cell-mediated immunity through the differentiation of a T_H1 or T_H2 response, or initiate humoral immunity through the activation of B cells. Thus, the ability of HPV to evade the immune response and persist in the epithelium for prolonged periods of time must stem from the capability of the virus to suppress any, or all, of the previously described components of the immune response.

1.3 HPV Immune Evasion

HPVs are small DNA viruses that display strict tissue tropism, infecting only the cutaneous or mucosal epithelium. The subtypes identified to date are classified as low-risk or high-risk based on their potential to cause benign or malignant proliferation of infected cells, respectively. High-risk HPVs have been correlated with at least 90% of cervical cancers and more than 50% of other anogenital cancers (113).

An *in vitro* characteristic of high-risk HPV infections is the ability to cause cellular immortalization by certain viral oncoproteins. The HPV genome is divided into a long control region, an early region, and a late region. The early region consists of the E1, E2, E4, E5, E6 and E7 proteins involved in the viral life cycle, while the late regions consists of structural proteins L1 and L2 [reviewed in (113)]. The E1 protein is necessary for viral replication, while E2 acts as a transcriptional regulator and also assists E1 in viral replication. While the exact function of E4 has yet to be identified, it has been speculated to play a role in disrupting the normal differentiation program of the epithelium. This function would assist viral proliferation in that keratinocytes of the epithelium normally undergo a process of programmed cell death to create the keratinized upper layers of skin. Cell death is not beneficial to the life cycle of the virus, but disrupting this differentiation program would result in continued cellular division and allow the virus to replicate. E5 is the main oncoprotein in bovine papillomaviruses; however, its role in HPV appears to be the formation of complexes with transmembrane proteins and their subsequent trafficking and degradation. The major transforming proteins of HPV are E6 and E7, and the involvement of these genes in cellular immortalization is a characteristic that distinguishes the high-risk HPVs from the low-risk types.

The sustained expression of E6 and E7 from initial HPV-positive lesions to malignant biopsies corroborates that expression of these genes plays a necessary role in the progression of infected cells to immortalized cells. As such, the functions of E6 and E7 have been studied extensively [reviewed in (15, 52, 114)]. E6 was discovered

to bind and subsequently degrade p53, an interaction mediated by E6-associated protein. The role of p53 is cell cycle arrest following DNA damage; thus, non-functioning p53 allows for chromosomal instability in HPV-infected cells. The importance of p53 degradation by E6 is further substantiated by the lack of p53 mutations discovered in HPV-related cancers. Through the binding and degradation of p53, E6 can drive cells with chromosomal instability through cellular division and inhibit normal p53-induced apoptosis. E6 has also been linked to the degradation of other proapoptotic proteins, Bak and c-Myc, as well as to the activation of telomerase. Thus, E6 expression enables cells to bypass cell cycle arrest and resist apoptosis – important modifications contributing to the eventual immortalization of HPV-infected cells. Expression of E6 alone is sufficient for immortalization of mammary epithelial cells; however, both E6 and E7 are required for efficient immortalization of keratinocytes. Similar to E6, E7 also disrupts cell cycle arrest but through the binding of the Rb protein. Hypo-phosphorylated Rb (pRb) inhibits E2F-dependent transcription of genes essential for cell division. Once hyperphosphorylated by cyclin-dependent kinases, Rb releases E2F and genes involved in cell division are activated. Binding of E7 to pRb hinders the interaction with E2F; thus, allowing DNA synthesis and cell cycle progression to occur in the absence of hyperphosphorylation. E7 has also been shown to regulate gene transcription and stimulate cell cycle progression through interactions with cyclin-dependent kinase inhibitors p21^{CIP-1} and p27^{KIP-1}, histone deacetylase complexes, cyclin and cyclin-dependent kinase complexes, and activator protein-1 (AP-1) transcription factors. The synergistic effects of E6

inhibiting apoptosis and E7 inducing cellular proliferation enables cells infected by high-risk HPVs to ultimately transform and immortalize. However, the progression of low-grade HPV-positive lesions to high-grade lesions and eventual invasive carcinoma is a process that takes years. In order for the infection to persist and precancerous lesions to progress to cervical carcinoma, it is essential for the virus to evade the host immune response.

Successful clearance of a viral infection requires the coordination of both the innate and adaptive immune responses. In the skin, immune mediators produced by injured keratinocytes function in many ways. Pro-inflammatory cytokines stimulate the secretion of additional immune signals. Both cytokines and chemokines, along with adhesion molecules, facilitate the recruitment of immune cells to the site of infection, while complement proteins, proteases, and IFNs function as antimicrobial and antiviral proteins. Antigen-presenting cells that migrate to the site of inflammation initiate the cytotoxic, cell-mediated, and humoral immune responses through the activation of T and B cells. Thus, the ability of an HPV infection to circumvent any of these steps of a successful immune response would contribute to the survival and propagation of HPV-infected cells.

The E6 and E7 genes of high-risk HPVs contribute to the virus's oncogenic potential to transform infected cells. Due to their importance in both inhibiting apoptosis and inducing cellular proliferation, many studies have also investigated the mechanisms by which E6 and E7 negatively impact the immune response. A few studies have correlated the expression of E6 and/or E7 with the repression of immune

signals important in initiating innate immunity. In a study examining IL-18 expression in normal keratinocyte (HaCaT), HPV-negative (C33A), HPV-16-positive, (SiHa, CaSki), and HPV-18-positive (HeLa) cell lines, the level of IL-18 was high in HaCaT cells, while moderate in C33A and SiHa cells, and undetectable in HeLa and CaSki cells (18). Upon further examination, expression of E6 in both the HaCaT and C33A cell lines inversely correlated with the expression of IL-18. Levels of p53 were reduced in the C33A cell lines, but not in the HaCaT cells suggesting that the repressive effect of E6 on IL-18 was p53-independent. IL-18 is a pro-inflammatory cytokine similar to IL-1 that induces IFN- γ production, important in the induction of CD4⁺ T cells. IFN- γ has also been correlated with increasing the sensitivity of LC to mature, augmenting the ability of the cells to properly process and present antigen (61). In a study investigating the role of E6 and E7 in primary human keratinocytes both genes, individually and cooperatively, were found to down-regulate the expression of IL-8, a potent chemoattractant for T cells (43). The mechanism underlying the repression of IL-8 was through the interaction of both E6 and E7 with co-activators of nuclear factor- κ B (NF- κ B), an important transcriptional activator of immune-related genes. Human keratinocytes derived from foreskins and cervical or vaginal tissue displayed high levels of MCP-1 when stimulated by IFN- γ ; however, the same cells immortalized by HPV-16 E6 and E7 failed to produce detectable amounts of MCP-1 even after IFN- γ treatment (51). Another study using cervical keratinocytes found that, following transfection with either E6 or E6/E7, these cells produced undetectable granulocyte/macrophage colony stimulating factor (GM-CSF)

and TNF- α (36). A similar trend was seen when investigating normal keratinocytes as well as HPV-negative cervical carcinoma (HT3) and HPV-positive (SiHa, CaSki) cell lines. The activity of NF- κ B was also reduced in the HPV-positive cell lines, suggesting a possible role of E6 in the alteration of NF- κ B signaling and subsequent production of GM-CSF and TNF- α . Since TNF- α functions as a pro-inflammatory cytokine, reduced levels of TNF- α would result in decreased induction of cytokines, chemokines, and adhesion molecules. Additionally, TNF- α is important in the activation of neutrophils, and GM-CSF is important in the maturation of dendritic cell precursors (6). Activation of neutrophils is important in sustaining an inflammatory response, and maturation of dendritic cells allows the antigen-presenting cells to emigrate out of tissue and activate naïve T lymphocytes. Thus, these studies to date have identified repressed levels of cytokines and chemokines in HPV-positive cell lines and have correlated the reduced levels with expression of E6 and/or E7 in these cells. The ability of high-risk HPVs to down-regulate these immune mediators could be an effective method of silencing the initial component of the innate immune response.

In addition to recruiting immune cells through the production of cytokines and chemokines, infected cells have mechanisms that assist in an antiviral response, primarily the production of IFNs. IFNs- α and - β inhibit viral replication within infected cells and also up-regulate the expression MHC class I molecules (10). Keratinocytes, the target cell of HPV infections, can process foreign antigen and present the peptide fragments on class I molecules to CD8⁺ T cells. The ensuing

activation of these cells initiates a cytotoxic immune response which kills other infected cells presenting the same antigen. IFNs- α and - β also induce the production of proteins that interfere with viral replication and degrade viral RNA (97). Thus, the ability to block this critical antiviral response is significant to the persistence of HPV infections. In a microarray study investigating cervical keratinocytes expressing HPV-16 E6 and/or E7, the expression of E6/E7 or E6 correlated with a decrease in the overall expression of IFN- α and - β and several IFN-inducible genes (70). Both E6 and E7 also decreased the expression of signal transducer and activator of transcription-1 (STAT-1), and inhibited the binding of STAT-1 to the IFN-stimulated response element (ISRE). Normal binding of the interferon-stimulated gene factor 3 (ISGF3) transcription complex (composed of STAT-1, STAT-2, and p48) to the ISRE results in activation of IFNs and IFN-inducible genes. In a yeast two-hybrid screen, HPV-16 E6 was found to interact with interferon regulatory factor-3 (IRF-3), an important transcriptional regulator of IFN- β which also binds to the ISRE (82). Additional experiments expressing E6 in primary human foreskin keratinocytes confirmed the ability of E6 to inhibit the transactivation function of IRF-3, resulting in decreased mRNA levels of IFN- β . Expression of E7 did not have an effect on IFN- β levels; however, another study showed that E7 inhibited the induction of IFN- α -inducible genes through the binding of E7 to p48, an important component of the ISGF3 transcription complex (8). HPV-16 E7 was also shown to bind to IRF-1, both in a yeast two-hybrid screen and in mouse fibroblast cells (NIH 3T3). The expression of E7 in NIH 3T3 cells inhibited the IRF-1-directed activity of the IFN- β promoter

(73). Interestingly, E7 from a low-risk HPV type (HPV-11) also showed similar ability to inhibit the activity of IRF-1, suggesting a role for low-risk HPV genes in suppressing the antiviral activity of IFNs. The E6 and E7 genes from other high-risk HPV types also interfere with components of the IFN signaling pathway. Similar to the experiments investigating HPV-16 E7 and IRF-1, HPV-18 E7 was also found to down-regulate IRF-1 activity in E7-inducible NIH 3T3 cells and HPV-18 E6/E7 transgenic mice (103). The inhibition of IRF-1 activity decreased the expression of IFN-responsive genes, such as IFN- β , transporter associated with antigen presentation (TAP)-1, and MCP-1. Expression of HPV-18 E6 in human epithelial-like cells inhibited the transactivation ability of ISGF3 in response to IFN- α treatment, and resulted in decreased expression of IFN-inducible genes ISG-15 and IFP-53 (57). Several IFN-responsive genes were also down-regulated in human foreskin keratinocytes that were immortalized with the genome of another high-risk HPV type (HPV-31). Of particular interest was the decreased expression of STAT-1, an important transcriptional activator of downstream genes induced by IFN- α and - β (16).

As stated previously, effective clearance of a viral infection involves the activation of T cells which requires the presentation of peptide by either infected cells or antigen-presenting cells, such as monocytes, macrophages, or LCs. Keratinocytes assist in antigen presentation through the up-regulation of class I molecules and I-CAM. Expression of class I molecules and accessory proteins, such as TAP-1, are down-regulated in HPV infections and cervical cancers [reviewed in (71, 108)]. Presentation of peptide fragments by class I molecules is normally assisted by TAP-1

and TAP-2. Specific correlation of HPV proteins with decreased expression of components of the antigen presentation pathway, such as TAP-1, has been limited to HPV-16 E7, HPV-18 E7, HPV-11 E6, and BPV-1 E5. Also important in initiating the antigen-specific immune response in T cells is the presentation of antigen by keratinocytes to LCs, and by LCs to naïve T lymphocytes. Studies aimed at investigating the relationship between keratinocytes and LCs during HPV infection have discovered either a lack of immune cells, or inadequate adhesion/co-stimulatory molecules necessary for antigen-presentation to LCs. LC density was significantly reduced in low-grade and high-grade cervical lesions, and cervical carcinomas (positive for HPV types 16, 18, 31, and 33) compared to normal tissue (44). Other studies corroborate decreased numbers of LCs in low-grade and high-grade lesions when compared to normal tissue (2, 21, 62, 105). Important to the adhesion between keratinocytes and LCs is the expression of I-CAM and E-cadherin. When HPV-positive cells (SiHa) grown to an epithelial layer of several cells in thickness were stained for E-cadherin and LCs, the organotypic cultures displayed no staining for E-cadherin and no infiltration of LCs (44). Following transfection with E-cadherin cDNA, the cultures expressed high levels of E-cadherin which correlated with increased anchorage of LCs in the upper layers of cells. E-cadherin staining was also weak or undetectable in HPV-16-positive cervical tissue compared to normal biopsies (62). In the same study, transfection of HPV-16 E6 resulted in significant reduction of E-cadherin on the surface of HaCaT cells, and less numbers of LCs. Although the reasons for decreased LC density in HPV infections have not been clarified (lack of

chemoattractants, decreased adhesion, untimely emigration out of tissue), the fact that tissues positive for HPV exhibit diminished numbers of LCs suggests that these tissues have impaired immune surveillance.

Measurement of T cell responses to HPV infections involves testing T cell proliferation and cytokine production (for CD4⁺ responses) or cytotoxic ability (for CD8⁺ response) to HPV proteins, synthetic peptides, or HPV-transformed cells. However, the results of studies using T cell proliferation to measure CD4⁺ T cell responses have been inconsistent [reviewed in (60, 93)]. Some studies show no difference in CD4⁺ T cell response towards HPV antigens (L1, E2, E5, E6, E7) between HPV-positive patients and controls, while others showed a higher response in the control group. Additionally, the presence of significant CD4⁺ responses sometimes correlated with either active clearance of an HPV infection or lesions in regression. More consistent data has arisen from studies investigating the cytokine production in patients which suggests that a T_H1 directed cell-mediated response may be important in clearing HPV infections. As stated previously, the production of IFN- γ , TNF- α , and IL-2 initiate cell-mediated inflammation, while the secretion of IL-4, -5, -6, -9, -10, and -13 is thought to suppress this response [reviewed in (92)]. A study comparing extensive and localized HPV disease found that patients with extensive HPV disease had lower levels of IL-2 and higher levels of IL-4 and IL-10 than those with localized disease, suggesting that the persistence of HPV shifts the T cell response towards a reaction that suppress cell-mediated immunity (19). This directed CD4⁺ response is further substantiated by studies investigating the immune

environment of intraepithelial lesions and genital warts. Comparisons of cytokine expression between high-grade and low-grade lesions found that the expression of IL-12p40 was lowest in the high-grade lesions, while IL-10 expression was highest in these same lesions (31). Spontaneously regressing warts were characterized by a massive infiltration of mainly CD4⁺ cells and production of IFN- γ , TNF- α , and IL-12p40 (20). Conversely, the non-regressing warts had a lack of immune cells. In another study of genital warts, those patients that responded well to IFN- γ treatment had higher levels of IL-2 and IFN- γ than non-responders which exhibited low levels of these same cytokines and also reduced CD4⁺ T cells (3). Similar to the studies of CD4⁺ T cell responses, the studies investigating CD8⁺ T cell responses towards specific HPV antigens have also shown variable results [reviewed in (60, 93)]. Some cases show patients with intraepithelial lesions or cervical cancer had active E6- and E7-specific CD8⁺ T cell responses, while others show a higher cytotoxic T cell response in patients with HPV infection but no lesions compared to patients with both HPV infection and lesions. Sometimes the responses were transient, and other positive CD8⁺ responses showed no direct correlation with regression of disease.

The results from studies investigating the host immune response to HPV infections suggest that HPV may have evolved numerous mechanisms to evade both the innate and adaptive immune responses. The first line of defense is the production of immune mediators which alert cells of the immune system to presence of the virus; however, HPV can down-regulate the expression of many cytokines and chemokines. The reduced expression of pro-inflammatory cytokines inhibits the ability of

keratinocytes to successfully present peptide to antigen-presenting cells. Additionally, the virus interferes with the antiviral response of IFNs within infected cells.

Repressed chemokine production results in less immune cells recruited to the site of infection. Additionally, the cytokine environment of HPV lesions may contribute to reduced susceptibility of T cell responses. Thus, it appears that an HPV infection can subvert the normal immune response and persist in the epithelium, contributing to the progression of disease.

1.4 MIP-3 α and Langerhans Cells

Following microbial infection, chemokines secreted by cells of the epidermis are instrumental in linking the innate and adaptive immune responses by recruiting antigen-presenting cells, such as macrophages and dendritic cells. Many subsets of dendritic cells exist, characterized by differences in developmental pathways, anatomical localization, and surface marker expression. Dendritic cells differentiate from bone marrow progenitors and circulate in peripheral blood as immature precursors. The immature dendritic cell precursors are able to respond to a range of chemokines (MIPs-1 α -1 β -3 α , MCPs-1/-3/-4, RANTES, TARC) depending on the expression of specific chemokine receptors (6). The release of chemokines by infected cells recruits immature dendritic cell precursors to the site of inflammation. Following uptake of antigen, dendritic cells move to draining lymphoid organs where they are converted to mature dendritic cells and subsequently prime naïve T lymphocytes.

LCs are a subset of dendritic cells which, as stated previously, differ from other subsets by their anatomical localization and surface marker expression. Immature LCs or LC precursors circulate through the blood and take up residence in the epidermis and oral/genital mucosa, where the cells continually monitor the tissue for damage and infection. The epidermis is thought to sustain a population of immature LCs through adhesion mediated by expression of E-cadherin on both LCs and keratinocytes (48, 100). There are conflicting reports on whether populations of immature LCs are maintained in the skin for long periods of time or continually replaced by LC precursors. However, it has been shown that LC precursors are recruited under conditions of local LC loss such as during inflammation or injury (39, 64). Additionally, the regular presence of LCs in the dermal lymphatics suggests that LCs emigrate from the epithelium, even in the absence of inflammatory stimuli (13). Whether in response to local LC loss due to emigration during inflammation or normal LC replenishment, the production of chemokines chemotactic for LC precursors is important for recruiting these cells to the epidermis.

Among the immune signals secreted by keratinocytes during the inflammatory response, MIP-3 α is the most potent chemokine for LC precursors and dermal dendritic cells (25). Liver and activation-induced chemokine/CCL20/MIP-3 α /Exodus-1 was cloned nearly simultaneously from three separate cDNA libraries (human liver, activated monocyte, and pancreatic islet cells) (38, 41, 83). Expression of MIP-3 α has been detected in a variety of tissues, to include cells of the lungs and small intestine, epidermal keratinocytes, and epithelial cells of the appendix, tonsils, colon, and cervix

[reviewed in (91)]. The level of expression in these tissues is constitutively low, but expression is increased under inflammatory conditions, such as atopic dermatitis, tonsils, psoriasis, and inflammatory bowel disease (25, 40, 47, 53, 69). In these same tissues, MIP-3 α expression could be enhanced by stimulation with pro-inflammatory cytokines, such as TNF- α , IL-1 α , and IL-1 β . Additionally, MIP-3 α production can be increased by TNF- α , IL-1 α , or IL-1 β in oral squamous cell carcinomas, intestinal epithelial cells, and keratinocytes (1, 25, 40, 47, 53, 69, 101).

The receptor for MIP-3 α , CC chemokine receptor 6 (CCR6), was first discovered in lymphocytes and dendritic cells [reviewed in (91)]. Chemotaxis experiments using CCR6-transfected cell lines revealed that MIP-3 α was strictly selective for CCR6 only and was unable to signal through any of the other chemokine receptors. This selective relationship between MIP-3 α and CCR6 is unique to chemokine/chemokine receptor signaling, as many chemokines have the capacity to recruit varying immune cells through binding of different chemokine receptors. As such, the subset of cells that express CCR6 is also highly selective, in that immature dendritic cells, memory T cells, and epidermal LC precursors appear to be the only immune cells that migrate in response to MIP-3 α because of their CCR6 expression (32, 54, 58, 80). Maturation of LCs, like dendritic cells, can be induced by exposure to an antigen, TNF- α , IFN- γ , IL-4, or CD40L, and triggers both a loss of responsiveness to MIP-3 α and an acquired responsiveness to MIP-3 β (24, 59, 85). Accordingly, these same cells express decreased CCR6 mRNA followed by an up-regulation of CCR7, known receptor for MIP-3 β . The CCR7 expression causes the

migration of mature cells to T cell areas of the body (14, 84, 85, 109). Following antigen processing and subsequent maturation through differential regulation of surface markers, LCs can traverse the epidermal basement membrane and migrate to either dermal lymphatics or peripheral lymphoid organs where antigen is presented to naïve T cells (7, 79). Following the emigration from the epidermis, antigen presentation to naïve T cells would initiate the secondary adaptive immune response.

1.5 NF- κ B Control of Immune Mediators

NF- κ B was first discovered in 1986 as a necessary transcription factor in B cells (94). Five members of the NF- κ B family have been identified so far: c-Rel, NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), and RelB [reviewed in (5, 30, 86)]. Each member contains regions responsible for interactions with inhibitors, nuclear translocation, and DNA binding. The family members form either homodimers or heterodimers which recognize and bind specific DNA consensus sequences. For example, the common NF- κ B dimer of p50/RelA recognizes 5'-GGGRNNYYCC-3'. Thus, the ability of different dimers to bind certain DNA sequences allows for fine-tuned regulation of gene transcription. Additional control of gene expression occurs through which dimers bind to DNA. Certain dimers are transcriptional active (p50/p65, p65/c-Rel) while others are repressive (p50/p50, p52/p52). NF- κ B is retained in the cytoplasm of cells associated with inhibitors of κ B (I κ B), such as I κ B α , I κ B β , I κ B ϵ , I κ B γ , and Bcl-3, which block the nuclear localization component of NF- κ B. Each I κ B combines with a particular set of NF- κ B dimers which differs among

cell types, and this interaction again provides a precise degree of specific gene regulation. Activation of NF- κ B begins with stimuli such as TNF- α , IL-1, IL-18, lipopolysaccharide (LPS), double-stranded RNA, or ultraviolet light. Different stimuli initiate diverse downstream signaling pathways. For example, the binding of LPS to Toll-like receptors recruits myeloid differentiation primary response gene 88 and Toll-interacting protein. TNF- α binding to the TNF receptor recruits the receptor-interacting protein and TNF receptor-associated factor. However, all signaling cascades converge on the IKK kinase complex, composed of IKK- α , IKK- β , and IKK- γ . The IKK complexes mediate phosphorylation of I κ B, which is subsequently ubiquitinated and targeted for degradation by the proteasome. The release of NF- κ B dimers from I κ B allows for translocation into the nucleus and binding to DNA.

However, regulation of gene expression by NF- κ B does not end following nuclear translocation and binding to DNA. Maximal gene expression often requires the recruitment of coactivators. One such coactivator is the cyclic AMP response element binding protein (CREB)-binding protein (CBP) and its homolog p300 which bind the rel family member p65. The interaction of CBP/p300 with p65 was originally discovered to enhance p65-activated expression of adhesion molecules E-selectin and VCAM, as well as the cyclin E-Cdk2 complex (29, 78). This association between CBP/p300 and p65 was also found to be augmented by phosphorylation of p65 by protein kinase A which exposes two CBP/p300 binding sites (111). Several other studies have revealed the interaction of CBP/p300 with other transcription factors (c-Jun, STAT2, E2F-1), p160 family of coactivators, and acetyltransferases (such as

CBP-associated factor, p/CAF) [reviewed in (96)]. The level of p50-activated NF- κ B transactivation is enhanced by the interaction of p50 with one of the p160 family members, steroid receptor-coactivator-1 (SRC-1) (68). Additionally, coactivators with intrinsic histone acetyltransferase (HAT) activity, such as CBP and p/CAF, can further regulate gene expression through the acetylation of core histones. Once histones are acetylated, DNA unwinds from the histones, making the consensus sequences more accessible to NF- κ B dimers and coactivators. It appears that NF- κ B transactivation often requires a combination of coactivators and HAT activity. For example, the p65/p50 heterodimer recruits CBP, SRC-1, and p/CAF, and gene expression required the HAT activity of p/CAF, but not of CBP (95). Thus, the set of dimers that translocates and binds to DNA, along with the requirement of specific coactivators and acetyltransferase activity, help regulate the level of expression of NF- κ B-target genes. Moreover, many genes that have NF- κ B consensus sequences also require the binding of additional transcription factors, such as CAAT/enhancer-binding protein (C/EBP) and AP-1. As a result, cells are able to tightly regulate when and to what degree genes, with NF- κ B consensus sequences, are expressed.

Over 150 genes have been identified as targets of NF- κ B, and their expression is involved in a myriad of cellular functions: cytokine/chemokine and growth factor production, expression of immune and other cell surface receptors, antigen presentation, cell adhesion, acute phase and stress responses, regulation of apoptosis, and expression of transcription factors (72). In addition to the number of NF- κ B-responsive genes that are related to immunity, the activation of NF- κ B by many

stimuli involved in the inflammatory response to injury or infection implies a central role for NF- κ B in transcriptional regulation of many genes involved in immune function. The importance of NF- κ B in initiating many aspects of the immune response is corroborated by studies of knockout mice [reviewed in (30)]. Investigations of p50 knockout mice revealed normal development, but non-responsive B cells. Similar to the p50 knockouts, c-Rel knockout mice showed no developmental abnormalities, but the proliferative response of both T and B cells to antigen was inhibited. RelB knockout mice also developed normally until shortly after birth when they had abnormal development of the thymus and spleen. Further complications in immune function resulted in premature death. In contrast, mice lacking RelA died during gestation, most likely due to severe apoptosis of liver cells. The result of these studies suggest that the p50 and c-Rel members are important for immune responses, while RelB and RelA are more involved in development. Limited studies using knockouts of the I κ B proteins revealed a crucial role for I κ B α in regulating induced NF- κ B responses. The I κ B α -knockout mice died shortly after birth due to degeneration of lymphoid tissue and skin abnormalities which were probably caused by overexpression of NF- κ B activity.

Consequently, many viruses have developed strategies to subvert NF- κ B activation in order to evade the host immune response as well as prevent apoptosis [reviewed in (86)]. Some viruses, such as vaccinia virus, African swine fever virus, and human immunodeficiency virus (HIV) encode their own homologs of components of the NF- κ B signaling cascade, or interfere with I κ B α degradation. Both strategies

would inhibit the immune response of infected cells. In contrast, other viruses, such as HIV, herpesviruses, hepatitis C virus, and encephalomyocarditis virus, activate NF- κ B in an attempt to promote cellular proliferation and viral transcription, and inhibit apoptosis of infected cells.

1.6 Specific Aims

The research described herein was undertaken to identify immune-related genes that are de-regulated by the expression of HPV-16 E6 and E7. Additionally, it was of interest to establish biological significance of any down-regulated immune targets as well as identify the mechanism underlying the ability of E6/E7 to repress an immune response. The following aims address immune dysregulation in E6/E7-expressing cells.

- (1) Quantitative PCR and ELISAs were used to identify and validate immune-related genes repressed in HPV-16 E6/E7-expressing keratinocytes.
- (2) The biological significance of decreased MIP-3 α expression in E6/E7-expressing cells was investigated using Langerhans precursor-like cells in migration assays.
- (3) The molecular mechanism underlying MIP-3 α repression was investigated through the use of mutational promoter analysis and expression of E6 and E7 mutants.

Chapter 2

Materials and Methods

Cells. Primary human foreskin keratinocytes (HFKs) were isolated from neonatal foreskins and cultured in EpiLife medium supplemented with human keratinocyte growth supplement (bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin, human epidermal growth factor) and penicillin/streptomycin/amphotericin B (PSA) solution (Cascade Biologics). Cells were cultured in EpiLife medium supplemented without bovine pituitary extract and hydrocortisone (hereafter called minimal EpiLife) when culture supernatants were harvested for use in ELISAs and migration assays.

U2OS cells were cultured in DMEM supplemented with 10% fetal calf serum (Fetalclone I, Hyclone).

Preparation of cell lines. Generation of stable cell lines expressing HPV-16 E6/E7, HPV-6 E6/E7, and HPV-11 E6/E7 was accomplished using the pBabe retroviral system (65). Retrovirus encoding either vector alone or E6/E7 from HPV-16, HPV-6, and HPV-11 was produced by transfecting Φ NX packaging cells (American Type Culture Collection) with vesicular stomatitis virus envelope gene and pBabe retroviral constructs by calcium phosphate method (BD Biosciences), according to the manufacturer's protocol. Retrovirus was concentrated by centrifugation for 90 min at 15,000 rpm, 4°C, 48 h after transfection. Virus pellets were resuspended in 2mL EpiLife overnight at 4°C and either used immediately to infect HFKs or frozen at -80°C for future use. Resuspended virus was used to infect HFKs in 8 μ g polybrene/mL of EpiLife for 5 h. Media was changed and cells were allowed to recover for 24 h and then selected with 1.25 μ g/mL puromycin for 2-4 days. Stable

cell lines were expanded for treatment with polyinosine-polycytidylic acid [Poly(I)·Poly(C)] (Amersham Biosciences).

Generation of E6 and E7 mutations. Mutagenesis of E6/E7 was performed on HPV-16 E6/E7 in pGEM7Zf using the QuikChange kit (Stratagene). The following primers were used to generate a stop codon at 16th amino acid in E7 gene of HPV-16 for E6/E7stop (forward GATTTGTAACCAGAGACAACTG; reverse CAGTTGTCTCTGGTTACAAATC) and a stop codon at 15th amino acid in E6 gene of HPV-16 for E6stop/E7 (forward CCACAGTAATGCACAGAGCTGC; reverse GCAGCTCTGTGCATTACTGTGG). HPV-16 E6(C66G/C136G) and HPV-16 E7.2, previously characterized as unable to bind CBP/p300 and pCAF (43, 75), were generated in combination with E7stop and E6stop respectively. The primers listed previously were used to mutate HPV-16 E6(C66G/C136G)/E7 in pGEM7ZF to generate E6(C66G/C136G)/E7stop. Mutagenesis of E7 was performed on HPV-16 E6stop/E7 in pGEM7Zf using the QuikChange kit (Stratagene). The following primers were used to generate an amino acid substitution of histidine to proline at position 2 (forward GCTGTAATCATGCCTGGAGATACACCTACA; reverse TGTAGGTGTATCTCCAGGCATGATTACAGC). The mutants were sequenced and subcloned into the pBabe retroviral vector.

Micro fluidic card analysis. 1×10^6 cells were seeded on 10-cm dishes 24 h prior to treatment with Poly(I)·Poly(C). Cells were treated with 100 μ g/mL Poly(I)·Poly(C) in antibiotic-free EpiLife for 16 h, washed 5 times with PBS, and refed with minimal EpiLife for 4 h. Cells were harvested and total RNA was extracted using the RNeasy

mini kit (Qiagen), according to the manufacturer's instructions. RNA was provided to the University of Rochester Functional Genomics Center for cDNA amplification and RT-PCR gene expression quantification using micro fluidic cards (Applied Biosystems) containing primers specific for a panel of genes implicated in the immune response. Following sample loading, cards were run on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and data was analyzed using the Sequence Detection System (SDS) 2.2 software using glyceraldehyde phosphate dehydrogenase (GAPDH) as the endogenous control and the control cell line (Babe) set as the calibrator sample (expression equal to 1). Each sample was run in quadruplicate in each experiment and paired t-test was used to generate p values. Results are expressed as the average fold reduction in gene expression for three experiments using three independent cell lines. Only those targets where fold reduction was statistically significant ($p < 0.05$) are listed in Table 1.

Measurement of immune targets and E6/E7 by semi-quantitative PCR. As described previously, 1×10^6 cells were seeded on 10-cm dishes 24 h prior to treatment with Poly(I)-Poly(C). Following treatment and addition of minimal EpiLife, cells were harvested, pelleted, and stored at -80°C until RNA isolation. Total RNA was extracted using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. cDNA amplification was carried out in $25\mu\text{L}$ reverse transcriptase (RT) reaction mixtures containing $3\mu\text{g}$ RNA, $400\mu\text{M}$ deoxyribonucleoside triphosphates, RNase out ribonuclease inhibitor (Invitrogen), 10ng random hexamer primer, 5U avian myeloblastosis virus RT (Promega), and 5X RT buffer (Promega). cDNA

obtained was used in 25 μ L semi-quantitative PCR reaction mixtures containing 2.5 μ L buffer (Promega), 1.5 μ L (1.5 mM) MgCl₂ (Promega), 400 μ M deoxyribonucleoside triphosphates, 1 μ L each (50pM) forward and reverse primers, and 0.25 U Taq polymerase (Promega). The protocol used included a denaturation step (94°C for 1 min) followed by amplification repeated 30 times (94°C for 15 s, T_m°C for 30 s, 72°C for 40 s). Primers used are listed with annealing temperatures (T_m) given in parentheses. The GAPDH primers work at a broad range of T_m's and can, therefore, be run at the same T_m as the target primers in each reaction.

IL-1 α forward (51°C): ATCCTTCTATCATGTAAGCTATGGC

IL-1 α reverse (51°C): TCCTCTGAGTCATTGGCGAT

IL-1 β forward (62°C): GGCGGCATCCAGCTACGAAT

IL-1 β reverse (62°C): CCTGGAGGTGGAGAGCTTTCAGTT

IL-6 forward (62°C): TGGTGTGCTGCTGCCTTC

IL-6 reverse (62°C): CTGCACAGCTCTGGCTTGTTC

IL-8 forward (51°C): CTGCAGCTCTGTGTGAAGGT

IL-8 reverse (51°C): CTCTGCACCCAGTTTTCCTT

GRO- α forward (56°C): TGCTGCTCCTGCTCCTGGTA

GRO- α reverse (56°C): GGATTGAGGCAAGCTTTCCG

ISG15 forward (58°C): TTCCAGCAGCGTCTGGCTGT

ISG15 reverse (58°C): CCCGCTCACTTGCTGCTTCA

MIP-3 α forward (51°C): ATTTATTGTGGGCTTCACACG

MIP-3 α forward (51°C): TGTGCAAGTGAAACCTCCAA

TNF- α forward (56°C): GAAAGCATGATCCGGGACGT

TNF- α reverse (56°C): CAGGCTTGTCACTGCGGGTT

TNF α IP3 forward (56°C): CACAGAGCTGGAAACGACGC

TNF α IP3 reverse (56°C): TCTGGAACCTGGACGCTGTG

E6 forward (51°C): GAGAGGATCCATGTTTCAGGACCCACAGG

E6 reverse (51°C): CATGAATCCTTACAGCTGGGTTTCTCTAC

E7 forward (47.5°C): CGGGATCCATCATGCATGGAGATAC

E7 reverse (47.5°C): GTGGATCCGGTTTCTGAGAACAGATGG

GAPDH forward: CCCCTCTGCTGATGCCCCATGTT

GAPDH reverse: GAGCTTCCCGTCTAGCTCAGGGAT

For semi-quantitative PCR on HPV-16 E6/E7, HPV-6 E6/E7, and HPV-11 E6/E7 cell lines, the protocol used included a denaturation step (95°C for 1 min) followed by amplification repeated 30 times (95°C for 20 s, T_m of 50°C for 30 s, 72°C for 45 s).

Primers used follow:

HPV-16 E6 forward: GAGAGGATCCATGTTTCAGGACCCACAGG

HPV-16 E7 reverse: GTGGATCCGGTTTCTGAGAACAGATGG

HPV-6 E6 forward: GCGGATCCATTATGGAAAGTGCA

HPV-6 E7 reverse: GCGAATTCTTATGTCTTCGG

HPV-11 E6 forward: GCGGATCCATTATGGAAAGTAAA

HPV-11 E7 reverse: GCGAATTCTTATGGTTTTGG

Measurement of MIP-3 α by quantitative PCR (qPCR). As described previously, 1 x 10⁶ cells were seeded on 10-cm dishes 24 h prior to treatment with Poly(I)·Poly(C).

Following treatment and addition of minimal EpiLife, cells were harvested for RNA isolation, and culture supernatants were collected for use in ELISAs and migration assays. Total RNA was extracted using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. cDNA amplification was carried out in 25 μ L RT reaction mixtures containing 3 μ g RNA, 400 μ M deoxyribonucleoside triphosphates, RNase out ribonuclease inhibitor (Invitrogen), 10ng random hexamer primer, 5U avian myeloblastosis virus RT (Promega), and 5X RT buffer (Promega). cDNA obtained was used in real-time qPCR reaction mixtures containing 2.5 μ L each of (1 μ M) forward and reverse primers, 12.5 μ L 2X iQ SYBR Green Supermix (BioRad), and 1 μ L cDNA template. The protocol used included a denaturation step (95°C for 30 s) followed by amplification repeated 45 times (95°C for 30 s, 60°C for 1 min, 68°C for 1 min). A melt curve analysis performed following every run confirmed the amplification of a single product and no primer dimer. In each experiment, reactions were carried out in quadruplicate for each sample and GAPDH was used for normalization. Primers used for real-time qPCR were obtained from Primer Bank (106):

MIP-3 α forward: TGCTGTACCAAGAGTTTGCTC

MIP-3 α reverse: CGCACACAGACAACTTTTTCTTT

GAPDH forward: TGTTGCCATCAATGACCCCTT

GAPDH reverse: CTCCACGACGTA CTCAGCG

Paired t-test was used to generate p values.

ELISAs. MIP-3 α was measured in culture supernatants collected from Poly(I)·Poly(C)-treated cells using the human MIP-3 α DuoSet ELISA Development kit (R & D Systems) according to the manufacturer's instructions. The lower limit of detection was 32pg/mL. Each sample was run in triplicate in each experiment and paired t-test was used to generate p values.

IL-6 was measured in culture supernatants using affinity purified anti-human IL-6 (eBioscience) at 2 μ g/mL as a coating antibody and biotin anti-human IL-6 (eBioscience) at 1 μ g/mL as the detection antibody. Recombinant human IL-6 (eBioscience) was used in serial dilutions for standards. The lower limit of detection was 12pg/mL. Each sample was run in triplicate in each experiment and paired t-test was used to generate p values.

Culture of Langerhans Precursor-like Cells (LPLCs). Mononuclear cells were isolated from peripheral blood of healthy donors by centrifugation on Lymphocyte Separation Medium (Cellgro). The mononuclear cell layer was collected from the interface and monocytes were purified by positive magnetic selection using CD14 microbeads and an MS separation column (Miltenyi Biotec). The purity of CD14⁺ cells was routinely >95%, as assessed by flow cytometry. Purified CD14⁺ monocytes were cultured in 24-well tissue culture plates (Costar) in complete medium [RPMI 1640-supplemented with L-glutamine (Cellgro), 1% penicillin/streptomycin, and 10% heat-inactivated fetal calf serum (Fetalclone I, Hyclone)] supplemented with 200ng/mL GM-CSF and 10ng/mL TGF- β 1 (Peprotech), or 80ng/mL GM-CSF and 10ng/mL IL-4 (Peprotech). At days 2 and 4, cells were fed with fresh medium plus

cytokines. At day 6, LPLCs were harvested, analyzed by flow cytometry, and used in migration assays.

Analysis of LPLC surface marker expression and phagocytic capacity. 1×10^5 cells were incubated for 30 min at 4°C in PBS/2% fetal calf serum (Fetalclone I, Hyclone)/1% sodium azide, with allophycocyanin-conjugated CD14 (eBioscience), phycoerythrin-conjugated CD83 (eBioscience), phycoerythrin-conjugated CCR6 (BD PharMingen), fluorescein isothiocyanate-conjugated HLA-DR (BD PharMingen), fluorescein isothiocyanate-conjugated CCR6 (R & D systems), allophycocyanin-conjugated CD1a (BD PharMingen), or phycoerythrin-conjugated CD1a (eBioscience) monoclonal antibodies at the appropriate concentration or with control isotype-matched irrelevant monoclonal antibodies at the same concentration. Cells were washed twice and analyzed with a FACS caliber (Becton-Dickinson) using CellQuest software.

1×10^5 cells were incubated with 10 µg/mL fluorescein isothiocyanate-conjugated ovalbulmin (Molecular Probes) for 30 min at 37°C or 4°C (control cells). Cells were washed twice and analyzed with a FACS caliber. Fluorescein intensity provided a measurement of phagocytic capacity of the cells.

Migration assay. Migration assays were carried out using 24-well Transwell plates with 5-µm pore size polycarbonate filters (Costar 3421). Briefly, 2×10^5 LPLCs in 100 µL medium were added to the upper wells and 600 µL of conditioned medium was added to the lower wells. Cells were allowed to migrate for 4 h at 37°C under 5% CO₂. Migrated cells and medium from the lower wells were collected, spun down for

5 min at 1200rpm, 4°C, resuspended in medium and counted using a hemacytometer. The number of cells that migrated in response to unconditioned medium was subtracted from the number of cells migrating in response to conditioned medium to control for nonspecific migration. Recombinant human MIP-3 α (Peprotech) and anti-human MIP-3 α antibody (R & D Systems) were used in the compensation and neutralization migration assays. Each sample was run in duplicate in each experiment and paired t-test was used to generate p values.

Reporter assays. HFKs were seeded in 6-well dishes at a density of 1×10^5 cells per well. The luciferase reporter constructs, kindly provided by Andrew Keates (53), consisted of an 849-bp fragment of the MIP-3 α promoter cloned into the pGL3-Basic firefly luciferase expression vector (MIP-3 α WT). The mutant reporter constructs were generated by either targeted substitutions in the NF- κ B (mNF- κ B) and C/EBP (mC/EBP) sites, or sequential 10 base pair substitutions in the AP-1 (mAP-1) and second Ets (mEts) sites. Cells were transfected 24 h later with 100ng MIP-3 α WT or mutated reporter constructs and 900ng pSG5, 900ng pSG5 E6, or 900ng pSG5 E7 using Fugene 6 (Roche). 24 h after transfection, cells were washed with 1x PBS, refed with PSA-free EpiLife, and left untreated or treated with 10 μ g/mL Poly(I)·Poly(C), 10ng/mL TNF- α (Peprotech), or 10ng/mL IL-1 β (Peprotech) for 6 h. Following treatment, cells were harvested, lysed, and measured for luciferase activity as described previously (63, 75). Assays were carried out in triplicate for each sample.

U2OS cells were seeded in 6-well dishes at a density of 1×10^5 cells per well 24 h prior to transfection. Cells were transfected with 400ng NF- κ B luciferase

promoter (NF- κ B luc) and 600ng pcDNA3, 600ng pcDNA3 E6, or 600ng pcDNA3 E7. The NF- κ B luciferase promoter contains three NF- κ B binding sites upstream of the luciferase gene. Media was changed 5 h after transfection. After an additional 48 h, cells were harvested, lysed, and measured for luciferase activity. Assays were carried out in triplicate for each sample.

Western blotting. Cells were harvested, pelleted, and stored at -80°C until lysed in radioimmunoprecipitation assay buffer [1x PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1:100 protease inhibitor mixture (Sigma)]. Following centrifugation, protein was quantitated by Bradford Assay. 100 μg of protein was loaded in 2x sample buffer and run on 8% (for p53 and actin) or 15% (for E7) SDS-polyacrylamide gels. Protein was transferred to 0.2 μm nitrocellulose membrane and membranes were blocked at room temperature in PBST (1x PBS/0.1% Tween 20) containing 5% milk for 1 h. Membranes were probed with p53 (BD Pharmingen) at 1:2000 dilution, E7 (Zymed) at 1:250 dilution, and actin (Santa Cruz Biotechnology) at 1:1000 dilution in blocking buffer. Blots were visualized on the ChemiImager 5500 (Alpha Innotech) and images were quantitated using the FluorImager software 2.1.

IFA. HFks were seeded on glass cover slips in 6-well dishes at a density of 1.5×10^5 cells per well 24 h prior to treatment. Media was changed to PSA-free EpiLife and cells were left untreated or treated with either 100ng/mL TNF- α (for 30 min) or 100 μg /mL Poly(I)·Poly(C) (for 3 h). Following treatment, cells were washed with 1x PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with 1x PBS, cells were permeabilized with 0.5% Triton-X100 in PBST (1x

PBS plus 0.2% Tween 20) for 10 min at room temperature. Cells were washed with PBST and then blocked in PBST plus 5% fetal calf serum (blocking solution) for 15 min at 37°C. Cells were incubated in blocking solution with 1:250 primary antibody, α -p65 (Santa Cruz), overnight at 4°C, washed with PBST, and incubated in blocking solution with 1:200 secondary antibody, AlexaFluor 488 (Molecular Probes) for 1 h at room temperature. Cells were washed two times with PBST and cover slips were mounted on slides for viewing by fluorescent microscopy.

Chapter 3

**Suppression of immune mediators by E6/E7 inhibits the migration of responding
immune cells**

3.1 Introduction

Initially, microarray technology was carried out on differentiated keratinocytes to identify genes de-regulated by the expression of HPV-16 E6/E7 compared to normal keratinocytes. As expected, many genes associated with cell cycle progression and correlated with chromosomal abnormalities were up-regulated in the E6/E7-expressing cells; however, expression of a cluster of immune-related genes was down-regulated in the E6/E7-expressing keratinocytes. This observation is of particularly interest because the immune genes identified in the microarray were cytokines, chemokines, and IFN-inducible genes, all of which are important in initiating an innate immune response to an HPV infection. Other HPV studies, such as microarrays and the use of HPV-immortalized cell lines, have identified similar immune targets that are repressed in HPV-positive cells compared to normal cells. Nevertheless, these studies tend to focus more on identifying the de-regulated genes and/or elucidating the molecular mechanism underlying the repression by E6 and/or E7. As such, we focused our research on not only validating the down-regulated immune genes, but on establishing a biological consequence of this de-regulation of immune products.

3.2 Results

3.2.1 Down-regulation of immune related genes in E6/E7-expressing keratinocytes.

cDNA microarray technology was used previously to identify differences in gene expression between control keratinocytes and keratinocytes expressing HPV-16

E6 and E7 during differentiation (76). HFKs were stably infected with retrovirus carrying either an empty vector (Babe) or a vector containing HPV-16 E6 and E7 in tandem (E6/E7). In order to mimic the biological environment in which HPV-16 replicates (i.e. the upper, differentiated layers of the epithelium), the keratinocytes were first differentiated in methylcellulose prior to extraction of RNA for the microarray chips. Cluster analysis classified the genes, observed as significantly up-regulated or down-regulated in E6/E7-expressing keratinocytes, into functional groups. Since the primary role of E6 and E7 is to induce keratinocyte proliferation in an environment where the cells are normally undergoing programmed cell death, it was not surprising that the majority of functional groups identified in the microarray revealed numerous genes up-regulated by E6/E7. However, several genes involved with immune regulation, IL-1 α , IL-8, GRO- α , IFN-stimulated gene 15 (ISG15), MIP-3 α , TNF- α , TNF α -inducible protein 3 (TNF α IP3), were identified as being down-regulated in the E6/E7-expressing cells. Semi-quantitative RT-PCR was used to validate not only the immune-related microarray targets, but also IL-1 β and IL-6, two additional cytokines produced by keratinocytes during the innate immune response. HFKs were stably infected with retrovirus carrying either vector alone (Babe) or a vector containing HPV-16 E6/E7 (E6/E7). In order to stimulate the production of cytokines and chemokines, keratinocytes were activated with Poly(I)·Poly(C), which has been shown to efficiently activate keratinocytes (55). Poly(I)·Poly(C), a synthetic double-stranded ribonucleotide, mimics an intermediate of a viral infection and is recognized by Toll-like receptor 3. Activation of Toll-like receptor 3 results in the

induction of NF- κ B activation and production of IFNs, cytokines, and chemokines. Control and E6/E7-expressing keratinocytes were activated with Poly(I)·Poly(C) for 16 h, washed, and refed with minimal EpiLife for 4 h. Following treatment, cells were harvested, RNA was purified, and cDNA was amplified for use in PCR reactions to provide semi-quantitative RT-PCR validation of the immune genes. As shown in Figure 3.1, the PCR results validated the genes shown to be down-regulated in E6/E7-expressing keratinocytes from the microarray. The additional cytokines selected for investigation, IL-1 β and IL-6, were also down-regulated in E6/E7-expressing cells. Results, following normalization to GAPDH levels, showed 1.3 to 3-fold reductions in mRNA levels of the immune genes in E6/E7-expressing cells compared to control cells.

To obtain a more accurate picture of immune response genes that are down-regulated by E6/E7, micro fluidic cards of 96 immune-related genes were used for real-time qPCR validation. Each cell line of control and E6/E7-expressing keratinocytes was generated by the retroviral infection of pooled neonatal foreskin keratinocytes; thus, each cell line possesses a mixture of unique genetic backgrounds. In order to control for genetic background effects, three experiments using cells from three different pooled foreskins were infected with retrovirus and treated with Poly(I)·Poly(C). Following treatment, cells were harvested and RNA was purified for cDNA amplification. The micro fluidic cards, containing 96 genes involved in the immune response, were run on the ABI PRISM 7900HT Sequence Detection System (SDS) and data was analyzed with SDS 2.2 software using GAPDH as the endogenous

control. Results from the micro fluidic cards provided quantitative validation of the immune genes known to be down-regulated in E6/E7-expressing keratinocytes from the microarray and also identified additional cytokines, chemokines, receptors, and wound healing factors that were down-regulated in Poly(I)·Poly(C)-stimulated E6/E7-expressing keratinocytes (Table 1). Many of these genes may not be direct targets of E6/E7, but rather the decrease in expression may be a result of reduced production of their inducers. For instance, IL-1 β induces the production of TNF- α , and IL-15 stimulates inducible nitric oxide synthetase (iNos) in keratinocytes (36, 110). The expression of RANTES, Endothelin 1 (EDN1), and Complement Component 3 (C3) in keratinocytes is increased after stimulation with TNF- α (56, 74, 102). Thus, it is possible that down-regulation of RANTES, EDN1, C3 and iNOS may be an indirect result of repression of IL-1 β and IL-15 by E6/E7.

Since one of the focused areas of this research was to ascertain a biological consequence of down-regulated immune genes, we selected MIP-3 α for further investigation due to its selective and potent role in the recruitment of LCs during the innate immune response. To examine the effect of E6/E7 on MIP-3 α mRNA and protein levels, control and E6/E7-expressing cells were stimulated with Poly(I)·Poly(C) for 16 h. In our hands, the use of Poly(I)·Poly(C) resulted in the strongest stimulation of MIP-3 α production, compared to LPS, TNF- α , and IL-1 β (Figure 3.2). HFKs were treated with 100 μ g/mL Poly(I)·Poly(C), 100ng/mL LPS, 100ng/mL TNF- α , or 100ng/mL IL-1 β for 1 h, 4 h, or 16 h. Following treatment, cells were washed with PBS, and refed with minimal EpiLife for 1 h or 4 h. While the

mRNA levels of MIP-3 α increased quickly after treatment for 1 h, the protein levels were low. Detection of higher levels of protein was needed for future experiments since we wanted to detect significant differences in production between control cells and E6/E7-expressing cells. The highest mRNA and protein levels of MIP-3 α were detected following 16 h of treatment with Poly(I)·Poly(C). Treatment with Poly(I)·Poly(C) for longer than 16 h resulted in increased cell death; therefore, stimulation of keratinocytes with Poly(I)·Poly(C) in this study involved treatment for 16 h followed by addition of minimal EpiLife for an extra 4 h. Following treatment, the cells were harvested for evaluation by real-time qPCR, and culture supernatants were collected for use in ELISA analysis and migration assays. Following Poly(I)·Poly(C) treatment of three independent cell lines, E6/E7-expressing keratinocytes showed an average 3-fold reduction in MIP-3 α mRNA levels compared to control cells (Figure 3.3A). We next examined the secretion of MIP-3 α by Poly(I)·Poly(C)-treated keratinocytes. As shown in Figure 3.3B, levels of secreted MIP-3 α protein parallel the levels of mRNA expression, such that there was a reduced level of MIP-3 α in cells expressing E6/E7 (123 pg/mL) compared to controls (228 pg/mL). Given that MIP-3 α functions as a potent chemoattractant for immature dendritic cells, memory T cells, and epidermal LC precursors, reduced production of MIP-3 α by E6/E7-expressing keratinocytes could explain the decreased numbers of LCs in both low-grade and high-grade cervical lesions compared to normal tissue. Without MIP-3 α to recruit antigen-presenting cells to the site of infection, HPV could persist undetected by the host immune system.

3.2.2 Characterization and migration of LPLCs.

To evaluate the effect of factors secreted by the keratinocytes on the migration of LPLCs, it was necessary to generate cells characteristic of epidermal LC precursors. The method for generating immature LCs or LC precursors is similar to the means by which cells characteristic of immature dendritic cells can be cultured. Both types of cells can be generated *in vitro* from either CD34⁺ hematopoietic progenitor cells from bone marrow or cord blood, or peripheral blood CD14⁺ monocytes. Culturing CD34⁺ cells with GM-CSF and TNF- α , or CD14⁺ monocytes with GM-CSF and IL-4 yields cells characteristic of immature dendritic cells [reviewed in (99)]. However, the use of IL-4 in culture suppresses the expression of CCR6 (26). As such, culturing CD14⁺ monocytes with GM-CSF and IL-4 yields cells with no expression of CCR6, but culturing monocytes in the presence of GM-CSF, IL-4, and transforming growth factor- β 1 (TGF- β 1), results in cells having minimal expression of CCR6 (28, 34). TGF- β 1 has been found to be critical for the development of LCs both *in vitro* and *in vivo* (11, 49). Several different culture conditions were utilized in order to generate a population of cells characteristic of LC precursors. Culturing CD14⁺ monocytes with GM-CSF and IL-4 yielded a population of cells with moderate expression of CD1a (a surface marker of immature dendritic cells), but low CD14 and CCR6 (Figure 3.4). In contrast, culturing CD14⁺ monocytes in the presence of GM-CSF and TGF- β 1 generated cells with low CD1a expression and high expression of CD14 and CCR6. Additionally, after 6 days in culture, the cells also showed low levels of CD83 and

high levels of class II molecules, consistent with immature status and the ability to uptake antigen. The phagocytic ability of these cells was confirmed by the capacity to take up fluorescent-conjugated ovalbumin. As shown in Figure 3.5, results from FACS analysis on CD14⁺ monocytes cultured for 6 days in the presence of GM-CSF and TGF- β 1 revealed cells that expressed surface markers and antigen uptake characteristic of LC precursors: CD14 (47% \pm 17), CCR6 (13% \pm 8), CD83 (10% \pm 4), class II molecules (95% \pm 2), antigen uptake (63% \pm 12). These results are consistent with other studies either using CD14⁺ monocytes in similar culture conditions or LC precursors (34, 39). Maturation of immature LCs can be induced by exposure to TNF- α , LPS, or CD40L. As such, addition of 100ng/mL LPS to the LPLC cultures for 24 h resulted in increased expression of CD14 and decreased antigen uptake and expression of class II molecules consistent with the ability of LPLCs to respond to LPS and begin maturation.(Figure 3.6).

3.2.3 Migration of LPLCs in response to culture supernatant from HPV-16 E6/E7-expressing keratinocytes.

The migratory response of the LPLCs to MIP-3 α was first tested to confirm that the level of CCR6 expression generated under the culture conditions described in this study would allow investigation of differences in migration between supernatants from control and E6/E7-expressing keratinocytes. In three independent experiments, the addition of 100ng/mL MIP-3 α to minimal media increased migration of LPLCs an average of 2.5-fold (Figure 3.7A). The migration of three independent LPLC cultures

was then tested against matched culture supernatants from three independent cell lines of Poly(I)·Poly(C)-treated control and E6/E7-expressing keratinocytes. As shown in Figure 3.7B, exposure to supernatant from E6/E7-expressing keratinocytes resulted in a 1.6-fold reduction in the number of migrating LPLCs.

3.2.4 Decreased migration of LPLCs is specific to the reduction of MIP-3 α by HPV-16 E6/E7-expressing keratinocytes.

Keratinocytes produce a number of immune mediators in response to an infection, many of which are chemotactic for LCs, T cells, and macrophages. In order to determine the role of MIP-3 α in the migratory response of LPLCs, MIP-3 α was added to conditioned media from E6/E7-expressing keratinocytes. The amount of exogenous MIP-3 α added to each migration assay was calculated from ELISAs carried out on the culture supernatants from Poly(I)·Poly(C)-treated control and E6/E7-expressing keratinocytes (Figure 3.3B). The number of migrating LPLCs increased, following addition of exogenous MIP-3 α to supernatant from E6/E7-expressing keratinocytes, to the level of LPLCs migrating in response to supernatant from control cells (Figure 3.8A). To further examine the effect of MIP-3 α on migrating LPLCs, neutralizing antibody to MIP-3 α was added to supernatant from Poly(I)·Poly(C)-treated control cells. Similar to the compensation assays, the amount of MIP-3 α to be neutralized in the migration assay was calculated from ELISAs carried out on culture supernatants (Figure 3.3B). The approximate amount of neutralizing antibody to be used was calculated using the Neutralization Dose₅₀ provided on the technical data

sheet. Use of the neutralizing antibody resulted in a reduction in the migration of LPLCs to the level of that seen with supernatant from E6/E7-expressing keratinocytes (Figure 3.8B). Thus, reduction in numbers of migrating LCs is due partially to decreased MIP-3 α production by E6/E7-expressing keratinocytes.

3.3 Discussion

Keratinocytes, the main target cell of HPV infections, play a key role in initiating the innate immune response. The cells function as producers of inflammatory cytokines, chemokines, and adhesion molecules which assist the recruitment of granulocytes, macrophages, and LCs. The ability of HPV proteins to inhibit these alert signals would aid the virus in establishing a persistent infection. Our results demonstrate that the expression of several immune-related genes is reduced in HPV-16 E6/E7-expressing keratinocytes compared to control cells. Many of these factors perform multiple functions and, as such, the direct effect of their dysregulation during an HPV infection may be unclear. For example, TNF- α induces the expression of adhesion molecules, recruits neutrophils, and stimulates the production of other cytokines and chemokines. IL-1 α and - β also stimulate the production of pro-inflammatory mediators and adhesion molecules, but also activate T lymphocytes by inducing expression of IL-2 and IL-2 receptor, and enhance B cell proliferation. Thus, the biological consequences of TNF- α and IL-1 down-regulation by HPV-expressing cells are quite diverse and impact both the innate and adaptive immune responses. In contrast, MIP-3 α is instrumental in initiating innate immunity

by means of its selective role as a chemoattractant for immature LCs. Hence, our studies focused on both verifying the down-regulation of MIP-3 α by E6/E7-expressing keratinocytes and correlating this repression with differential migration of LPLCs.

Our results showed that E6/E7-expressing keratinocytes express less MIP-3 α mRNA and protein than control cells. Prior to investigating the effect of decreased MIP-3 α on the migration of LC precursors, it was necessary to identify culture conditions which would generate a population of cells characteristic of immature LC precursors. Immature LC precursors have not completely differentiated towards immature LCs; thus, these cells retain a moderate level of CD14 expression. However, these cells express CCR6 which allows for migration to MIP-3 α , and low CD83 which indicates an immature status. In addition to low CD83, a high expression level of class II molecules is also indicative of the ability of LC precursors to uptake antigen once they have migrated to the site of antigen entry. While many conditions used in other studies generate cells characteristic of immature dendritic cells from either CD14⁺ or CD34⁺ cells, the culture conditions of GM-CSF and TGF- β 1 used in this study generated cells characteristic of immature LC precursors from CD14⁺ cells. The use of LPLCs in migration assays not only confirmed a migratory response to MIP-3 α , but also demonstrated that reduced migration of LPLCs in response to culture supernatants of E6/E7-expressing cells is specific to the decreased production of MIP-3 α . While it is possible to hypothesize that the global suppression of many immune-related targets would negatively impact host innate immunity, we have demonstrated a biological consequence of reduced MIP-3 α expression. The result of decreased MIP-

3 α production by E6/E7-expressing keratinocytes results in reduced migration of LPLCs. Other studies have demonstrated decreased LC density associated with HPV-positive lesions and cervical carcinoma. Thus, tissues with persistent HPV infection have reduced immune surveillance which may be attributed, in part, to reduced expression of MIP-3 α .

Figure 3.1 RT-PCR target validation of genes identified in microarray. Primary HFKs stably expressing vector alone (Babe) or HPV-16 E6/E7 (E6/E7) were treated with 100 μ g/mL Poly(I)-Poly(C) for 16 h. Following treatment, cells were washed and refed with minimal EpiLife for 4 h. RNA was harvested from the cells and mRNA levels for IL-1 α , IL-1 β , IL-6, IL-8, GRO- α , ISG15, MIP-3 α , TNF- α , and TNF α IP3 were determined by RT-PCR. Results are normalized to GAPDH (shown to the right of each RT-PCR result).

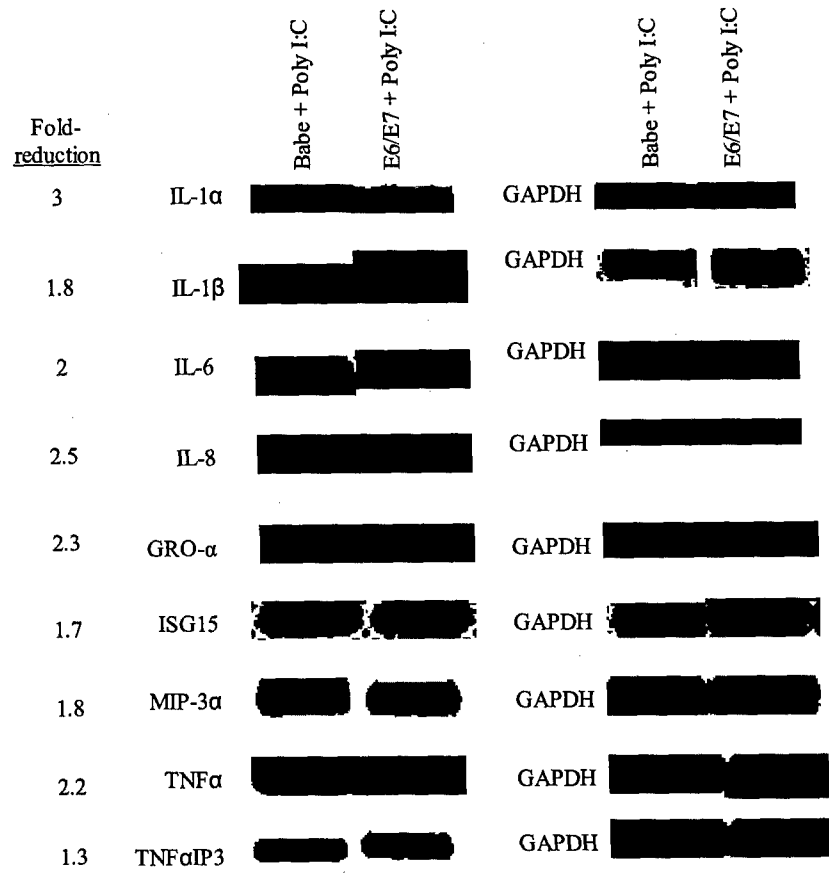


TABLE 1. Immune-related genes down-regulated in E6/E7-expressing keratinocytes

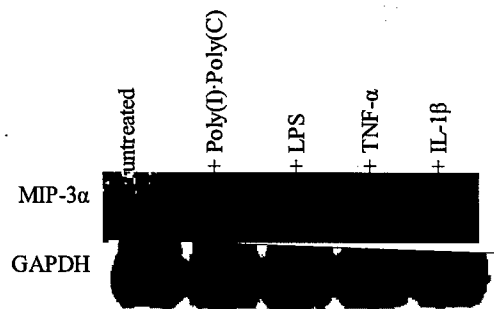
Gene name	Accession number	Average fold decrease in E6/E7 cells
CD28 ^a	NM_006139	50
CD25 ^a	NM_000417	25
CD80 ^a	NM_005191	20
iNOS	NM_000625	11
CYP7A1	NM_000761	10
LT α	NM_000595	6.7
CD54 (ICAM)	NM_000201	5.9
RANTES	NM_00298	5.6
MIP-1 α	NM_002983	4
ITAC	NM_005409	3.6
MIP-3 α ^b	NM_004591	3
IP10	NM_001565	3
CD40	NM_001250	3
C3	NM_000064	2.8
IL-8	NM_000584	2.8
FasL	NM_000639	2.6
M-CSF	NM_005211	2.6
IL-15	NM_000585	2.4
HO-1	NM_002133	2.4
ECE-1	NM_001397	2.4
Fas	NM_000043	2.4
EDN1	NM_001955	2.3
IL-6	NM_000600	2.1
NF κ B2(p49)	NM_002502	2.1
IL-1 β	NM_000576	1.9
MADH-7	NM_005904	1.5
BCL-XL	NM_001191	1.5
Stat3	NM_139276	1.5
BAX	NM_004324	1.4

^adenotes expression in 2 of 3 micro fluidic cards

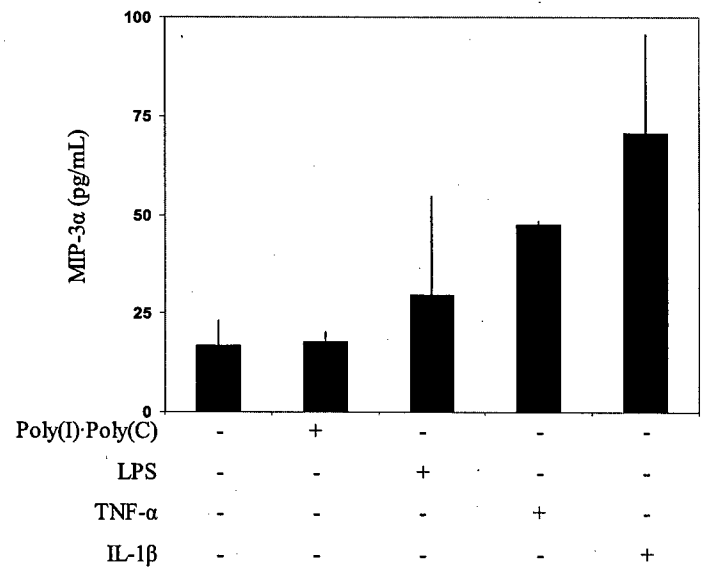
^bnot a target gene included on micro fluidic cards; value expressed is a result of separate real-time PCR validation

Figure 3.2 MIP-3 α expression in human foreskin keratinocytes under different stimuli. 1×10^6 keratinocytes were seeded on 10-cm dishes 24 h prior to treatment. Keratinocytes were refed with antibiotic-free media and left untreated or treated with 100 μ g/mL Poly(I)·Poly(C), 100ng/mL LPS, 100ng/mL TNF- α , or 100ng/mL IL-1 β for 1 h (A and B) or 16 h (C and D). Following treatment, cells were washed and refed with minimal EpiLife for 1 h (A and B) or 4 h (C and D). (A and C) RNA was harvested from the cells and mRNA levels for MIP-3 α were determined by RT-PCR. (B and D) Culture supernatants were harvested and levels of secreted MIP-3 α were measured by ELISA.

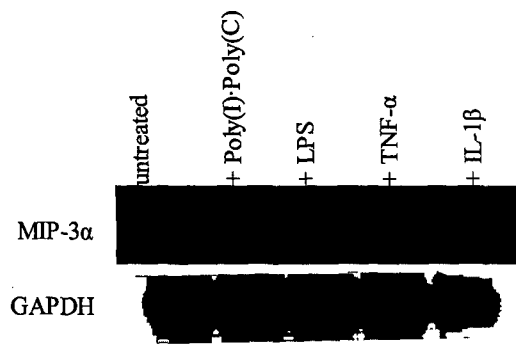
A



B



C



D

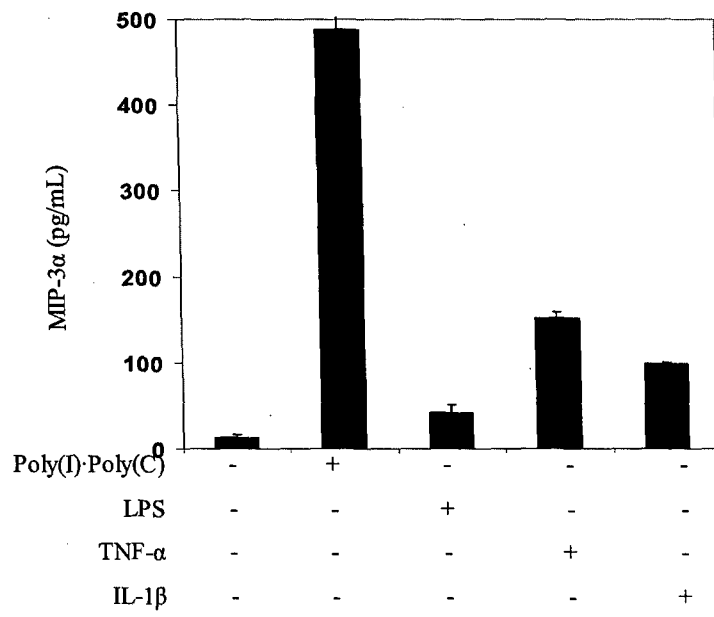
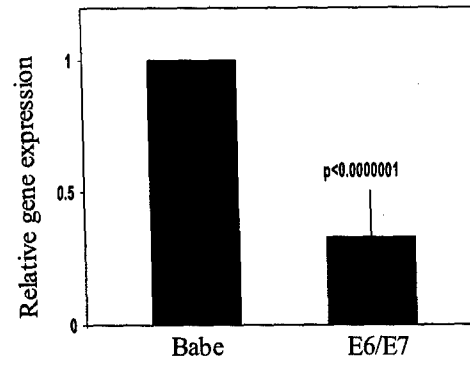


Figure 3.3 MIP-3 α expression in control and HPV-16 E6/E7-expressing keratinocytes measured by real-time qPCR and ELISA. Primary HFKs stably expressing vector alone (Babe) or HPV-16 E6/E7 (E6/E7) were treated with 100 μ g/mL Poly(I)·Poly(C) for 16 h. Following treatment, cells were washed and refed with minimal EpiLife for 4 h. (A) RNA was harvested from the cells and mRNA levels for MIP-3 α were determined by real-time qPCR. Results are normalized to GAPDH and expressed as the mean \pm SD of three experiments using three independent cell lines. (B) Culture supernatants were harvested and levels of secreted MIP-3 α were measured by ELISA. Results are expressed as the mean \pm SD of three experiments using three independent cell lines.

A



B

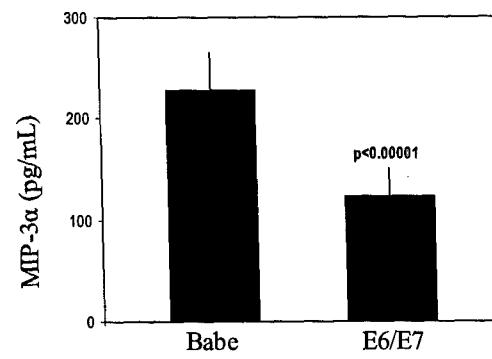


Figure 3.4 Use of GM-CSF and IL-4 in cultures of CD14⁺ monocytes does not generate CCR6⁺ LPLCs. CD14⁺ monocytes were isolated from peripheral blood as described in Materials and Methods and cultured in complete media supplemented with 80ng/mL GM-CSF and 10ng/mL IL-4. On day 6, cells were stained with anti-CD14, CCR6, or CD1a mAbs and were analyzed with a FACScan using CellQuest software.

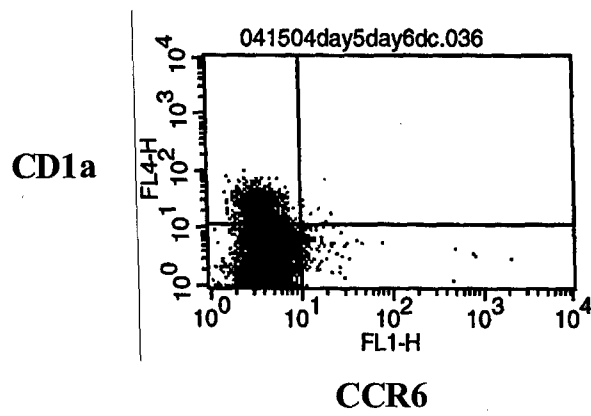
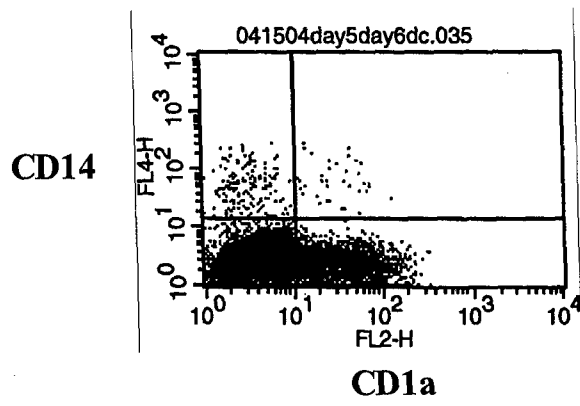


Figure 3.5 Characterization of LPLCs. LPLCs were generated from CD14⁺ peripheral blood monocytes as described in Materials and Methods and cultured in complete media supplemented with 200ng/mL GM-CSF and 10ng/mL TGF- β 1. On day 6, LPLCs were stained with anti-CD14, CCR6, CD83, MHCII mAbs or incubated with conjugated ovalbumin and were analyzed with a FACScan using CellQuest software. Open histograms indicate cell staining with the isotype-matched negative control antibody.

CD14



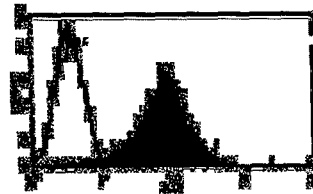
CCR6



CD83



MHCII



Antigen uptake



Figure 3.6 Addition of LPS to LPLC culture decreases immature status of LPLCs. LPLCs were generated from CD14⁺ peripheral blood monocytes as described in Materials and Methods and cultured in complete media supplemented with 200ng/mL GM-CSF and 10ng/mL TGF- β 1. On day 6, 100ng/mL LPS was added to duplicate wells 24 h prior to staining for FACS analysis. LPLCs were stained with anti-CD14, CCR6, CD83, MHCII mAbs or incubated with conjugated ovalbumin and were analyzed with a FACScan using CellQuest software. Open histograms indicate cell staining with the isotype-matched negative control antibody.

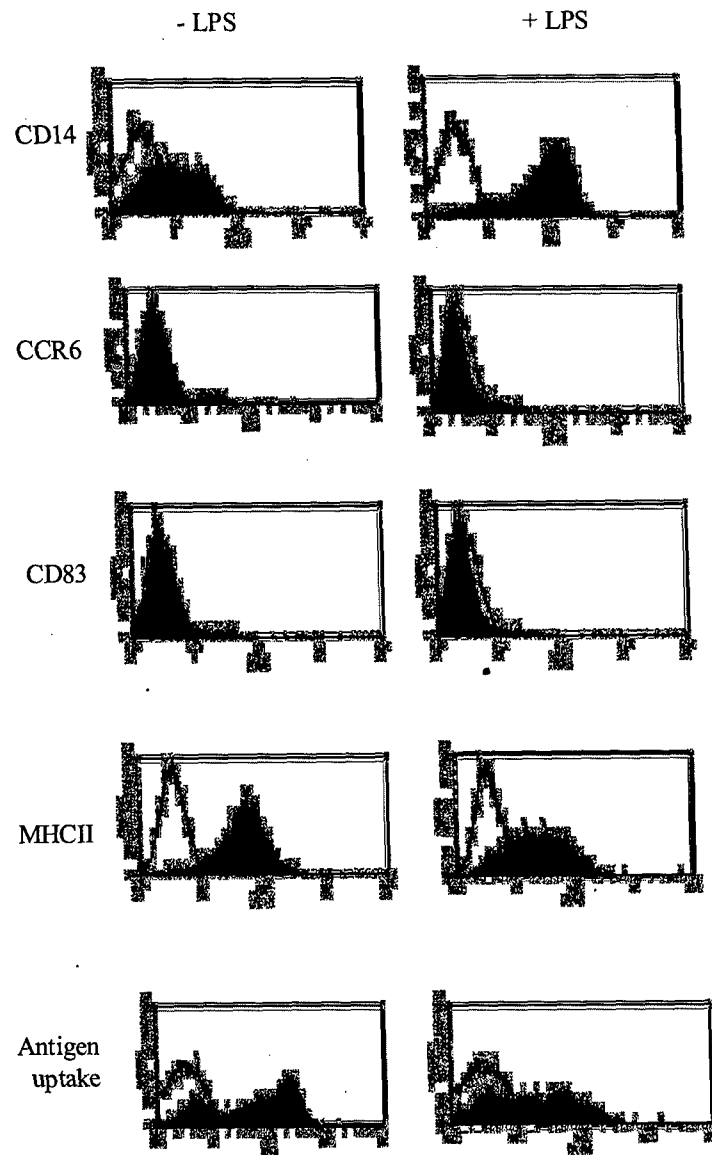


Figure 3.7 Migration of LPLCs. LPLCs were generated as described in Materials and Methods. On day 6, LPLCs were used in multiwell chamber assays. (A) Minimal media alone or with the addition of 100ng/mL exogenous MIP-3 α was used in multiwell chamber assays. Values are expressed as migration of LPLCs in response to media plus MIP-3 α relative to migration in response to media alone. Results are expressed as the mean \pm SD of three experiments using three independent LPLC cultures. (B) Primary HFKs stably expressing vector alone (Babe) or HPV-16 E6/E7 (E6/E7) were treated with Poly(I)·Poly(C). Following treatment, cells were washed with PBS and refed with minimal media for 4 h. Culture supernatants were collected and used in multiwell chamber assays. Values are expressed as migration of LPLCs in response to E6/E7 supernatant relative to migration in response to supernatant from control cells (Babe). Results are expressed as the mean \pm SD of three experiments using three independent cell lines.

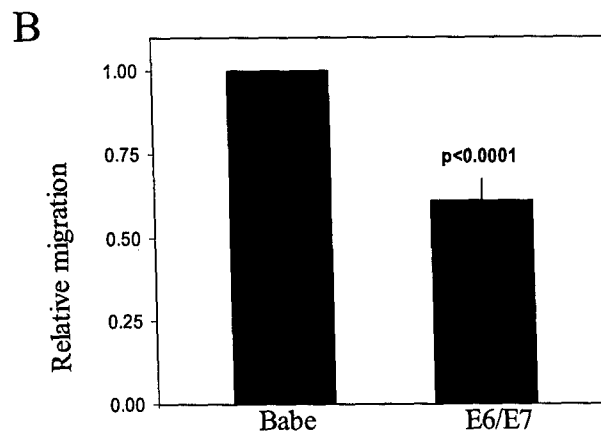
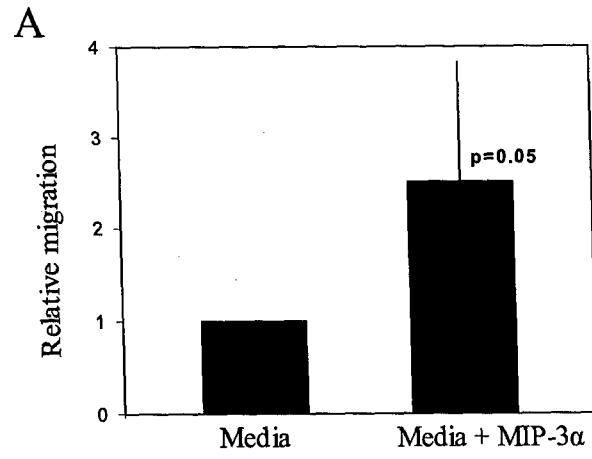
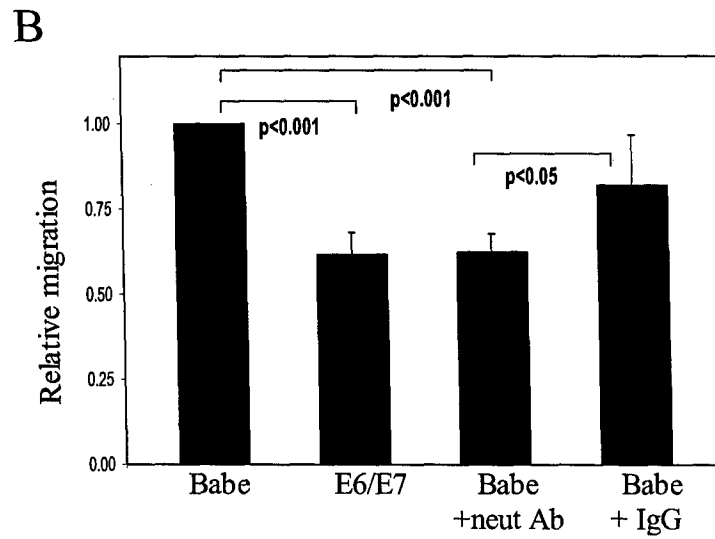
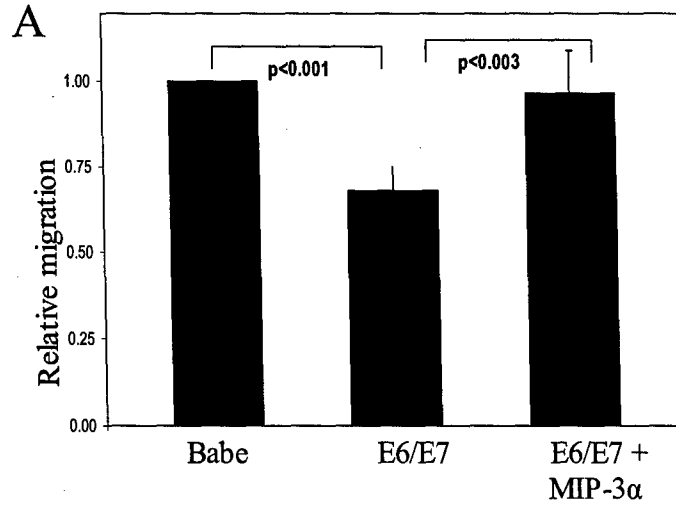


Figure 3.8 Effect of MIP-3 α on migration of LPLCs. Primary HFKs stably expressing vector alone (Babe) or HPV-16 E6/E7 (E6/E7) were treated with Poly(I)·Poly(C). Following treatment, cells were washed with PBS and refed with minimal EpiLife for 4 h. Culture supernatants were collected and used in multiwell chamber assays. Values are expressed as migration of LPLCs in test samples relative to migration in response to supernatant from control cells (Babe). (A) Migration of LPLCs was increased upon addition of MIP-3 α to E6/E7 supernatant. Results are expressed as the mean \pm SD of three experiments using three independent cell lines. (B) Migration of LPLCs was inhibited when MIP-3 α neutralizing antibody was added to control (Babe) supernatant. An equivalent concentration of goat IgG was added to duplicate wells as an internal control. Results are expressed as the mean \pm SD of three experiments using three independent cell lines.



Chapter 4

**Disruption of NF- κ B signaling by HPV-16 E6/E7 accounts for reduced MIP-3 α
expression in E6/E7-expressing keratinocytes**

4.1 Introduction

We previously demonstrated that the expression of numerous immune-related genes was reduced in HPV-16 E6/E7-expressing keratinocytes. All of the genes identified are important in many aspects of the innate immune response, such as induction of inflammation and adhesion molecules, as well as recruitment and costimulation of immune cells. The ability of E6/E7 to hinder the induction of immune molecules in an HPV-infected cell would allow the virus to persist in the epithelium undetected by the host immune system. Critical to this primary response is the release of chemokines which recruit immature LCs to the site of infection. Upon antigen processing, LCs present foreign peptide to T lymphocytes and activate a specific immune response devoted to elimination of HPV-infected cells. MIP-3 α functions as a potent chemoattractant of immature LCs, and our previous results showed that mRNA levels of MIP-3 α are reduced in E6/E7-expressing keratinocytes. As a result of decreased MIP-3 α protein produced by these cells, there was a reduced number of migrating LPLCs. In an attempt to understand the mechanism underlying this repression of MIP-3 α production, we investigated the individual contributions of E6 and E7 from HPV-16, as well as the combined involvement of E6 and E7 from low-risk HPV types 6 and 11. Additionally, we examined possible abrogation of the NF- κ B pathway by E6 and E7. Given that NF- κ B is a central regulator of many genes involved in immune function, the ability of E6/E7 to disrupt this signaling pathway could explain the extensive suppression of immune mediators we detected early in our study.

4.2 Results

4.2.1 Contributions of E6 and E7 from HPV-16 to MIP-3 α repression.

In order to determine possible mechanism(s) through which HPV-16 E6/E7 down-regulate MIP-3 α expression, it is important to investigate the individual contributions of E6 and E7 to this repression. Since the interactions of E6 and E7 with p53 and Rb, respectively, are critical in de-regulating transcription of cellular genes important in cell cycle progression and apoptosis, these same interactions could also participate in dysregulation of immune genes in HPV-infected cells. In order to investigate the individual effects of E6 or E7 to the repression of MIP-3 α , cell lines that expressed significant levels of each oncoprotein were generated. Retroviral constructs containing stop codons in either E6 or E7 of HPV-16 were used to produce keratinocytes expressing higher levels of E6 (E6/E7stop) or E7 (E6stop/E7) than when expressed alone (Figure 4.1). Semi-quantitative PCR and western blots confirmed levels of E6 and E7 mRNA and protein in control (Babe), E6/E7- (E6/E7), E6- (E6 or E6/E7stop), and E7- (E7 or E6stop/E7) expressing cells. Results from semi-quantitative PCR revealed higher levels of E6 mRNA in the E6/E7stop cell line than the E6 cell line, as well as higher levels of E7 mRNA in the E6stop/E7 cell line than the E7 cell line (Figure 4.1B). Protein levels of E7 correlated with the mRNA levels in that more E7 was detected in the E6stop/E7 cell line than the E7 cell line. Without the availability of an antibody that can detect HPV-16 E6 protein levels expressed in human cells, p53 levels are used as an indirect indication of E6 levels. HPV-16 E6 can bind and degrade p53 (45, 88), as indicated by lower levels of p53 in the E6/E7

and E6/E7stop cell lines. A higher level of p53 in the E6 cell line corresponds with the low level of E6 mRNA observed in the same cell line; therefore, with less E6 expressed in the cell, there is less degradation of p53. The highest level of p53 was expressed in the E6stop/E7 cell line which is related to both the absence of any E6 as well as the documented ability of E7 to stabilize p53 (23). Thus, retroviral constructs encoding E6/E7stop and E6stop/E7 were used to infect keratinocytes since the use of these constructs generated cell lines expressing higher levels of E6 and E7 than the use of constructs encoding E6 or E7 individually.

To determine the effects of E6 or E7 on the down-regulation of MIP-3 α , control, E6/E7, E6 (E6/E7stop), and E7 (E6stop/E7)-expressing cells were stimulated with Poly(I)·Poly(C) as described previously. Following treatment, cells were harvested for evaluation by real-time qPCR, and culture supernatants were collected for use in ELISA analysis. The results following treatment of three independent cell lines showed that E6/E7-expressing keratinocytes had the greatest repression (75%) in MIP-3 α mRNA levels compared to control cells, followed by E6-expressing cells (62%) and E7-expressing cells (58%) (Figure 4.2A). Similar to the data shown in Figure 3.3, the levels of secreted MIP-3 α protein parallel the mRNA levels, with E6/E7-expressing keratinocytes producing the least amount of MIP-3 α (84pg/mL) compared to control cells (304pg/mL), followed by E6-expressing cells (88pg/mL) and E7-expressing cells (178pg/mL) (Figure 4.2B). Analysis of mRNA and protein levels indicates that E6 may contribute slightly more than E7 to the repression of MIP-

3 α ; however, the expression of both E6 and E7 in keratinocytes results in the greatest decrease in MIP-3 α production.

4.2.2 The ability to down-regulate MIP-3 α is a common feature of E6 and E7 proteins from high- and low-risk HPV types.

Of particular interest is whether repression of MIP-3 α is unique to the high-risk HPV types. In addition to the ability of E6/E7 to immortalize cells, the high-risk HPVs, particularly type 16 and 18, are highly correlated with high-grade cervical lesions and cervical cancers. In contrast, E6/E7 from low-risk HPV types (6 and 11) cannot immortalize cells (89), but are still associated with genital warts and low-grade cervical lesions. As such, the dysregulation of cell cycle genes caused by E6 and E7 differs between the high-risk and low-risk HPV types. The ability of E6/E7 from high-risk HPV types to disrupt normal cell cycle progression and programmed cell death contributes to the progression of lesions to invasive carcinoma. However, the disruption of gene regulation by E6/E7 of low-risk HPV types differs from that of the high-risk types. If MIP-3 α levels are reduced in both low-risk and high-risk HPV types, this result would indicate that the mechanism underlying MIP-3 α repression is a function of E6/E7 that is common to both low-risk and high-risk HPVs. To examine whether or not suppression of MIP-3 α is unique to only the high-risk HPV types, cell lines were generated carrying HPV-16 E6/E7, HPV-6 E6/E7, or HPV-11 E6/E7. The cell lines and control keratinocytes were treated with Poly(I)·Poly(C) as before, then cells and culture supernatants were harvested for qPCR and ELISA analysis. Real-

time qPCR and ELISA results revealed similar reductions in MIP-3 α mRNA levels among all three HPV types, as well as decreased production of MIP-3 α protein when compared to control cells (Figure 4.3). Although not correlated with progression of malignant disease, the capacity for low-risk HPVs to evade the host immune response would still be beneficial to the persistence and propagation of these viruses in genital warts and low-grade lesions.

Results showing MIP-3 α suppression in cells expressing E6 or E7 individually (Figure 4.2) suggest that both E6 and E7 contribute to the repression of MIP-3 α ; however, the mechanism underlying this inhibition is not known. For high-risk HPVs, their oncogenic potential has often been linked to the ability of E6 to bind and degrade p53, and of E7 to bind to pRb and abrogate the functions of the Rb family members (42, 45, 77, 88). Therefore, one possible mechanism of the effect of HPV-16 E6 and E7 on MIP-3 α expression would be through their interactions with p53 and Rb. However, E6 of HPV-6 and HPV-11 shows no binding to or degradation of p53 (87, 107), and E7 of HPV-6 and HPV-11, while binding to Rb, do so to a lesser extent than that seen with E7 of high-risk HPVs (37, 67). If the down-regulation of MIP-3 α occurred through a p53- or Rb-dependent pathway, reductions in MIP-3 α production would not be displayed by cell lines expressing E6/E7 from HPV-6 or -11. Thus, the ability of E6/E7 from both low-risk and high-risk HPV types to repress MIP-3 α transcription suggests that the inhibition of MIP-3 α is through a mechanism independent of the effects of E6 and E7 on p53 and Rb.

4.2.3 NF- κ B is required for MIP-3 α expression in keratinocytes.

Identifying the transcription factors necessary for gene expression is important in investigating how genes may be dysregulated by viral proteins. As shown in Figure 4.4A, the promoter region of MIP-3 α has been identified to possibly contain binding sites for several transcription factors, such as NF- κ B, CAAT/enhancer-binding protein (C/EBP), AP-1, stimulating protein-1 (Sp-1), and epithelium-specific Ets nuclear factor (Ets) (91). Initial experiments using a luciferase reporter gene containing the full-length MIP-3 α promoter demonstrated that MIP-3 α promoter activity in primary human keratinocytes can be enhanced by the addition of Poly(I)·Poly(C), TNF- α , and IL-1 β (Figure 4.4B). Our results are consistent with those of other studies that have shown an upregulation of MIP-3 α expression in keratinocytes, melanocytes, endothelial cells, fibroblasts, colon carcinoma cells, and mouse skin following addition of TNF- α , IL-1 α , and/or IL-1 β (25, 40, 53, 69, 101). Similar reporter assays were then carried out using the full-length MIP-3 α promoter with mutated transcription factor binding sites in order to verify which transcription factors are important for basal and stimulated MIP-3 α activity in primary keratinocytes. NF- κ B and C/EBP mutants were generated by targeted substitution mutations in the respective binding sites, and the AP-1 and Ets mutants were created using sequential 10 base pair nucleotide substitutions (53). Figures 4.4D-F show that keratinocytes transfected with the MIP-3 α promoter constructs containing mutations in the C/EBP, Ets, and AP-1 binding sites exhibit similar basal activity as the full-length MIP-3 α promoter construct. Additionally, MIP-3 α promoter activity can be increased by the

addition of Poly(I)·Poly(C), TNF- α , and IL-1 β . In contrast, use of the promoter construct containing a mutated NF- κ B binding site demonstrated reduced basal MIP-3 α activity as well as a lack of induction by Poly(I)·Poly(C), TNF- α , and IL-1 β (Figure 4.4C). Due to the fact that MIP-3 α expression has been shown to be increased by the pro-inflammatory cytokines TNF- α and IL-1 in several different cell types, we wanted to confirm that the reduced MIP-3 α expression in our study was not an indirect result of decreased TNF- α and/or IL-1 production caused by E6/E7. Luciferase reporter assays using the full-length MIP-3 α promoter demonstrated that both E6 and E7 can repress MIP-3 α promoter activity even in the presence or absence of exogenously added TNF- α and IL-1 β (Figure 4.5). Similar results were obtained when adding either exogenous TNF- α or IL-1 β . These results suggest that expression of E6/E7 was having a direct effect on MIP-3 α transcription, and not indirectly through their down-regulation of TNF- α and IL-1 β .

Recent work by other groups has revealed a critical role for NF- κ B in the expression of MIP-3 α . For example, mice pretreated with gliotoxin showed dramatically reduced MIP-3 α expression following LPS injections (98). Gliotoxin is thought to hamper NF- κ B activation by inhibiting I κ B degradation. Additionally, reporter assays using an I κ B α super repressor expression plasmid in HeLa cells completely inhibited TNF-stimulated MIP-3 α promoter activity. Similar results were obtained using the MIP-3 α promoter and I κ B α super repressor in HeLa cells and an inducible-Tax human T cell leukemia cell line (46). Tax, an accessory molecule encoded for in the genome human T cell leukemia virus, is thought to be responsible

for the infiltration of lymphocytes observed during disease, primarily through its transactivation of genes involved in chemoattraction. Other studies also demonstrated the requirement of intact NF- κ B binding sites for basal and IL-1 β -stimulated MIP-3 α activity in colon carcinoma cells, as well as TNF- α -induced activity in human melanoma cells (35, 53). Work accomplished by these groups and others has revealed the role of p50 and p65 rel family members in MIP-3 α activity. Results from cDNA subtractive hybridization demonstrated that MIP-3 α was a p65-dependent transcriptional target of NF- κ B (98). Electrophoretic mobility shift assays confirmed the binding of p50/p65 heterodimers to the NF- κ B binding site of the human MIP-3 α promoter following treatment with IL-1 β or TNF- α (35, 53), and TNF- α /IL-1 β -induced binding of p50/p65 and p65/p65 dimers to the NF- κ B binding site of the mouse MIP-3 α promoter (27). The human and mouse MIP-3 α promoters share ~75% homology, and only the TATA box, NF- κ B site, and one Ets site are conserved between the two promoters. The fact that the NF- κ B site is one of only two transcription factor binding sites conserved between the mouse and human promoters gives credence to the importance of NF- κ B transactivation for MIP-3 α activity. Additionally, work involving overexpression of p65 in MIP-3 α reporter assays resulted in p65 increasing MIP-3 α promoter activity in assays using the full-length promoter, but not in assays using the promoter with a mutated NF- κ B site (27, 35).

So far, our results indicated that NF- κ B was an important transcription factor for basal and stimulated MIP-3 α activity in primary human keratinocytes. The previously described work from other groups confirmed the importance of NF- κ B in

MIP-3 α promoter activity and expression in other cells, as well as identified the p50/p65 heterodimer as the NF- κ B rel family members responsible for this transactivation. Therefore, the down-regulation of MIP-3 α observed in E6/E7-expressing cells could result from interference in the NF- κ B signaling pathway. As stated previously, activation of the NF- κ B pathway results in phosphorylation of I κ B and subsequent release and translocation of rel family member dimers to the nucleus where they bind NF- κ B consensus sequences. Treatment of control, HPV-16 E6/E7, HPV-16 E6/E7stop (E6, HPV-16 E6stop/E7 (E7), HPV-6 E6/E7, and HPV-11 E6/E7-expressing keratinocytes with either TNF- α or Poly(I)·Poly(C) revealed no disruption of p65 translocation following stimulation (Figure 4.6). However, luciferase assays using an NF- κ B reporter construct, containing three NF- κ B sites upstream of the luciferase gene, demonstrated that E6 and E7 can decrease NF- κ B activity (Figure 4.7). Similar work accomplished by Patel et al. (75) demonstrated that HPV-16 E6 binds to three regions of CBP/p300, an important coactivator of NF- κ B activity. As a result, E6 was able to inhibit the transcriptional activity of CBP/p300 and decrease the ability of p300 to activate NF- κ B-responsive promoter elements. This interaction between E6 and CBP/p300 was further shown to repress IL-8 promoter activity, as E6 was able to compete with both p65 and SRC-1 for binding to CBP (43). In the same study, HPV-16 E7 was shown to bind to p/CAF and inhibits its coactivation of the IL-8 promoter. Additional studies demonstrate that binding of E7 to p/CAF reduces its acetyltransferase activity, and binding of E7 to p300 suppresses the transcriptional coactivation function of p300 (4, 9). Since our results indicate that p65 translocated to

the nucleus following stimulation of control and E6/E7-expressing cells, we investigated whether the repression of MIP-3 α occurred through the binding of E6 and E7 to NF- κ B coactivators.

Two mutations of E6 and E7 have been identified that disrupt their binding to NF- κ B coactivators, CBP/p300 and p/CAF. Mutating the 66th and 136th amino acids of HPV-16 E6 from cysteine to glycine abrogates the ability of E6 to bind to CBP/p300 (75). An amino acid substitution at position 2 of HPV-16 E7 from histidine to proline generates an E7 mutant that exhibits less binding to p/CAF compared to wild-type HPV-16 E7 (43). In addition to control cells and E6/E7-expressing keratinocytes, cell lines were generated expressing E6 alone (E6/E7stop), E6 C66G/C136G (E6 C66G/C136G/E7stop), E7 alone (E6stop/E7), and E7.2 (E6stop/E7.2). Results following Poly(I)-Poly(C) treatment of three independent cell lines showed statistically significant repression of MIP-3 α mRNA in the E6/E7 and E6-expressing keratinocytes compared to control cells, but MIP-3 α mRNA level in the cell line expressing E6 C66G/C136G was close to the level of control cells (Figure 4.8A). Similar results were observed when using E6/E7, E7, and E7.2-expressing keratinocytes (Figure 4.8B), suggesting that the interaction of HPV-16 E6 and E7 with CBP/p300 and p/CAF contributes to the overall repression of MIP-3 α production in keratinocytes. E6 and E7 of low-risk HPV types have also been found to bind p300 and p/CAF (4, 9, 75), thus suggesting that the interaction of E6 and E7 with these coactivators could be the mechanism underlying the repression of MIP-3 α observed in HPV-16, HPV-6 and HPV-11 E6/E7-expressing cell lines.

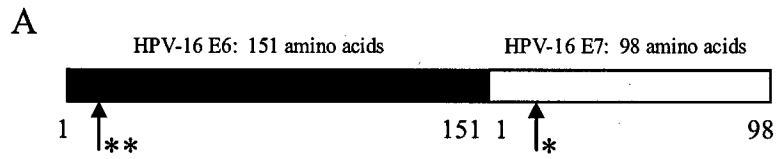
4.3 Discussion

Previous work demonstrated that expression of both E6 and E7 from HPV-16 in primary human keratinocytes decreased MIP-3 α production. In order to elucidate the mechanism underlying this repression, it is necessary to investigate whether E6 and E7 contribute individually or cooperatively to the down-regulation of MIP-3 α . In other studies, such as those examining dysregulation of genes responsible for cell cycle progression, E6 and E7 would most likely be contributing to the overall phenotype of infected cells through their respective effects on p53 and Rb. Our results suggested that E6 and E7 could individually repress MIP-3 α expression, but a greater effect was seen when both E6 and E7 were expressed together in cells. Therefore, the down-regulation may have occurred through p53- and Rb-dependent mechanisms, but results showing similar reductions of MIP-3 α in keratinocytes expressing E6/E7 from low-risk HPV types indicated that the mechanism was actually independent of p53 and Rb.

Many of the NF- κ B-responsive genes that have been identified are induced by pro-inflammatory cytokines, bacterial products, or viruses. Additionally, NF- κ B is an important transcriptional regulator of immune-related genes, such as cytokines, chemokines, adhesion molecules, cell surface receptors, and wound healing factors (30, 72, 86). Not surprisingly, previous work on MIP-3 α expression identified an NF- κ B binding site in the promoter that is required for basal and stimulated activity. Also, activation of the NF- κ B pathway by TNF- α or IL-1 β results in the binding of a

p50/p65 heterodimer to the NF- κ B site in the MIP-3 α promoter. Our results demonstrate that in primary human keratinocytes, an intact NF- κ B binding site is required for basal MIP-3 α promoter activity and induction by Poly(I)·Poly(C), TNF- α , and IL-1 β . However, while E6 and E7 inhibit NF- κ B activity, p65 can still translocate to the nucleus of control and E6/E7-expressing cells. As stated previously, binding to DNA consensus sequences is not always sufficient for complete gene transcription. NF- κ B transactivation often requires a combination of coactivators and HAT activity. The p50/p65 heterodimer recruits CBP, SRC-1, and p/CAF, and requires the HAT activity of p/CAF (95). HPV-16 E6 has been shown to compete with p65 and SRC-1 for binding to CBP, and HPV-16 E7 decreases the HAT activity of p/CAF by binding to it (43, 75). We, therefore, investigated the effects of E6 and E7 mutants that showed a lack of binding to CBP/p300 and decreased binding to p/CAF, respectively. While E6- and E7-expressing keratinocytes produced significantly less MIP-3 α mRNA than control cells, the MIP-3 α mRNA levels of keratinocytes expressing the E6 and E7 mutants were similar to the level in control cells. Thus, the interaction of E6 and E7 with NF- κ B coactivators could account for the down-regulation of MIP-3 α as well as several additional NF- κ B-responsive immune genes identified in earlier experiments. This interference of maximal NF- κ B transactivation could also explain the repression of MIP-3 α detected in keratinocytes expressing E6/E7 from low-risk HPV types since these proteins can also bind p300 and p/CAF (4, 9, 75).

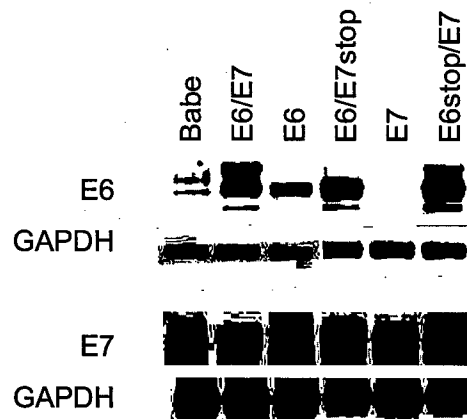
Figure 4.1 HPV-16 E6/E7stop and HPV-16 E6stop/E7 exhibit higher levels of E6 and E7 than HPV-16 E6 and HPV-16 E7 expressed alone. (A) Schematic of HPV-16 E6 and E7 showing location of stop codons used to generate E6/E7stop and E6stop/E7. Primary HFKs stably expressing vector alone (Babe), HPV-16 E6/E7 (E6/E7), HPV-16 E6 (E6 or E6/E7stop), or HPV-16 E7 (E7 or E6stop/E7) were harvested for RNA and protein levels of E6 and E7. (B) RNA was harvested from the cells and mRNA levels for E6 and E7 were determined by semi-quantitative PCR. (C) Western blot assays were performed with 100µg of total protein. Blots were probed for total p53, E7, and actin (as a loading control).



* E6/E7stop: 16th amino acid of E7 changed to stop codon

** E6stop/E7: 15th amino acid of E6 changed to stop codon

B



C

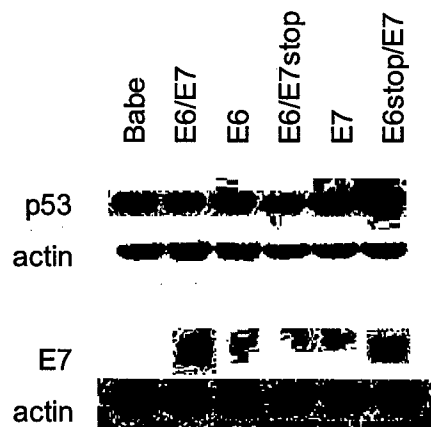
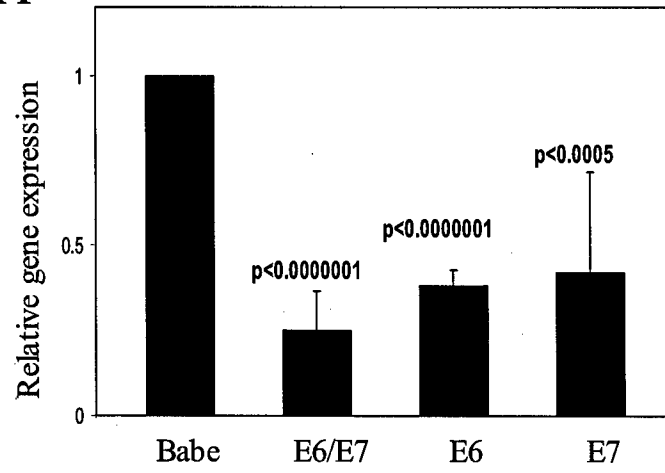


Figure 4.2 Effect of HPV-16 E6 and E7 on MIP-3 α mRNA and protein levels.

Primary HFKs stably expressing vector alone (Babe), HPV-16 E6/E7 (E6/E7), HPV-16 E6/E7stop (E6), or HPV-16 E6stop/E7 (E7) were treated with 100 μ g/mL Poly(I)·Poly(C) for 16 h. Following treatment, cells were washed and refed with minimal EpiLife for 4 h. (A) RNA was harvested from the cells and mRNA levels for MIP-3 α were determined by real-time qPCR. Results are expressed as the mean \pm SD of three experiments using three independent cell lines. (B) Culture supernatants were harvested and levels of secreted MIP-3 α were measured by ELISA. Results are expressed as the mean \pm SD of three experiments using three independent cell lines.

A



B

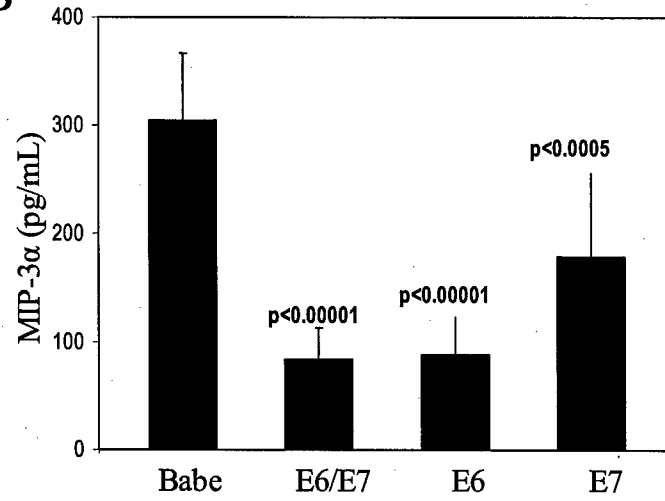
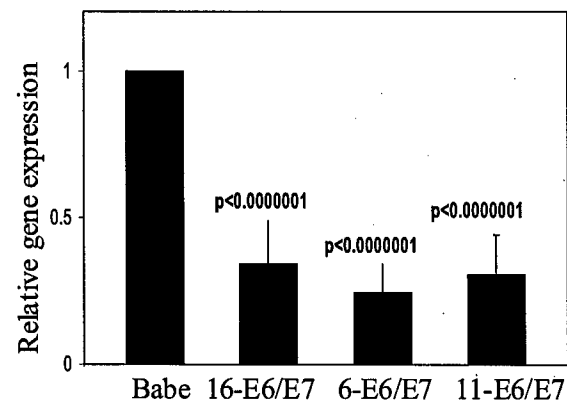
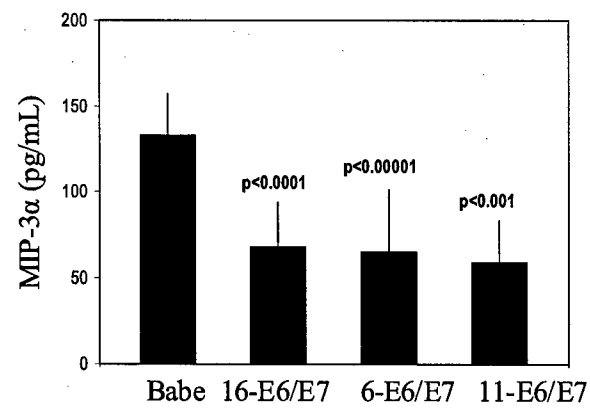


Figure 4.3 MIP-3 α expression in control, HPV-16 E6/E7, HPV-6 E6/E7 and HPV-11 E6/E7-expressing keratinocytes measured by real-time qPCR and ELISA. Primary HFKs stably expressing vector alone (Babe), HPV-16 E6/E7 (16-E6/E7), HPV-6 E6/E7 (6-E6/E7), or HPV-11 E6/E7 (11-E6/E7) were treated with 100 μ g/mL Poly(I)·Poly(C) for 16 h. Following treatment, cells were washed and refed with minimal EpiLife for 4 h. (A) RNA was harvested from the cells and mRNA levels for MIP-3 α were determined by real-time qPCR. Results are expressed as the mean \pm SD of three experiments using two independent cell lines. (B) Culture supernatants were harvested and levels of secreted MIP-3 α were measured by ELISA. Results are expressed as the mean \pm SD of three experiments using two independent cell lines. (C) Semi-quantitative PCR results showing expression of E6/E7 in the 16-E6/E7, 6-E6/E7, and 11-E6/E7 cell lines.

A



B



C

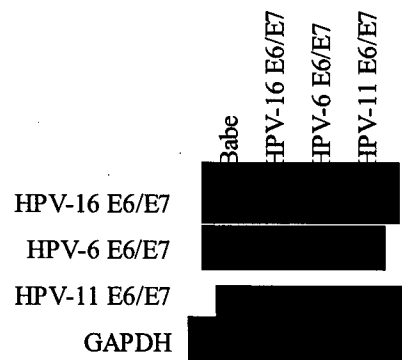
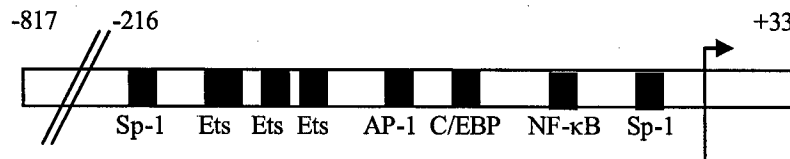
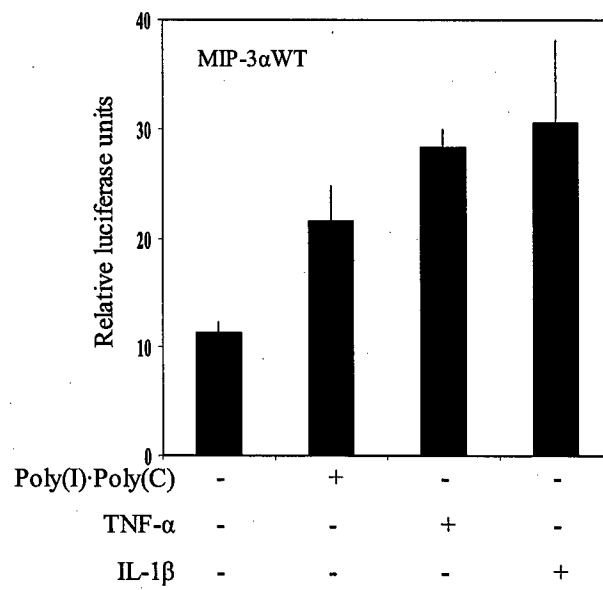


Figure 4.4 MIP-3 α activity in keratinocytes requires NF- κ B signaling. (A) Schematic of human MIP-3 α promoter showing location of transcription factor binding sites. 1×10^5 keratinocytes were seeded in 6-well dishes and transfected 24 h later with (B) 100ng MIP-3 α reporter construct containing the full-length MIP-3 α promoter (MIP-3 α WT), or 100ng MIP-3 α reporter constructs containing the full-length MIP-3 α promoter with mutations in the (C) NF- κ B binding site (mNF- κ B), (D) C/EBP binding site (mC/EBP), (E) second Ets binding site (mEts), or (F) AP-1 binding site (mAP-1) using Fugene 6. 24 h after transfection, cells were washed with 1x PBS and treated with media alone or media containing 10 μ g/mL Poly(I)·Poly(C), 10ng/mL TNF- α , or 10ng/mL IL-1 β for 6 h. Following treatment, cells were harvested, lysed, and measured for luciferase activity. Results are representative of two independent experiments.

A



B



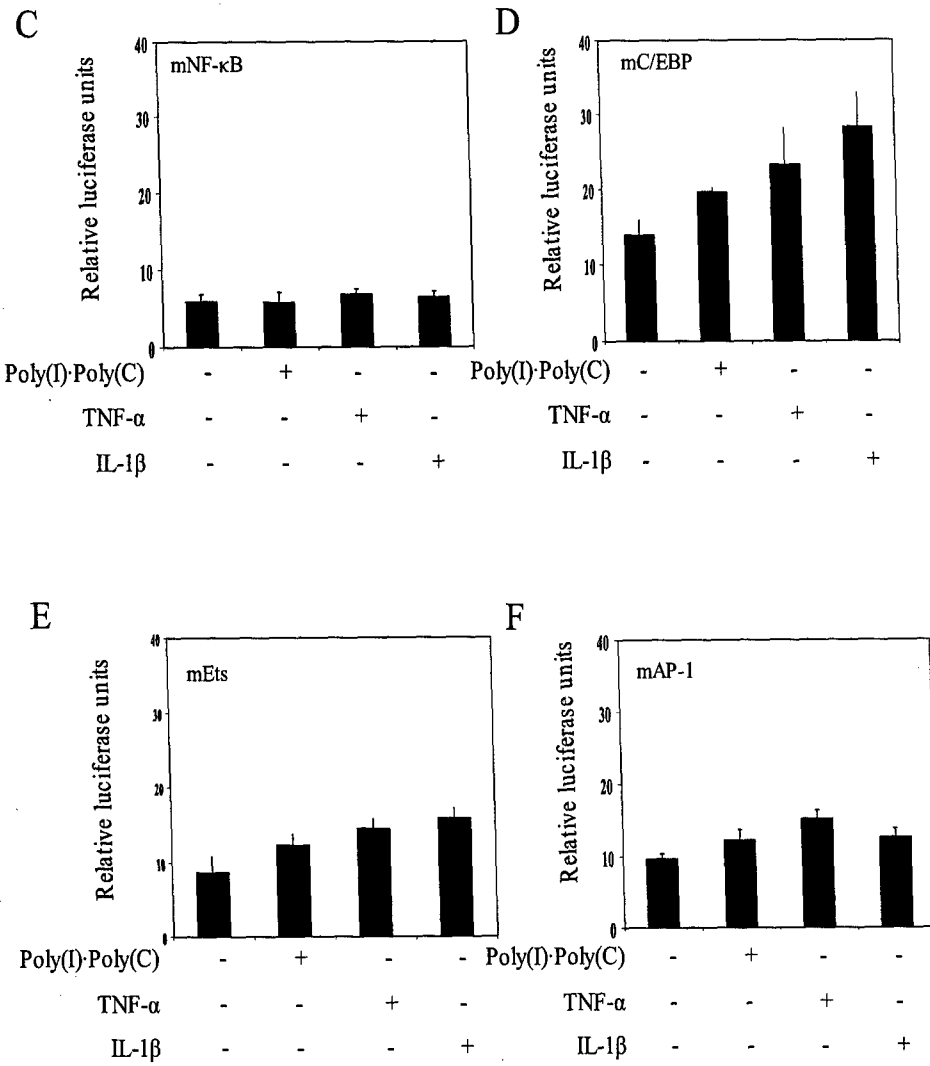
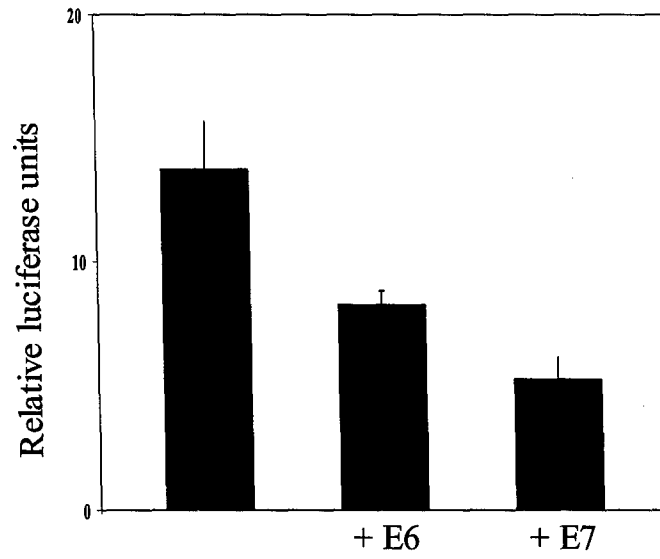


Figure 4.5 HPV-16 E6 and E7 repress MIP-3 α basal and stimulated promoter activity. 1×10^5 keratinocytes were seeded in 6-well dishes and transfected 24 h later with 100ng MIP-3 α WT and 900ng pSG5, pSG5 E6 (+ E6) or pSG5 E7 (+ E7). 24 h after transfection, cells were washed with 1x PBS and treated with (A) media alone or (B) media containing 10ng/mL TNF- α and 10ng/mL IL-1 β for 6 h. Following treatment, cells were harvested, lysed, and measured for luciferase activity. Results are representative of three independent experiments.

A



B

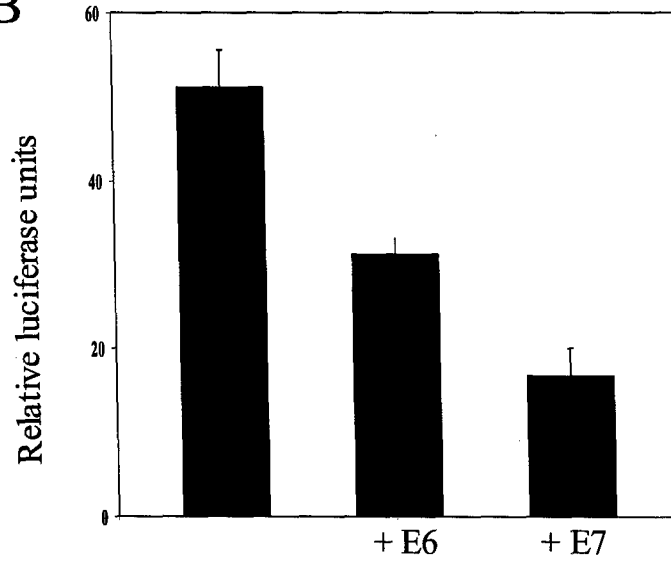
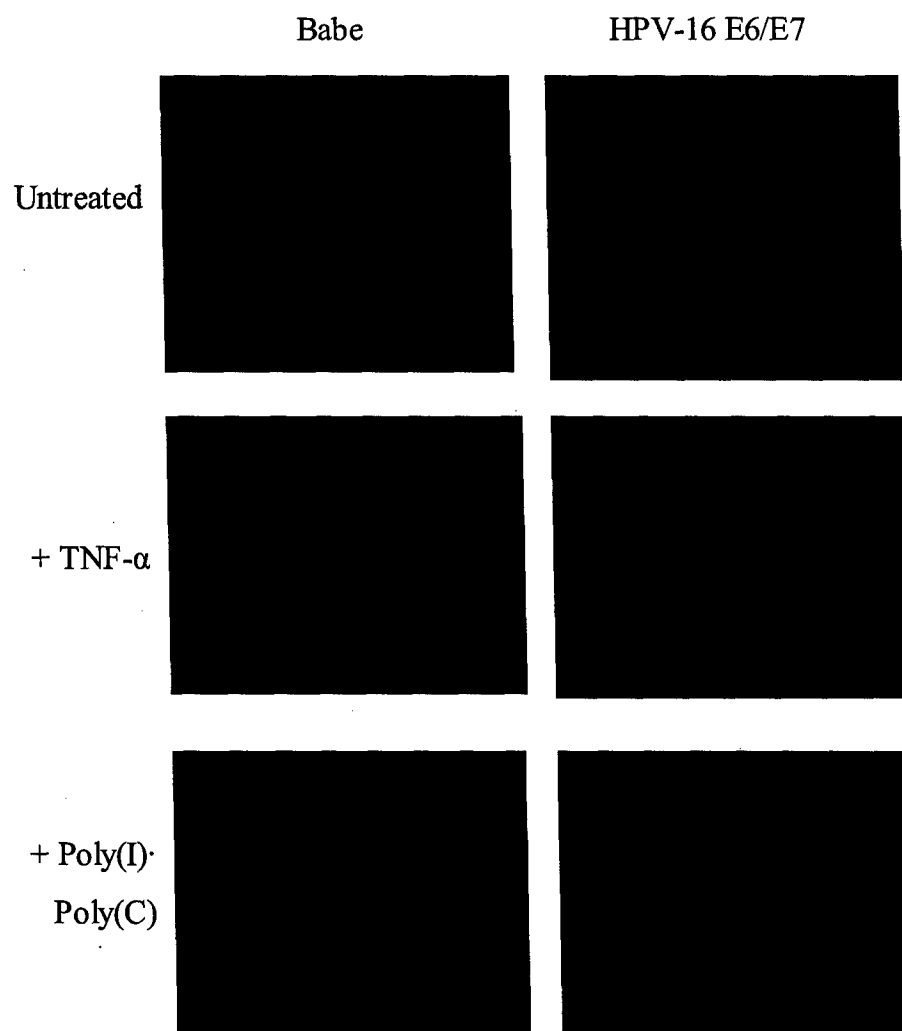
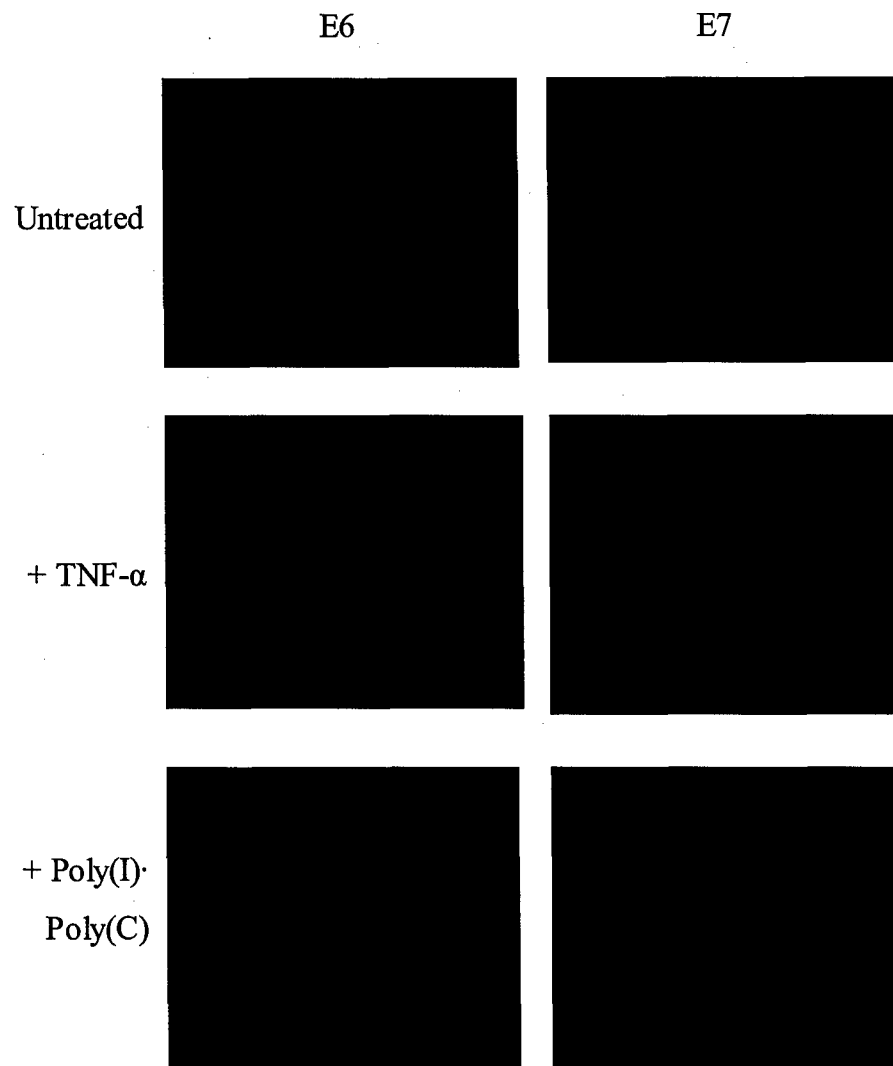


Figure 4.6 Expression of E6 and E7 does not inhibit p65 translocation to the nucleus. 1.5×10^5 keratinocytes were seeded on glass cover slips in 6-well dishes 24 h before treatment. Keratinocytes were treated with media alone (untreated), 100ng/mL TNF- α for 30 min (+ TNF- α), or 100 μ g/mL Poly(I)·Poly(C) for 3 h (+Poly(I)·Poly(C)). Following treatment, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in 1x PBS. Cells were incubated with α -p65 at 1:250 dilution overnight and then incubated with AlexaFluor 488 secondary antibody at 1:200 dilution for 1 h. Following mounting of cover slips onto microscope slides, images were visualized with fluorescence microscopy.





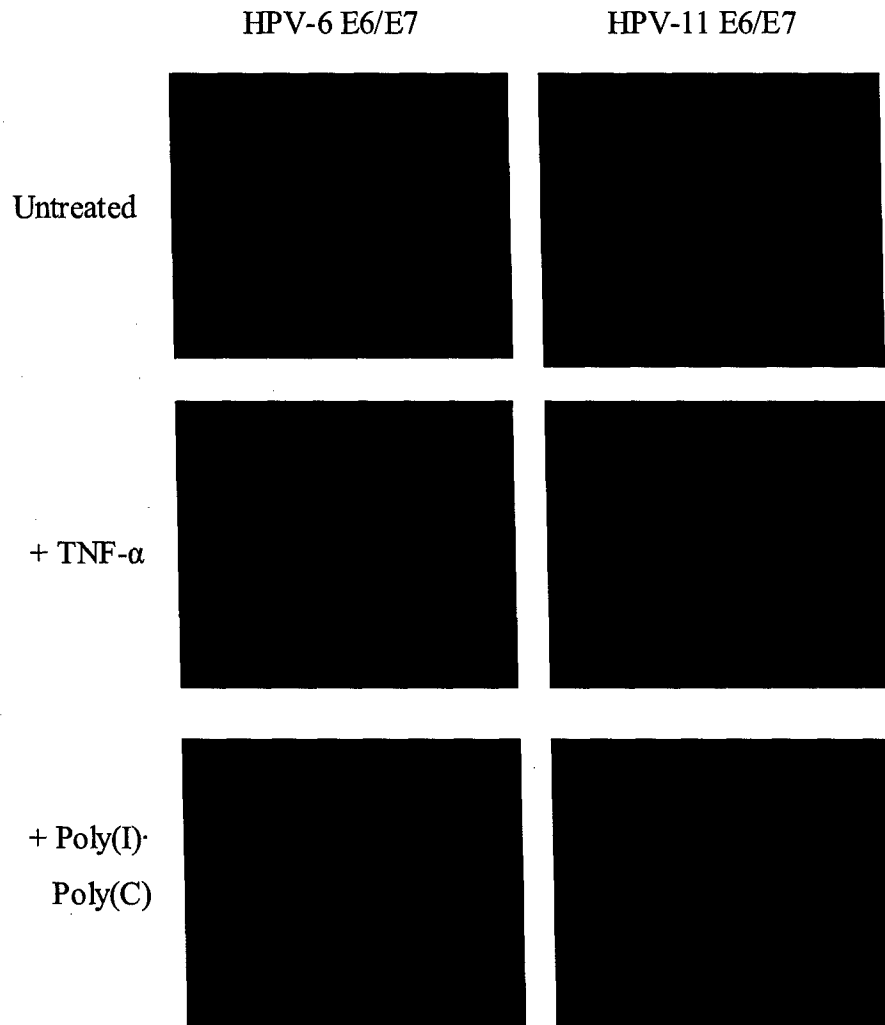


Figure 4.7 HPV-16 E6 and E7 repress NF- κ B activity. 1×10^5 U2OS cells were seeded in 6-well dishes and transfected 24 h later with 400ng NF- κ B luc and 600ng pcDNA3, pcDNA3 E6 (+ E6), or pcDNA3 E7 (+ E7) using Lipofectamine 2000. 48 h after transfection, cells were harvested, lysed, and measured for luciferase activity. Results are representative of three independent experiments.

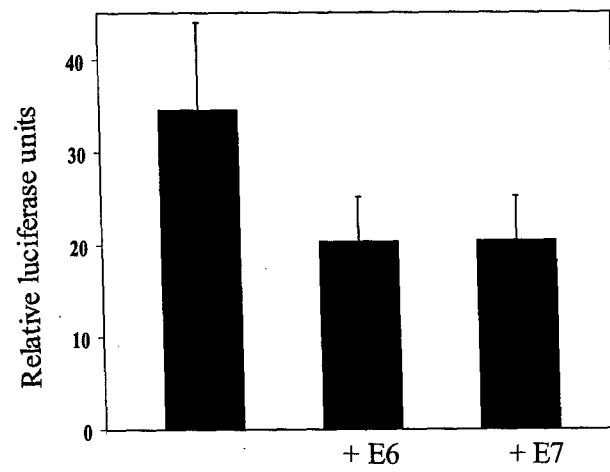
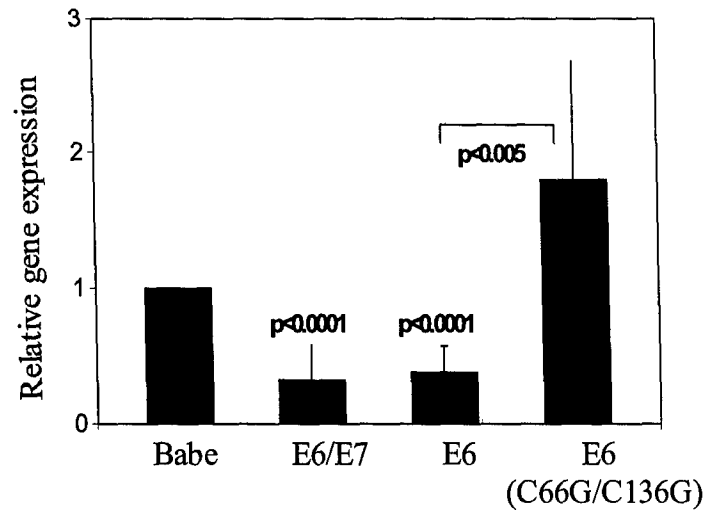


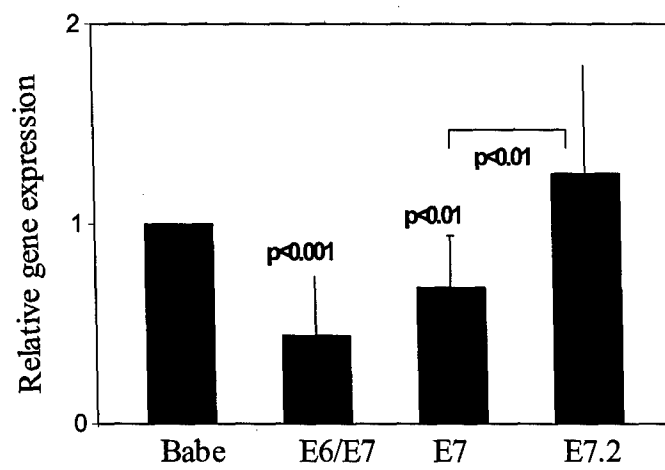
Figure 4.8 Mutations of HPV-16 E6 and E7 do not repress MIP-3 α expression.

Primary HFKs stably expressing (A) vector alone (Babe), HPV-16 E6/E7 (E6/E7), HPV-16 E6/E7stop (E6), HPV-16 E6(C66G/C136G)/E7stop (E6(C66G/C136G)) and (B) vector alone (Babe), HPV-16 E6/E7 (E6/E7), HPV-16 E6stop/E7 (E7), HPV-16 E6stop/E7.2 (E7.2) were treated with 100 μ g/mL Poly(I)·Poly(C) for 16 h. Following treatment, cells were washed and refed with minimal EpiLife for 4 h. RNA was harvested from the cells and mRNA levels for MIP-3 α were determined by real-time qPCR. Results are expressed as the mean \pm SD of three experiments using two independent cell lines.

A



B



Chapter 5

Discussion and Summary

Although highly correlated with the development of premalignant and malignant disease of the lower genital tract and oral cavity, infection with high-risk HPVs is not sufficient for the progression of disease. Many factors, including immune status, contribute to viral persistence and advancement to more severe stages of disease. The role of the immune system in controlling HPV infection has been corroborated by studies showing increased prevalence and persistence of infections in individuals with suppressed immune function, such as transplant recipients and HIV-positive individuals [reviewed in (93)]. The squamous epithelium that lines the reproductive tract, the oral cavity and the skin provides the first line defense against bacterial and viral pathogens. However, if this barrier is compromised through injury or infection, the innate immune response of the skin responds through the release of cytokines, chemokines, antimicrobial molecules, and complement proteins by monocytes, macrophages, professional antigen presenting cells, and keratinocytes (12, 50, 104). Keratinocytes play an active role in the innate immune response as cellular sources of cytokines in the epithelium (IL-1, -6, -7, -10, -12, -15, -18, -20) which not only influence the migration of immune cells, but also stimulate the further release of cytokines by keratinocytes and immigrating immune cells (33). Also important in the initiation of the immune response are chemokines, such as MIPs, MCPs, IL-8, GRO- α /- β /- γ , IP-10, and RANTES which are chemotactic for leukocytes, T lymphocytes, monocytes, eosinophils, and dendritic cells (10). Immune cells recruited to the site of inflammation further promote the immune response through the production of additional immune mediators, and the uptake of foreign antigen. Following

processing of foreign antigen, the antigen-presenting cells emigrate out of the tissue and back to lymphoid organs where naïve T lymphocytes are activated through the presentation of peptide fragments.

The potential ability of HPV to subvert many aspects of the host immune response is a characteristic shared by several other viruses [reviewed in (22)]. Orthopoxviruses, such as vaccinia virus and cowpox virus, encode homologues of IFN and cytokine receptors which inhibit the antiviral function of these immune mediators. Human herpesvirus 8 (HHV-8) encodes viral proteins that are homologous to MIP-1 α , MIP-1 β , and RANTES. These virally encoded chemokines bind to the respective chemokine receptor, CCR5, which is commonly used by HIV to gain entry into cells. Thus, HHV-8 inhibits the ability of HIV to infect cells already infected by HHV-8. Additionally, HHV-8 encodes a protein with homology to human IL-6 which contributes to inhibition of apoptosis. Herpes simplex virus not only inhibits the antiviral effects of complement by binding to one of the components, but the virus also encodes a protein which binds to TAP and, as such, hampers presentation via class I molecules. Several other viruses, such as adenovirus, human cytomegalovirus, HIV, and Epstein-Barr virus, interfere with the processing and presentation of antigen by class I molecules. While many of these viruses and others have also evolved strategies for evading T cell responses, the primary focus of our study was on the initial immune response to an infection which involves the release cytokines and chemokines and recruitment of antigen-presenting cells. However, additional preliminary data

(presented in Table 1) does suggest that HPV has developed mechanisms for subverting other aspects of the immune response.

Immune surveillance of the epithelium, the tissue targeted by HPV, is maintained by immature LCs which function as professional antigen-presenting cells and stimulate T lymphocytes following antigen uptake (7, 79). The expression of CCR6 by immature LCs and LC precursors allows their selective migration in response to MIP-3 α (32, 54, 58, 80), a potent chemokine released by inflamed epithelium (14, 69). The regular presence of LCs in dermal lymphatics suggests that the resident population of immature LCs in the epithelium emigrates out of tissue even in the absence of inflammatory stimuli (13). However, the requirement for LC precursors in the epithelium increases during inflammation since the resident immature LCs would emigrate out during the course of an immune response. Nevertheless, in HPV lesions there is a reduction in many of the immune cells needed to mount an effective immune response. Studies of cervical biopsies reveal that the number of LCs present in cervical tissue is decreased in low-grade and high-grade cervical lesions compared to normal tissue (2, 21, 44, 105) which suggests that areas of HPV persistence also have decreased immune surveillance. The reduced levels of immune cells in lesions would allow persistence of the viruses and consequently the lesions for prolonged periods, which appears to be required for the eventual malignant conversion. Results from this study demonstrate that expression of HPV-16 E6 and E7 in human keratinocytes down-regulates the level of MIP-3 α mRNA and protein. The decreased MIP-3 α production by E6/E7-expressing keratinocytes reduces the

migration of immature LPLCs. In addition, both E6 and E7 expressed individually in keratinocytes can repress MIP-3 α mRNA and protein levels; however, the greatest reduction is observed when E6 and E7 are expressed together. Interestingly, the E6/E7 proteins from the low-risk HPV types 6 and 11 are also capable of modulating MIP-3 α expression. This feature of low-risk HPV types may account for the persistence of genital warts and low-grade lesions in some infected individuals.

However, E6 and E7 from HPV-16 appear to have an immune-modulatory role that is more broad than the simple effects on MIP-3 α , as indicated by the down-regulation of numerous other cytokines, chemokines, wound healing factors, immune receptors, and adhesion molecules presented in this study (Table 1). Many of the immune factors identified in Table 1 bind to receptors found on a variety of immune cells and can, thus, direct the migration and influence the function of these cells. For example, immature dendritic cells migrate in response to RANTES, IL-8, and MIP-1 α (6) during the innate immune response, and T cell responses are regulated by IL-8 and I-CAM (93). The overall reduction in the expression of these and other proteins by E6/E7 may account for the low numbers of different types of immune cells in lesions caused by the virus. With cell types normally involved in initiating both the innate and adaptive immune responses unresponsive to an HPV infection, the virus would be better able to persist in the epithelium unobserved by the immune system. This correlation between HPV expression and a reduction in other immune factors has been substantiated by studies investigating clinical samples. Groups examining the immune environment of cervical intraepithelial neoplasia samples compared to normal tissue

found decreases in HLA-DR (class II molecules), CD54, TNF- α , GM-CSF, and E-cadherin as well as increases in IL-10, an immunosuppressive cytokine (31, 36, 44, 66). Additionally, the expression of HPV-16 E6 and E7 has been directly linked to the down-regulation of other immune mediators, such as IL-18, IL-8, and MCP-1 (18, 43, 51).

The activation of many immune response genes is mediated by NF- κ B, a transcription factor that is activated by bacterial products, viral infections, and pro-inflammatory cytokines such as TNF- α and IL-1 β . Upon activation, dimers of rel family member proteins are released from I κ B and translocate to the nucleus where binding to DNA consensus sequences occurs. In the case of MIP-3 α , activation by TNF- α or IL-1 β releases the p50/p65 heterodimer which binds to the NF- κ B binding site on the MIP-3 α promoter. Our results demonstrate that, upon activation with TNF- α or Poly(I)·Poly(C), p65 still translocates to the nucleus in control and E6/E7-expressing cells. However, E6 and E7 can repress NF- κ B activity which suggests that these proteins interfere with NF- κ B transactivation downstream of p65 translocation. Work on NF- κ B transactivation has revealed that the process often requires the contributions of coactivators and HAT activity. The p50/p65 heterodimer has been shown to recruit the coactivators CBP, SRC-1, and p/CAF, and also requires the HAT activity of p/CAF (95). Interestingly, groups have shown that HPV-16 E6 and E7 bind to CBP/p300 and p/CAF, and inhibit their transcriptional activity (4, 9, 43, 75, 112). Specifically, the interaction of E6 and E7 with CBP/p300 and p/CAF, respectively, resulted in decreased IL-8 promoter activity (43). We investigated this interaction as a

possible mechanism by which E6 and E7 repress MIP-3 α expression. Using keratinocytes that expressed a mutant of E6 that does not bind to CBP/p300 and an E7 mutant that exhibits decreased binding to p/CAF, we observed MIP-3 α mRNA levels near the level seen in control cells. Thus, it appears that the interaction of E6 and E7 with these NF- κ B coactivators is a potential mechanism underlying the reduced MIP-3 α expression observed in our study. This mechanism may also account for the ability of E6 and E7 from both high and low-risk viruses to modulate MIP-3 α production, as E6 and E7 of low-risk HPV types have been shown to also bind p300 and p/CAF (4, 9, 43, 75). Additionally, nearly two-thirds of the targets identified as being down-regulated in E6/E7-expressing keratinocytes (Table 1) are target genes of the NF- κ B signaling cascade (30, 72). The ability of E6 and E7 to inhibit expression of several immune-related genes through abrogation of the NF- κ B pathway would present a critical advantage to the life cycle of the virus. Through the down-regulation of cytokines, chemokines, adhesion molecules, and immune receptors, HPV-infected cells could escape detection of nearly every stage of the host immune response and remain undetected in the epithelium, a requirement for viral persistence and disease progression.

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Appendices

Appendix A IL-6 expression in control and HPV-16 E6/E7-expressing keratinocytes measured by ELISA. Primary HFKs stably expressing vector alone (Babe), HPV-16 E6/E7 (E6/E7), HPV-16 E6 (E6), or HPV-16 E7 (E7) were treated with 100 μ g/mL Poly(I)·Poly(C) for 4 h. Following treatment, cells were washed and refed with minimal EpiLife for 4 h. Culture supernatants were harvested and levels of secreted IL-6 were measured by ELISA. Results are expressed as the mean \pm SD of three experiments using three independent cell lines.

