# IDENTIFICATION AND FUNCTIONAL ANALYSIS OF HPV-16 E7 HLA-DR RESTRICTED EPITOPES IN PATIENTS WITH CERVICAL NEOPLASIA OR CANCER

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Dominic Warrino, PhD

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Abstract

To generate an effective cellular immune response, it is necessary to elicit both antigen-specific CTL ($CD8^+$) and Th ($CD4^+$) T cell recognition. The design of an effective therapeutic vaccine must incorporate means by which to generate novel T cell responses or enhance existing responses of a clinically-preferred functional type. In the cancer setting, one vaccine strategy is to target the immune system to specifically recognize tumor-associated antigens (TAAs). TAAs can be sub-categorized in many ways (i.e. onco-viral, mutated self-proteins, overexpressed and fetal-like proteins). Thus far, in part based on the historical prioritization applied to epitope searches, the number of defined CTL epitopes greatly outnumbers that of Th epitopes. Our goal in the current studies was to define and then characterize functional $CD4^+$ T cell responses directed against the HPV-16 E7 oncoprotein in patients with cervical intraepithelial neoplasia (CIN) or cancer using dendritic cell (DC)-based vaccine strategies.

The importance of this work stems from the unequivocal linkage between oncogenic HPV-infection and the development of cervical carcinoma. Over 50% of all cervical carcinomas are HPV-16 positive, making it the most salient HPV type for integration into therapeutic vaccine designs. For cellular transformation to occur and be maintained, expression of the HPV early region gene products E6 and E7 is mandatory. Due to this unique requirement for

sustained expression of the E6 and E7 proteins in transformed cells, these proteins make excellent candidates for protective or therapeutic vaccinations. While numerous HPV-16 E7-derived CTL epitopes have been identified over the past several years, surprisingly, only a single Th epitope has been reported thus far. In this thesis, I have defined three novel, naturally-processed and -presented epitopes derived from the HPV-16 E7 oncoprotein that are recognized by CD4+ T helper cells in patients with cervical intraepithelial neoplasia (CIN) or cervical carcinoma. Since the functional polarization state of the E7-specific CD4+ T cells remains of the Th1-type until the development of cancer *in situ* in these patients, DC-based vaccines that include E7-derived peptides or the whole E7 protein, and which are capable of selectively maintaining or enhancing Type-1 immunity may prove clinically beneficial in preventing or treating HPV-16+ malignancies, including cervical cancer.

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# PREFACE

## Acknowledgements

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# 1. Introduction

## 1.1 HPVs and Cervical Cancer

Cervical cancer is the second most common cancer among women world wide, killing approximately 250,000 women per year (1), (2). The human papillomavirus (HPV) has been linked to cervical cancer, and is the first cancer recognized by the World Health Organization (WHO) to be 100% attributable to a viral infection (3). HPV has been classified by genotypes into high-risk and low-risk subsets. Acute intravaginal infection with mucosal high-risk HPV subsets (HPV-16, -18, -31, -33, and -45) results in cervical intraepithelial neoplasia (CIN), of which approximately 1% progress to invasive cancer (1). The Progression of CIN I to cervical carcinoma is depicted in Figures 1-4. Fortunately, more than 95% of HPV infections of the anogenital tract resolve over three to five years (4). HPV-16 is responsible for more than 50% of cervical cancers worldwide (5), and it is estimated that less than 5% of healthy individuals infected with HPV-16 progress to cervical cancer (6). Cutaneous high-risk HPV genotypes, such as HPV-5 and -8, cause warts and may promote squamous skin cancer (SCC), while low-risk cutaneous (HPV-1 and –2) and mucosal (HPV-6 and –11) genotypes appear to cause skin and genital warts, respectively (7).

The present treatment for cervical cancer combines surgery or radiotherapy with adjuvant chemotherapy and has almost a 100% cure rate for Federation International of Gynecology and Obstetrics (FIGO) if the tumors are stage I low-grade, with invasion confined to the inner half of the myometrium (8). Individuals with advanced FIGO stage tumors that have spread beyond the

pelvis, however, are currently regarded as incurable, with the majority of these patients living in developing countries. Indeed, more than 80% of cervical cancer occurs in developing countries (1), which lack the proper cytology screening that has resulted in the remarkable reduction in the cervical cancer incidence in more developed countries.

### 1.2 Transformation of cells by HPVs

HPVs are small double-stranded DNA viruses, which infect stratified epithelium. HPV-associated cancers arise from a single spontaneous integration event of the viral genome into a host cell chromosome. Integration is a terminal event for the viral life cycle (9). The viruses enter the epithelium through microlesions and infect the basal epithelial cells where they maintain a copy number of 50-100 genomes per cell (10). Upon cell division, one daughter cell will remain part of the basal epithelium, while the other daughter cell will migrate up to the next level and start to differentiate (11). At this stage the viral DNA will segregate with the two daughter cells and replicate to maintain the 50-100 copies per cell. One daughter cell starts to differentiate, which is a problem for the virus, because it needs the replication machinery of the cell in order to maintain viral DNA synthesis, with terminally differentiated cells exhibiting reduced levels of requisite transcriptional enzymes (11). Therefore, the virus needs to stimulate G1 to S-phase progression in a cell programmed to terminally differentiate in order to provide the correct environment for viral DNA replication (9). The virus has evolved to trigger cell differentiation, allowing the mRNA coding for the capsid proteins, which is only switched in differentiated cells to be transcribed. Following, uncoupling of G1 to S-phase progression from differentiation, the viral message is transferred to the newly divided cells (11). As the infected cells move up through the epithelium and partially differentiate, the viral genome is amplified to

thousands of copies per cell in the granular layer and late gene transcription and translation occur near the top of the epithelium with viral particle assembly taking place in the cornified tissue layer (12). Infected cells are sloughed off from the top of the epithelium releasing HPV particles and are transmitted directly to other individuals by contact during sexual intercourse. Alternatively, for HPVs that infect cutaneous cells, the infected cells may remain present in the skin for extended periods of time before the virus is transmitted to a new epithelial surface (9). It should be stressed that the basis for these models are derived from experiments performed in tissue culture. It remains unclear how a given daughter cell transmigrates the epithelium, as outlined above, while the other daughter cell continues to divided in the basal layer and to provide a reservoir of viral DNA for further cell divisions (10). What is known is that infected cells of the basal layer are not lysed by virion production, but instead, these cells can continue to proliferate. This differentiation dependence allows the latently infected cells to persist in the basal layers of the mucosa for several years (10). This protracted period of HPV transcriptional maintenance prior to occult tumor development provides confidence that appropriately designed HPV vaccines may prove effective in preventing and treating HPV-related malignancies, such as cervical cancer.

### 1.3 HPV onco-proteins and the activities of E7

Insights into the mechanisms by which HPV infection can result in malignancy has come from the observation that three onco-proteins encoded by HPVs, E6, E7, and to a lesser extent E5, target host factors that control the cell cycle and proliferation. The replication proteins E1 and E2 are required for episomal replication of the genome in human keratinocytes and when either of these open reading frames is disrupted, the genome integrates into the host chromosome (13). However, extensive experiments have demonstrated that high-risk HPV E6 and E7

proteins are sufficient to induce immortalization of primary human keratinocytes in tissue culture (14-16). In contrast, low-risk HPV E6 and E7 proteins are unable to immortalize cells *in vitro,* although they may be able to moderately extend the life span of HPV-infected cells (17).

The activity of the high-risk HPV E7 protein has been extensively studied. E7 proteins of the high-risk types are primarily nuclear proteins of approximately 100 amino acids in length that dimerize through a zinc-finger motif in the C-terminus (18). The primary activity of high-risk E7 proteins is to facilitate progression of the cell cycle into S phase by binding with members of the retinoblastoma (Rb) tumor repressor family proteins (19). The role of the Rb protein in cell cycle regulation is to promote transition from G1 into S phase. In normal cells, early G1 phase Rb is hypophosphorylated and becomes increasingly phosphorylated towards S phase. The hypophosphorylated form of Rb binds the transcription factor E2F and actively represseses transcription from promoters containing E2F sites (20). A large number of genes for DNA synthesis are regulated by E2F, such as DNA polymerase alpha and thymidine kinase, which are transcribed in a cell cycle-dependent manner (21), (22). When the E7 protein binds to hypophosphorylated Rb, it prevents Rb from binding to E2F and thereby, promotes cell cycle progression (23). In normal epithelia cells, it is believed that Rb mediates cell cycle exit following differentiation, but in infected cells the binding of E7 to Rb promotes cell cycle progression in differentiated cells and allows for productive replication of HPV genes. Interestingly, to efficiently overcome cell cycle arrest, Rb appears to be targeted for E7-induced ubiquitin-mediated degradation, but this fails to occur when a low-risk HPV E7 binds Rb (24). Of note, E7 binds to other Rb family proteins p107 and p130, which negatively regulate E2F transcription (25), (26). E7 from high-risk HPV types have demonstrated the ability to diminish the inhibitory activities of the cyclin-dependent kinase inhibitors (CKIs) such as p21 (27, 28) and

p27 (29). Recently, it was reported that inactivation of both Rb and p21 by E7 is necessary to prevent cell cycle arrest (30). Other mechanisms of preventing cell cycle arrest, which are independent of Rb, include E7 binding to histone deacetylase-1 (HDAC-1) and the AP-1 family of transcription factors, c-Jun, JunB, JunD and c-Fos (31).

The E6 protein is the first gene expressed during HPV infection and acts together with E7 to induce changes leading to immortalization (32). One important function of E6 is to bind the tumor suppressor p53 and target it for ubiquitin-mediated degradation. p53 is expressed in response to DNA-damaging agents or unscheduled induction of DNA replication resulting in cell cycle arrest or apoptosis (33-35). The over-expression of p53 is a major impairment for viral replication, since HPV depends on the host cell DNA machinery and must stimulate S phase progression for replication. Therefore, it is not surprising that HPV E6 and E7 function together as the only HPV proteins necessary for immortalizing cells. Due to the inherent dependency of cervical carcinomas on maintained expression of the E6 and E7 proteins, these viral proteins represent very attractive targets for preventative and therapeutic treatments, such as vaccines. Numerous approaches are being investigated for the development of a HPV vaccine including peptide-based, protein-based, DNA-based, viral-vector based, bacterial vector-based, cell-based, dendritic cell-based, modified tumor-based, and VLP based (36). The major obstacle preventing the traditional approach of developing a heat-inactivated or attenuated viral vaccine is the inability to propagate the HPVs in culture or in animal systems.

### 1.4 Cellular Immunity to HPV : Th1/Th2/Treg in cervical cancer

While $CD8^+$ anti-tumor T cells have typically taken center stage as the pre-eminent immune element believed required for tumor rejection (37), (38), specific CD4+ T cells appear

crucial at the time of CTL priming if secondary expansion and durable memory in $CD8^+$ T cell responses are to occur (39), (40). Furthermore, in experimental models, anti-tumor $CD4^+$ T effector cells have the capacity to mediate the regression of MHC class I-loss tumors, that can no longer be regulated by specific CTLs (41). Notably, the quality and functional nature of patient anti-tumor $CD4^+$ T cell responses have been suggested as major prognostic indicators of disease progression and immunotherapeutic responsiveness (42), (43).

Mature murine and human CD4+ T helper cells can be segregated into 3 principal functional categories (i.e. Th1, Th2 and Th3/Treg) based on their patterns of effector cytokine secretion (44), (45). Th1-type $CD4^+$ T cells are delineated by their signature secretion of IFN-γ (and IL-2) and their propensity to support cellular immunity, while Th2-type cells produce high levels of IL-4, IL-5 and/or IL-13 and have been traditionally linked to humoral immune responses (46), (47). The Th3/Treg subsets of $CD4^+$ T cells appears to play the role of an antigen-specific T "suppressor" cell capable of silencing both Th1- and Th2-type immunity, in part due to secretion of the immunosuppressive cytokines IL-10 and TGF-β1 (48). Many Treg cells constitutively bear a $CD4^+CD25^+$ phenotype *in situ* and appear responsible for protection against autoimmunity in healthy normal donors, representing fully 5-10% of peripheral blood $CD4^+$ T cells (49). It should be noted that only a subset of total $CD4^+CD25^+$ T cells in the circulation mediates immunosuppression, with the level of CTLA-4 and GITR co-expression by these cells correlating with their degree of suppressor function (50).

The pathogenesis of carcinogenic HPV infections of the cervix results in early induction of peripheral tolerance of tissue infiltrating lymphocytes and an imbalanced Th2 response to HPV early virus proteins (51). It is estimated that 70% of patients with cervical cancer have a

predominant Th2 response to the HPV E6 and E7 antigens (51). Many previous reports addressing the issue of $CD4^+$ T cell functional polarization in cervical cancer patients has been equivocal (52), (53). In some studies, freshly-isolated cervical cancer TILs may exhibit a predominant Th2-type phenotype associated with the locoregional production of IL-4 and IL-10 (53), (54). These cytokines are affiliated with enhanced humoral (i.e. antibody) responses and with inhibition of "professional" antigen-presenting cell (i.e. dendritic cell) function, respectively, and are often inversely correlated with effective induction or dysfunction of cellular T cell-mediated immunity. However, Santin et al. (55) have shown in 15 patients with cervical cancer that isolated TIL are strongly polarized to a Type-1 differentiation pattern (i.e. production of IL-2 and IFN-γ), but may be suppressed *in vivo* by strong production of IL-4/IL-10 within the tumor microenvironment (53), (55). These data support a mixed Th1/Th2-type $CD4^+$ T cell infiltrate that may overall display Type-2 function *in situ*.

In order to evaluate the polarization status of tumor-specific $CD4^+$ T cells in cancer-bearing patients in a more refined manner, we have recently defined a series of epitopes derived from the HPV-16 E7 protein that are recognized by $CD4^+$ T cells, and have applied these in state-of-the-art ELISPOT assays to discriminate the functional status of anti-tumor Th cells. We demonstrated that peripheral blood $CD4^+$ T cells isolated from patients with cervical cancer exhibit profound dysfunction in their specific Th1-type immune responses. Indeed, IFN-γ responses were rarely observed, while IL-5 (Th2-type) responses were quite common against HPV-16 E7-derived $CD4^+$ T cell epitopes. Notably, TGF-β1 secretion by $CD4^+$ T cells in response to HPV-16 E7 peptides was observed in approximately 12% of advanced stage patients, and when this occurred, neither IFN-γ nor IL-5 responses were evident, suggesting the likely functional dominance of specific T-reg cells in these patients. It is important to stress that

Th1-type immune dysfunction was noted only within the tumor epitope reactive $CD4^+$ T cell repertoire, but not within the repertoire of $CD4^+$ T cells recognizing HLA-DR restricted epitopes derived from viral pathogens, such as influenza or EBV (which remained strongly Th1-type polarized in these patients).

### 1.5 Dendritic cell-based vaccines and therapies for cancer

DCs have been shown to derive from numerous lineages. Generally, DCs, which have been used in vaccine protocols, have been derived from monocytes stimulated with Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and IL-4 or from CD34+ precursors. A phenotypically immature DC can efficiently phagocytose both apoptotic and necrotic cell debris, microbe and particulate antigens and may through macropinocytosis, take up soluble proteins. After antigen capture and maturation, DCs promote antigen-specific T cell activation and survival within secondary lymphoid sites (56, 57). DCs have multiple roles and may dynamically shift their phenotype in response to the local inflammatory environment. The understanding of subtleties in DC phenotypes will be important in ultimately determining the optimal phenotype for use in clinical trials and in allowing one to compare results obtained in different protocols. Immature DCs capture antigens by several pathways such as, 1) macropinocytosis, 2) receptor-mediated endocytosis via mannose receptors and via Type I (CD64) and Type II (CD32) $F_c$–$\gamma$ receptors, 3) phagocytosis of particulates such as latex beads, apoptotic and necrotic fragments (involving CD36 and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins), (58-60) 4) internalization of heat-shock protein gp96 and Hsp 70 via Toll-like receptor 2/4 or CD9 (61-63). The captured antigen is

delivered to Major Histocompatibility Complex (MHC) class II compartments, processed and then directed to the cell surface in peptide-MHC complexes that can elicit $CD4^+$ T cell responses. Furthermore, DCs are also able to present peptides derived from exogenous antigens in MHC class I complexes through 'cross-priming' mechanisms (64-66). Thus, DCs can stimulate both $CD4^+$ and $CD8^+$ T cells by specialized processing of exogenous antigens. DCs can also facilitate antigen-specific B cell activation (67).

Mature DCs develop extensive cytoplasmic veils, exhibit reduced antigen uptake capacity, express different cytokine genes and display even higher levels of co-stimulatory molecules, as well as higher levels of class II MHC presenting molecules than immature DCs (68). DC maturation can be induced following interaction with diverse stimuli (bacterial products such as lipopolysaccharide (LPS) or Staphylococcus aureus, Cowan-I strain (SAC), activated $CD4^+$ T cells expressing CD40 ligand, apoptotic body uptake, monocyte-conditioned media (macrophage supernatant), recombinant cytokines including TNF-$\alpha$ or IL-1$\beta$ and heat-shock proteins (69, 70). Recently, it has also been demonstrated that DC maturation and activation can be induced by direct interaction with either naive or activated $CD8^+$ T cells (71, 72). Mature human DCs are perhaps best identified by expression of CD83 and the p55 actin-bundling protein, fascin (73). Interestingly, better prognosis in cancer are associated with increased infiltration by DCs and notably by $CD83^+$ DCs (74), (75). Peripheral blood DCs may either migrate directly into the tumor or recruited mononuclear cells may differentiate into DCs in the local tumor environment under cytokine stimulation and phagocytose tumor-associated antigens. Antigen (notably particulate) uptake also promotes immature DCs to mature. Mature DCs are then prompted to migrate to secondary lymphoid organs by their CCR7 chemokine receptors. There they present processed Ag to naive T cells. The initial contact between DCs and T cells

may be mediated by a transient, high affinity interaction between DC-SIGN on DCs and the intracellular adhesion molecule ICAM-3 on T cells (76). Subsequently, this initial contact is strengthened via interactions through other adhesion molecules and their corresponding ligands, ICAM-1/LFA-1 and LFA-3/CD2 (77, 78). The induction of specific T cell responses by mature DCs critically depends on the DCs-T cells crosstalk involving CD40 and co-stimulatory molecules (B7-1, B7-2 *et al.*). Activation of naive T cells occurs only after priming by APCs exhibiting high levels of antigen, co-stimulatory molecules or in the presence of pro-inflammatory cytokines. T cells may become activated after exceeding a critical threshold of antigen- MHC crosslinking of their T cell receptors (TCRs). The involvement of co-stimulatory and adhesion molecule interactions, particularly if these are sustained over time, modulates the threshold antigen dose for activation (79). Just as lymphocytes are composed of different subsets with specific functions (T cells, B cells and Natural Killer [NK] cells), DCs are composed of distinct subsets that can induce different types of immune responses.

In mice, three distinct pathways of DC development are likely to exist: myeloid DCs, lymphoid DCs and plasmacytoid DCs (77, 80-82). Myeloid DCs can be grown with GM-CSF from precursors that yield both granulocytes and monocytes (83). Lymphoid DCs, distinguished by CD8α expression, can be generated from precursors that can also develop into T cells, B cells and NK cells (84, 85). Ikaros knockout mice lack T, B and NK cells as well as DCs, again implicating a role for a lymphoid lineage in the development of DCs (86). However, some have questioned a distinct developmental pathway for lymphoid DCs since recent reports have demonstrated that both $CD8\alpha^+$ and $CD8\alpha^-$ DCs can arise from clonogenic common myeloid progenitors, suggesting that CD8α expression is not always indicative of 'lymphoid' origin, and that phenotypic and functional differences among DC subsets might reflect in maturation status

(87), (88). While myeloid DCs and Langerhans cells (LCs) share many common surface markers, they can be distinguished developmentally. Genetic studies support the notion of separate development pathways in mice deficient in RelB (89). RelB-/- mice, in addition to their defects in NK and B cell development, have impaired production of mature DCs, although LCs develop normally in these mice (90-92). Murine plasmacytoid DCs can be differentially isolated based on their expression of CD11c, B220 (CD45R) and Thy1.2 (CD90). These cells lack expression of myeloid (CD11b) antigens and CD8α, a marker used to isolate lymphoid DCs. Mobilization of mice with Flt3 ligand (Flt3L) or Flt3L and GM-CSF, which are haematopoietic factors that specifically enhance DC growth, results in strikingly increased numbers of plasmacytoid DCs in bone marrow and spleen (82).

In humans, $CD34^+$ haematopietic cells (HPC), the representative DC progenitors, can be evolved into two discrete myeloid DC populations, the epidermal LC and the interstitial DC (intDC), in cultures containing GM-CSF and TNF-α (39, 77, 93, 94). While both LC and intDC subsets can produce IL-12 upon CD40 ligation, only intDC can produce IL-10 (94). DCs can also arise from a subset of $CD34^+$ HPC committed to the lymphoid lineage (95). However, as is also observed in mice, there is no clonal evidence that would permit one to formally establish the existence of a unique lymphoid DC lineage. Three subsets of DC precursors circulate in the human blood: $CD14^+$ monocytes, lineage negative $CD11c^+$ precursor DCs and $CD11c^-$ precursor DCs (77, 80, 96). Monocytes can differentiate into cells displaying features of immature DCs or macrophages in response to GM-CSF and IL-4 or Macrophage- Colony Stimulating Factor (M-CSF), respectively (92, 96, 97). $CD11c^+$ subset contains precursors of intDCs, LC and macrophages (98). Distinct factors regulate the survival and differentiation of $CD11c^-$IL-3R$\alpha^+$ DC precursors, also known as plasmacytoid T cells or plasmacytoid monocytes (99). These cells

are critically dependent on IL-3 for survival and on CD40L for maturation. $CD11c^{-}IL3R\alpha^{+}$ plasmacytoid DCs have recently been demonstrated to be the principal IFN-γ producing cells (100), (101). In mice, activated lymphoid DCs make higher levels of IL-12 than myeloid DCs (102), (103) and induce naive T cells to produce IFN-γ in association with Th1-type responses (102), (104, 105). Mouse myeloid DCs induce T cells to produce IL-4 in support of Th2-type responses (104), (106). While human DCs can also polarize naive T cell responses, the type of DC associated with Th1- or Th2- type responses seem opposite to those observed in mice. Human myeloid DCs secrete larger amounts of IL-12 and favor Th1-type responses (107), (101), while plasmacytoid $CD11c^{-}$ DCs induce T cell to produce Th2 cytokines, particularly IL-10 (101, 107, 108). However, the extent of T cell polarization may also be related to the stage of DC differentiation/maturation (101, 109) and not simply reflect linkage with a strict phenotypically-defined subset of DCs (109), (110), suggesting that different protocols of DC preparation may affect outcome of vaccinations.

### 1.6 Dendritic cell-based cancer therapy: delivery and vaccination schedules

DCs represent attractive vectors for tumor immunotherapy due to their unique properties, including a high degree of antigen capture and antigen presenting capacity that support extremely efficient induction and maintenance of specific cell-mediated immune responses. Numerous strategies exist for priming DCs to serve as biologic adjuvants: 1) DC pulsed with synthetic peptides and eluted peptides, 2) DC transfected with: cDNA or RNA encoding known tumor-associated antigen, 3) Tumor cell product-based vaccines DC pulsed with: Tumor lysate, Apoptotic body/necrotic body tumor RNA, DC-derived exosomes, Heat-shock protein, DC fused

to tumor cells. Mayordomo *et al.* reported that synthetic peptide-pulsed DCs elicit potent anti-tumor immunity when injected as vaccines (111). Pulsing synthetic peptides derived from known tumor-antigens such as MART-1 (Melanoma Antigen Recognized by T-cells 1)/Melan-A, tyrosinase, carcinoembryonic antigen (CEA), gp100, Her-2/neu or MUC1 expressed on human DCs have also been shown to yield effective therapeutic vaccines in the clinical setting (112)). Some of these peptide epitopes have been modified using single amino acid substitution in the MHC anchor positions of the peptide in order to give stronger binding affinity for the MHC class I molecules (that is, agonist epitopes), thus increasing their ability to induce CTL responses. The subsequent clinical application of these modified peptides has been associated with enhanced responses in human trials (113). However, several disadvantages exist in the use of defined tumor antigens. In addition to the overwhelming lack of known tumor rejection antigens (TRAs) for most human tumors, loaded peptides only reside on the DC cell surface for a short period (hours) and most antigenic peptides are only applicable for patients who express a defined specific Human Leukocyte Antigen (HLA) haplotype capable of presenting the given epitope (114).

In contrast, gene-based strategies employing cDNA encoding tumor-associated antigens do not require prior knowledge of the responder MHC haplotype or of specific MHC-presented peptide epitopes. Human DCs genetically engineered to express the melanoma antigen MART-1 are able to generate peptide-specific, class I-restricted cytotoxic T lymphocytes in cultures of peripheral blood leukocytes from normal donors (115). Heiser *et al.* reported that the vaccination with DCs transfected with prostate specific antigen (PSA) mRNA elicits PSA specific CTL responses against metastatic prostate tumors, with no evidence of side effects in metastatic prostate cancer patients (116). DC-based strategies that do not require prior knowledge of the

responder MHC haplotype or of the relevant MHC restricted peptide epitope have also been developed using whole tumors as a source of antigen. DCs can be transduced with recombinant viruses such as retroviral or adenoviral vectors, or transfected with whole tumor RNA (117-119). Other approaches utilizing whole tumors as a source of antigens have been developed using DCs loaded with tumor lysates, acid-eluted tumor peptides or dying tumor cells (apoptotic bodies and necrotic cells) (120-122).

In another strategy, cell-fusion techniques have been used to make hybrids comprising a fused tumor cell and a DC; these hybrids may concurrently express tumor DNA encoded antigens and the potent stimulating capabilities of the parental DCs (123, 124). Exosomes are small, membrane-bound vesicles (vesicles that contain high amounts of MHC, CD86 and tumor related peptide) released from tumor cells that may be taken up and presented by DCs (125). Tumor-peptide-pulsed, DC-derived exosomes have been successfully used to prime specific CTLs *in vivo* and, when used as a vaccine, to eradicate or suppress the growth of established murine tumors (126). The disadvantage of this technique includes the requirement to isolate sufficient quantities of patient tumor from which to generate exosomes.

To enhance the anti-tumor immunity elicited by DC-based vaccination, cytokine gene-engineered DCs or co-administration of Th1 cytokines have been examined in murine tumor models. Using gene-based strategies, it has been shown that co-expression of known melanoma antigens with IL-12 or IFN-γ enhances the magnitude of antigen-specific CTL reactivity in murine tumor models (127, 128). As alternative approaches to enhance DC-based vaccination, systemic administration of Th1 cytokines such as IL-2 and IL-12 have also been shown to enhance the therapeutic effectiveness of DC-based vaccination (129, 130). Such combinational therapeutic approaches may ultimately be required to improve the clinical efficacy of DC-based

immunotherapy. The delivery of DCs directly into tumors is an attractive alternative approach in stimulating improved anti-tumor T cell responses *in situ*, via cross-priming. Both human and murine DCs express TNF family ligands (Fas ligand, TRAIL, TNF and Lymphotoxin (131-135) and may mediate tumoricidal activity. Subsequent uptake and cross-presentation of tumor epitopes may provide a potent immunogen *in situ*. The ability to mobilize large numbers of DCs into the tumor might also be accomplished by administration of Flt3- ligand or GM-CSF, which have both been recently evaluated (108).

Flt3-ligand is a member of a small family of fms-like tyrosine kinase signaling growth factors that includes M-CSF and the c-kit ligand. These factors stimulate the proliferation of haematopoietic progenitor cells by binding to and activating tyrosine kinase receptors (136, 137). Expression of Flt3-ligand receptor is restricted to the most primitive haematopoietic progenitor cells and Flt3-ligand stimulates the expansion and mobilization of progenitor cells (138). Lynch *et al.* reported that Flt3-ligand induces at least transient tumor regression in a mouse sarcoma model (139). In humans, normal individuals tolerate Flt3-ligand without significant toxicities and DC numbers in the peripheral blood increase by more than 20-fold (102). To date, Flt3-ligand has not been investigated in human clinical trials as a cancer therapy.

### 1.7 Vaccine-Induced Immunity to HPVs

One approach to treating various cancers is to target T cell responses to tumor associated antigens (TAA) that are expressed on the surface of neoplastic cells in the context of MHC I and/or MHC II molecules. In the case of cancers induced by HPV infections, the oncogenic viral proteins (such as E6 and E7) are ideal tumor associated antigens, since they are the only viral proteins that must be expressed by the transformed cell and given their derivation from a xenogenic source, they are highly-immunogenic. Dendritic cells (DCs), the most potent antigen-

presenting cell, are ideal cells for testing the *in vitro* efficacy of a given TAA (such as high-risk HPV E6/E7) to elicit specific $CD8^+$ and $CD4^+$ T effector cells.

Dendritic cells are unique antigen presenting cells because they are the only cells with the capacity to induce primary immune responses, thus permitting establishment of immunological memory (80, 140). DCs circulate throughout the periphery as precursors and enter the tissue becoming immature DCs (iDCs). Upon encounter with antigen in a pro-inflammatory microenvironment, iDCs are induced to mature and to traffic to the tissue-draining lymph node(s), where they home to T cell-rich zones. T cells recognizing cognate antigen presented in MHC complexes expressed by DCs exhibiting elevated levels of co-stimulatory molecules, results in the mutual activation of both the DC (cytokine secretion) and the rare-event, antigen-specific T cell (77). After several rounds of replication, effector and memory T cells develop that may play important long-term regulatory roles against (HPV) virally-infected/transformed cells. Indeed, in animal models and human studies, HPV-$16^+$ tumors may undergo regression in individuals vaccinated with autologous HPV-16 E6/E7 peptide-pulsed DCs, synthetic E6/E7 peptides in adjuvant, or HPV-16 Viral-Like Particles (VLPs; refs. (141), (142), in concert with increased frequencies of tumor-specific T cells. However, a major obstacle identified in a significant proportion of $HPV^+$ CIN or cervical cancer patients is that, despite the presence of detectable anti-HPV-16 E7 T cell responses, lesions are not resolved, due to immune escape mechanisms acting at the effector T cell and/or target cell levels (143, 144). Of note, HPV-infected cervical epithelial exhibit HLA class I dysregulation (i.e. downregulation) in approximately 90% of tumors (145). Antigen processing defects in cervical carcinomas may also further limit the presentation of HPV E6/E7-derived epitopes required to generate CTL responses and to allow for tumor cell recognition by effector anti-E6/E7 T cells (146). Notably,

$CD4^+$ T cells can provide "help" in the promotion and maintenance of specific $CD8^+$ T cell immunity during chronic viral infections (45, 46), and can serve as complementary effector mechanisms that are capable of mediating the regression of MHC class I-deficient tumors (147-149). Hence, it appears prudent to research vaccines that are capable of augmenting both anti-HPV $CD4^+$ and $CD8^+$ T cells in order to effect optimal preventative or therapeutic benefit to the patient.

Since the vast majority of research related to the immunology of cervical cancer antigens has been focused on defining $CD8^+$ T cell epitopes (150-152), it is perhaps not surprising that this is also true for cervical cancer. Intensive work has been done to characterize HPV-16 E7 class I restricted epitopes using VLPs, E7-fusion proteins, intact E7 protein, or E7 synthetic peptides (153-156). The study of $CD4^+$ T cell responses and their specificities have received far less attention, which is confounding given the central role they have in regulating most anti-viral immune responses (157), (158). Given our perception as to the importance of $CD4^+$ T cell responses in the ultimate efficacy of any anti-HPV vaccine for cancer, others and we have focused upon, and recently reported the identification of several HLA-DR presented epitopes derived from the HPV-16 E7 protein (52, 58). Based on these results, the central region of the HPV-16 E7 protein ($E7_{35-77}$)) appears to represent the major immunogenic region and contains at least 3 distinct Th epitopes; within this region (DR15/E7(50-62), DR3/E7(43-77), DQ2/E7(35-50)) (159).

### 1.8 Current HPV preventative and therapeutic vaccines

A preventative vaccine should immunologically mimic the infection they prevent. Prophylactic viral vaccines are designed to induce neutralizing antibodies, which reduce the

number of cells infected and prevent disease after challenge with virus. A successful vaccine must prime the adoptive immune system to recall specific effector functions in order to prevent future infections. A pioneering animal study for HPV vaccines showed vaccinations that induced papillomavirus specific antibody prevented infection with virus (160). As mentioned above, the inability to propagate virus for vaccines from *in vitro* culture has led to the development alternative approaches including VLPs. The 100% efficacy of preventing persistent high-risk HPV-16 infection in a recent clinical trial using HPV-16 L1 VLPs (161) is unequivocally the leading preventative vaccine.

The majority of clinical trials for the treatment of HPV have been carried out in patients with late-stage disease, and these individuals are typically immune-compromised due to treatment with radiotherapy and/or chemotherapy. To date, a pilot study of 15 patients vaccinated with DCs fed recombinant HPV-16 and –18 proteins and matured with cytokine cocktail (IL-1β, IL-6, TNF-α, and $PGE_2$) is the only clinical trial for the treatment of cervical cancer using DCs (Table 1) (162). Three clinical trials for CIN patients utilize HPV-16 E7 peptide (163, 164) and HPV-16 E6-E7 fusion protein (165) for vaccinations are also being performed, with the currently available information summarized in Table 1. Additionally, two clinical trials for cervical cancer patients vaccinated with recombinant HPV-16 protein (166, 167) and one vaccinated with peptide and adjuvant (168) have demonstrated only limited efficacy (Table 1). No clinical trials utilizing VLPs for immunotherapy have been performed to date. However, with the engineering of the VLP to contain onco-proteins, such as E7, future immunotherapies for CIN and cervical carcinoma appear promising. The combination of VLPs fed to autologous DCs and modulated by adding exogenous factors, specifically the DC1 polarizing cytokines IL-12 and IFN-γ (169), would be presumed most likely to promote

enhanced Th1-type immunity, that many believe will be most effective in treating HPV-related malignancies.

**Scope of This Thesis**

We have evaluated $CD4^+$ T cells isolated from the peripheral blood of normal donors, CIN patients and cervical cancer patients, for their quantitative and qualitative functional responses against novel HPV-16 E7-derived peptide epitopes that we have defined. We observed that while HPV-$16^+$ CIN patients frequently displayed Type-1 $CD4^+$ T cell responses to E7 epitopes, this was comparatively rare for HPV-$16^+$ patients with cervical cancer. Instead, cervical cancer patients displayed predominant Type-2 Th responses against E7-derived epitopes. Subsequent analyses suggest that poly-epitope specific, anti-E7 $CD4^+$ T cell responses can be elicited from these patients using vaccines based on autologous DCs and HPV-16 E7 $CD4^+$ epitopes, L1L2-E7 VLPs or rE7 protein. These data suggest that DC-E7 based vaccines may promote therapeutically-important immunity in the majority of treated patients, particularly in a setting where Type-1 immunity may be preferentially elicited in the cervical cancer-bearing host, or in CIN+ patients where Th immune deviation has not yet occurred.

**Preface Chapter 2**

Using DC-based *in vitro* vaccinations as a platform, our goal was to define CD4+ T cell epitopes derived from the HPV-16 E7 oncoprotein. We tested CIN and cervical cancer patients for the presence of T-helper cell reactivity to six HPV-16 E7 peptides predicted to be presented my HLA class II molecules. By analyzing known HPV-infected individuals, the likelihood of identifying circulating HPV-specific Th cells in the peripheral blood was anticipated to be greatly increased. We identified three novel HPV-16 E7 ($E7_{1-12}$, $E7_{48-62}$, and $E7_{62-75}$) peptide epitopes that were recognized by patient CD4+ T cells. Based on the donor haplotype information, the HPV-16 $E7_{1-12}$ and $E7_{62-75}$ epitopes are presented by HLA-DR4 and -DR15, while the HPV-16 $E7_{48-62}$ epitope is presented by multiple non-HLA-DR4 (HLA-DR3, -11, and –15) class II alleles. By analyzing the CD4+ T cell functional responses in both IFN-γ and IL-5 ELISPOT assays, we were also able to determine that Th1-type (i.e. IFN-γ) dominated responses to HPV-16 E7 epitopes was common in patients with pre-malignant CIN lesions, while Type-2 (i.e. IL-5) dominated immunity was prevalent in patients with cervical cancer.

The studies in **Chapter 2** support the immunogenicity of the HPV-16 $E7_{1-12}$, $E7_{48-62}$, and $E7_{62-75}$ peptides, but does not stringently address whether these epitopes are naturally processed and presented by patient DCs or tumor cells. This issue is more formally addressed in **Chapter 3,** where recombinant E7 protein/DC-based vaccines are evaluated to elicit epitope-specific Th cell responses.

## 2.0 Disease-Stage Variance in Functional CD4$^+$ T Cell Responses Against Novel Pan-HLA-DR Presented HPV-16 E7 Epitopes

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## 2.1 ABSTRACT

Given the anticipated clinical importance of helper and regulatory $CD4^+$ T cells reactive against HPV-16 E7 in the cervical carcinoma setting, we performed this study to identify novel E7-derived T helper (Th) epitopes and to characterize functional anti-E7 Th responses in normal donors and patients with CIN I-II or cervical cancer.

Candidate pan-HLA-DR binding peptides were identified and synthesized based on results obtained using a predictive computer algorithm, then applied in short-term *in vitro* T cell sensitization assays. Using IFNγ/IL-5 ELISPOT assays as readouts for Th1-type and Th2-type $CD4^+$ T cell responses, respectively, we identified 3 E7-derived T helper epitopes ($E7_{1-12}$, $E7_{48-62}$ and $E7_{62-75}$), two of which are novel.

Normal donor $CD4^+$ T cells failed to react against these E7 peptides, while patients with pre-malignant CIN I-III lesions displayed preferential Th1-type responses against all three E7 epitopes. Th1-type responses were still observed to the $E7_{48-62}$, but not the $E7_{1-12}$ and $E7_{62-75}$ peptides in cancer patients, where these latter two epitopes evoked Th2-type responses. Notably all responders to the $E7_{1-12}$ and $E7_{62-75}$ peptides expressed the HLA-DR4 or -DR15 alleles, while all responders to the $E7_{48-62}$ peptide failed to express the HLA-DR4 allele.

Our results are consistent with a model in which cervical cancer progression is linked to an undesirable Th1- to Th2-type shift in functional $CD4^+$ T cell responses to two novel E7-derived epitopes. These peptides may prove important in vaccines to promote and maintain protective Th1-type anti-HPV immunity and for the immune monitoring of treated patients harboring HPV-$16^+$ malignancies.

## 2.2 INTRODUCTION

Human papillomaviruses (HPVs) have been linked to cervical dysplasia and cervical cancer, and are associated with 12% of all cancers in women (2). An estimated 30-60% of sexually active men and women are infected with genital HPVs, although most are asymptomatic (170). There are approximately 370,000 cases of cervical cancer diagnosed worldwide each year and a 50% mortality rate has been reported (171, 172).

HPV is a papillomavirus consisting of a non-enveloped, 55-nm, icosahederal-shaped virion, with HPV genotypes divided into several risk groups. The low-risk genotypes, such as HPV-6 and HPV-11, are detected in benign genital warts and low-grade squamous intraepithelial lesions (SIL), but are rarely found in invasive carcinomas (7). HPV DNA of high-risk genotypes (HPV-16, -18, -31, -33, -45, or -56) are detected in nearly all cases of cancers of the cervix, with HPV-16 observed in 50-60% of $HPV^+$ carcinomas(7, 173). Expression of early viral proteins, such as HPV-16 E6 and E7 are essential in promoting and maintaining the transformation of cells by binding to and inactivating p53 and pRB, respectively (14, 15, 25, 174-177).

Why some individuals clear the virus, while others do not, remains unknown. The observed increase in frequency of $HPV^+$ lesions in immunosuppressed individuals however, suggests that the immune system may play a important protective role (178), with T cell-mediated immunity appearing crucial to the control and eradication of HPV-transformed tumors (179). While most attention has been traditionally dedicated to the study of anti-HPV $CD8^+$ CTL responses, it is also clear that $CD4^+$ T cell recognition of E6- and/or E7-derived peptides may be critical for optimal prophylactic or therapeutic efficacy against HPV-related malignancies. In the current study, we have identified or confirmed three E7-derived Th epitopes and analyzed the magnitude and polarization of specific $CD4^+$ T cell responses in the peripheral blood of normal donors and patients with cervical intraepithelial neoplasia (CIN) or cervical carcinoma. We have

observed that in contrast to the undisturbed Th1-type responses against the $E7_{48-62}$ epitope in all donor cohorts, $CD4^+$ T cell responses against the $E7_{1-12}$ and $E7_{62-75}$ epitopes showed a cancer-related bias away from clinically preferred Th1-type and towards a potentially undesirable Th2-type immunity.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Peptide Selection and Synthesis.

The HPV-16 E7 protein (GeneBank Accession AAB70738) was scanned using a MHC Class II HLA-DR peptide binding algorithm (180), with predicted high-affinity HLA-DR binding peptides reported in Table 2. Predicted E7 epitopes and the known malarial circumsporozooite$_{326\text{-}345}$ ($CS_{326\text{-}345}$) Th epitope (181) were synthesized using fMOC chemistry by the University of Pittsburgh Cancer Institute's (UPCI) Peptide Synthesis Faculty. Peptides were >96% pure based on high-performance liquid chromatography, with identities validated by mass spectrometric (MS/MS) analysis performed by the UPCI Protein Sequencing Facility.

### 2.3.2 Isolation of Peripheral Blood T cells and DCs.

Patient information is provided in Table 3. Briefly, the CIN or invasive cancer status of patients were determined by pathology review. No patient had received any adjunctive (chemo-, radio-, or immuno-) therapy within 6 weeks prior to blood donation. Vaginal hysterectomy patients were clinically treated for various reasons unrelated to CIN or invasive cervical cancer, and were considered normal in the context of the current study. Normal donors (ND) are asymptomatic women with no history of CIN. Fifty to one-hundred milliliters of heparinized donor blood was obtained with informed consent under an IRB-approved protocol and diluted 1:2 in PBS, prior to being centrifuged (550 x g for 25 min at RT) on discontinuous ficoll-hypaque gradients (Cellgro$^{TM}$, Mediatech, Inc., Herndon, VA) per the manufacturer's instructions. Bouyant PBMCs at the gradient interface were recovered and washed three times with PBS (BioWhittacker, Walkersville, MD) to remove residual platelets and ficoll-hypaque. The generation of donor DCs from adherent mononuclear cells and the isolation and cryo-

preservation of autologous $CD4^+$ T cell from non-adherent cells were performed as previously described (182).

### 2.3.3 HLA-DR Typing.

The HLA-$DR4^+$ status of peripheral blood monocytes was confirmed by flow cytometry as previously described (182). Patient HLA-DR genotyping was performed using the Dynal Allset+™ SSP DR "low resolution" kit (Dynal Inc., Lake Success, NY), with template DNA extracted from patient lymphocytes expanded for 5 days in the presence of 25 μg/ml phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, MO). DNA extraction was performed using the DNeasy Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol.

### 2.3.4 Induction of Th Effector Lymphocytes.

On day 7 of DC culture, autologous $CD4^+$ T cells were thawed in 10 ml of RPMI 1640 containing 10% FBS supplemented with 20 Units/ml DNase I (type II, from bovine pancreas, Sigma) to increase harvested cell yield and to reduce clumping. Harvested, non-adherent DCs ($2 \times 10^5$) were co-cultured with $2 \times 10^6$ thawed, autologous $CD4^+$ T cells in the presence of 10 μM E7-derived synthetic peptides in RPMI-1640 media containing 10% FBS. No exogenous cytokines were added to these cultures, in order to prevent any Th1 polarizing effects. Responder $CD4^+$ T cells were then harvested on day 7-10 and analyzed for HPV-16 E7 peptide-specific reactivity in ELISPOT assays.

### 2.3.5 IFN-γ and IL-5 ELISPOT assay for Peptide-Reactive $CD4^+$ T cell Responses.

To evaluate the frequencies of peripheral blood $CD4^+$ T cells recognizing peptide epitopes, ELISPOT assays for IFN-γ and IL-5 were performed as previously described(182, 183). Briefly, $10^5$ $CD4^+$ T cells and autologous thawed DCs ($2x10^4$ cells) were seeded in ELISPOT wells. Synthetic peptides (stocks at 1 mg/ml PBS) were then added to appropriate wells at a final concentration of 10 μg/ml. Negative control wells contained $CD4^+$ T cells and DCs pulsed with $CS_{326-345}$ peptide, with DCs alone serving as the APC control. Data are reported as actual numbers of E7-specific T cell spots above T cell background responses to the negative control $CS_{326-345}$ peptide. Additionally, when sufficient T cells were available, $CD4^+$ T cells co-cultured with non-peptide pulsed DCs were included as an additional negative control. The non-peptide pulsed DC and $CS_{326-345}$ peptide-pulsed DC groups varied at most by 5 spots/$10^5$ CD4+ T cells which was statistically insignificant (data not shown). Positive controls wells contained T cells plated in the presence of 5 μg/ml PHA (Sigma). All determinations were performed in triplicate, with spots imaged using the Zeiss AutoImager (and statistical comparisons determined using a Student two-tailed T-test analysis). The data are reported as the mean (+/- SD) number of IFN-γ or IL-5 spots per $10^5$ responder $CD4^+$ T cells analyzed.

### 2.3.6 TGF-β and IL-10 ELISAs.

Supernatants were harvested from ELISPOT plates at the endpoint of the culture period, pooled for a single stimulus (i.e. a given peptide, etc.) and, then frozen at -20°C until being analyzed by cytokine-specific ELISA. Cytokine capture and detection antibodies and recombinant cytokines for the TGF-β ELISA were purchased from BD-Pharmingen (San Diego, CA), while the IL-10 ELISA was purchased from Mabtech (Stockholm, Sweden) and used per

the manufacturer's instructions. The lower limit of detection for the TGF-β and IL-10 assays were 50 pg/ml and 12 pg/ml, respectively.

### 2.3.7 PCR Analysis.

PCR analysis for HPV-16 E6, HPV-16 E6/E7, and HPV L1 DNA was performed on patient's loop electrosurgical excision procedure (LEEP) biopsies. Extraction of DNA from the biopsies was performed using the DNeasy Tissue Kit (Qiagen, Valencia, CA) according to provided manufacturer's protocol. The following primer sets were used: HPV-16 E6 (forward: ATGCACCAAAAGAGAACTGC, reverse: TTACAGCTGGGTTTCTCTAC, product size 477bp with cycles: melting 94$^{o}$C for 45 sec, annealing 59$^{o}$C for 45 sec, extension 72$^{o}$C for 1 min, for 38 cycles), HPV-16 E6/E7 (forward: ATGCACCAAAAGAGAACTGC, reverse: TGCCCATTAACAGGTCTTCC, product size 735 bp with cycles: melting 94$^{o}$C for 45 sec, annealing 59$^{o}$C for 45 sec, extension 72$^{o}$C for 1 min, for 40 cycles), HPV L1 capsid (forward: GCMCAGGGWCATAAYAATGG, reverse: CGTCCMARRGGAWACTGACT, product size 450 bp, with cycles same as E6/E7, M denotes A and C; R denotes A and G; W denotes A and T; and Y denotes C and T). The capsid primers specifically amplify the L1 gene for the majority of (but not all) oncogenic HPV genotypes (184). A patient was defined as HPV-16$^{+}$ if they were qualitatively positive by PCR using either the E6 or E6/E7 primers sets. Hence, we report a given patient's HPV genotype status as HPV-16$^{+}$, HPV-16$^{-}$/HPV-L1$^{+}$, or HPV-16$^{-}$/HPV-L1$^{-}$.

### 2.3.8 Statistical Analysis.

Statistical significance of differences was determined between ELISPOT data sets using a Student's two-tailed T test, with statistical significance defined at $p < 0.05$.

## 2.4.RESULTS

### 2.4.1. Selection and screening of candidate HLA-DR-binding peptides derived from the E7 protein.

Our investigation was designed to identify novel HPV-16 E7 derived peptides capable of being presented in a pan-DR manner to $CD4^+$ T cells, and to characterize functional anti-E7 Th responses in patients with HPV-related dysplasia (CIN I-III) or cervical cancer. We subjected the cDNA sequence of the HPV-16 E7 protein to a computer algorithm analysis designed to identify peptides likely to bind a broad range of HLA-DR alleles (i.e. pan-DR binding). Given the relatively high frequency of HLA-DR4 in the general population (~20%) and our past success in identifying HLA-DR4-presented epitopes derived from melanoma and renal cancer antigens (181-183), we initially required that HLA-DR4 be among the range of class II alleles the selected peptides would be predicted to bind to.

Nine amino acid-long 'core' sequences were evaluated and scored for predicted binding to nine distinct HLA-DR4 sub-alleles, with individual scores summed for the nine sub-alleles (Table 2). The highest aggregate scoring sequence was taken to represent the peptide most likely to bind in a pan-DR4 manner. Peptides $E7_{1-12}$, $E7_{11-25}$, $E7_{62-75}$, $E7_{72-86}$, and $E7_{83-97}$ each contained predicted HLA-DR4-binding peptide nonamers, while peptide $E7_{48-62}$ that failed to contain a predicted HLA-DR4 binding sequence, was selected for analysis due to its otherwise strong predicted pan-DR binding capacity (Table 2). These six peptides were synthesized and subsequently analyzed for T cell recognition *in vitro*.

### 2.4.2. $CD4^+$ T cells isolated from the majority of patients evaluated respond to HPV-16 E7-derived peptides.

Peripheral blood $CD4^+$ T cells were isolated from 28 patients with CIN I-III or cervical carcinoma, and from 5 age-matched controls (3 normal individuals undergoing vaginal hysterectomy and 2 normal donors with no history of CIN; see Table 3 for donor characteristics). After a single round of *in vitro* stimulation (IVS) with autologous DCs pulsed with a given E7 peptide, the resulting $CD4^+$ T cells were screened for peptide-specific responses in IFN-γ (Th1-type responses) and IL-5 (Th2-type responses) ELISPOT assays. Figure 5 depicts the Th1- vs. Th2-type $CD4^+$ T cell responses of all donors against the $E7_{1-12}$ and $E7_{62-75}$ peptides, reported as spots per $10^5$ $CD4^+$ T cells analyzed. A corresponding analysis of $CD4^+$ T cell response to the $E7_{48-62}$ peptide is provided in Figure 6**.** These data are also summarized in a qualitative (+/-) format in Table 2 for each individual evaluated. The results for peptides $E7_{11-25}$, $E7_{72-86}$, and $E7_{83-97}$ have been omitted due to the lack of donor responses to these peptides (data not shown).

Eighteen of the 28 CIN/cancer patients responded significantly to at least one HPV-16 E7 peptide in either the IFN-γ (14/28) and/or IL-5 (6/27, 1 patient not tested) ELISPOT assays (Table 3). In the majority (15 of 18) of cases where patients did respond to HPV-16 E7 peptides, they reacted against a single peptide. Reactivity to two E7 peptides was observed in only 2 patients, and reactivity to all three E7 peptides was noted for a single patient with cervical cancer (patient #35). Overall, 6, 8 and 8 patients were responsive to the $E7_{1-12}$, $E7_{48-62}$ and $E7_{62-75}$ peptides, respectively. Of note, 0/3 patients undergoing vaginal hysterectomies and 0/2 normal donors were reactive against any of the E7-derived peptides evaluated.

With regard to disease-stage, Type-1 (IFN-γ) responses against any of the three E7 peptides were observed in 5/11 CIN I, 3/6 CIN II, 3/5 CIN III and 3/6 cervical cancer patients.

In contrast, Type-2 (IL-5) responses were not seen in CIN I/II patients, but were observed in 1/5 CIN III and 5/5 (patient #27 was not evaluated) cervical cancer patients. Notably, $CD4^+$ T cell responses appeared Th1- or Th2-type polarized in the vast majority of responders, with mixed Th1 and Th2 reactivity to E7 peptides evident in only 2 patients with cervical cancer (patients #12 and #35). We were able to evaluate HPV genotype status by performing a PCR-based analysis of DNA extracted from LEEP biopsies in 16 of the 18 patients responsive to E7-derived peptides. Of these 16 patients, 12 screened as HPV-$16^+$, 3 patients (#24, #29, #32) exhibited an HPV-$16^-$/HPV-$L1^+$ genotype and a single patient (#31) was evaluated as HPV-$16^-$/HPV-$L1^-$. Plasma was available for 3 of these HPV-$16^-$ patients, and in 2 of 3 cases, anti-HPV-16 E7 IgG antibodies were detected by ELISA (data not shown).

### 2.4.3. Peptides $E7_{1-12}$ and $E7_{62-75}$ are primarily recognized by HLA-$DR4^+$ CIN patients and HLA-$DR15^+$ patients with cervical cancer.

As indicated above, $CD4^+$ T cells isolated from 6 of 28 patients exhibited statistically significant (ELISPOT) responses against the HPV-16 $E7_{1-12}$, with Type-1 responses observed in 4 CIN I/II patients and Type-2 responses identified in two patients with cervical carcinoma (Figure 5 and Table 3). Strikingly, all six of these responding patients expressed the HLA-DR4 (4/6) and/or -DR15 (2/6) class II molecules, and were typed as HPV-$16^+$ (with the exception of patient #29 who tested as HPV-$16^-$/HPV-$L1^+$) by RT-PCR.

Similarly, 7 of 8 patients with $CD4^+$ T cells reactive against the HPV-16 $E7_{62-75}$ peptide were either HLA-$DR4^+$ or -$DR15^+$ (Table 2), with Type-1 responses observed in 3 patients with CIN II/III and Type-2 responses identified in 5 patients with cervical carcinoma (Figure 5). Cancer patient #27, who expresses neither HLA-$DR4^+$ nor HLA-$DR15^+$, was responsive to the

HPV-16 $E7_{62-75}$ epitope in the IFN-γ ELISPOT assay (but was not tested for IL-5 response due to an insufficient number of T cells). All responders tested positive for expression of HPV-16, with the exception of CIN II patient #24, who was typed as HPV-$16^-$/HPV-$L1^+$. This patient's (#24) plasma also failed to contain detectable levels of anti-HPV-16 E7 IgG antibodies by ELISA (data not shown).

### 2.4.4. Peptide $E7_{48-62}$ appears to be primarily recognized by patients who do not express HLA-DR4.

Eight of 28 patients evaluated with CIN I-III or cervical carcinoma displayed statistically significant $CD4^+$ T cell responses to the $E7_{48-62}$ peptide in either the IFN-γ or IL-5 ELISPOT assays, as summarized in Table 3. In marked contrast to Th responses against the $E7_{1-12}$ and $E7_{63-75}$ peptides, none of the responders to peptide $E7_{48-62}$ were HLA-$DR4^+$ (Figure 6). Hence, 8 of 18 (44.4%) evaluable $DR4^-$ women exhibited $CD4^+$ T cells reactive against this peptide. Seven of these 8 responder patients displayed Th1-type biased reactivity to the $E7_{48-62}$ peptide, with CIN III patient #28 representing the sole Th2-type responder to this epitope. Interestingly, two HLA-$DR15^+$ cancer patients displayed strong Th1-type immunity to the $E7_{48-62}$ peptide, and in the case of patient #35, this response occurred in concert with Th2-biased responses against the $E7_{1-12}$ and $E7_{62-75}$ peptides (Table 3). LEEP biopsies were available for 7/8 responder patients. PCR analyses determined the genotype status of 5 patients as HPV-$16^+$, 1 patient (#32) as HPV-$16^-$/HPV-$L1^+$ and 1 patient (#31) as HPV-$16^-$/HPV-$L1^-$ (Table 3). Based on analysis of their plasma, these latter two patients displayed detectable levels of anti-HPV-16 E7 IgG antibodies by ELISA (data not shown).

## 2.5 DISCUSSION

In the current report, we have identified two novel HPV-16 E7-derived Th epitopes contained within the E7 $_{1-12}$ and E7 $_{62-74}$ peptides. Each epitope is seemingly restricted by at least HLA-DR4 and -DR15, and is recognized by $CD4^+$ T cells isolated from CIN and cervical cancer patients, but not normal donors. We also synthesized (and evaluated $CD4^+$ T cell responsiveness) the HLA-DR15-presented $E7_{48-62}$ epitope previously defined by Van der Burg et. al. (159), based on our algorithm analysis result suggesting that this peptide was likely to be pan-DR presented. Our study revealed that the $E7_{48-62}$ peptide is recognized by $CD4^+$ T cells isolated from a high frequency (i.e. approximately 44%) of HLA-$DR4^-$ CIN or cervical cancer patients evaluated in this study, including but not restricted to, HLA-$DR15^+$ patients. Theoretically, this data argues for the pan-class II presentation of this epitope on multiple HLA-DR, -DP or -DQ molecules (other than HLA-DR4). Alternatively, this peptide can be presented by HLA-DR4 and is either an immunodominant antagonist or it preferentially promotes Th responses other than Th1- or Th2-type (i.e. T regulatory, etc.). These two possibilities are unlikely since this peptide is predicted to bind to HLA-DR4 very poorly, if at all. Furthermore, in extended studies, we analyzed the supernatants harvested from ELISPOT wells by ELISA to determine whether E7 peptides elicited TGF-β and IL-10 production (an index of T regulatory function, ref. 103) from any of these donors. We observed E7 peptide-specific TGF-β production from $CD4^+$ T cells isolated from only two patients (CIN II (patient #1): 102 pg/ml and Cancer patient #39: 54pg/ml) that exceeded the 50 pg/ml lower limit of detection for this ELISA. Detectable IL-10 production (i.e. > 12 pg/ml, the lower limit for the IL-10 ELISA) was observed only for Cancer patient #39 (25 pg/ml). Interestingly, this patient was also one of the 2 patients producing TGF-β in response to E7-derived peptides. We were unable to evaluate the other TGF-β producer

(patient #1) for IL-10 production due to insufficiency in T cell numbers required for the assay. In aggregate, the available data support the pan-class II (but non-HLA-DR4) presented nature of the $E7_{48-62}$ peptide.

IFN-γ and IL-5 ELISPOT assays were employed to monitor the functional polarization of patient $CD4^+$ T cell responses against HPV-16 E7-derived peptides. We observed that a high number of CIN I-III patients (11/22) displayed Th1-type immunity to one or more E7 peptides, but that only 1/22 CIN I-III patients displayed detectable Th2-type responses to these same peptides. In marked contrast, 5 of 6 patients with cervical cancer (i.e. patient #27 could not be evaluated for IL-5) displayed Th2-type responses to at least one E7-derived Th epitopes, with 2/5 exhibiting mixed Th1-/Th2-type immune responses. These data suggest that Th2-type dominated $CD4^+$ T cell responses against HPV-16 E7 epitopes may correlate with advanced disease status in these individuals, a finding consistent with results reported for tumor antigen-specific Th responses in patients with advanced stage cancers of alternate histologies (182, 185-189).

It should be stressed that polarized Th2-type $CD4^+$ T cell responses were specific for the HPV-16 $E7_{1-12}$ and $E7_{62-75}$ peptides tested and do not reflect the general tendency of these donors with advanced stage malignancy to respond in a generic Th2-biased fashion. Indeed, mitogen (PHA) control spot frequencies obtained for both the IFN-γ and IL-5 ELISPOT assays using $CD4^+$ T cells were indiscriminant between patients with cancer, CIN patients, and (normal) vaginal hysterectomy patients. In addition, cancer patients exhibited strong Th1-type immunity to the pan-DR-presented EBV Th epitope EBNA-$2_{280-290}$ (ref. (190), data not shown). Perhaps most salient, cervical cancer patient #35 displayed coordinate Th2-type responses to the $E7_{1-12}$ and $E7_{62-75}$ peptides and Th1-type reactivity against the $E7_{48-62}$ epitope.

The mechanism(s) by which Th2-type skewing may occur in anti-E7 $CD4^+$ T cell responses of cervical carcinoma patients remains unknown, but could include: 1) chronic antigenic restimulation (i.e. repetitive re-infection, refs. (191-195)) *in situ* that may delete Th1-type responders (182, 196) 2) dominant locoregional DC2-type antigen presenting cell function in the advanced cancer setting that favors Th2-type immunity (107) and/or 3) a generalized Th2-polarizing cytokine microenvironment at the tumor site and within the tumor draining lymph nodes of affected patients (197). The maintenance of Th1-type immunity against the pan-DR-presented EBV Th epitope (and against the $E7_{48-62}$ peptide in patient #35) argues against dominant DC2-mediated functional, or global Th2-type cytokine-mediated, skewing of peripheral blood $CD4^+$ T cell responses in cervical cancer patients. However, these important issues will require intense prospective evaluation to determine their unequivocal roles in shaping the HPV-specific immune response in high-risk and progressor patients.

Our analysis of donor $CD4^+$ T cells using ELISAs specific for IL-10 and TGF-β revealed weak evidence for anti-E7 Treg-type activity in only 2/28 patients (CIN II patient #1 and Cancer patient #39). This suggests, at least in the peripheral circulation of patients, that the detection of Treg-type CD4+ T cells reactive against HPV-16 E7-derived epitopes is not a common event. Such responses, if they exist in patients, may be more dominant in cervical mucosal sites or the secondary lymphoid organs that drain these tissues (198).

Coordinate analysis of $CD4^+$ T cell responsiveness to HPV-16 E7-derived peptides and HPV-16 genotype status of LEEP biopsies indicated that in 6/7 cases for the $E7_{1-12}$ peptide, 5/7 (evaluable) cases for the $E7_{48-62}$ peptide and 7/8 cases for the $E7_{62-75}$ peptide, the responding patients were HPV-$16^+$. A total of 3 patients (#24 CIN III, #29 CIN I, #32 CIN II) that reacted against E7 peptides were genotyped as HPV-$16^-$/HPV-$L1^+$ indicating current infection by

alternate HPV types, and a single CIN II patient (#31) who reacted against the $E7_{48-62}$ peptide was genotyped as HPV-$16^-$/HPV-$L1^-$. We would hypothesize that each of these latter 4 patients had at one time been infected with HPV-16, but had cleared the virus, potentially via specific T cell dependent immunity. This latter possibility is consistent with the detection of (T-dependent) IgG, anti-E7 antibody levels in the plasma of patients #31 and 32 by ELISA (data not shown, patient #29 could not be evaluated). After clearing HPV-16, a subsequent re-infection with alternate HPV genotypes could have resulted in HPV-$L1^+$ status for the CIN lesion in 3 of these 4 patients. We currently cannot provide a convincing rationale for the results obtained in patient #31, unless the biopsy tissue provided for analysis failed to contain a sufficient quantity of the CIN II lesion within the LEEP biopsy, resulting in a positive control signal (for β-actin) but a failure to detect the HPV-16 or HPV-L1 genes. The lack of an L1-specific signal could also occur if the patient's CIN lesion were infected by a high risk HPV type that is not amplified by our selected PCR primers. Finally, the data derived from CIN III patient #24 who exhibited Th1-type $CD4^+$ T cells reactive against the $E7_{62-75}$ peptide, but who failed to display positive HPV-16 PCR or serology results (data not shown) remains an enigma.

One surprising finding in this study was that 5 out of 6 (83%) cancer patients evaluated were tissue typed as HLA-$DR15^+$, although this allele is typically only observed in the general population at a frequency of 17-29%, depending on donor ethnicity (199). Of interest in this regard, recent reports argue that HLA-DR15 may be associated with increased susceptibility to cervical cancer (200-202). We will continue to monitor whether the HLA-DR15 allele is expressed by a disproportionately high frequency of cervical cancer patients in our prospective studies.

Overall, we believe that the $E7_{1-12}$ and $E7_{62-75}$ peptides represent good candidate epitopes for implementation in vaccines for (at least) HLA-$DR4^+$ or -$DR15^+$ individuals to prevent or treat cervical cancer. Based on the high-frequency of responders against the $E7_{48-62}$ epitope in HLA-DR4-negative patients, the inclusion of this peptide in a polyepitope vaccine (including the $E7_{1-12}$ and $E7_{62-75}$ epitopes), would complement and broaden patient coverage. This would allow for the majority of patients (i.e. > 70%) with HPV-$16^+$ CIN or cervical carcinomas to be treated by such a modality. Furthermore, given an expanding number of cancer histologies reported to be HPV-positive, HPV-16 E7 peptide-based vaccines may prove clinically applicable to an even larger patient population. When provided with an adjuvant capable of preferentially promoting Th1-type immunity, E7 peptide-based vaccines would be anticipated to stimulate IFN-γ secreting, anti-E7 $CD4^+$ effector T cells. These Type-1 Th cells would, in turn, be expected to enhance the frequencies and functionality of anti-HPV-16 reactive $CD4^+$ and $CD8^+$ T cells *in situ* and to recruit these T cells into HPV-$16^+$ tissues (via a DTH-type mechanism), yielding enhanced clinical efficacy. These peptides will also likely prove useful in the longitudinal immunomonitoring of functionally evolving $CD4^+$ T cell responses in patients with, or at high-risk to develop, cervical carcinoma or other HPV-related malignancies.

**Preface Chapter 3**

Having defined three HLA-DR-restricted Th epitopes derived from the HPV-16 E7 protein in **Chapter 2**, we next explored the potential of using recombinant E7 in vaccine formulations to discern: 1) whether all three epitopes are naturally-processed and presented by host DCs, 2) whether whole protein vaccines are able to elicit poly-specific anti-E7 Th responses in patients with CIN or cervical cancer and 3) whether recombinant E7 protein- or E7 peptide-based vaccines are preferred for the effective induction of E7-specific Th cells *in vitro*. **Chapter 3** outlines the use of DC/viral-like particle (VLP) vaccines to elicit specific CD4+ T cell responses *in vitro*. We observed that this approach is effective in promoting Th responses against all three E7-derived epitopes in a subset of donors and importantly, that the use of E7 recombinant protein- vs. peptide-based vaccines does not alter the functional polarization (i.e. Th1-type vs. Th2-type) of the CD4+ T cell immune response. Since cancer patients, but not patients with CIN, continued to display Type-2 dominated immunity to the $E7_{1-12}$ and $E7_{62-75}$ epitopes, further modification of DC-based vaccines will be warranted to yield predictable and preferential Type-1 immunity.

## 3.0 DCs Infected by Human Papillomavirus L1L2-E7 Viral-Like Particles (VLPs) Elicit Polyspecific Anti-E7 CD4+ T Cell Responses from CIN and Cervical Cancer Patients *In Vitro*

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Running Title: CD4+ T Cell Responses Induced by DC-VLP Vaccines

Key Words: Dendritic cells, cervical cancer, cervical intraepithelial neoplasia, polarization, Viral-like particle

## 3.1 ABSTRACT

Human papillomavirus (HPV) has been casually linked to cervical cancer and cervical intraepithelial neoplasia (CIN), making HPV-based vaccines logical candidates for prevention or amelioration of these conditions. The inability to culture HPV or to generate attenuated vaccines has however, necessitated the development of novel immunization strategies to prevent and treat HPV-associated malignancies. We evaluated the ability of recombinant HPV-16 L1L2-E7 virus-like particles (VLPs) to stimulate E7-specific CD4+ T cell responses from normal donors and patients with CIN lesions or cervical carcinoma when employed in DC-based vaccines *in vitro*. We have determined that VLPs partially mature DCs and that DC-L1L2-E7 VLP vaccines elicit CD4+ T cell responses against three distinct, pan-DR-presented HPV-16 E7-derived epitopes to a degree comparable to or exceeding that observed for DCs pulsed with synthetic E7-derived peptides or recombinant E7 (rE7). Notably, DC-L1L2-E7 VLP vaccines stimulated, but did not alter the functional polarization state of specific anti-E7 $CD4^+$ T cells. Patients with pre-malignant CIN I-III lesions displayed predominant Th1-type anti-E7 immunity, while those with cervical cancer were more commonly Th2-type biased. Since $CD4^+$ T cells isolated from more than 90% (17 of 18) of patients evaluated responded to DC-L1L2E7 VLP vaccines *in vitro*, such vaccines may represent promising preventative or therapeutic treatment strategies for patients with HPV-16-associated malignancies.

## 3.2 INTRODUCTION

The human papillomaviruses (HPV) have been causally-linked to cervical dysplasia and cervical cancer, which represents the third most common cancer (12%) among women worldwide (2). Approximately 370,000 cases of cervical cancer are newly diagnosed each year, with a 50% mortality rate reported(171, 175). An estimated 30-60% of sexually active men and women are infected with genital HPVs, and these infections are typically resolved asymptomatically (170). The low-risk HPV genotypes, such as HPV-6 and HPV-11, are detected in benign genital warts and low-grade squamous intraepithelial lesions (SIL), but rarely in invasive carcinomas (7). High-risk HPV genotypes (HPV-16, -18, -31, -33, -45 or –56) have been detected in nearly all cancers of the cervix, with HPV-16 identified in 50-60% of $HPV^+$ carcinomas (7, 173).

Why some individuals clear the virus while others do not is only now being resolved (203), but the observed increase in frequency of $HPV^+$ lesions in immunosuppressed individuals suggests that a functional immune system plays a critical regulatory role (178). Indeed, T cell-mediated immunity appears crucial to the control and eradication of HPV-transformed tumors (179). However, even in cases where a patient harboring $HPV^+$ CIN or carcinoma lesions develop HPV-specific $CD8^+$ T cell responses, this immunity may prove clinically ineffective due to tumor-induced immune deviation and tumor immune escape mechanisms (144, 155). For instance, $HPV^+$ tumor cells exhibit suppressed levels of HLA class I expression in approximately 90% of cases, making them refractory to specific $CD8^+$ T cell killing (170). In this regard, it may be particularly prudent to promote both specific $CD4^+$ and $CD8^+$ T cell-mediated immunity to HPV in order to gain optimal therapeutic benefits. $CD4^+$ T cell-mediated "help" appears important in the maintenance of effective CTL responses (204), particularly in the setting of

chronic viral infections (179), which may serve as a relevant paradigm for indolent tumors. Furthermore, in murine tumor models, $CD4^+$ T cells specific for tumor antigens have been shown capable of mediating the rejection of MHC class I-negative tumor cells that cannot be eradicated by specific $CD8^+$ CTLs (205, 206).

Since the oncogenic E6 and E7 gene products are the only HPV-encoded proteins required for cellular transformation (15), they represent logical targets for the development of prophylactic/therapeutic vaccines for the prevention and treatment of HPV-related malignancies. However, HPVs remain very hard to propagate *in vitro*, making attenuated viral vaccines logistically untenable. This has mandated the evaluation of alternative strategies, including vaccines based on synthetic E6/E7 CTL-recognized peptides, recombinant protein, DNA, and chimeric Virus-Like Particles (cVLP) (36). CVLPs formed by spontaneous assembly of the L1 and L2 capsid proteins of HPV have proven attractive for vaccination purposes since they are highly-immunogenic, have no inherent transforming capacity (207) and have been shown to be safe and effective in preventing the development of CIN in clinical trials (161). Since patients develop strong neutralizing anti-L1/-L2 antibody responses, the efficacy of repeated VLP-based vaccination designed to boost HPV-specific T cell responses is considerably reduced (208), however, this effect may be circumvented by vaccinating patients with autologous dendritic cells infected with VLPs. In the current study, we evaluated the ability of DCs infected with cVLPs composed of the HPV-16 L1 and L2-E7 (fusion) proteins to elicit E7-specific $CD4^+$ T cell responses *in vitro*, with a focus on responses directed against pan-HLA-DR presented E7 epitopes that we have recently defined[1].

**FOOTNOTES**

[1] Warrino, D.E., Olson, W.C., Knapp, W.T., Scarrow, M.I., D'Ambrosio-Brennan, L.J., Guido, R.S., Edwards, R.P. Kast, W.M., and Storkus, W.J. Disease-Stage Variance in Functional $CD4^+$ T Cell Responses Against Novel Pan-DR Presented HPV-16 E7 Epitopes. Clin. Cancer Res., in press, 2004.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Peptides

The pan-HLA-DR presented HPV-16 $E7_{1-12}$, $E7_{48-62}$, $E7_{62-75}$ peptides, as well as the pan-DR presented malarial circumsporozooite ($CS_{326-345}$) peptide were synthesized using fMOC chemistry by the University of Pittsburgh Cancer Institute's (UPCI) Peptide Synthesis Faculty, as previously described (181). Peptides were >90% pure based on high-performance liquid chromatography, with identities validated by mass spectrometric (MS/MS) analyses performed by the UPCI Protein Sequencing Facility.

### 3.3.2 Generation and Purification of Recombinant HPV-16 E7 Protein.

Total RNA was isolated from the HPV-$16^+$ Caski cell line (the kind gift of Dr. Saleem Kahn, U. of Pittsburgh) and converted to cDNA using the TRIzol® Reagent (Invitrogen, Carlsbad, CA) and the GeneAmp Kit (Roche, Basel, Switzerland), respectively, per the manufacturer's instructions. The following primer set was used for HPV-16 E7; forward: ATGCATGGAGATACACCTACATT, reverse: TGGTTTCTGAGAACAGATGG, product size 300bp with cycles: melting 94°C for 45 sec, annealing 65°C for 45 sec, extension 72°C for 1 min, for 38 cycles. This PCR product was then sub-cloned into the pBAD TOPO® TA expression vector (Invitrogen) according to the manufacturer's protocol, yielding a HPV-16 E7 fusion product containing a C-terminal poly-histidine tag for isolation purposes. After sequencing the insert for identity (DNA Sequencing Facility, UPCI), competent bacteria were transformed and expanded in LB Broth (Invitrogen). Bacteria and inclusion bodies were disrupted using 8M Urea (Sigma), with protein dialyzed against PBS. Recombinant E7 protein was isolated using a HisTrap™ (Pharmacia) column and eluted in 300 mM imidazole, per the

manufacturer's instructions. After dialysis against PBS, and confirmation of correct Mr by Western Blotting using both anti-His (Sigma) and anti-HPV-16 E7 (Zymed, San Francisco, CA), the protein was frozen at –80$^{o}$C at 0.5 mg/ml (Figure 7). The protein was >90 % pure based on an analysis of Coomassie-stained gels (Bio-Rad, Hercules, CA).

### 3.3.3 Generation and purification of HPV16 E7 Virus-like Particles (VLPs).

VLPs L1L2-E7 and VLP L1L2 were provided by Dr. Da Silva, who produced and qualified as previously described (155). Each batch was tested by Western blot for presence of L1, L2, and in the case of chimeric particles, also for L2 fusion protein (E7 protein). Each batch was tested by transmission electron microscopy as described (155) for the presence of particles. For production of green fluorescence protein (GFP)-VLPs, the GFP gene was cloned from pEGFP-plasmid (Clonetech, Palo Alto, CA) with the primers (GFP forward) 5' ATGGTGAGCAAGGGCGAGG-3' and (GFP reverse *Xba*I) 5' GCGTCTAGATTGTACAGCTCG-3' by PCR. The mutated L2 gene (lacking the stop codon) was cloned by PCR with the primers (L2 forward) 5' CCCATGCGACACAAACGTTCTGCAAAACGC-3' and (L2 reverse no stop) 5'-ATCGGCAGCCAAAGAGACATCTG-3'. PCR products of both reactions were purified and ligated at equimolar amounts with T4-DNA-Ligase (BD PharMingen), and L2-GFP fusion product was cloned by PCR with primers GFP reverse *Xba*I and L2 forward. Resulting PCR product was purified and cloned into pZero (Clonetech) via *Eco*RV and *Xba*I sites, sequenced, and subsequently subcloned into pFASTBAC from where chimeric VLP particles were produced as described previously (155). Presence of L2-GFP fusion protein in purified chimeric VLPs was confirmed by Western blot analysis with anti-GFP Ab (BD PharMingen).

Crude insect cell lysates for control experiments were generated by collecting supernatant of sonicated insect cell pellets after centrifugation. Before use, the supernatants were treated with END-X B15 (Cape Cod Associates, Falmouth, MA) according to manufacturers instructions for removal of endotoxins.

### 3.3.4 Isolation of Peripheral Blood T cells and DCs.

Patient information is provided in Table 4. Briefly, the CIN or invasive cancer status of patients were determined by pathology review. No patient had received any adjunctive (chemo-, radio-, or immuno-) therapy within 6 weeks prior to blood donation. Vaginal hysterectomy patients were clinically treated for various reasons unrelated to CIN or invasive cervical cancer, and were considered normal donors in the context of the current study. Additional normal age-matched donors (ND) included asymptomatic women with no history of CIN. Fifty to one-hundred mililiters of heparinized donor blood was obtained with informed consent under an IRB-approved protocol and diluted 1:2 in PBS, prior to being centrifuged (550 x g for 25 min at RT) on discontinuous ficoll-hypaque gradients (CellgroTM, Mediatech, Inc., Herndon, VA) per the manufacturer's instructions. Bouyant PBMCs at the gradient interface were recovered and washed three times with PBS (BioWhittacker, Walkersville, MD) to remove residual platelets and ficoll-hypaque. The generation of donor DCs from adherent mononuclear cells and the isolation and cryo-preservation of autologous $CD4^+$ T cell from non-adherent cells were performed as previously described (182).

### 3.3.5 HLA-DR Typing.

The HLA-DR4$^{+}$ status of peripheral blood monocytes was confirmed by flow cytometry using the 359-13F10 mAb as previously described (183). Patient HLA-DR genotyping was performed using the Dynal Allset+™ SSP DR "low resolution" kit (Dynal Inc., Lake Success, NY), with template DNA extracted from patient lymphocytes expanded for 5 days in the presence of 25 μg/ml phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, MO). DNA extraction was performed using the DNeasy Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol.

### 3.3.6 PCR Analysis.

PCR analysis for HPV-16 E6, HPV-16 E6/E7, and HPV L1 DNA was performed on patient's loop electrosurgical excision procedure (LEEP) biopsies. Extraction of DNA from the biopsies was performed using the DNeasy Tissue Kit (Qiagen, Volencia, CA) according to the manufacturer's protocol. The following primer sets were used: HPV-16 E6 (forward: ATGCACCAAAAGAGAACTGC, reverse: TTACAGCTGGGTTTCTCTAC, product size 477bp with cycles: melting 94$^{o}$C for 45 sec, annealing 59$^{o}$C for 45 sec, extension 72$^{o}$C for 1 min, for 38 cycles), HPV-16 E6/E7 (forward: ATGCACCAAAAGAGAACTGC, reverse: TGCCCATTAACAGGTCTTCC, product size 735 bp with cycles: melting 94$^{o}$C for 45 sec, annealing 59$^{o}$C for 45 sec, extension 72$^{o}$C for 1 min, for 40 cycles), HPV L1 capsid (forward: GCMCAGGGWCATAAYAATGG, reverse: CGTCCMARRGGAWACTGACT, product size 450 bp, with cycles same as E6/E7, M denotes A and C; R denotes A and G; W denotes A and T; and Y denotes C and T). The capsid primers specifically amplify the L1 gene for the majority of

(but not all) oncogenic HPV genotypes (184). A patient was defined as HPV-16$^+$ if they were qualitatively positive by PCR using either the E6 or E6/E7 primer sets. Hence, we report a given patient's HPV genotype status as HPV-16$^+$, HPV-16$^-$/HPV-L1$^+$, or HPV-16$^-$/HPV-L1$^-$.

### 3.3.7 Induction of Th Effector Lymphocytes.

On day 7 of DC culture, autologous CD4$^+$ T cells were thawed at 37ºC water bath and added to 10 ml of RPMI 1640 media containing 10% FBS and supplemented with 20 U/ml DNase I (type II, from bovine pancreas, Sigma) to increase harvested cell yield and to reduce clumping. Harvested, non-adherent DCs (2 x $10^5$) were co-cultured with 2 x $10^6$ thawed, autologous CD4$^+$ T cells in the presence of 10 µg E7-derived synthetic peptides, 10 µg HPV-16 VLP L1L2-E7, 10 µg HPV-16 VLP L1L2 (control), or 10 µg rE7 full length protein, in RPMI-1640 media containing 10% FBS. No exogenous cytokines were added to these cultures. Responder CD4$^+$ T cells were then harvested on day 7-10 of culture and analyzed for HPV-16 E7 peptide-specific reactivity in ELISPOT or ELISA assays.

### 3.3.8 Immunofluorescence Microscopy of DC infection by VLP

Normal donor Day five iDCs were grown overnight on sterile 12mm glass circular cover slips (Corning Inc.) in 24 well plates (Corning Inc.) in DC media. Following day the DCs were fed 10ug/ml GFP-VLPs for 2hrs. Cells were washed and fixed with 2% Paraformaldehyde and subsequently the cover slip (with cells adhered) were removed from the 24 well plate. The coverslips were coated with 1:1000 Triton X-100/PBS (Sigma) and incubated at room temperature (RT) for 30 minutes. The coverslips were washed 5x with 0.5%BSA (Sigma) and 0.15% Glycine (Sigma) in PBS (BSA) and then blocked with 1:20 dilution normal mouse serum

(Sigma) in 0.5% BSA and incubated for 40 minutes at RT. The coverslips were washed 5 times with BSA and then incubated for 1hr with a 1:100 dilution of L243 in BSA at RT. Coverslips were washed 5 times with BSA and incubated for 1hr with a 1:1000 dilution of Cy3 Fluor® goat anti-mouse IgG (Molecula Probes, Eugene, Oregon) in BSA. The coverslips were washed 5 times with BSA and followed by 5 washes with PBS alone. The coverslip was then stained with Hoescht (Sigma) for 30 seconds and washed once with PBS. To a glass slide one drop of gelvatol (the kind gift of Dr. Simon Watkins, U. of Pittsburgh) was added and the coverslip was placed face down on the gelvatol. Imaging was done on an Olympus Provis Light Microscope and analysis was done on Adobe Photoshop.

### 3.3.9 IFN-γ and IL-5 ELISPOT assays.

To evaluate the frequencies of peripheral blood $CD4^+$ T cells recognizing peptide epitopes, ELISPOT assays for IFN-γ and IL-5 were performed as previously described (182, 183). Briefly, $10^5$ $CD4^+$ T cells and autologous thawed DCs ($2x10^4$ cells) were seeded in multi-screen hemagglutinin antigen plates. Synthetic peptides (stocks at 1 mg/ml PBS) were then added to appropriate wells at a final concentration of 10 μg/ml. Negative control wells contained $CD4^+$ T cells and DCs pulsed with $CS_{326-345}$ peptide, with DCs alone serving as the APC control. Data are reported as actual numbers of E7-specific T cell spots above T cell background responses to the negative control $CS_{326-345}$ peptide. Additionally, when sufficient patient's cells were available, $CD4^+$ T cells and DCs with no peptide were also employed as a negative control group. In all cases, this control group yielded results that were statistically indistinguishable from the DC+$CS_{326-345}$ peptide control group (data not shown). Positive control wells contained T cells plated in the presence of 5 μg/ml PHA (Sigma). All determinations were performed in triplicate,

with spots imaged using the Zeiss AutoImager (and statistical comparisons determined using a Student two-tailed T-test analysis). The data are reported as the mean (+/- SD) number of IFN-γ or IL-5 spots per 100,000 responder $CD4^+$ T cells analyzed.

### 3.3.10 TGF-β, IL-10, and IL-12p70 ELISAs.

Supernatants were harvested from ELISPOT plates at the endpoint of the culture period, pooled for a single stimulus (i.e. a given peptide, etc.) and, then frozen at -20°C until being analyzed for TGF-β and IL-10 production by cytokine-specific ELISA. Cytokine capture and detection antibodies and recombinant cytokines for the TGF-β ELISA were purchased from BD-Pharmingen (San Diego, CA), while the IL-10 ELISA was purchased from Mabtech (Stockholm, Sweden) and used per the manufacturer's instructions. The lower limit of detection for the TGF-β and IL-10 assays was 50 pg/ml and 37 pg/ml, respectively. Supernatants were harvested from day 5 iDCs fed 10 μg/ml VLP, iDCs stimulated with 250 ng/ml of LPS, 100 μg/ml of TNF-α, and untreated control iDCs cultured in 24 well plates with 500,000 iDC/ml for 48 hours and, then frozen at -20°C until being analyzed for IL-12p70 and IL-10 by cytokine-specific ELISA. Cytokine capture and detection antibodies and recombinant cytokines for the IL-12p70 ELISA were purchased from BD-Pharmingen (San Diego, CA) and used per the manufacturer's instructions. The lower limit of detection for the IL-12p70 assays was 37 pg/ml.

### 3.3.11 HPV-16 E7 IgG1 and IgG4 ELISA.

High-binding 96 well flat-bottom EIA/RIA plates (Corning Inc., Corning, NY) were coated overnight at 4°C with 100 μl of PBS containing 2 μg/ml recombinant HPV-16 E7 protein.

Plates were then manually washed 3 times with PBS and blocked for 2h at RT with 100 μl of 1% BSA (Sigma) in PBS (w/v). Plates were then washed 5 times with 0.5% Tween (Sigma) in PBS (w/v) and coated with 100 μl of patient plasma diluted 1/25 and 1/250 in 0.5% Tween/PBS. After incubating for 2h at RT, plates were again washed 5 times with 0.5% Tween/PBS, followed by addition of 100 μl of a 1/1000 dilution of biotinylated mouse anti-human IgG1 or anti-human IgG4 (Sigma) in 0.5% Tween/PBS, followed by incubation for 1h at RT. After 5 more washes with 0.5% Tween/PBS, 100 μl of a 1/1000 dilution of peroxidase-coupled steptavidin (Sigma) in 0.5% Tween/PBS was added to each well and plates incubated for 1h at RT. Finally after 7 washes in 0.5% Tween/PBS, 100 μl of TMB peroxidase substrate (KPL, Gaithersburg, MD) per the manufacturer's instructions was added to each well, plates incubated for 5-30 min. at RT, with the reaction stopped by addition of 50 μl of 2N sulfuric acid (Labchem Inc., Pittsburgh, PA). $OD_{450nm}$ readings were taken immediately using a Dynex MRX ELISA Reader (Chantilly, VA). Assays were standardized against purified human IgG1,κ or IgG4,κ (Sigma), with results reported in ng of IgG1/ml and IgG4/ml of patient plasma.

### 3.3.12 DQ-BSA uptake and analysis.

DC were harvested on day 5 and pulsed for 48h with 10 μg VLP, 100 ng/ml TNF-α, 100 ng/ml TNF-α + 10 μg VLP, or control DCs left untreated. The four DC sample cell populations were stained individually (25,000-30,000 cells/tube) with 10 μg/ml of DQ-BSA (Molecular Probes) in Aim-V media. After being incubated for 4 hours at either 4°C or 37°C, and washed twice with FACS buffer, the cells were immediately analyzed by flow cytometry.

### 3.3.13 Flow Cytometry Analysis.

Two-color flow cytometry analysis was performed as described previously, using a FACScan (Becton Dickinson) equipped with a single 488-nm argon ion laser. DC were harvested on day 5 and pulsed for 48h with media containing 10 μg VLP, 100 ng/ml TNF-α, 250 ng/ml LPS, or control media. At least 50,000 events were acquired for each sample, with data analyzed using the WinMDI software program (Version 2.8, Joseph Trotter, Scripps Inst., La Jolla, CA).

### 3.3.14 Statistical Analysis.

Statistical significance of differences was determined between ELISPOT data sets using a Student's two-tailed T test and between HPV-16 E7 IgG data sets using the Mann-Whitney rank sum test, with statistical significance defined as $p < 0.05$.

## 3.4 RESULTS

### 3.4.1 DCs take up (are infected by) VLPs and appear to co-localize to class II compartment.

The ability of DCs to take up HPV-16 VLPs and generate epitopes for presentation in MHC II molecules was critical for our study to define VLP derived HPV-16 E7 $CD4^+$ T cell epitopes. Normal donor day 5 iDCs were fed GFP-VLPs for 2 hours and stained for MHC class II and examined by immunofluorescence microscopy for co-localization. As shown in figure 8, it appears that the VLP is co-localizing with MHC class II resulting in yellow fluorescence when the images are overlaid. This is consistent with phagocytosis by iDCs.

### 3.4.2 $CD4^+$ T cells isolated from the majority of CIN or cervical cancer patients respond to in vitro DC-based vaccines incorporating HPV-16 E7-derived peptides or VLPs.

Peripheral blood $CD4^+$ T cells were MACs-isolated from 17 patients with CIN I-III or cervical carcinoma, and from four age-matched control donors (1 normal individual undergoing vaginal hysterectomy and three normal, asymptomatic donors with no history of CIN; see Table 4 for donor characteristics). These T cells were then stimulated with autologous DCs pulsed with a given E7 peptide, HPV-16 L1L2-E7 VLPs, control HPV-16 L1L2 VLPs, or recombinant E7 protein (rE7; when sufficient numbers of cells were available) and cultured for one week. The resulting $CD4^+$ T cells were then analyzed for peptide-specific responses in IFN-γ (Th1-type) and IL-5 (Th2-type) ELISPOT assays. Figure 9 depicts the $CD4^+$ T cell responses of 21 donors against the $E7_{1-12}$, $E7_{48-62}$, and $E7_{62-75}$ peptides after stimulation for one week with DC-L1L2-E7 vs. DC-peptide vaccines, with data reported as spots per $10^5$ $CD4^+$ T cells analyzed. These data are also summarized in a qualitative (+/-) format in Table 5 for each individual

evaluated. Overall, 16 of the 17 CIN/cancer patients responded in a statistically significant manner to at least one HPV-16 E7-derived Th epitope in either the IFN-γ (12/16) and/or IL-5 (8/16) ELISPOT assays (Table 5). In a majority (9 of 16) of these cases, patients responded to a single E7 epitope, with reactivity to two E7 peptides observed in 4 patients, and reactivity to all three E7 peptides observed in three patients. Overall, 7, 8, and 11 patients were responsive to the $E7_{1-12}$, $E7_{48-62}$ and $E7_{62-75}$ peptides, respectively. Of note, none of the four normal donors analyzed exhibited $CD4^+$ T cell that were reactive against E7-derived peptides.

With regard to disease-stage, Type-1 (IFN-γ) $CD4^+$ T cell responses against any of the three E7 Th epitopes were observed in 10/10 (100%) CIN patients and 2/7 (28%) cervical cancer patients. In contrast, Type-2 (IL-5) responses were seen in 2/10 (20%) CIN patients, but were observed in 6/7 (86%) of the cervical cancer patients. Notably, $CD4^+$ T cell responses appeared strongly Th1- or Th2-type polarized in the vast majority of responders, with mixed Th1-/Th2-type (i.e. Th0-like) reactivity to E7 peptides evident in only 2 patients with cervical cancer (patients #35 and #49) and 2 patients with CIN II (patients #43 and #50). We were able to evaluate HPV genotype status by performing a PCR-based analysis of DNA extracted from LEEP biopsies in 15 of the 17 CIN/cancer patients. Of these 15 patients, 12 screened as HPV-$16^+$, while 2 patients exhibited an HPV-$16^-$/HPV-$L1^+$ genotype. In addition, the presence of anti-E7 IgG1 and IgG4 antibodies was evaluated for 15 of these 17 patients from whom plasma had been obtained. All 11 evaluable patients that had screened as HPV-$16^+$ by RT-PCR also had elevated levels of circulating anti-E7 IgG1 and/or IgG4 antibodies in their plasma (Table 4). Of the two HPV-$16^-$/HPV-$L1^+$ patients, plasma was only available for patient #24, in whom levels of anti-E7 IgG1 and IgG4 antibodies were undetectable.

In the majority of cases where patients displayed $CD4^+$ T cell responses against HPV-16 E7-derived epitopes, Th cells with that specificity were evoked *in vitro* by both DC-peptide and DC/L1L2-E7 VLP, but not by DC/L1L2 VLP vaccines. In two cases (patients #20 and #24), $CD4^+$ T cells simulated by DC/L1L2-E7 VLP vaccines reacted against all three E7 epitopes, while peptide-based vaccines in these patients yielded Th responses against only a single epitope. Additionally, patient CIN II #50 generated anti-E7 Th responses against the DC/L1L2-E7 VLP vaccine but not against the peptide-based vaccine. Conversely, for patient #35, single peptide vaccinations proved successful, while stimulations with the DC/L1L2-E7 vaccine promoted a detectable response only against the $E7_{48-62}$ epitope. Notably, in all cases where DC/peptide and DC/L1L2-E7 vaccines were both successful in eliciting epitope-specific Th responses, the polarized nature (i.e. IFN-γ vs. IL-5 biased) of these responses was identical.

### 3.4.3 Comparison of VLP-, protein- and peptide-based vaccines for promoting E7-specific Th responses in vitro.

Our data to this point suggested that VLP-based *in vitro* vaccines were either equivalent to, or in some cases, superior to peptide-based vaccines in eliciting E7-specific T helper cell responses (based on the range of stimulated $CD4^+$ T cell specificities observed). We next wished to ask, in cases of VLP superiority to peptides, if this was attributable to the application of full-length E7 in the VLP construct. To test whether the inclusion of full-length E7 or the activating effects of VLPs were responsible for improved efficacy, we pulsed DCs with recombinant E7 (rE7) protein alone or with L1L2 VLPs, or with L1L2-E7 VLPs alone, and used these Ag-charged DCs as *in vitro* vaccines (Table 5). Responder $CD4^+$ T cells were harvested and analyzed on day 7 of culture using IFN-γ and IL-5 ELISPOT assays against E7 peptide-

pulsed, autologous immature DCs. Due to limited cell numbers, we were only able to evaluate eight patients and two normal donors in these comparative assays. We observed $CD4^+$ T cell reactivity to E7 peptides in 4 of these 8 patients after stimulation with DC-rE7 (Table 5), with responses being of the Th1-type in 3 of 4 CIN patients. One CIN patient exhibited Th0-type anti-E7 $CD4^+$ T cell responses. Of note, in patient #43, the magnitude of $CD4^+$ T cell responses to the $E7_{48-62}$ epitope (the only E7 epitope recognized by this donor) was comparable regardless of whether DC-L1L2-E7 or DC-rE7 +/- DC-L1L2 (or even DC-peptide) was used in the IVS protocol (Figure 10).

### 3.4.4 Effects of VLPs on DC function and phenotype.

While the inclusion of L1L2 VLPs in the DC-rE7 vaccines failed to enhance anti-E7 $CD4^+$ T cell responses, supporting a lack of "adjuvant" effect by the VLPs in this setting, we additionally chose to analyze the direct effect of VLP infection on DC phenotype. As VLPs have been previously reported to activate human DCs, we analyzed the impact of VLP infection on DC uptake and processing of the fluorogenic substrate DQ (dye-quenched)-green BSA (209), as well as, DC expression of MHC/co-stimulator molecules and secretion of cytokines as indices of activation/maturation.

Untreated DCs, DCs infected with VLPs, DCs cultured with the maturation factor TNF-α and DCs co-cultured with VLPs and TNF-α were each analyzed for DQ-BSA uptake and catalysis as a surrogate measure for exogenous antigen presentation. As a negative control, a subset of each of the four samples were fed DQ green BSA and incubated at 4°C to inhibit metabolic uptake. As depicted in Figure 11, when compared to control untreated (immature

DCs), the mean fluorescence intensity of all other groups was reduced, consistent with the ability of these culture conditions to activate/mature DCs. Both L1L2-E7 VLP infection and TNF-α treatment of DCs yielded a comparable level of reduction (approximately 90%) in DQ-BSA signal intensity, while a combination of both stimuli appeared to result in the greatest degree of DC activation/maturation.

DCs in each of these treatment cohorts were also analyzed by flow cytometry for comparative expression of the MHC class I and II, CD86, and CD40 molecules. Treatment of DCs with 100 ng/ml TNF-α was used as a maturation control, while untreated-unpulsed iDCs served as a negative control. IgG isotype controls were used for gating and compensation. The results depicted in Figure 12 indicate that infection of DCs with L1L2E7 VLPs increased expression of the co-stimulatory molecule CD86, MHC class I and MHC class II molecules and the activation marker CD40, and this degree of increase was comparable to that observed for known maturation stimuli (i.e. TNF-α).

## 3.5 DISCUSSION

In our current report, we have compared the *in vitro* efficacy of vaccines consisting of autologous DCs pulsed with three forms of HPV-16 E7 antigen (synthetic peptide, recombinant protein, VLP) to elicit E7 epitope-specific $CD4^+$ T cell responses from 17 patients with CIN I-III or cervical carcinoma and four normal donor controls. In most cases, DC-L1L2-E7 VLP vaccines proved either equivalent to, or superior to, DC-peptide vaccines in promoting stronger E7 epitope-specific responses and they were capable of coordinately stimulating $CD4^+$ T cell responses against multiple E7-derived epitopes simultaneously in these IVS protocols in a subset of patients.

Notably, the functional polarization of $CD4^+$ T cells directed against E7 epitopes was not qualitatively altered by the use of DC-L1L2 VLP vaccines vs. DC-peptide (or DC-rE7) vaccines. Thus, Th1-type immunity to E7 epitopes was typically observed for patients with CIN I-III, while Th2-type responses were commonly noted for patients with cervical cancer. These data are in keeping with our hypothesis that Th2-type dominated $CD4^+$ T cell responses against HPV-16 E7 epitopes may correlate with advanced disease status in these individuals, a finding consistent with results reported for tumor antigen-specific Th responses in patients with advanced stage cancers of alternate histologies[1] (182), (185-189). It should be stressed that polarized Th2-type $CD4^+$ T cell responses were specific for the HPV-16 $E7_{1-12}$ and $E7_{62-75}$ peptides tested and do not reflect the general tendency of these donors with advanced stage malignancy to respond in a generic Th2-biased fashion. Indeed, mitogen (PHA) control spot frequencies obtained for both the IFN-γ and IL-5 ELISPOT assays using $CD4^+$ T cells were indiscriminant between patients with cancer, CIN patients, and (normal) vaginal hysterectomy

patients (data not shown). In addition, cancer patients exhibited strong Th1-type immunity to the pan-DR-presented EBV Th epitope EBNA-$2_{280-290}$ (ref. (190), data not shown). Perhaps most salient, cervical cancer patient #35 displayed coordinate Th2-type responses to the $E7_{1-12}$ and $E7_{62-75}$ peptides and Th1-type reactivity against the $E7_{48-62}$ epitope. Overall, these results suggest that VLP-based vaccines do not affect DC polarization to a degree that is capable of modulating the patient's baseline functional $CD4^+$ T cell responses and that additional factors may need to be applied to DC-VLP vaccines to promote biased Th1-type polarization of responder $CD4^+$ T cells in order to provide the greatest degree of therapeutic benefit to cancer patients (210). In this regard, we have recently defined a novel protocol to produce potent Type-1 polarized DC1s *in vitro* from patient monocytes and are currently evaluating the impact of DC1-L1L2-E7 VLP vaccines on the functional bias anti-E7 $CD4^+$ T cell responses *in vitro*. (Wesa et. al. submitted 2004)

In extended studies, we analyzed the supernatants harvested from responder $CD4^+$ T cell ELISPOT wells by ELISA to determine whether E7 peptides elicited TGF-β and IL-10 production (i.e. indices of T regulatory function, ref. (182)) from any of these donors. We observed detectable E7 peptide-specific TGF-β production from $CD4^+$ T cells isolated from cancer patient #44 (51 pg/ml) and cancer patient #48 (93 pg/ml; data not shown). IL-10 production by anti-E7 $CD4^+$ T cells was not detected in any of the donors evaluated in this study. We feel that if HPV-16 E7 $CD4^+$ T cell regulatory responses exist, they are sufficiently rare and/or undetectable in our assay system.

While our data argue against VLPs impacting the function phenotype of DCs (by comparison to peptide-pulsed DC cohorts), we did observe evidence that VLPs may at least partially activate and mature DCs. Notably, VLPs caused a decrease in antigen

uptake/processing (DQ-BSA assay) and an increase in expression of MHC Class I and II, as well as CD40 and CD86 co-stimulatory molecules by treated DCs. However, we did not detect differences in DC expression of the CD80 marker (data not shown). This result differs slightly from a previous report (ref. (155)) that demonstrated CD80 upregulation in addition to MHC class I and II, CD40, and CD86 being upregulated by VLPs. This difference could reflect variations in study design (i.e. types of media, use of day 4 vs. day 5 cultured DCs, cytokines employed during the "maturation" cultures).

Coordinate analysis of $CD4^+$ T cell responsiveness to HPV-16 E7-derived peptides and HPV-16 genotype status of LEEP biopsies indicated that in 4/5 cases for the $E7_{1-12}$ peptide, 4/5 cases for the $E7_{48-62}$ peptide and 4/5 cases (evaluable to date) for the $E7_{62-75}$ peptide, the responding patients were HPV-$16^+$. Two patients (#24 CIN III and #29 CIN I) that reacted against E7 peptides were genotyped as HPV-$16^-$/HPV-$L1^+$, indicating current infection by alternate HPV types, and no patients who reacted against the HPV-16 E7 epitopes were genotyped as HPV-$16^-$/HPV-$L1^-$. We would hypothesize that the two HPV-$16^-$/HPV-L1+ patients had at one time been infected by HPV-16, but they had since cleared the virus, potentially via specific T cell dependent immunity. After clearing HPV-16, a subsequent re-infection with alternate HPV genotypes could have resulted in HPV-$L1^+$ status for the CIN lesion in these patients. The data derived from CIN III patient #24 who exhibited Th1-type $CD4^+$ T cells reactive against the HPV-16 E7 peptide epitopes, but who failed to display positive HPV-16 PCR or anti-E7 serology results remains an enigma. Unfortunately, no plasma was available to test patient #29 for the presence of HPV-16 E7 IgG antibodies.

The mechanism(s) by which *in situ* Th2-type skewing may occur in anti-E7 $CD4^+$ T cell responses of cervical carcinoma patients remains unclear, but could include: 1) chronic antigenic

restimulation (i.e. repetitive re-infection, refs. (191-195) *in situ* that may delete Th1-type responders (182, 196) 2) dominant locoregional DC2-type antigen presenting cell function in the advanced cancer setting that favors Th2-type immunity (195) and/or 3) a generalized Th2-polarizing cytokine microenvironment at the tumor site and within the tumor draining lymph nodes of affected patients (197). The maintenance of Th1-type immunity against the pan-DR-presented EBV Th epitope (and against the $E7_{48-62}$ peptide in patients #35 and #49) argues against dominant DC2-mediated functional, or global Th2-type cytokine-mediated, skewing of peripheral blood $CD4^+$ T cell responses in cervical cancer patients. However, these important issues will require intense prospective evaluation to determine their unequivocal roles in shaping the HPV-specific immune response in high-risk and progressor patients.

Of great clinical interest, only 1 of 18 patients with CIN I-III or cervical cancer failed to exhibit $CD4^+$ T cell responses to at least one of our pan-DR-presented E7 epitopes after *in vitro* vaccination with autologous DC-based vaccines. As we evaluated all-comers to this analysis, this argues that such vaccines may be applied without consideration of patient HLA class II haplotypes, with the expectation that >90% of treated patients will prove responsive to the these vaccines. DCs pulsed with E7 peptides, L1L2-E7 VLPs or rE7 protein all appeared effective in promoting T cell responses that in most cases appeared qualitatively regulated more by the $CD4^+$ T cell repertoire present in a given patient rather than the form of E7 antigen applied in the DC-based vaccine. Based on the data set provided, the VLP-based vaccines might be preferred overall for clinical implication however, given the greater level of E7-specific responses observed in comparative studies against peptide-based vaccines and the ability of DC-VLP vaccines to promote poly-specific responses in some patients where peptide-based vaccines failed to do so. When combined in adoptive DC1-based vaccines or DC0-based vaccines

supplemented by type-1 biasing cytokines, L1L2-E7 VLPs may exhibit optimal therapeutic benefit given their potential to coordinately stimulate Th1-type $CD4^+$ and CTLs specific for E7 and to avoid neutralizing anti-L1/L2 antibodies that may be present in the sera of patients with CIN or cervical cancer.

# 4.0 SUMMARY AND CONCLUSIONS

Antigen-specific $CD4^+$ T cells play important roles in clearing viral infections and in preventing/treating cancer, yet little is known with regard to the specificity and functional nature of Th responses in the cancer setting. Indeed, while a plethora of $CD8^+$ T cell epitopes have been defined that derive from tumor-associated antigens (TAA), the number a defined $CD4^+$ T cell tumor epitopes is rather small. We chose to investigate the HPV-16 E7 onco-protein as a target of $CD4^+$ T cell recognition for a variety of reasons, including the lack of defined $CD4^+$ T cell epitopes from this transforming protein and its association expression in greater than 50% of all cervical cancers. In addition to defining novel $CD4^+$ T cell epitopes within the HPV-16 E7 protein, we examined the functional polarization state of anti-E7 Th responses in various control and HPV-$16^+$ patient cohorts using refined *in vitro* analyses. By identifying and qualifying the nature of anti-E7 $CD4^+$ T cell reactivity in such individuals, we expect to develop more effective E7-directed vaccines that may protect or treat against HPV-16-associated malignancies.

The initial phase of my studies involved the dissection of the 98aa HPV-16 E7 protein into candidate peptide epitopes, based on algorithm-predicted binding of these sequences to a subset of HLA-DR alleles, with a focus on the common HLA-DR4 allele. The basic experimental protocol employed in these studies involved the loading of monocyte-derived immature dendritic cells (DC) with HPV-16 E7-derived peptides, and the use of these APCs to stimulate autologous $CD4^+$ T cells purified from donor peripheral blood. Following a single round of *in vitro* stimulation, the responder $CD4^+$ T cells were then assayed for reactivity against HPV-16 E7 epitopes using IFN-$\gamma$ (Type-1) and IL-5 (Type-2) ELISPOT assays using autologous immature DCs as APCs. By structuring the *in vitro* assays in this way, we could determine both the specificity and the functional nature (i.e. polarization state) of patient E7-reactive $CD4^+$ T

cells. The screen was initiated using seven predicted Th epitopes, but was ultimately trimmed to three ($E7_{1-12}$, $E7_{48-62}$, and $E7_{62-75}$) relevant immunogenic epitopes after evaluating responsiveness of Th cells isolated from a large number of normal donors, CIN patients, and cervical cancer patients. Additionally, these studies allowed us to conclude that the $E7_{1-12}$ and $E7_{62-75}$ epitopes were likely HLA-DR4 and –DR15 restricted, while the $E7_{48-62}$ peptide was presented in a "pan-DR" manner (but not by HLA-DR4). Lastly, we are were able to demonstrate that advanced stage cervical cancer patients exhibit a predominant Th2-type response to HPV-16 E7-derived epitopes, while early stage patients displayed predominantly Th1-type immunity to HPV-16 E7. This is a very novel and important finding and agrees with a trend in Th immune responses seen in patients with advanced disease stage compared to early disease stage patients in other cancer types (such as renal cell carcinoma and melanoma; ref.(182)). These results suggest an evolving immune response that converts from a hypothetically beneficial Th1-type to a less beneficial Th2-type effector bias as cervical cancer develop and progress in afflicted patients.

The next phase was to determine the best *in vitro* method of inducing HPV-16 E7 $CD4^+$ T cell responses, as a prelude to developing specific vaccines for patients with HPV-16-associated malignancies. In this work, we compared three methods of inducing HPV-16 E7 $CD4^+$ T cell responses, namely autologous DCs pulsed with: 1) HPV-16 E7 peptides, 2) HPV-16 L1L2E7 Virus-like Particles (VLPs), or 3) Full-length recombinant HPV-16 E7 protein (when sufficient numbers of T cells were available for all of these analyses). Importantly, our experimental model used immature DCs in order to determine the magnitude, specificity and polarization of anti-E7 $CD4^+$ T cell responses in our *in vitro* vaccine formulations. This was in part predicated on our previous findings that immature DCs would not alter the functional polarization state of responder $CD4^+$ T cells in our IVS protocols[1] and the knowledge that iDCs

(vs. mature DCs) were efficient at taking up and processing cVLPs (i.e. L1L2-E7 cVLPs) and recombinant proteins, such as rE7 (155). Our cumulative data suggest that VLP-based vaccines were either equivalent to, or slightly superior to, peptide-based vaccines, based on the range of stimulated $CD4^+$ T cell specificities recognized by stimulated $CD4^+$ T cells observed in follow-up assays. It should be noted, however, that we did observe more IL-5 responses from E7-stimulated $CD4^+$ T cells when peptide-based vaccines were used vs. VLP-based vaccines. The limited data set from rE7-based vaccines suggests that it too is equivalent to peptide-based vaccines. Additionally, we showed that VLPs partially mature the iDCs, which may represent a potential *in vivo* advantage verses peptides in vaccine formulations. This by no means suggests that HPV peptide-based vaccines are inferior, but may suggest that additional Type-1 biasing adjuvants might need to be applied in this context. Clearly, for the treatment of cervical cancer patients with peptide-based vaccines is an attractive option since pre-existing antibodies (against the L1 and L2 capsid proteins contained in VLPs), which form immune complexes with VLPs may cause vaccine clearance and limited efficacy in promoting specific T cell responses to L1/L2-E7 cVLPs (208). For this same reason, peptide-based vaccines can be administered multiple times without significant concerns for generating neutralizing antibody responses, unlike VLPs, where it may prove necessary to boost with heterologous VLPs in order promote an increase in specific immunity (211). Lastly, a previous report showed VLPs when used to treat iDCs could lead to full maturation of, and IL-12p70 production from the resulting APCs (155). In our study, we could not reproduce all aspects of this work, indeed, our data can only support the partial maturation of DCs by VLPs, with very low to undetectable levels of IL-12p70 (or IL-10) being promoted from the treated cells as measured by cytokine-specific ELISAs. This variance in results could be due to patient variability or to subtle differences in the DC culturing

conditions (i.e. media types used, the day of culture on which the iDCs were harvested, FBS, and levels and schedule of supportive cytokines added to DC cultures).

While our results were striking, it was necessary to determine if patient $CD4^+$ T cell responses to HPV-16 E7 epitopes correlated with their HPV-16 status. We extracted genomic DNA from vaginal hysterectomy biopsies, patient LEEP biopsies (CIN patients), and cervical cancer biopsies and tested for HPV-16 status by PCR using specific primers. To further expand the genostatus assessment, we performed PCR analyses for HPV L1, using a primer pair that was relevant for almost all transforming HPV types. The vast majority of patients who displayed anti-E7 Th responses were HPV-$16^+$/HPV $L1^+$. In rare cases where a responder was HPV-$16^-$/HPV-$L1^+$ or HPV-$16^-$/HPV-$L1^-$, we attribute the response to *de novo* recognition of T cells primed in the single IVS using DCs, or that the biopsy was in fact HPV-$16^+$ or $L1^+$, but that the portion of tissue available for our analysis lacked HPV integrations. As an alternative to PCR analysis, which determines the current state of HPV infection, we used the HPV-16 rE7 whole protein as bait in ELISA assays in order to quantitate the level of anti-E7 IgG Abs present in the plasma of patients. If positive, this assay would indicate that a given HPV-$16^-$/$L1^-$ patient was likely infected at some previous time, but had since cleared HPV-16. We assessed plasma for its level of IgG1 (Th1-type) and IgG4 (Th2-type) HPV-16 anti-E7 specific antibodies. Somewhat surprising, we did not observe any correlation between Th1-type $CD4^+$ T cell responders and high levels of IgG1 anti-HPV-16 E7 antibody detection, or Th2-type $CD4^+$ T cell responders strong IgG4 anti-HPV-16 E7 antibody levels. Notably, antibody levels do not provide stringent indices with regard to the number of activated (antigen-specific) B and supportive Th-type T cells and may explain why no correlation was detected between the ELISPOT-based T cell assays and the ELISA-based antibody assays.

We also tested the culture supernatants harvested from ELISPOT wells for CD4+ T cell production of IL-10 and TGF-β by ELISA as indices for the presence of E7-specific regulatory-type $CD4^+$ T cells in these patients. Although one cancer patient was determined to produce minimal, but detectable levels of IL-10 and TGF-β in response to E7 peptides, and was simultaneously deficient in E7-specific IL-5 and IFN-γ responses (i.e. T-reg cells are dominant over Type-1 or Type-2 responses), we believe that our *in vitro* vaccines (peptide-based, VLP-based, and rE7 protein-based) do not promote prevalent T-reg responses. A further analysis using tetramer staining, may allow us to corroborate such $CD4^+$ T cell populations for the presence of $tetramer^+$ T cells bearing a T-reg phenotype (i.e. $foxP3^+$, $GITR^+$, etc.).

Having defined HLA-DR restricted HPV-16 E7 epitopes and shown that Th2-type anti-E7 responses predominate in cancer patients, we believe the next step towards defining a therapeutically beneficial vaccine will be to increasingly bias Type-1 immunity in an E7-specific manner. While we anticipated that Type-1 responses might be a common observation in $CD4^+$ T cell cultures stimulated with DC-VLP vaccines *in vitro*, Th2-type responses remained prevalent among cancer patients. This suggests that the degree of DC maturation along the DC1 pathway is incomplete after cVLP-pulsing of these APCs and suggests that alternative approaches should be actively pursued. One such approach is to use polarized DC1s as the APCs to "infect" with L1/L2-E7 cVLPs and to stimulate patient CD4+ T cells *in vitro* (and possibly *in vivo*?). Numerous studies have now shown that DC1-based vaccines promote Th1-type specific CD4+ T cell responses *in vitro* (210, 212), and may have the potential to overcome Th2/Treg-type biased responses (Wesa et al., Manuscript submitted for publication, 2004) that are present endogenously in patients with cervical cancer.

The results reported in this thesis will have a variety of clinical ramifications. Based on the data to date and the current trend in the field of clinical immunology, we would argue that the best preventative vaccines for CIN and cervical cancer are VLP-based. The 100% prevention efficacy in CIN patients on clinical trials clearly distinguishes VLPs as the best approach for preventative vaccines. Further clinical trials will define the duration of immunity to HPV infection after immunization with cVLPs in healthy, immune compromised men and women, and children prior to sexual activity, which will help to better define the role and optimal use of cVLPs for the prevention of CIN and cervical cancer. Continuing studies should better define the role of HPV in other cancers, such as head and neck cancers and the potential utility of E7-based vaccines in these disease histologies.

This is not to suggest that cVLPs are the only effective preventative vaccine. A study vaccinating healthy donors with a HPV-16 L2-E6-E7 fusion protein (in the absence of a defined adjuvant) resulted in the induction of detectable levels of specific Abs, T-cell proliferation, and IFN-γ (ELISPOT) secretion in response to HPV-16 proteins, all in association with resistance to HPV infections (213). Previous reports have also demonstrated that peptide based vaccines do not generate high antigen-specific immunity and refinements of an HPV vaccine are necessary, but did show that HPV peptide-based vaccines could be utilized as an effective therapy (163, 168).

Regardless of whether cVLPs, rE7 or E7 peptides are employed as the immunogen, we currently believe that a preferred vaccine will include polarized DC1s. The use of DC1s allows one to regulate the dosing of antigen applied to stimulate T cells *in vivo* and overcomes the technical short-coming of cVLPs and their associated induction of neutralizing antibodies. Even though novel prime-boost strategies using heterologous VLPs and vaccines using the Venezuelan

equine encephalitis virus replicon particles to deliver the HPV onco-proteins may promote enhanced immunogenicity (211, 214), the use of *ex vivo* "infected" DCs obviates concerns that professional APCs are accessed by these vectors *in situ*. Optimizing HPV DNA-based vaccines have had some moderate success *in vitro* (215) and *in vivo* (216), but DNA-based vaccines have inherent dangers including the integration of viral genomic DNA into host cells, which in the case of HPV-16 E7 cDNA could lead to cellular transformation.

Our group, using an animal model, has previously demonstrated the ability to prevent and treat established HPV-induced tumors using DCs pulsed with HPV-16-derived E7 peptides (111). A comparable study has also demonstrated the ability to induce HPV-16 specific CTL and HPV specific T-helper responses in the peripheral blood of cervical cancer patients after vaccination with iDCs pulsed with autologous or allogeneic HPV-16+ tumor lysates (217), but concluded that the use of immature DC for anti-cancer vaccination may not be optimum. In follow up experiments, the authors generated human monocyte derived DC primed with tumor lysates and matured with the novel synthetic dsRNA analogue Ampligen®, which has shown great promise *in vitro,* with future clinical trials currently pending (218). The authors anticipate that this approach may offer a more effective alternative for optimizing Th1-type anti-cancer T-cell responses in HPV-related malignancy. We feel that the generation of polarized DC1s using IFN-γ and a TLR-agonist (such as LPS, poly I:C, CpGs), which are currently being used in clinical trials for treatment of other cancers, are superior to synthetic dsRNA because of the inherit dangers of RNA and DNA based vaccines inserting oncogenes such as E7. However, currently no consensus exists with respect to the key issues for DC-based vaccines such as (1) loading method for optimum immune responses and (2) the optimum means of activating/maturation of the DC phenotype. Other important unknowns include the optimum

route of DC administration, DC dosage schedule, and the DC dose is yet to be determined for optimal clinical efficacy.

Lastly, a recent study using autologous dendritic cells pulsed with HPV E7 protein induced T cell responses in a portion of late stage cervical cancer patients (162). The authors stated that boosting of immune responses by adjuvants would be mandatory in future trials. Although a number of adjuvants exist, such as alum (Th2 polarizing), incomplete Freund's adjuvant (IFA), KLH (Keyhole Limpet Hemocyanin), and Montanide ISA-51, among others, we feel the best adjuvant is an autologous, polarized DC-1.

Currently, VLP-based vaccines for the prevention of HPV can protect against only 70% of cervical cancers (HPV-16 and HPV-18 collectively account for 70% of all cervical cancers). Studies will be necessary to broaden the range of HPV genotypes that are protected against and better define the duration of protection from HPV-related infections. Results from these trials will enable scientists to determine the appropriate target groups of young women at highest risk to vaccinate. The most obvious groups to vaccinate with VLPs for prevention of HPV related disease are adolescent girls prior to the onset of sexual activity. However, difficultly exists in determining what age adolescents begin to have sexual activity and therefore, what the appropriate standardized age to vaccinate should be. The downside is a moral one, in that vaccines protective vaccines may be misinterpreted resulting in adolescents believing that they are freely-licensed for sexual activity and immune against all STDs. Additionally, the optimal age to vaccinate and over what period of time to re-boost has yet to be determined and may involve multiple years. An extended issue is whether to vaccinate adolescent males, who can carry and disseminate the disease, while remaining asymptomatic.

Thus, a major obstacle in the development of preventative HPV vaccines is the necessity to educate healthcare professionals and public-health authorities about the benefits of this approach and that the benefits outweigh the potential costs. This is particularly true in the case of developing countries and countries with high rates of HPV related infections, where people are unaware that papillomaviruses cause cancer. Cervical cancer can largely be prevented in developed countries with cytology screening, and the introduction of vaccines that protect against 70% of cervical cancers should result in fewer HPV related disease. However, studies for further management of HPV abnormalities after vaccination will need to be carefully planned and coordinated in order to result in the long-term goal of eliminating HPV related cancers.

Therapeutic vaccines for the treatment of existing HPV infections are currently experimental in nature. We feel that the HPV peptide-based vaccines and VLP-based vaccines may hold the key to overcoming the immunologic challenges imposed by HPV related malignancies. Those challenges include the poor (MHC) presentation of viral nucleo-protein antigens that are expressed at low levels and the weak natural immune response to HPV-infected tissues, that may result in poor trafficking of effector T cell populations to non-inflamed infected sites (219). The ability to induce HPV-specific immune responses in humans has already been demonstrated by a number of studies (161, 163, 168, 213, 216), but the trafficking of effector T cells to the cancer sites is an obstacle that has yet to be overcome. We feel that peptide-based vaccines and VLP-based vaccines utilizing polarized DC1s is potentially an effective Type-1 immunotherapy that supports the trafficking of Type-1 effector T cells into HPV-$16^{+}$ tissues due to locoregional production of chemokines such as IP-10, Mig and ITAC that recruit $CXCR3^{+}$ Type-1 T cells.

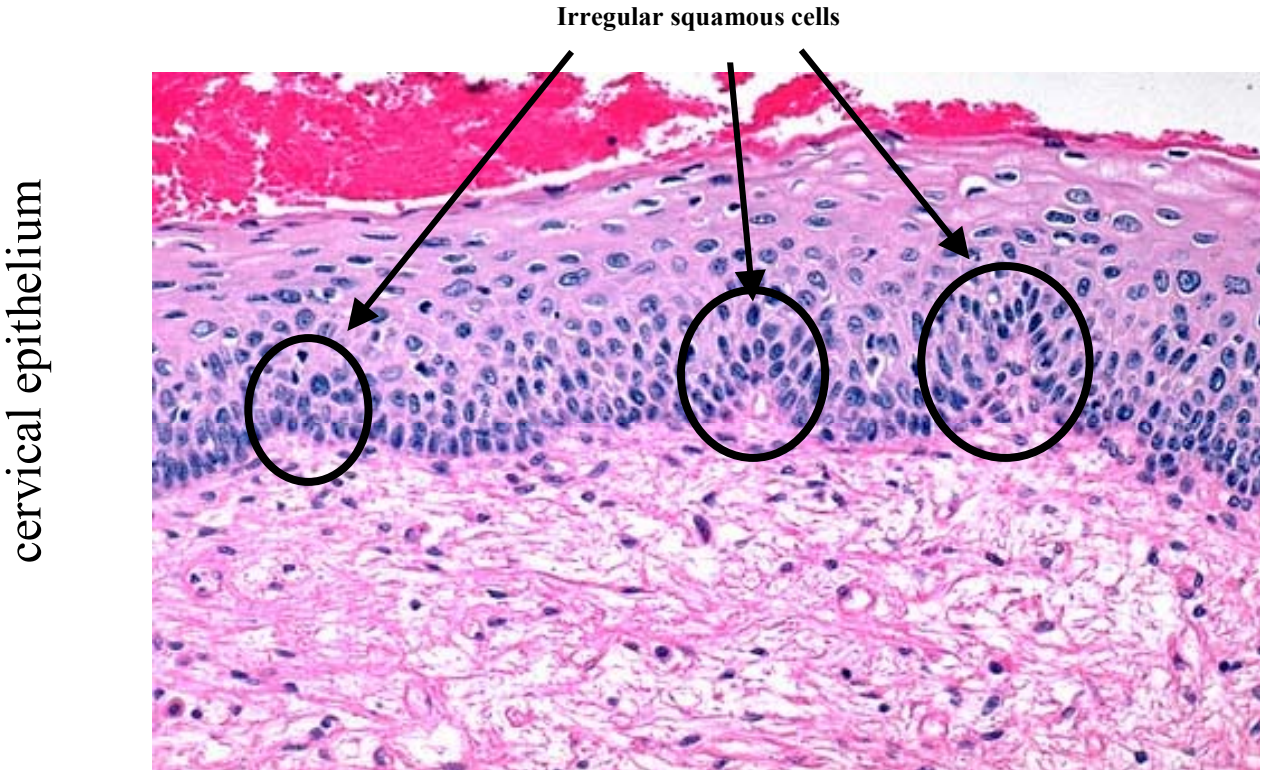


**Figure 1. CIN I**

The histology of cervical intraepithelial neoplasia I (CIN I) is defined as dysplastic changes of the lower third of the cervical epithelium. The cytology of CIN I can be determined by squamous cells with enlarged, irregular, hyperchromatic nuclei and cytoplasm similar to that of normal superficial squamous epithelial cells. In the figure below the dysplastic squamous cells are restricted to the lower third of the cervical epithelium and as indicated by the arrows and circles the dysplastic squamous cells are spread throughout the lower third of the cervical epithelium. CIN I can be associated with any HPV type. Pictures in figures 1-4 were obtained with permission from the wevsite http://www.cytology.com/home.htm.

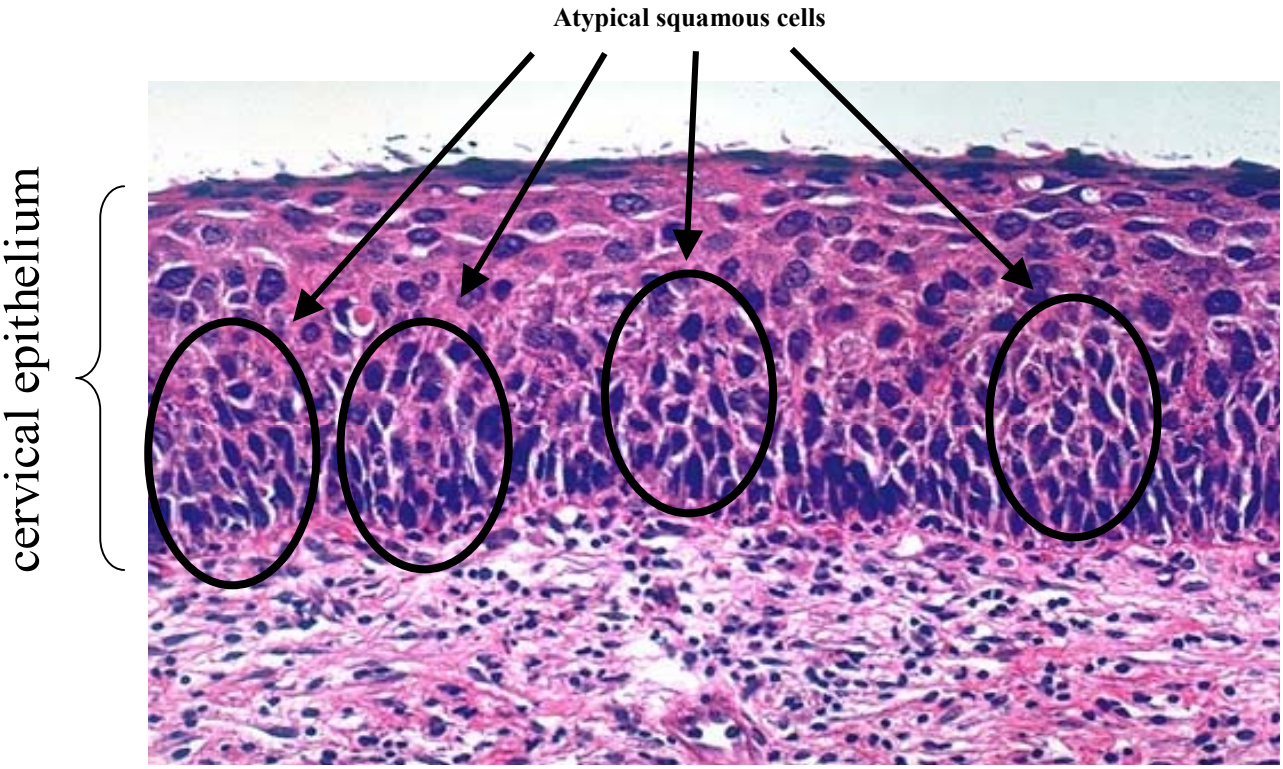


**Figure 2. CIN II**

The histology of CIN II is defined as proliferation of atypical squamous cells occupy between one third and two thirds of the thickness of the cervical epithelium. The cytology of CIN II is identified by squamous cells with enlarged, irregular, hyperchromatic nuclei, and less cytoplasm, which is similar to that of intermediate squamous epithelial cell. As indicated in the figure below the lower two thirds of the cervical epithelium contain numerous atypical squamous cells. CIN II is associated with “high-risk" HPV types (16, 18, 31, 33, 45) intermediate-risk” HPV types ( 35, 51, 52).

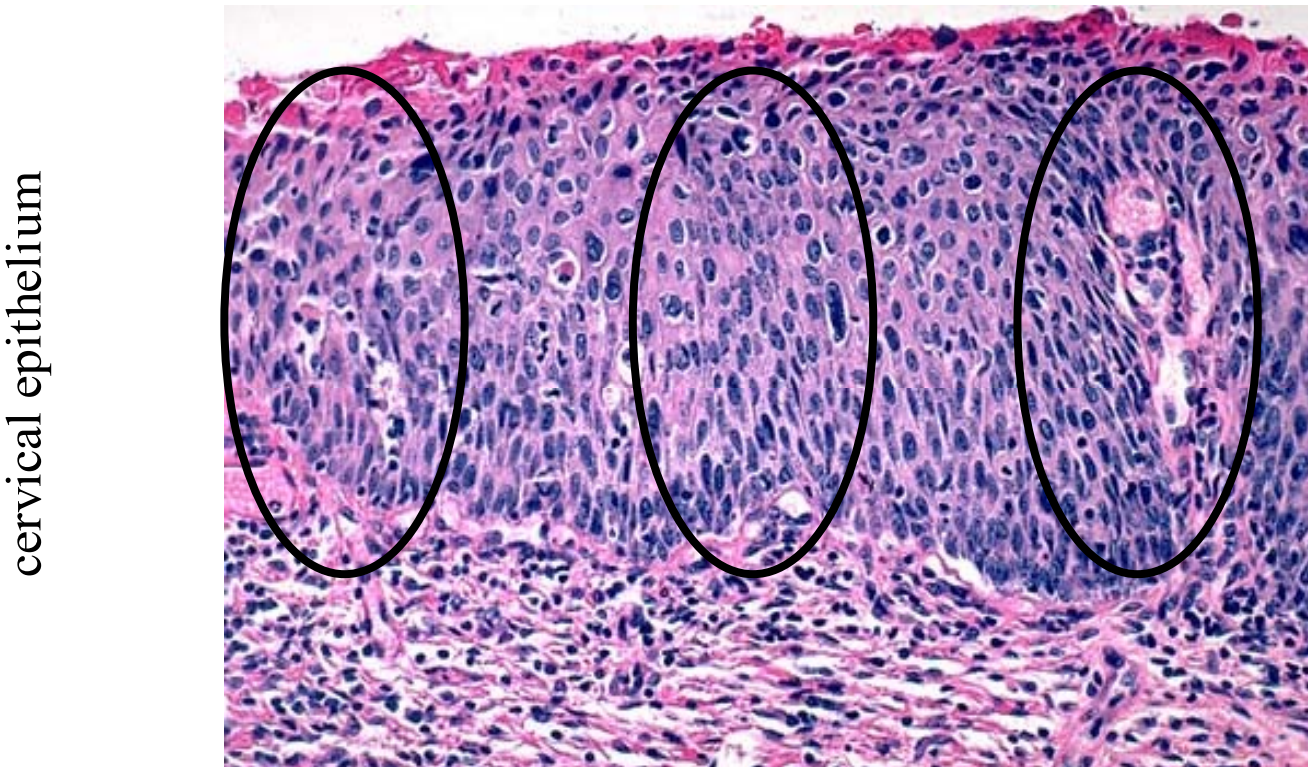


**Figure 3. CIN III**

The histology of CIN III is distinguished by more than two thirds of the thickness of the epithelium is replaced by atypical immature parabasal cells with marked cellular crowding, hyperchromasia and loss of polarity. The cytology of CIN III is described as cells with enlarged, hyperchromatic, irregular nuclei and less cytoplasm. As indicated by the circles the atypical parabasal squamous cells are traversing the entire cervical epithelium. Similar to CIN II, CIN III is associated with “high-risk" HPV types (16, 18, 31, 33, 45) intermediate-risk” HPV types ( 35, 51, 52).

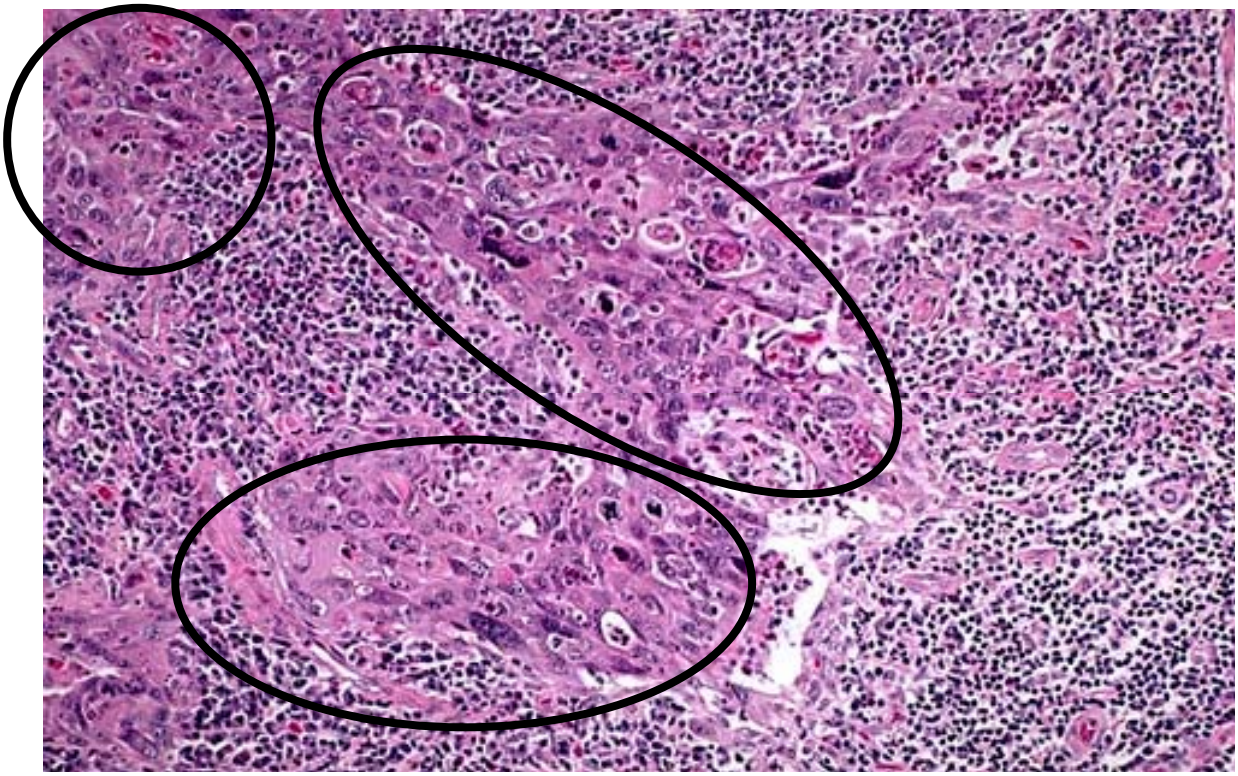

**Figure 4. Cervical Carcinoma**

The morphology of the cervical epithelium is completely lost. Most invasive cervical cancers are composed of nests or solid sheets of oval to polygonal cells with abundant eosinophilic cytoplasm and nuclei with variable size and shape. Well-differentiated tumors are composed predominantly of mature squamous neoplastic cells, which is indicated by the circles. Cervical cancer is caused by high-risk" HPV types (16, 18, 31, 33, 45), with greater than 50% of all cervical cancers being HPV-16 positive.

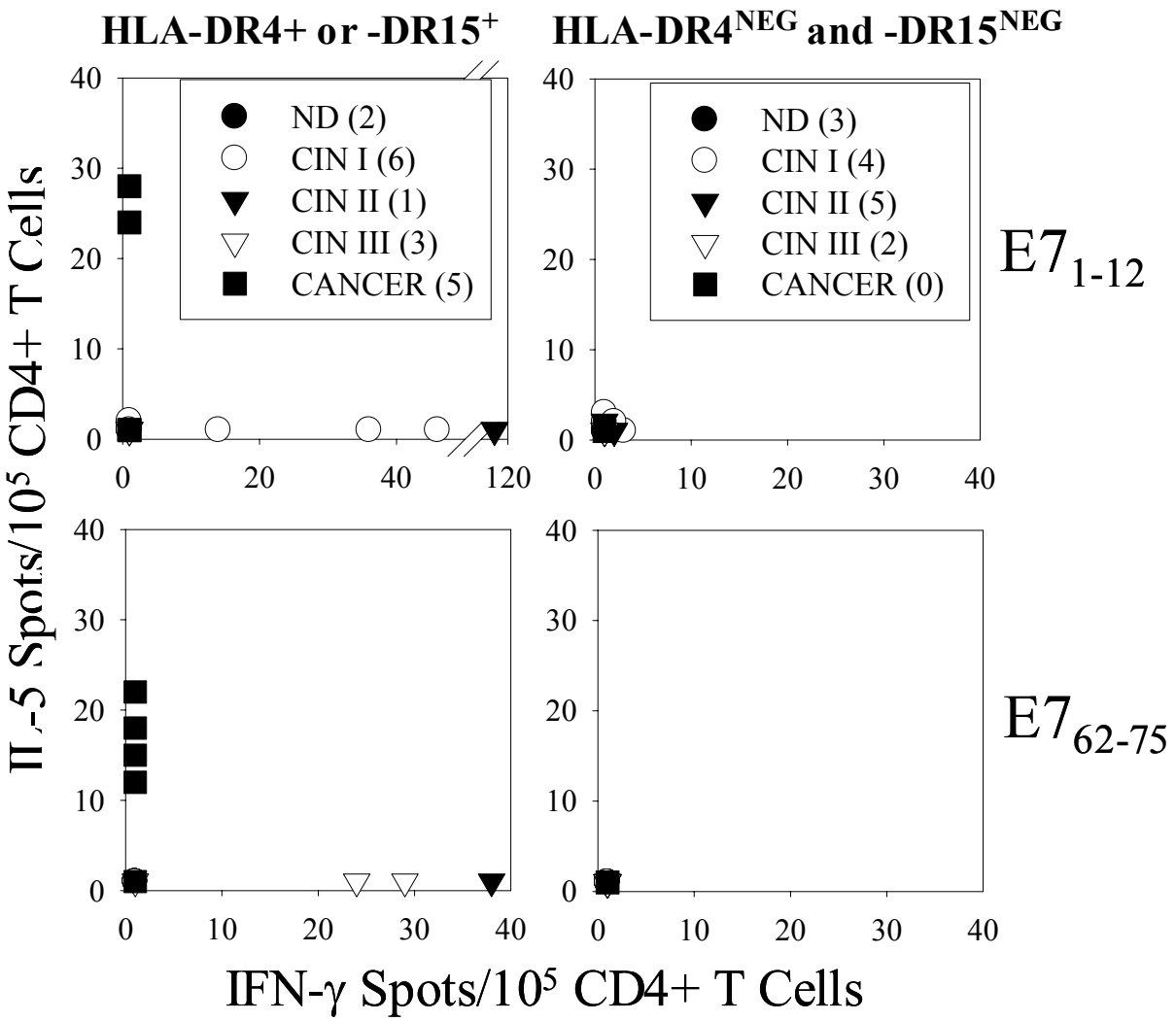


**Figure 5. $CD4^+$ T cell responses against the HPV-16 $E7_{1-12}$ and $E7_{62-75}$ peptide epitopes display disease stage-dependent Th1-/Th2-bias in patients expressing either HLA-DR4 or -DR15.**

Peripheral blood $CD4^+$ T cells were isolated from patients or normal donors (ND) and stimulated for 5-7d with autologous DCs plus individual HPV-16 E7 peptides, as described in the legend of Table 2. Responder $CD4^+$ T cells were then analyzed for reactivity against autologous DCs pulsed with the indicated HPV-16 E7 peptides in IFN-γ and IL-5 ELISPOT assays. For each peptide analyzed, patient responders have been segregated based on whether they expressed the HLA-DR4 and/or -DR15 class II alleles (left panels) or failed to express these alleles (right panels). Each symbol in a panel represents an individual patient's data, with patient disease-stage cohorts defined by the type of symbol, as indicated. The number of patients in each cohort is indicated in parentheses for each of the HLA-segregated groups. The IFN-γ and IL-5 ELISPOT data are reported as spot numbers per $10^5$ $CD4^+$ T cells analyzed.

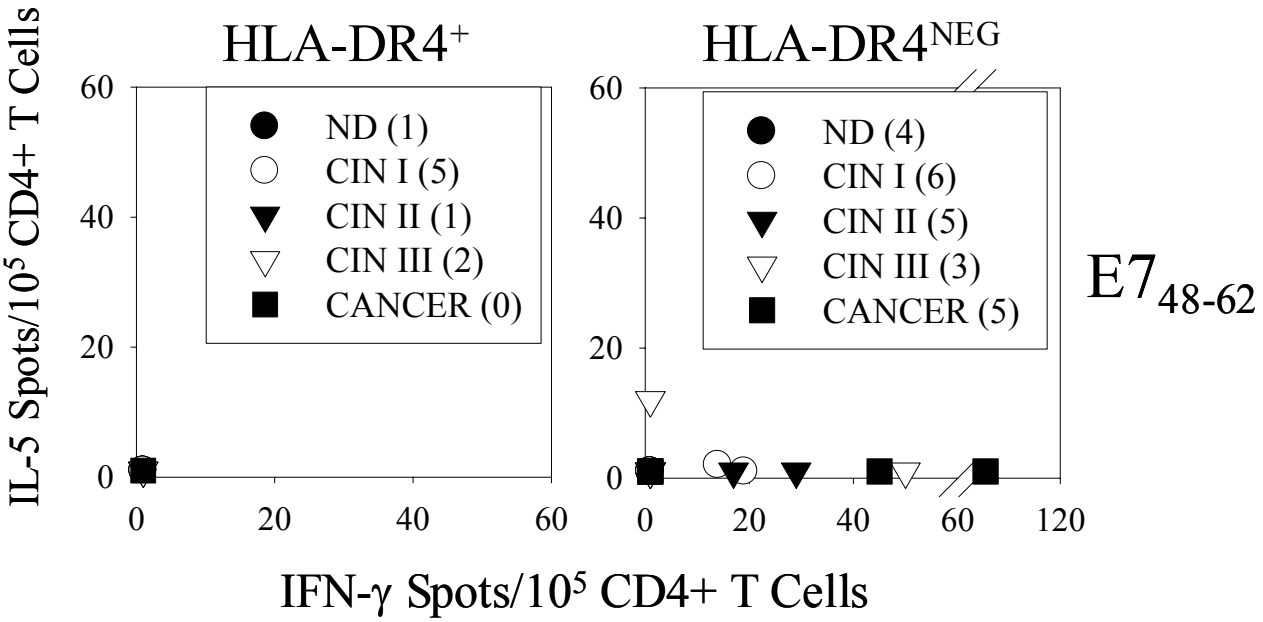


**Figure 6. The HPV-16 $E7_{48-62}$ epitope is recognized in a predominantly Th1-biased manner by $CD4^+$ T cell isolated from HLA-DR4-negative patients.**

Using the experimental protocol outlined in the Figure 1 legend and text, we analyzed the Th1- vs. Th2-type response of patient $CD4^+$ T cells against the E7 48-62 peptide presented by autologous DCs in IFN-γ and IL-5 ELISPOT assays. For each peptide analyzed, patient responders have been segregated based on whether they expressed (left panel) or failed to express (right panel) the HLA-DR4 class II allele. Each symbol in a panel represents an individual patient's data, with patient disease-stage cohorts defined by the type of symbol, as indicated. The number of patients in each cohort is indicated in parentheses for each of the HLA-segregated groups. The IFN-γ and IL-5 ELISPOT data are reported as spot numbers per $10^5$ $CD4^+$ T cells analyzed.

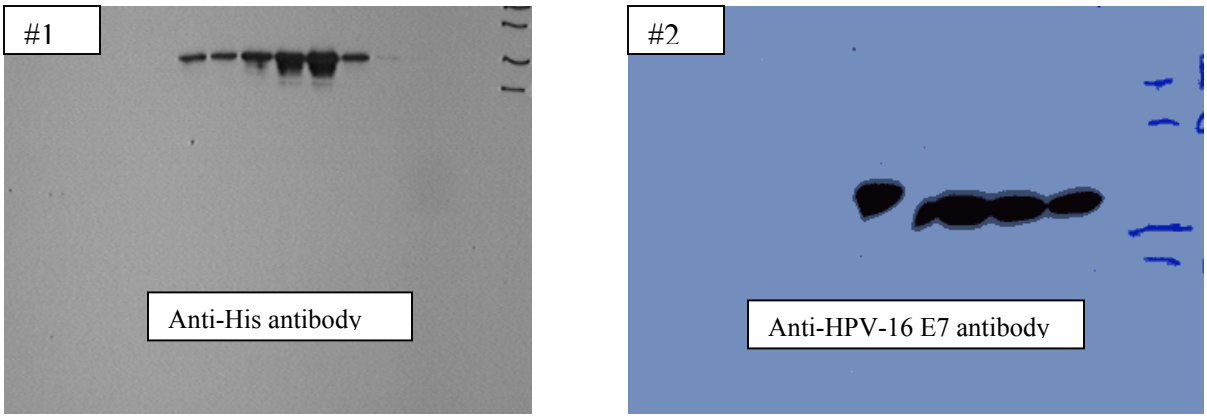


**Figure 7. Western Blots of purified HPV-16 E7 recombinant protein.**

Western blots were performed as described in the materials and methods. The HPV-16 E7 recombinant protein was engineered to contain an 8 residue Histidine tag that was used for purification purposes. Blot #1 shows the results of the anti-His antibody at a 1:10,000 dilution for eluted fractions 5-10. Blot #2 shows the results for HPV-16 anti-E7 antibody at a 1:5,000 dilution for eluted fractions 6-9. Both results show the rE7 protein is at the correct molecular weight, which are approximately 20-kilo Daltons.

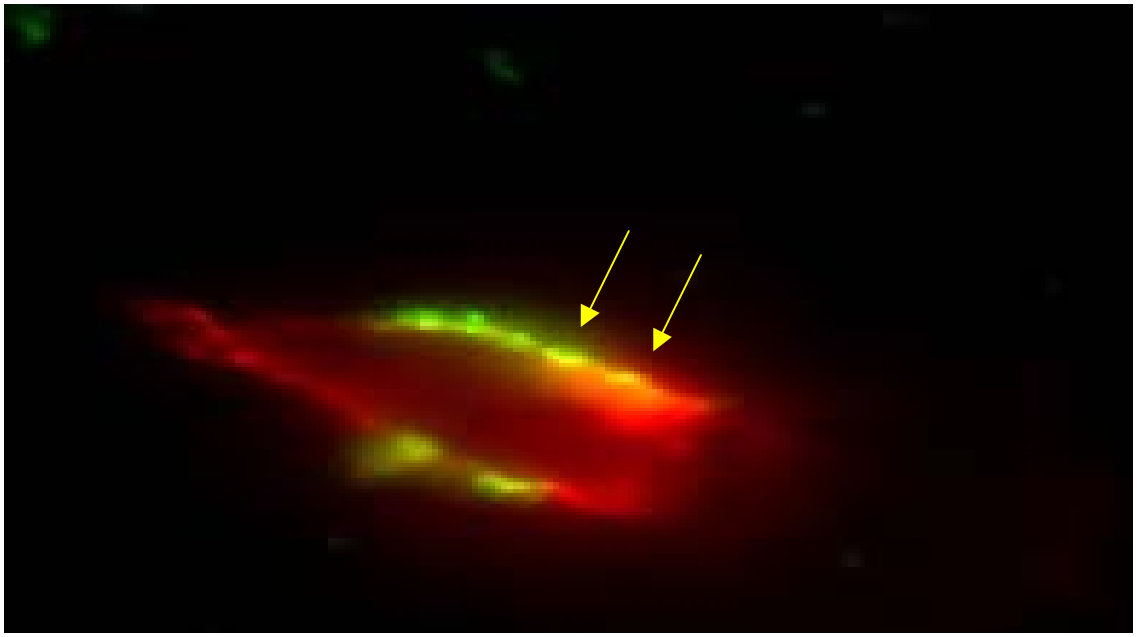

**Figure 8. cVLPs infect DCs.**

GFP labeled L1L2VLPs (Green) were fed to normal donor day five iDCs for 2hr and additionally stained for MHC Class II (Red) as described in the materials and methods. Using an overlay, the cVLPs and MHC Class II co-localize resulting in yellow fluorescence, which is illustrated by the arrows.

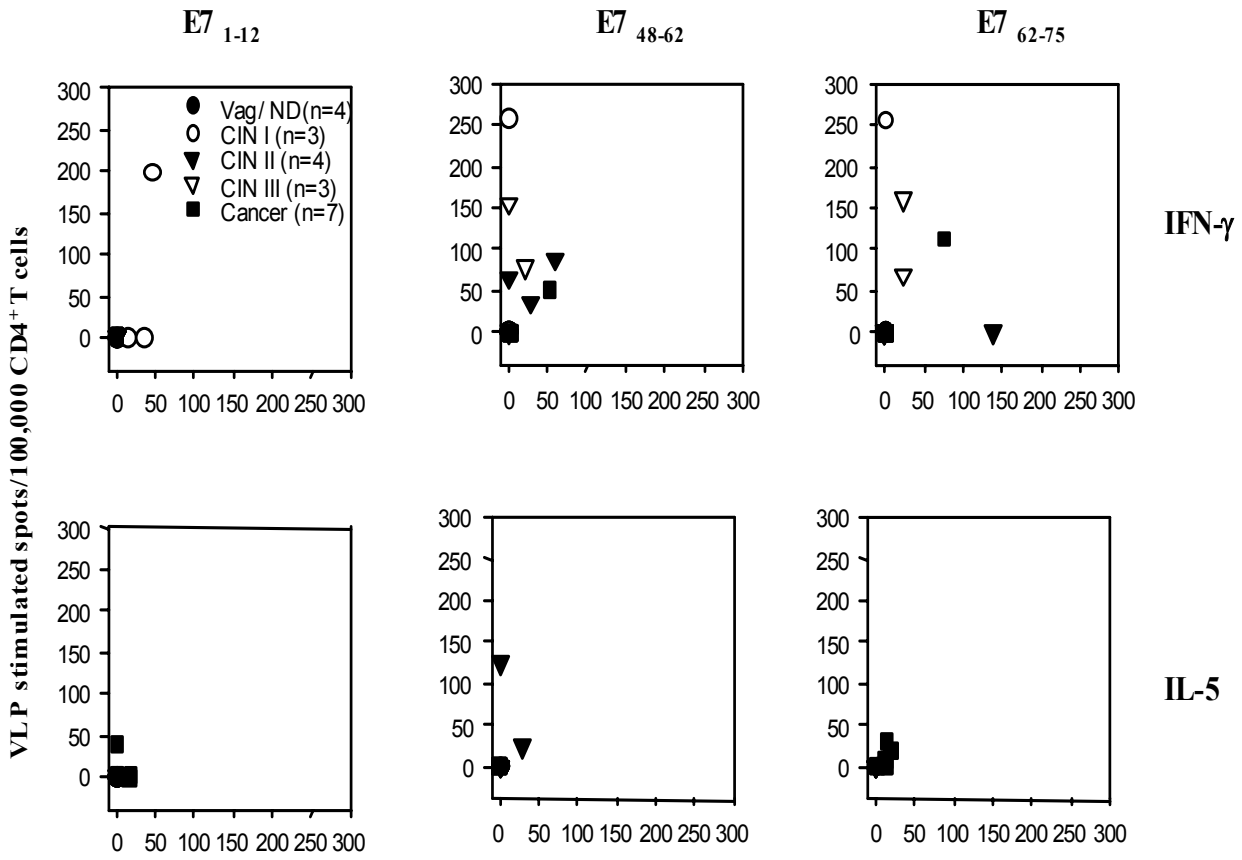


**Figure 9. Anti-E7 $CD4^+$ T cell responses are elicited by autologous DCs infected with L1L2-E7 VLPs or pulsed with pan-DR-presented E7 peptides.**

Peripheral blood $CD4^+$ T cells were isolated from patients or normal donors (ND) and stimulated for 5-7d with autologous DCs pulsed with individual HPV-16 E7 peptides or with HPV-16 VLPs as described in Materials and Methods. Responder $CD4^+$ T cells was then analyzed for reactivity against autologous DCs pulsed with the indicated HPV-16 E7 peptides in IFN-γ and IL-5 ELISPOT assays. Each symbol in a panel represents an individual patient's data, with patient disease-stage cohorts defined by the type of symbol, as indicated. The number of patients in each cohort is indicated in parentheses for each of the groups. The IFN-γ and IL-5 ELISPOT data are reported as spot numbers per $10^5$ $CD4^+$ T cells analyzed.

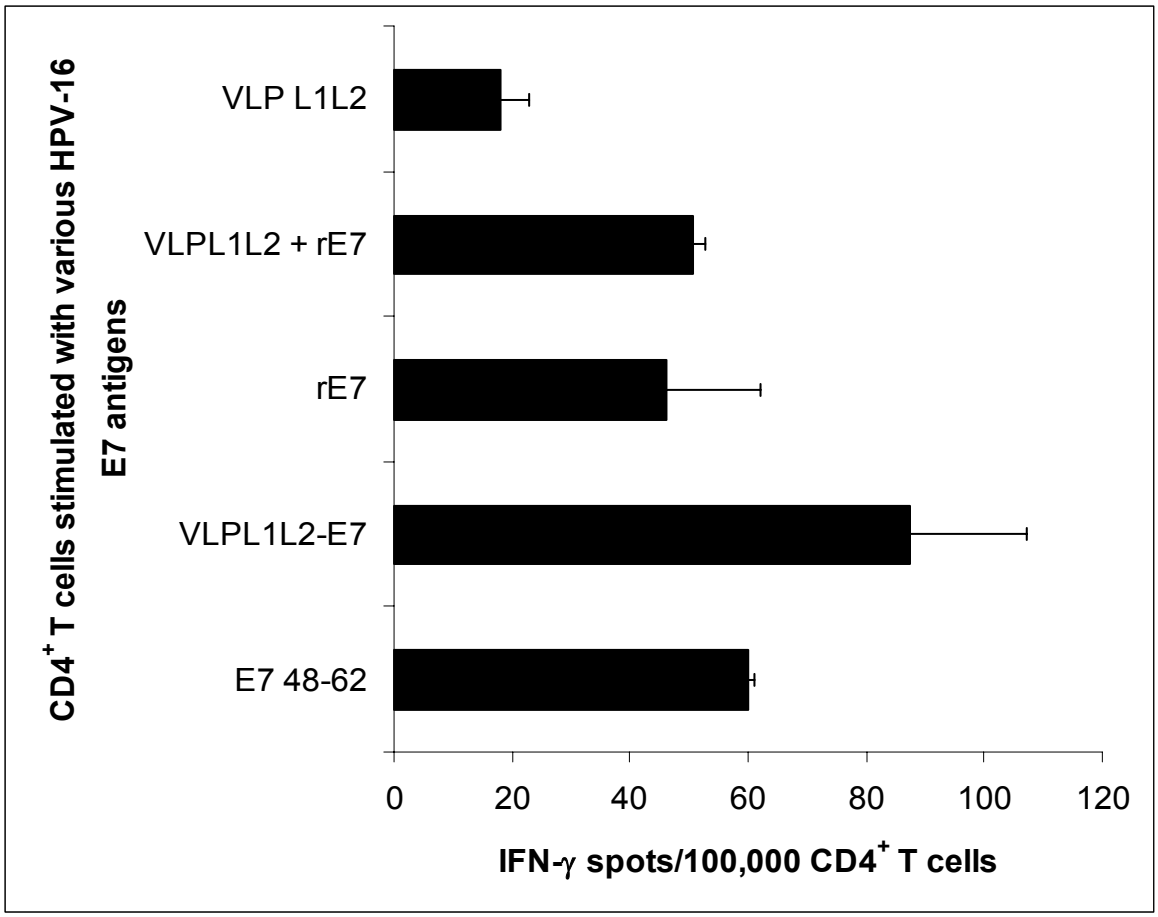


**Figure 10. DC-L1L2-E7 VLP and DC-rE7 vaccines effectively elicit Th1-type CD4+ T cell responses and rE7 vaccines are not improved by "co-infection" with L1L2 VLPs.**

Using the experimental protocol outlined in the Figure 1 legend and text, we analyzed the Th1-type CD4+ T cell responses of patient #43 against the $E7_{48-62}$ epitope after one week IVS using autologous DCs pulsed with 10 μg/ml of $E7_{48-62}$ peptide, 10 μg/ml L1L2-E7 VLP, 10 μg/ml L1L2 VLP, 10 μg/ml rE7 or 10 μg/ml rE7 + 10 μg/ml L1L2 VLP. T cell responses were evaluated in triplicate determinations in IFN-γ ELISPOT assays, with results reported as mean +/- SD.

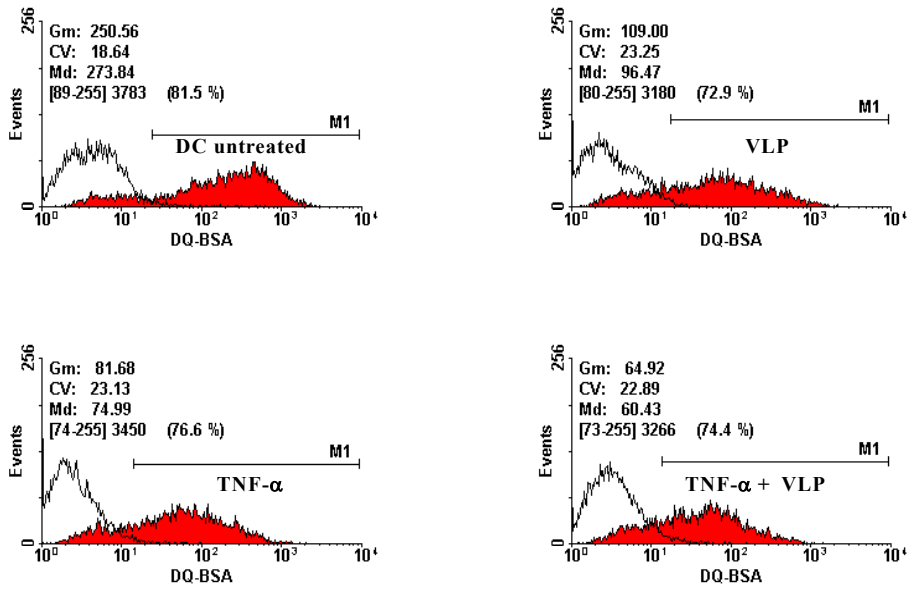


**Figure 11. Effects of VLP infection on exogenous DQ-BSA uptake and processing.**

Using the experimental protocol outlined in the Figure 1 legend and text, we analyzed the Th1-type CD4+ T cell responses of patient #43 against the $E7_{48-62}$ epitope after one week IVS using autologous DCs pulsed with 10 μg/ml of $E7_{48-62}$ peptide, 10 μg/ml L1L2-E7 VLP, 10 μg/ml L1L2 VLP, 10 μg/ml rE7 or 10 μg/ml rE7 + 10 μg/ml L1L2 VLP. T cell responses were evaluated in triplicate determinations in IFN-γ ELISPOT assays, with results reported as mean +/- SD.

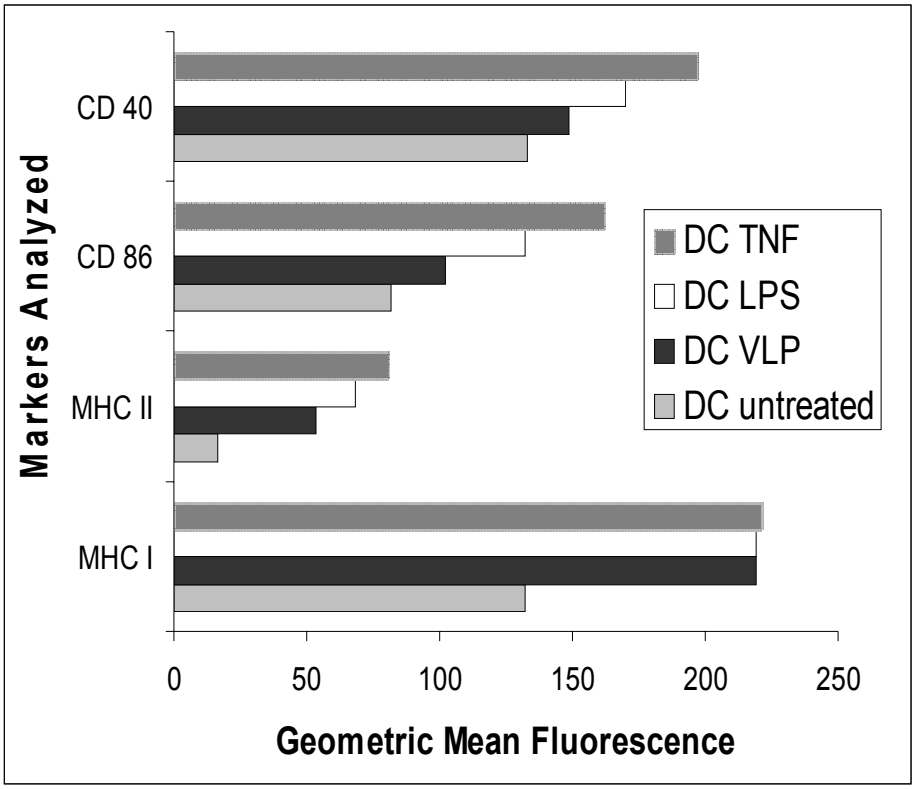


**Figure 12. Effects of VLP on DC maturation markers.**

DCs were harvested after 48h culture without or with VLPs and/or TNF-α as described in Figure 1, stained with mAbs specific for the indicated markers and analyzed by flow cytometry. Data presented are representative of 3 independent experiments performed.

**Table 1. Clinical Trials of HPV-16 Immunotherapies**

CIN, cervical intraepithelial neoplasia; CR, complete response; PR, partial response; CTL, cytotoxic T lymphocyte; DTH, delayed-type hypersensitivity; E, early; ELISPOT, enzyme-linked immunospot; GST, glutathione S-transferase; HPV, human papillomavirus; IFA, incomplete freund's adjuvant.

| Therapy | Antigen Type | Patient Cohort | Immune response | Efficacy | Comments | Ref |
|---|---|---|---|---|---|---|
| poly (lactide-co-glycolide) microparticles (ZYC101) | HPV-16 $E7_{83-94}$ | CIN II/III HLA-A2 | 11/15 had bulk T cell response measured by ELISA for IFN-$\gamma$ | 5/12 CRs | No CD4 data | 58 |
| Peptide + IFA | HPV-16 $E7_{12-20}$ HPV-16 $E7_{86-93}$ | CIN II/III HLA-A2 | 10/16 CTL responsive No DTH | 9 of 17 evaluable patients had PR or CR of their CIN lesions | CD4 response unchanged | 51 |
| Protein Iscomatrix adjuvant | HPV-16 E6-E7 fusion protein | CIN I-III | 12/20 had bulk T cell response measured by ELISA for IFN-$\gamma$ | No change in cervical histology 13/14 lower HPV copy number | No CD4 data | 59 |
| Protein Algammulin adjuvant | E7-GST fusion protein | Cervical Cancer | Antibody and DTH | No alteration in natural history of disease | No T cell data | 61 |
| Vaccinia virus TA-HPV, Xenova | HPV-16 E6-E7 fusion protein | Cervical Cancer | 1/8 CTL responsive 3/8 Antibody | Outcome not documented | No CD4 data | 60 |
| Peptide oil + Water adjuvant | HPV-16 $E7_{12-20}$ HPV-16 $E7_{86-93}$ DR helper epitope | Cervical Cancer | No CTL response | 2/17 stable disease | No CD4 data | 62 |
| Dendritic Cell | HPV-16 and -18 E7 protein | Cervical Cancer | 3/11 had bulk T cell response measured by ELIspot for IFN-$\gamma$ | No objective clinical response | No CD4 data | 57 |

**Table 2. Predicted pan-DR binding peptides derived from the HPV-16 E7 protein.**

The cumulative DR4 score is the sum of the following nine DR4 alleles: DRβ1*0401, DRβ1*0402, DRβ1*0404, DRβ1*0405, DRβ1*0408, DRβ1*0410, DRβ1*0421, DRβ1*0423, DRβ1*0426. The highest theoretical cumulative DR4 score achievable by any peptide is 81. The top five DR4 scores are shown above and were synthesized and subsequently used to screen patients for $CD4^+$ T cell responses. Other high scoring class II alleles (for at least one of the 5 peptides) were DRβ1*0101, DRβ1*0301, DRβ1*0701, DRβ1*0801, DRβ1*1101, DRβ1*1301, DRβ1*1501, and DRβ5*0101. An allele was designated as "high scoring" if a given peptide's predicted result was at least 10% of the maximal theoretical score for the HLA-DR allele being evaluated. The $E7_{48-62}$ peptide did not contain a predicted DR4-binding sequence, but was selected for analysis due to a predicted high cumulative score for the broad range of non-DR4 alleles (i.e. suggested to be a pan-DR binder). [a]DRβ3*0101 and DRβ4*0101 were not analyzed.

| **Peptide Position** | **Peptide Sequence** | **Cumulative DR4 Score** | **Other High-Scoring HLA-DR Alleles[a]** |
|---|---|---|---|
| E7 $_{1-12}$ | MHGDTPTLHEYD | 6.52 | DR3 |
| E7 $_{11-25}$ | YMLDLQPETTDLYCY | 10.5 | DR3, DR15 |
| E7 $_{48-62}$ | DRAHYNIVTFCCKCD | 0 | DR3, DR7, DR8, DR11, DR13, DR15, DRB5 |
| E7 $_{62-75}$ | DSTLRLCVQSTHVD | 14.3 | DR3, DR8, DR13 |
| E7 $_{72-86}$ | THVDIRTLEDLLMGT | 16.4 | DR3, DR7, DR11, DR13, DR15 |
| E7 $_{83-97}$ | LMGTLGIVCPICSQK | 19.88 | DR1, DR3, DR7, DR8, DR11, DR13, DR15, DRB5 |

**Table 3. Patient Characteristics and Th Responses to HPV-16 E7 peptides.**

Patient clinical disease stage (patient ID# in brackets), age, HLA-DR typing, HPV (geno- and Ab) typing and $CD4^+$ T cell responsiveness to HPV-16 E7-derived peptides are indicated. HPV and HLA-DR genotype status were determined by PCR, as outlined in Material and Methods, with data qualitatively reported as +/-. For HPV genotype status, HPV-16-specific (E6 and E6/E7) and pan-HPV (L1 capsid) primers were employed. IFN-γ/IL-5 ELISPOT assays were performed using $10^5$ $CD4^+$ T cells and 2 x $10^4$ thawed autologous monocyte-derived DCs and 10 μg/ml final concentration of specific peptide, as described in Materials and Methods. $CD4^+$ T cells were subjected to one round of *in vitro* stimulation using autologous, immature DCs and 10 μg/ml of specific peptide, and then assayed in the ELISPOT assay on day 10-14. Plus (+) signs appearing in the E7 columns indicate statistically significant ($p < 0.05$) spot numbers in response to E7 peptides vs. the Malarial CS (negative control) peptide background. Minus (–) signs in the E7 columns indicate no significant response to E7 peptides vs. the CS control peptide. Mean and SD are provided for the age of each cohort of patients or normal donors. The IFN-γ vs. IL-5 response data is tabulated for each peptide within each donor cohort. NT= not tested.

**Table 3 Patient Characteristics and Th Responses to HPV-16 E7 peptides. (Cont.)**

| | | HLA Genotype: | | HPV Genotype: | | $CD4^+$ T Cell Response Against (IFNγ/IL-5): | | |
|---|---|---|---|---|---|---|---|---|
| **Status** | **Age** | **HLA-DRβ1** | **HLA-DRβ (3,4,5)** | **HPV-16** | **HPV L1** | **$E7_{1-12}$** | **$E7_{48-62}$** | **$E7_{62-75}$** |
| **(N = 5)** | | | | | | | | |
| ND [01] | 28 | 11,13 | 3 | NT | NT | -/- | -/- | -/- |
| ND [03] | 30 | 03,12 | 3 | NT | NT | -/- | -/- | -/- |
| Vag Hyst [10] | 53 | 04,11or13 | 3 | - | - | -/- | -/- | -/- |
| Vag Hyst [14] | 46 | 07,13 | 3,4 | - | - | -/- | -/- | -/- |
| Vag Hyst [21] | 31 | 03,15 | 3,5 | - | + | -/- | -/- | -/- |
| | 37.6 +/-11.1 | | | | | 0/0 | 0/0 | 0/0 |
| **(N =11)** | | | | | | | | |
| CIN I [9] | 19 | NT | NT | - | - | -/- | -/- | -/- |
| CIN I [13] | 28 | 04,07 | 4 | - | - | -/- | -/- | -/- |
| CIN I [15] | 55 | NT | NT | - | + | -/- | -/- | -/- |
| CIN I [19] | 44 | 01,15 | 5 | + | + | -/- | -/- | -/- |
| CIN I [20] | 50 | 04,16 | 4,5 | + | + | +/- | -/- | -/- |
| CIN I [22] | 47 | 07,08 | 4 | + | + | -/- | -/- | -/- |
| CIN I [28] | 81 | 07,11 | 3,4 | NT | NT | -/- | +/- | -/- |
| CIN I [29] | 26 | 03,04 | 3,4 | - | + | +/- | -/- | -/- |
| CIN I [32] | 52 | 11 | 3 | - | + | -/- | +/- | -/- |
| CIN I [36] | 65 | 4,15 | 4 | + | + | +/- | -/- | -/- |
| CIN I/II [40] | 25 | 03,14 | 3 | NT | NT | -/- | -/- | -/- |
| | 44.7 +/- 19.0 | | | | | 3/0 | 2/0 | 0/0 |
| **(N = 6)** | | | | | | | | |
| CIN II [1] | 20 | NT | NT | + | - | -/- | -/- | -/- |
| CIN II [2] | 20 | 03,13 | 3 | - | - | -/- | -/- | -/- |
| CIN II [7] | 22 | 03,04 | 3,4 | + | + | +/- | -/- | +/- |
| CIN II [25] | 34 | 03,13 | 3 | + | + | -/- | +/- | -/- |
| CIN II [31] | 44 | 03,11 | 3 | - | - | -/- | +/- | -/- |
| CIN II [33] | 39 | 01,13 | 3 | - | - | -/- | -/- | -/- |
| | 29.8 +/- 10.6 | | | | | 1/0 | 2/0 | 1/0 |
| **(N =5)** | | | | | | | | |
| CIN III [6) | 46 | 03,07 | 3,4 | + | - | -/- | +/- | -/- |
| CIN III [8] | 28 | 14,15 | 3,5 | + | + | -/- | -/+ | -/- |
| CIN III [18] | 30 | 04,11 | 3,4 | + | - | -/- | -/- | +/- |
| CIN III [24] | 34 | 04,09 | 4 | - | + | -/- | -/- | +/- |
| CIN III [37] | 57 | 07,14 | 3,4 | + | + | -/- | -/- | -/- |
| | 39.0 +/- 12.2 | | | | | 0/0 | 1/1 | 2/0 |
| **(N = 6)** | | | | | | | | |
| CANCER [12] | 34 | 07,15 | 4,5 | + | + | -/- | +/- | -/+ |
| CANCER [23] | 37 | 03,15 | 3,5 | + | + | -/- | -/- | -/+ |
| CANCER [27] | 40 | 01,14 | 3 | NT | NT | -/NT | -/NT | +/NT |
| CANCER [35] | 44 | 15 | 5 | + | + | -/+ | +/- | -/+ |
| CANCER [38] | 50 | 07,15 | 5 | + | + | -/- | -/- | -/+ |
| CANCER [39] | 48 | 01,15 | 5 | + | + | -/+ | -/- | -/- |
| | 42.2 +/- 6.3 | | | | | 0/2 | 2/0 | 1/4 |

**Table 4. Patient Characteristics, HPV status, and IgG levels.**

Patient clinical disease stage and [ID #], age, HLA-DR typing, HPV (geno- and Ab) typing and $CD4^+$ T cell responsiveness to HPV-16 E7-derived peptides are indicated. HPV and HLA-DR genotype status were determined by PCR, as outlined in Material and Methods, with data qualitatively reported as +/-. For HPV genotype status, HPV-16-specific (E6 and E6/E7) and pan-HPV (L1 capsid) primers were employed. Anti-HPV-16 E7-specific IgG1 and IgG4 antibody concentrations were determined in specific ELISAs against recombinant E7 protein as bait. NT= not tested.

| | **Status** | **Age** | **HLA-DRβ1** | **HLA-DRβ (3,4,5)** | **HPV-16** | **HPV L1** | **IgG1 (ng/ml)** | **IgG4 (ng/ml)** |
|---|---|---|---|---|---|---|---|---|
| n=4 | | | | | | | | |
| | ND [03] | 30 | 03,12 | 3 | NT | NT | 1 | 15 |
| | ND [09] | 49 | 15,16 | 5 | NT | NT | NT | NT |
| | ND [10] | 40 | NT | NT | NT | NT | NT | NT |
| | Vag Hyst [21] | 31 | 03,15 | 3,5 | – | + | 1 | 61 |
| n=3 | | | | | | | | |
| | CIN I [20] | 50 | 04,16 | 4,5 | + | + | 779 | 152 |
| | CIN I [29] | 26 | 03,04 | 3,4 | – | + | NT | NT |
| | CIN I [36] | 65 | 4,15 | 4 | + | + | 994 | 324 |
| n=4 | | | | | | | | |
| | CIN II [25] | 34 | 03,13 | 3 | + | + | NT | NT |
| | CIN II [43] | 33 | 11,15 | 3,5 | NT | NT | 170 | 241 |
| | CIN II [45] | 29 | 03,07 | 3,4 | – | – | 43 | 871 |
| | CIN II [50] | 32 | 08,15 | 5 | + | + | 1 | 14 |
| n=3 | | | | | | | | |
| | CIN III [18] | 30 | 04,11 | 3,4 | + | – | 25 | 1276 |
| | CIN III [24] | 34 | 04,09 | 4 | – | + | 1 | 1 |
| | CIN III [51] | 74 | 07,16 | 4 | + | – | 1.7 | 1113 |
| n=7 | | | | | | | | |
| | CANCER [23] | 37 | 03,15 | 3,5 | + | + | 582 | 24 |
| | CANCER [35] | 44 | 15 | 5 | + | + | 850 | 94 |
| | CANCER [38] | 50 | 07,15 | 5 | + | + | 903 | 1 |
| | CANCER [44] | 74 | 0103,13 | 3 | + | + | 284 | 1 |
| | CANCER [46] | 61 | 07,14 | 4 | + | – | 1 | 7 |
| | CANCER [48] | 62 | 04,16 | 3,4 | NT | NT | 311 | 55 |
| | CANCER [49] | 39 | 04, 11/13 | 3,4 | + | – | 11 | 1 |

**Table 5. Patients' Functional Th Responses to HPV-16 E7 peptides.**

IFN-γ/IL-5 ELISPOT assays were performed using $10^5$ $CD4^+$ T cells and 2 x $10^4$ thawed autologous monocyte-derived DCs and 10 μg/ml (final concentration) of specific peptide, as described in Materials and Methods. $CD4^+$ T cells were subjected to one round of *in vitro* stimulation using autologous, immature DCs and 10 μg/ml of specific peptide, and then assayed in the ELISPOT assay on day 10-14. Plus (+) signs appearing in the E7 columns indicate statistically significant ($p < 0.05$) spot numbers in response to E7 peptides vs. the Malarial CS (negative control) peptide background. Additionally, plus signs appearing in the VLP columns indicate statistically significant spot numbers in response to VLPL1L2-E7 vs. VLPL1L2 (control VLP). Minus (–) signs in the E7 columns indicate no significant response to E7 peptides vs. the CS control peptide. NT= not tested.

| | **Status** | **$E7_{1-12}$** | **$E7_{48-62}$** | **$E7_{62-75}$** | **$VLP_{1-12}$** | **$VLP_{48-62}$** | **$VLP_{62-75}$** | **$rE7_{1-12}$** | **$rE7_{48-62}$** | **$rE7_{62-75}$** |
|---|---|---|---|---|---|---|---|---|---|---|
| n=4 | | | | | | | | | | |
| | ND [03] | -/- | -/- | -/- | -/- | -/- | -/- | NT | NT | NT |
| | ND [09] | -/- | -/- | -/- | -/- | -/- | -/- | -/- | -/- | -/- |
| | ND [10] | -/- | -/- | -/- | -/- | -/- | -/- | -/- | -/- | -/- |
| | Vag Hyst [21] | -/- | -/- | -/- | -/- | -/- | -/- | NT | NT | NT |
| n=3 | | | | | | | | | | |
| | CIN I [20] | +/- | -/- | -/- | +/- | +/- | +/- | NT | NT | NT |
| | CIN I [29] | +/- | -/- | -/- | -/- | -/- | -/- | NT | NT | NT |
| | CIN I [36] | +/- | -/- | -/- | -/- | -/- | -/- | NT | NT | NT |
| n=4 | | | | | | | | | | |
| | CIN II [25] | -/- | +/- | -/- | -/- | +/- | -/- | NT | NT | NT |
| | CIN II [43] | -/- | +/+ | -/- | -/- | +/+ | -/- | -/- | +/- | -/- |
| | CIN II [45] | -/- | -/- | -/- | NT | NT | NT | -/- | +/- | +/- |
| | CIN II [50] | -/- | -/- | -/- | -/- | +/+ | +/- | -/- | +/+ | -/- |
| n=3 | | | | | | | | | | |
| | CIN III [18] | -/- | -/- | +/- | -/- | -/- | +/- | NT | NT | NT |
| | CIN III [24] | -/- | -/- | +/- | +/- | +/- | +/- | NT | NT | NT |
| | CIN III [51] | -/- | +/- | -/- | -/- | +/- | -/- | -/- | +/- | -/- |
| n=7 | | | | | | | | | | |
| | CANCER [23] | -/- | -/- | -/+ | -/- | -/- | -/+ | NT | NT | NT |
| | CANCER [35] | -/+ | +/- | -/+ | -/- | +/- | -/- | NT | NT | NT |
| | CANCER [38] | -/- | -/- | -/+ | -/- | -/- | -/+ | NT | NT | NT |
| | CANCER [44] | -/- | -/- | -/- | -/- | -/- | -/- | -/- | -/- | -/- |
| | CANCER [46] | -/+ | -/- | -/+ | NT | NT | NT | -/- | -/- | -/- |
| | CANCER [48] | -/- | -/- | -/+ | -/- | -/- | -/- | -/- | -/- | -/- |
| | CANCER [49] | -/- | -/- | +/- | -/+ | -/- | +/- | -/- | -/- | -/- |

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