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EFFECTS OF HUMAN PAPILLOMAVIRUS-SPECIFIC IMMUNITY ON THE OUTCOME OF HPV INFECTIONS IN WOMEN

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To my family

ABSTRACT

Anna Paaso

Effects of human papillomavirus-specific immunity on the outcome of HPV infections in women

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The host's inadequate HPV-specific cell-mediated immune system might be crucial in HPV-specific tumor development.

This thesis is part of the longitudinal Finnish Family HPV Study and is focused on HPV16-specific cell-mediated immunity in women. PBMCs were stimulated with HPV16 E2, E6 and E7 peptides, and subsequent cytokine secretion was assessed. Women were divided into four subgroups based on their oral or genital HPV status and HPV serology collected during the 6 years follow up. The genotype-specific concordance between cervical and oral HPV infection and HPV serology was also assessed. The effect of the mothers' cervical intraepithelial neoplasia on their offspring's HPV16-specific cell-mediated immunity was evaluated.

The results showed that HPV infection in either the cervix or oral mucosa resulted in similar HPV16-specific cell-mediated immunity. The lack of HPV16 E2- and E6-specific T memory cells and Th2 cytokines predisposed women for persistent oral HPV16 infection, whereas women who remained oral HPV16-negative during the FU responded more frequently to HPV16 E2 and E6 peptides and had a more prominent profile of Th1 cytokines. In addition, children showed HPV16-specific proliferation. No concordance was found between HPV L1 serology and the presence of HPV DNA in either the cervix or the oral mucosa. Women who cleared their infection had the highest antibody titers.

HPV16-specific T memory cells were common in both HPV16 DNA-positive and -negative women and in their offspring. The Th2 cytokine profile might also predispose to persistent oral HPV16 infection. High HPV16 antibody titers might be a sign of clearance of cervical HPV infection.

Keywords: human papillomavirus, HPV genotype 16, women, cell-mediated immunity, T cells, cytokines, HPV serology, offspring

TIIVISTELMÄ

Anna Paaso

Ihmisen papilloomavirusinfektion aiheuttaman immuunivasteen merkitys taudin kulkuun naisilla

Turun yliopisto, Lääketieteellinen tiedekunta, Hammaslääketieteen laitos, Suupatologian ja suuradiologian osasto, Suun terveystieteiden tohtoriohjelma (FINDOS), Turun yliopistollinen keskussairaala, Naistenklinikka

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Pitkäaikainen ihmisen papilloomavirusinfektio (HPV) on merkittävin yksittäinen riskitekijä eräiden syöpien synnyssä. Lisäksi isännän HPV-spesifisen soluvälitteisen immuniteetin puutteellinen toiminta on oleellinen tekijä kasvaimen kehittymisessä.

Tämä väitöskirjatyö on osa monivuotista seurantatutkimusta, joka alun perin suunniteltiin selventämään HPV-infektioiden dynamiikkaa 329 perheessä. Väitöskirjatyössä tutkittiin HPV16-spesifistä soluvälitteistä immuniteettia naisilla, jotka oli jaettu alaryhmiin heidän 6 vuoden seurannan aikaisten HPV16-spesifisten näytteidensä perusteella. Naiset olivat HPV16-DNA-positiivisia joko genitaalin tai suun limakalvoilta kerättyjen näytteiden suhteen. Kontrolliryhmät muodostuivat naisista, jotka olivat olleet koko seurannan ajan HPV-DNA-negatiivisia vastaavien näytteiden suhteen. Lisäksi tutkittiin äitien kohdunkaulan syövän esiasteiden ilmenemisen vaikutusta heidän lastensa HPV16-spesifisen soluvälitteisen immuniteetin syntyyn. Tutkimuksessa määritettiin myös HPV-spesifisten vasta-aineiden tasoja sekä vastaavuutta HPV-DNA:n kanssa eri aikapisteissä.

Genitaali- ja suun alueen HPV16-infektioiden huomattiin aiheuttavan hyvin samanlaiset soluvälitteiset vasteet. HPV16-spesifisiä T-muistisoluja mitattiin naisilta, jotka olivat olleet seurannan aikana HPV16-positiivisia, kuten myös kontrolliryhmään kuuluvilta naisilta sekä lapsilta. Naisilla, joilla oli pitkäaikainen HPV16-infektio suussa, havaittiin enemmän Th2-tyypin sytokiinituotantoa sekä HPV16 E2- ja E6-proliferaatiota kuin kontrolleilla. HPV-tyyppispesifisiä L1-vasta-aineita ei havaittu samanaikaisesti vastaavan HPV-tyypin DNA:n kanssa. Korkeimmat vasta-ainetasot havaittiin naisilla, joiden genitaalialueen HPV-infektio parani seurannan aikana.

HPV16-spesifisten muistisolujen esiintyminen sekä HPV16-positiivisilla että negatiivisilla naisilla on yleistä, kuten myös lapsilla. Th2-tyypin sytokiinituotanto saattaa ennustaa pitkäaikaista suun HPV16-infektiota. Korkeat HPV-vasta-ainetasot saattavat olla ennusmerkki genitaalialueen HPV-infektoin paranemisesta.

Avainsanat: ihmisen papilloomavirus, HPV-genotyyppi 16, naiset, soluvälitteinen immuniteetti, T-solut, sytokiinit, HPV-serologia, lapsi

TABLE OF CONTENTS

ABBREVIATIONS

APC	antigen-presenting cell
CC	cervical cancer
CD	cluster of differentiation
CIN	cervical intraepithelial neoplasia
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
E	early
FOXP3	forkhead box P3
FU	follow-up
HLA	human leukocyte antigen
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HR	high-risk
IFN	interferon
Ig	immunoglobulin
IL	interleukin
L	late
LC	Langerhans cell
LR	low-risk
LST	lymphocyte stimulation test
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MRM	memory response mix
NK	natural killer cell
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
SCC	squamous cell carcinoma
SI	stimulation index
STD	sexually transmitted disease
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
T reg	regulatory T cell
VLP	virus-like particle

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV:

I Paaso A*, Louvanto K*, Syrjänen K, Waterboer T, Grénman S, Pawlita M, Syrjänen S. Lack of type-specific concordance between human papillomavirus (HPV) serology and HPV DNA detection in the uterine cervix and oral mucosa. J Gen Virol. 2011 Sep;92 (Pt9):2034-46. *Equal contribution

II Koskimaa HM*, Paaso A*, Welters MJ, Grénman S, Syrjänen K, van der Burg SH, Syrjänen S. Human papillomavirus 16 E2-, E6- and E7-specific T-cell responses in children and their mothers who developed incident CIN during a 14-year follow-up of the Finnish Family HPV cohort. J Transl Med. 2014 Feb 13;12:44. *Equal contribution

III Paaso A, Koskimaa HM, Welters MJ, Grénman S, Syrjänen K, van der Burg SH, Syrjänen S. Cell mediated immunity against HPV16 E2, E6 and E7 peptides in women with incident CIN and in constantly HPV-negative women followed-up for 10-years. J Transl Med. 2015 May 20;13:163.

IV Paaso A, Koskimaa HM, Welters MJ, Kero K, Rautava J, Syrjänen K, van der Burg SH, Syrjänen S. The interferon-γ associated cell-mediated immune responses to HPV16 E2 and E6 discern oral HPV16 DNA negative women from those with a persistent oral HPV16 infection. Submitted.

1 INTRODUCTION

Human papillomaviruses (HPV) can infect mucosal epithelia and skin, and subgroups of these viruses are potential risk factors for squamous cell carcinomas at different anatomic sites. Women have more HPV-induced cancers than men, and cervical cancer (CC) is the third most common cancer among women worldwide.

The majority of individuals will clear their HPV infections over time, but in some cases the HPV infection remains persistent. Over the course of years, the high-risk (HR)-HPV infection might progress to precancer lesions and even cancer. Of essential importance is the HPV's ability to effectively escape the host's immune system during the infection, which leads to inefficient adaptive immune responses. In some individuals, the antibody presenting cells (APCs) of the innate immune system do not recognize HPV or its structural proteins, which leads to insufficient activation of the adaptive immune system and gives the virus free access to the host cells. An HPV infection interferes with the cell cycling and deoxyribonucleic acid (DNA) repair systems, and can therefore lead to transformations of the infected cells. The cells will gradually acquire a growth advance among all other host cells, leading to precancer lesions and their progression to malignancy.

The E6 and E7 oncogenes of the HR-HPVs have many ways to modify the HPV-specific immune reaction in favor of the virus. At present, HPV-specific innate immunity is well known, but less is known about cell-mediated immunity. It is well-established that memory T cells and Th type 1 reactions are essential for recovery from HPV infection. There is inconsistent information about the role of T regs and Th17 cells in the clearance of an HPV infection.

The original studies of this thesis are focused mainly on cell-mediated immune responses in HPV16 DNA-positive women belonging to the Finnish Family HPV Study and the appropriate controls who remained HPV16 DNA-negative during the follow-up (FU). The Finnish Family HPV Study is a unique, longitudinal FU study that has been run since 1998. In addition, the significance of a mother's genital HPV status on her child's HPV16-specific cell-mediated immunity was assessed, and the pair-wise comparison of HPV DNA positivity and HPV seropositivity was also analyzed.

2 REVIEW OF THE LITERATURE

2.1 HUMAN PAPILLOMAVIRUSES

2.1.1 Classification

Human papillomaviruses (HPV) belong to the *Papillomaviridae* taxonomic family, which contains 29 genera. In total, papillomaviruses can be found in more than 20 mammals, birds, snakes and reptiles. HPVs are highly host- and tissue-specific and infrequently transmitted between species. Nowadays, over 180 HPV types and more than 60 animal papillomaviruses (PV) have been sequenced (de Villiers, 2013, de Villiers et al., 2004, Bernard, 2013). HPV types are divided into five genera, of which the alpha papillomaviruses are the most studied genus, because nearly all mucosal HPV genotypes belong to the alpha papillomaviruses (Figure 1) (Bzhalava et al., 2013, Doorbar et al., 2012). The beta papillomaviruses include most of the HPV genotypes that infect the skin, and some provide an increased risk for non-melanoma skin cancers (Bzhalava et al., 2013).

Briefly, HPV can be classified in three different ways: 1) by the sequence homology of the L1 gene, 2) by the capacity for inducing cancer (high (HR) or low (LR) risk in addition to probably high-risk, 3) by their tissue tropisms either to mucosal or skin epithelia. In addition, the lifecycles of high- and low-risk HPV genotypes are different, resulting in dissimilar immune responses.

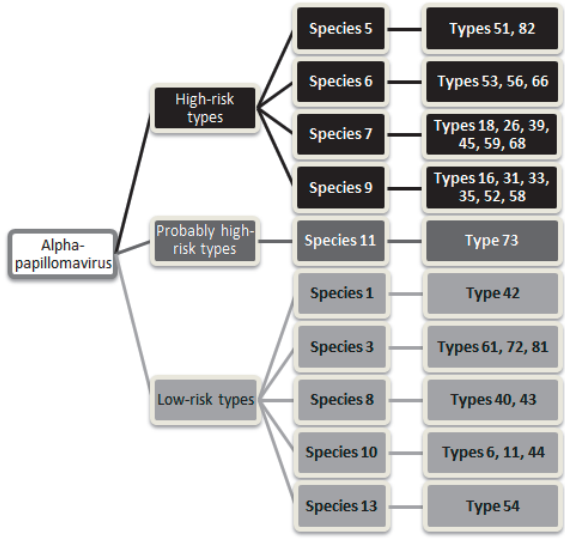

Figure 1. Classification of alpha papillomaviruses. Only the most important HPV types of alpha papillomaviruses are given.

2.1.2 HPV genomic organization

HPV is approximately 55 nm in diameter. It is a small non-enveloped virus with double-stranded DNA (Williams et al., 1961). The genome consists of about 8000 base-pairs in an icosahedral protein capsid composed of 72 capsomers (Kirnbauer et al., 1992).

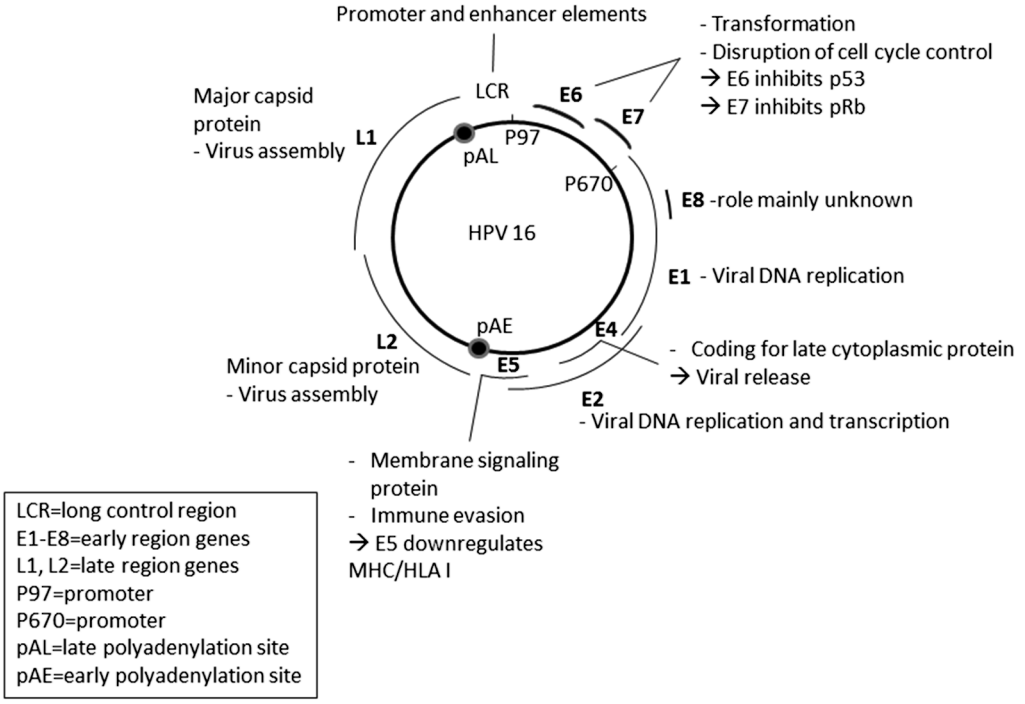

Figure 2. Genomic organization of HPV16.

The HPV genome consists of 9 genes (open reading frames) which are early (E1, E2, E4, E5, E6, E7 and E8) and late genes (L1 and L2), in addition to the LCR (long control region) (Figure 2). E6 and E7 are the oncogenes which modulate the transformation process and disrupt the cell cycle control (zur Hausen, 2000). The role of E5 in the progression of HPV-induced disease has also been acknowledged (Alonso and Reed, 2002, Kivi et al., 2008, Maufort et al., 2010, Kivi et al., 2012). The early genes E1 and E2 modify viral transcription and replication. The two structural genes, L1 and L2, are responsible for packaging the viral DNA into capsids in fully differentiated epithelial cells (Münger and Howley, 2002). The role of E8 is mainly unknown at the moment. It has been studied only in some high-risk-HPV genotypes such as HPV16 and HPV18, which express a spliced mRNA that links the E8 gene to the E2 gene encoding an E8^E2C protein (Stubenrauch et al., 2000). In undifferentiated keratinocytes, E8^E2C inhibits genome replication and it also might have the capacity to limit the productive replication of HPV16 in differentiated cells, leading to a persistent infection (Straub et al., 2015).

Table 1. Functions of alpha papillomavirus proteins.

Protein	Functions
L1	Major viral capsid protein, self-assembly in capsomers, interacting with cell receptors
L2	Minor viral capsid protein, virus assembly, viral entry into cell
E1	Viral DNA replication, ATP depent helicase, binds to specific DNA elements in the viral origin with the assist of second viral protein, E2
E2	Site-specific binding protein, viral DNA replication, transcription factor of E6 and E7, segregation of viral genome during cell division
E4	Expressed as a fusion protein E1^E4 during the late stages of viral lifecycle, binds to cytokeratin filaments and disrupts their functions, contributes to viral release and transmission
E5	Small transmembrane protein, activation of epidermal growth factor receptor (EGFR) and other protein kinases, inhibiting apoptosis, role in immune responses
E6	Immortalization and transformation of infected epithelial cells, disrupting normal cell growth and proliferation by binding to protein p53, inducing telomerase and preventing cell differentation, involved in immune evasion
E7	Immortalization and transformation of infected epithelial cells, disrupting normal cell growth and proliferation by binding to protein pRb (chromosomal instability)
E8	Functions mainly unknown

2.2 HPV LIFECYCLE

2.2.1 Epithelia in HPV infections

HPV infection is usually found in the skin or the anogenital tract, but also in the oral, oropharyngeal, laryngeal and sinonasal mucosa. HPV can also infect the urinary tract, the conjunctiva of the eye, the esophagus and the bronchial mucosa (Syrjänen and Syrjänen, 2000).

In the cervix, the transformation zone (junctional area of the squamous and columnar epithelium) is prone to HPV infection (Crum, 2000). The stratified squamous epithelium of the cervix consists of a basal layer, a parabasal layer, an intermediate layer and a superficial layer. The basement membrane and the lamina propria/stroma are under the epithelium (Figure 4).

The oral mucosa is covered by the stratified squamous epithelium. The oral mucosa can be categorized into three groups according to their function and histology: 1) masticatory mucosa, which is keratinized, stratified squamous epithelium found on the dorsum of the tongue, hard palate and attached gingiva; 2) lining mucosa, which is

non-keratinized stratified squamous epithelium covering the e.g. buccal mucosa and floor of the mouth; and 3) specialized mucosa, which can be found in the dorsal part of the tongue where the taste buds and lingual papillae are located. Based on the experimental studies, HPV has different abilities to infect different mucosal sites of the oral cavity (Parsons and Kidd, 1943). The reservoir of oral HPV infection is not known. In the oropharynx, the cryptal epithelia has been shown to be the target tissue of HPV (Begum et al., 2005).

2.2.2 Productive lifecycle

The lifecycle of HPV is tightly regulated by the differentiation of the host epithelial cell, because HPV does not have its own DNA polymerase activity for viral genome replication (Doorbar et al., 2012, Kajitani et al., 2012, Stubenrauch and Laimins, 1999, Alani and Münger, 1998). HPV is able to escape from the host's immune system because HPV infections do not cause any cell death (necrosis), as the viral mature particles are shed from the surface of the epithelium. Thus, in a productive HPV infection there is no inflammation, which silences the activation of the cellular (innate and adaptive) immune responses (Kupper and Fuhlbrigge, 2004, Boccardo et al., 2010).

An HPV infection requires epithelial wounding or microwounding which allows the virus to access the basal epithelial cells (Culp et al., 2006). Cell division during wound healing is essential for the entry of the virus genome into the nucleus. (Pyeon et al., 2009). After binding to the host cell, the L1 and L2 proteins undergo conformational changes which bring cleavage to the amino-terminus of L2 at the cell surface by the protease furin (Kines et al., 2009). After this, HPV is transferred to a cell-surface receptor and internalized into the host cell nucleus (Popa et al., 2015). The correct nuclear entry of the viral genomes is ensured by the L2 protein-DNA complex and the L1 is localized in the endosome affecting lysosomal degradation (Bergant Marušič et al., 2012, Schelhaas et al., 2012).

In the normal, uninfected epithelium, only the basal and parabasal cells are proliferating. Normally a basal cell divides, producing two daughter cells, and one of the daughter cells starts its differentiation toward the epithelial surface. HPV genes are expressed in different stages during the cell differentiation, as summarized in Figure 3. In the HPV-infected basal cells, the viral genome is maintained in an episomal form with a low copy number (50-200 copies/cell) (Doorbar et al., 2012). The viral proteins E1, E2, E6 and E7 are expressed at low levels in the lower epithelial layers (Maglennon et al., 2011). In the upper layers of the epithelium, the expression of E6 and E7 allows the infected cell to enter the S-phase when the viral copy number starts to rise. Other HPV proteins are also expressed in the upper layers of the epithelium. Finally, the cell exits from the cell cycle after the expression of minor coat protein L2. The major coat protein allows HPV genome packaging (Doorbar, 2006).

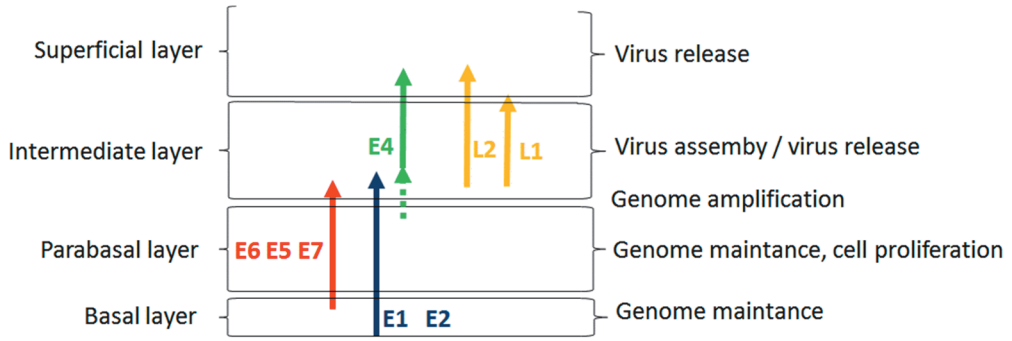

Figure 3. Productive lifecycle of HPV. E1, E2 proteins are response for genome maintenance when HPV is replicating together with its host cell. E1, E2, E5, E6 and E7 proteins are expressed at low levels in non-dividing cells (parabasal layer) and E6, E7, E1, E2 and E5 proteins are expressed at high levels in differentiating cells (at 1000 copies/cell) in the intermediate layer. L1, L2 proteins are responsible for assembly and release of virus particles in the superficial layer.

2.2.3 Lifecycle in tumor cells

HPV can occasionally evade the host immune system, which may lead to a chronic/persistent HPV infection. Persistent HPV infection is linked to the chronic inflammation which is an important cofactor for cancer development (Boccardo et al., 2010, Bernard et al., 1989, Romanczuk et al., 1990, Dowhanick et al., 1995). The essential step in the progression of an infected cell toward transformation and immortality is the integration of the viral genome into the host DNA (Moody and Laimins, 2010). The integration leads to the upregulation of E6 and E7 mRNA expression by the E2 gene (Moody and Laimins, 2010). Cells with integrated viral DNA impart a selective growth advantage over cells that have only episomal copies of viral DNA, because integrated copies show increased stability of the E6 and E7 mRNAs (Jeon and Lambert, 1995). The high-level expression of E6/E7 genes and accumulation of genetic errors finally leads to cancer progression (Pett et al., 2004). The E5 protein also has oncogenic functions such as interfering in the processing of classical MHC molecules to the cell surface and compromising the presentation of viral peptides at the surface on the host's epithelial cell (Ashrafi et al., 2006). HPV DNA might also present an integrated and episomal form in the same cell (mixed form). Overexpression of E6 degrades the p53 tumor suppressor protein, which leads to deregulation of the cell cycle checkpoints. In an HPV-infected cell, the E7 interrupts the retinoblastoma (pRb) protein to interact with E2F, which promotes the entry into cell division (Ghittoni et al., 2010, Sasagawa et al., 2012).

The expression levels of the E6 and E7 genes increase from intraepithelial neoplasia grade 1 to 3 which is in the cervix known as cervical intraepithelial neoplasia (CIN) and in the anus as anal intraepithelial neoplasia (AIN) (Doorbar et al., 2015). Changes in gene expression are directly connected to the neoplastic phenotype (Figure 5).

Persistent deregulation in host cell gene expression might lead to an accumulation of secondary genetic changes which are facilitated by the high expression of the E6 and E7 proteins.

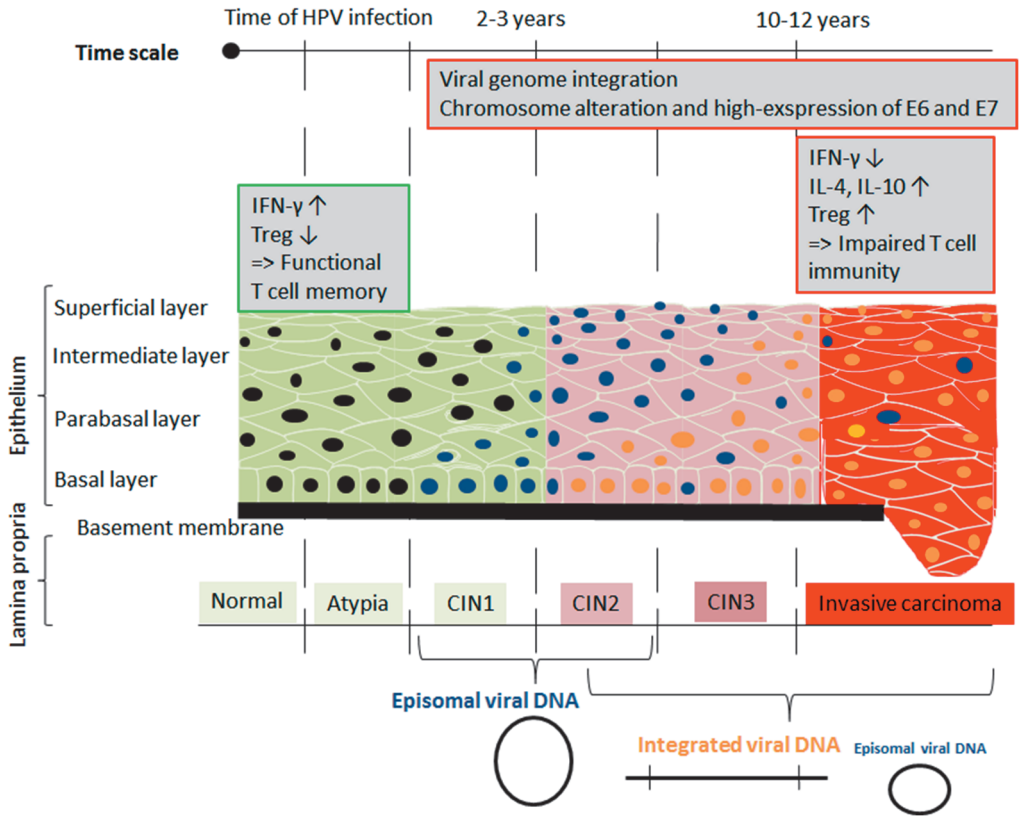

Figure 4. Schematic presentation of the progression of HR-HPV infection toward invasive cervical carcinoma. The green color indicates normal epithelium progressing to mild dysplasia (CIN1), the pink epithelium shows the progression from moderate dysplasia (CIN2) to severe dysplasia (CIN3) and the red area presents an invasive carcinoma.

The presence of external factors such as hormones and tobacco nitrosamines also has an effect on the HPV lifecycle. For example, experimental studies have shown that estrogen contributes to the onset, persistence and malignant progression of cervical cancer (CC) in a human papillomavirus-transgenic mouse model (Kumar et al., 2016, Gariglio et al., 2009, Brake and Lambert, 2005), while E7 overexpression together with the chemical carcinogen 4-nitroquinoline-1-oxide (4-NQO) induced head and neck cancers in mice (Jabbar et al., 2010, Strati and Lambert, 2007).

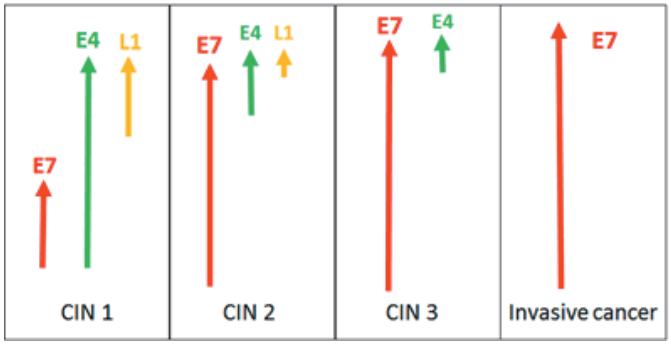

Figure 5. Changes in expressions of HPV proteins during cancer progression.

2.3 HPV INFECTIONS IN WOMEN

2.3.1 Asymptomatic HPV infections

HPV infections are the most common sexually transmitted infections (STIs) globally (Chesson et al., 2014). Nonsexual modes of HPV infections have also been identified. For example, HPV can be transmitted vertically from a mother to her offspring, possibly via the placenta or the birth canal or horizontally via the mother or other caretakers (Sarkola et al., 2008, Syrjänen, 2010a, Medeiros et al., 2005). HPV-infected individuals might unknowingly transmit the virus because most HPV infections are asymptomatic or subclinical. In most cases, an HPV infection will clear in approximately two years, but in the minority of infected subjects, an HPV infection might persist because of inadequate immune responses and viral DNA integration into the host's genome (Franco et al., 1999, Ho et al., 1998). HPV infections are common all around the world but globally variable. HPV16 is the most common genotype in HPV-infected tissues and has been shown to have the longest duration of viral persistence (Louvanto et al., 2010a). The frequency of asymptomatic HPV16 infection in the cervix is most common in Europe followed by South America, Asia and Sub-Saharan Africa (Clifford et al., 2005).

It has been estimated that the lifetime acquisition of a cervical HPV infection is around 80% of women (Syrjänen and Syrjänen, 1990). Women under 25 years have the highest prevalence of HPV infections (Franceschi et al., 2006).

2.3.2 Mucosal LR-HPV infections in the anogenital tract

Anogenital condylomas are globally prevalent and over 90% of them are caused by HPV6 and/or HPV11 infections (Bhatia et al., 2013). The productive infection of LR-HPV genotypes causes proliferation of the epithelial squamous cells, which leads to benign wart-like tumors. Condylomas are the most common clinical manifestation of non-oncogenic HPV infections. Genital warts are more common in women than men

(Lacey et al., 2006). Usually the warts clear within one to two years of infection through the host's cell-mediated immunity (Stanley, 2009). Although the genital warts are benign, they often cause psychological stress and significant medical costs.

2.3.3 Mucosal LR-HPV infections in the oral cavity

Focal epithelial hyperplasias, oral papillomas and oral condylomas are the most common manifestations of oral HPV infections (Rautava and Syrjänen, 2011, Syrjänen, 2003). LR-HPV types 6 and 11 are the main types, causing recurrent respiratory papillomatosis (RRP) in the head and neck region. This is a rare condition characterized by the recurrent growth of benign papillomas in the respiratory tract. The recurrent nature and location in the airways of the begin lesion require frequent surgical removal to keep the respiratory tract free from obstruction (Sittel, 2014). Approximately half of oral papillomas are caused by HPV, mostly by HPV6/11.

2.3.4 Cervical cancer

An HPV infection is the main etiological cause for CC and approximately 90% of CC is caused by high-risk HPVs (Doorbar, 2006). Globally, around 528 000 patients are annually diagnosed with cancer of the cervix uteri and 266 000 patients die of this disease. Over 80% of cases are diagnosed in developing countries (Jemal et al., 2011, Kling and Zeichner, 2010, Parkin and Bray, 2006). HPV types 16 and 18 are the most common causes of CC, being involved in over 70% of cases, followed by HPV genotypes 45, 31 and 33 (Muñoz et al., 2004, de Sanjose et al., 2010). HPV16, 18 and 45 are more likely to be integrated into the human genome than other genotypes. However, the other HR-HPVs have not been studied as intensively for their physical state in different lesions (de Sanjose et al., 2010). The integration of the HPV genome into the host genome is one crucial step in malignant transformation and might be an early event in CC development (Peitsaro et al., 2002, Schmitz et al., 2012). Progression from an infection to cancer is estimated to take 10-20 years, but the progression can happen in a short timeframe (Syrjänen et al., 1985). Several studies have shown that intervention in terms of the eradication of CIN can prevent cancer precursor lesions (Baloglu et al., 2010, Verguts et al., 2006, Draeby-Kristiansen et al., 1991). Screening with a Papanicolaou (pap) and/or HPV test has shown to be effective in identifying individuals at risk for CC (Saraiya et al., 2010, Schiffman et al., 2011).

The highest risk for CC is among women in Sub-Saharan Africa, partly because of the lack of a healthcare system. In Finland, the prevalence and associated mortality rates for CC have unfortunately slightly increased during the last 10 years. Despite this, the incidence of CC is still low, 4.32/100 000 cases, and the mortality 1.04/100 000 cases in the year 2012 (GLOBOCAN 2012 v1.0, http://globocan.iarc.fr, accessed on 20/5/2016).

2.3.5 Other anogenital cancers

Molecular and epidemiological studies have shown that HPV also has a causal role in vaginal, vulvar and anal cancers in women, but the risk of cervical cancer is higher (Chaturvedi, 2010). Individuals with human immunodeficiency virus (HIV) infection are at an increased risk for HPV-induced cancers such as anal and head and neck cancers (Stier and Baranoski, 2008).

HPV 16 is the predominant genotype in anal squamous cell carcinoma (SCC) (Williams et al., 1996). Anal cancers are predominantly SCCs, adenocarcinomas and basaloid or cloacogenic carcinomas, because the lower third of the canal is squamous epithelium with a specialized translational zone of epithelium in between. Anal cancer is fairly more common in women than in men and most common in the older population (De Vuyst et al., 2009).

HPV is also a potential risk factor for vulvar and vaginal cancers. Vulvar cancers are mostly SCC (Hill-Daniel and Roett, 2015). Vulvar intraepithelial neoplasia (VIN) is a precursor lesion of vulvar SCC. The prevalence of VINs starts to rise at the age of 20-29 years for vulvar cancer, but vaginal intraepithelial neoplasia is relatively uncommon in women under the age of 50 years. The highest peak of vulvar and vaginal cancers are in women at the age of 70 or older. The incidence of vulvar and vaginal cancers starts to increase at the age of 40-49 years and also at the age of 60-69 years, respectively (Nygård et al., 2014).

2.3.6 Head and neck cancers

HPV also causes a subset of head and neck squamous cell carcinomas (HNSCC). HNSCCs are a heterogeneous group of cancers deriving from different anatomical areas. The most common HNSCCs are squamous cell carcinoma of the oral cavity, larynx, oropharynx, hypopharynx, nasopharynx and sinonasal tract (Syrjänen, 2010b, Syrjänen and Syrjänen, 2013, Gama et al., 2016). HPV16 is the main HPV genotype in HNSCCs, accounting 87% of the oropharyngeal, 68% of oral and 69% of laryngeal carcinomas (Ndiaye et al., 2014, Castellsagué et al., 2016, Dayyani et al., 2010, Kreimer et al., 2005). HPV-positive HNSCCs develop differently from smoking and alcohol-associated HPV-negative cancers. HPV-positive HNSCCs have better survival rates than HPV-negative HNSCCs, although HPV-positive HNSCCs are often diagnosed at a late stage (Dale et al., 2015, Tahtali et al., 2013, Wong et al., 2016, Dayyani et al., 2010).

2.4 INNATE IMMUNITY IN HPV INFECTION

HPV has a very smart way to survive, because the virus can induce a chronic infection, infrequently kill the host cell and regularly shed infectious viruses from the epithelial

surfaces ready to infect naïve individuals. While managing to do all that, the HPV must efficiently evade the innate immune response which delays the activation of the adaptive immune response. Innate immunity is the first line of defense against extra- and intracellular pathogens or molecules. After activation of the innate immunity, the host cells start to produce different inflammatory cytokines and other molecules to protect the host. Antigen-presenting cells (APC) are mediators between the innate and adaptive immunity, introducing the antigens via a complex event to the naïve T cells in the local lymph node. HPV is able to hide from the host's immune system mainly through the action of the E6 and E7 proteins. The E5 protein also has a silencing effect on the immune response. Although the majority of HPV infections will clear, a minority of infections will persist and may finally progress to viral-induced neoplasia (Kanodia et al., 2007, Doorbar et al., 2012).

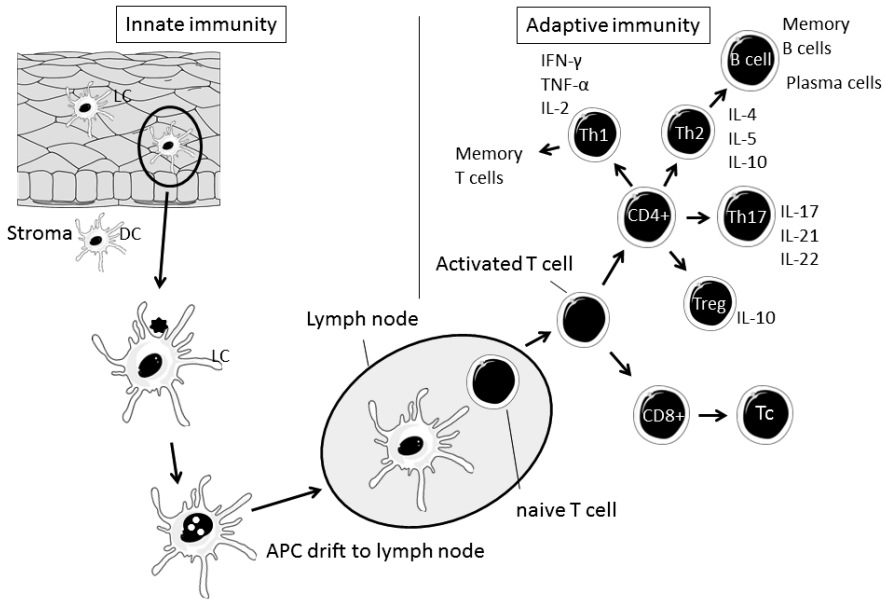

Figure 6. Innate immunity activates adaptive immunity as a result of HPV infection.

Cell death or injury of the host cells activates the innate immunity after an HPV infection. The innate immunity contains dendritic (DC), Langerhans (LC), natural killer (NK), natural killer T (NKT) cells (also part of the adaptive immunity) and keratinocytes, among others (Colonna et al., 2011, Amador-Molina et al., 2013). The innate immunity has no specific memory, but it is responsible for promoting an effective adaptive immune response against an HPV infection.

Instantly after the virus comes into contact with the mucosal epithelium, the innate immunity starts to process the effective immune response via soluble and cellular effectors. HPV has the ability to interrupt many innate immunity processes to cause inadequate immune responses, which is more precisely described in the sections below.

2.4.1 Dendritic cells

DCs act in priming adaptive immune responses and also in inducing self-tolerance. Two functional states of DCs can be found, immature and mature, and only mature DCs have the ability to prime an immune response (Reis e Sousa, 2006). In the epidermis and other stratified squamous epithelia, the DCs are called LCs (Steinman and Idoyaga, 2010). DCs are usually activated by viral capsid entry, but studies have indicated that an HPV infection does not activate the LCs in the squamous epithelia. LCs are immature DCs and are responsible for presenting the antigens for a naïve T cell in the lymph node, activating the adaptive immunity (Liu and Nussenzweig, 2010, Fausch et al., 2002). In the transformation zone of the cervix, the number of LCs is decreased as an impact of the HPV infection. The impaired function of LCs in HPV lesions might have an impact on the duration of viral persistence (Scott et al., 2001).

APC (LCs and DCs) adhesion and migration can be edited by the HPV-specific oncoproteins E6 and E7 which are able to downregulate E-cadherin to disrupt the adhesion of keratinocytes to LCs (Hubert et al., 2005). The IL-10 secreted by keratinocytes acts as an inhibitor of LC migration to lymph nodes, and high levels of IL-10 expression have been detected in squamous intraepithelial lesions (SIL) but totally absent in the normal epithelium (Scott et al., 2001, Gottfried et al., 2008). In addition, the downregulation of TNF-α might cause a weak immune response as a consequence of silencing the pro-inflammatory activities (Mota et al., 1999).

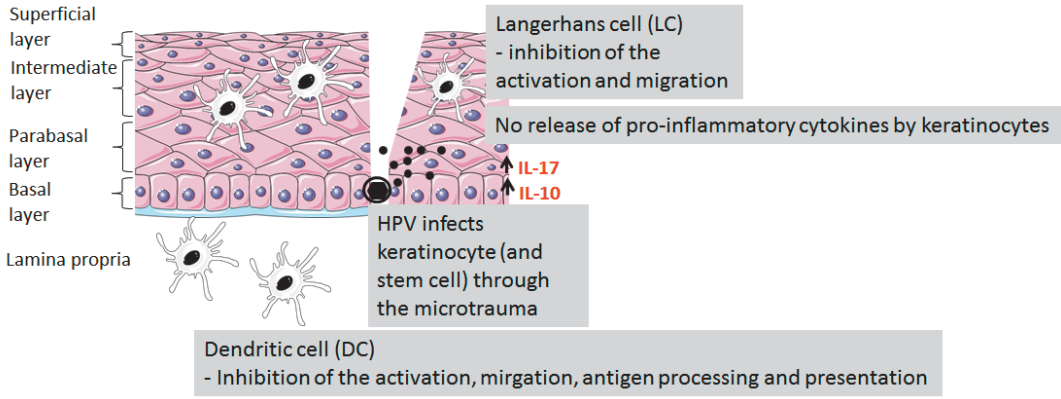

Figure 7. Deactivated functions of innate immunity in DCs and LCs after HPV infection.

2.4.2 Natural killer cells

NK cells are lymphocytes which can recognize and destroy infected or damaged cells in a nonspecific manner. They release cytotoxic granules onto the surface of target cells and kill the cells by apoptosis. The cytokines TNF-α and IFN-γ can be secreted by NK cells, enhancing the inflammatory and immune responses. NK cells also have the ability to express inhibitory receptors that interact with MHC class I proteins in host

cells. After the inhibitory receptors bind to MHC class I molecules, the levels of MHC class I will be reduced, slowing down the inhibitory signaling and favoring NK cell activation (Joncker and Raulet, 2008).

HPV has several mechanisms to avoid NK cell activity, and the role of NK cells in the natural history of HPV infection is still partly unknown. Only two mechanisms by which NK cells target HPV are mentioned here. The HPV-infected cells can be directly eliminated by NK cells (Renoux et al., 2011). HPV might also affect the NK cells by deregulating the NK receptors which are responsible for activating the NK cells. The action of deregulating receptors like NKp30 and NKp46 is commonly found in women with CC or HPV-infected mucosa (Bryceson and Long, 2008, Garcia-Iglesias et al., 2009).

2.4.3 Keratinocytes

HPV has several ways to modify the immune system to become more tolerant to the HPV infection. Most importantly, HPV replication and the release of mature viral particles do not result in cell death, because the life cycle of HPV is closely linked with the differentiation of the host keratinocytes, and therefore HPV can remain silent and hidden for a long time (Doorbar et al., 2012). HPV oncoproteins E6 and E7 inhibit IFN synthesis in the keratinocytes (Moody and Laimins, 2010). The HR-HPV infection also induces regulatory T cell infiltration and IL-10 production. In the infected host cells, the MHC class I proteins are expressed at low levels by the downregulation of the HPV16 E5 protein, which leads to impaired cytotoxic T lymphocyte (CTL) function (Ashrafi et al., 2006, Gruener et al., 2007). In addition, HR-HPV infection can lead to the CD4 and CD8 T lymphocytes in CIN2 or CIN3 lesions functioning ineffectively (Song et al., 2015).

After the activation of the viral infection, the basal keratinocytes in the cervix secrete low levels of a variety of cytokines, such as IFN-β and IFN-α (Le Bon and Tough, 2002). These cytokines are antiviral, antiproliferative and antiangiogenic and act like a bridge between innate and adaptive immunity. One mechanism in the immune evasion of HPV is the ability to modify cytokine levels to downregulate the pro-inflammatory response of cervical keratinocytes (Stanley, 2012). The interferon response is closely related to the regression of HPV lesions. Several studies have shown that the HPV oncoproteins E6 and E7 also reduce IFN secretion from keratinocytes by interacting directly with molecules belonging to the interferon signaling pathways (Song et al., 2015, Richards et al., 2014, Scott et al., 2009, Barnard et al., 2000, Park et al., 2000).

2.4.4 Toll-like receptors

Toll-like receptors (TLRs) are germ line-encoded receptors of the innate immune system, more accurately pathogen recognition receptors (PRRs), which allow the APC

to sense danger (Kawai and Akira, 2010). TLRs can be expressed either on the cell surface or intracellularly. In human cells there are 10 different TLRs recognizing bacterial (TLR 1, 2, 4, 5, 6) or viral (TLR 3, 7, 8, 9) products (Barton and Kagan, 2009). Activation of TLRs on keratinocytes in female genital tract promotes the production of type I interferons and other Th1–type cytotoxic responses which generate a powerful pro-inflammatory environment (Yang et al., 2005, Miller and Modlin, 2007, Nasu and Narahara, 2010). HPV18 E6 and E7 proteins have been shown to downregulate TLR9 expression at the infection site to allow viral escape from the host's immune system (Hasan, 2014). Instead, the TLR3/5/8 pathways in keratinocytes are activated during HPV infection and high TLR8 levels have been found in CC (Zhang et al., 2014). TLR4 expression levels have been found to be higher in HPV16-infected cells than HPV18-infected cells. Similarly, TLR4 levels are higher in CC lesions than in CIN lesions. Overexpression of TLR4 in HPV-infected cells has been associated with apoptotic resistance (Wang et al., 2014).

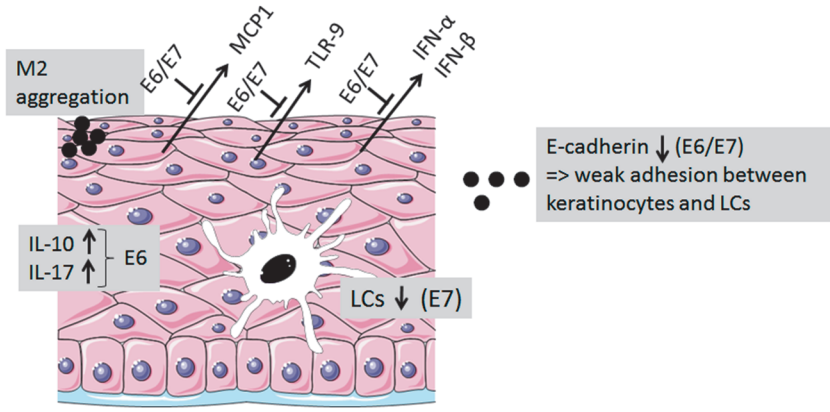

Figure 8. Microenvironment of keratinocytes modified by E6 and E7 proteins of HR-HPV. Abbreviations: M2=macrophages with anti-inflammatory functions, MCP=monocyte chemoattractant protein, TLR=toll-like receptor, IFN=interferon.

2.4.5 Marcophages

Macrophages have a crucial role in both innate and initiating adaptive immune responses and can be divided into two main groups: M1 macrophages, which exhibit inflammatory functions and rapidly kill pathogens, and M2 macrophages, which exhibit anti-inflammatory functions. In addition, there are several other types of macrophages depending on the signals in their microenvironment (Biswas and Mantovani, 2010). The number of M2 macrophages might significantly increase in a cervical lesion on progression, allowing efficient cancer cell proliferation, angiogenesis and restriction of defense mechanisms (Song et al., 2015). M2 cells can induce naïve T cell differentiation into T regulatory cells via IL-10 secretion licensing the tumor growth (Bolpetti et al., 2010).

2.5 ADAPTIVE IMMUNITY IN HPV INFECTION

Adaptive immunity is highly specialized and pathogen-specific. It has the ability to respond more vigorously to repeated exposures to the same microbe than innate immunity because of the memory cells. Adaptive immunity consists of two main cell types: T and B lymphocytes. T lymphocytes are divided into groups based on their distinct functions: Th 1 and 2 lymphocytes, T regulatory lymphocytes (T regs) and T helper 17 cells (Th17) which are all CD4-positive. T cytotoxic lymphocytes (CTL) are CD8-positive. B lymphocytes produce antibodies for creating humoral immunity.

HPV infection achieves an inadequate adaptive immune response mainly because the virus can evade the immune system for an elongated time period. Thus the innate immune system fails to activate the more effective adaptive immune system. Functionally working T helper cells are assumed to be most important in the regression of the HR-HPV lesion. In non-regressing genital warts and CIN lesions, the number of intraepithelial and stromal lymphocytes has been shown to decline and the ratio of CD4+/CD8+ lymphocytes to change (Stanley, 2009, Blanchet et al., 2007)

2.5.1 T cell responses

T cell activation is important in the regression of HPV-induced lesions. The equilibrium between Th1 and Th2 cells should be constant during intracellular or extracellular attacks by viral infections in the epithelia. The reduced number of CD4+ cells might provide an induction for cancer development. CD4+ and CD8+ T cells are activated by antigens which are presented by MHC class II and I molecules to the naïve T cells, respectively (Neurath et al., 2002). Several studies have shown that the equilibrium between Th1 and Th2 is unbalanced in an HPV lesion (Peghini et al., 2012, van der Burg et al., 2007, Welters et al., 2003, van der Burg et al., 2001, Xu et al., 2009). In patients with intraepithelial and invasive cervical HPV lesions, the Th2 cytokine profile is more prevalent, which leads to suppression of cellular immunity and progression of the lesion (Feng et al., 2012, Bais et al., 2005, Barbisan et al., 2012). An interesting observation is that both Th1 and Th2 phenotypes are suppressed in HPV lesions, which might be a consequence of the activity of the T reg cells (Deligeoroglou et al., 2013).

Th1-type cells produce IFN-γ-, TNF-α, and IL-2 cytokines to stimulate cellular responses, while Th2-type cells produce IL-4-, IL-5-, IL-10- and IL-13, among others, to stimulate humoral responses (Mosmann and Sad, 1996). As described earlier, certain cytokines such as TNF-α and IL-10 can be produced by different cell types, even by keratinocytes. According to current concepts, HPV infection normally achieves a Th1 response, and the Th2 response dominates only when the activation of the immune

system has been inappropriate (de Jong et al., 2004, Sasagawa et al., 2012). Typically, IFN-γ levels are decreased and IL-10 levels are increased both locally in the CIN2/3 and CC lesions and in the plasma (Farhat et al., 2009, Scott et al., 2009, Hu et al., 2015, Bais et al., 2005). The increased IL-10 production, mainly from lymphocytes but also from keratinocytes, might decrease the immune recognition of HPV-associated tumors by downregulating the expression of MHC class I and/or class II molecules (Scott et al., 2001, O'Garra and Vieira, 2007). The diminished expression of MHC class II molecules might lead to insufficient T cell responses.

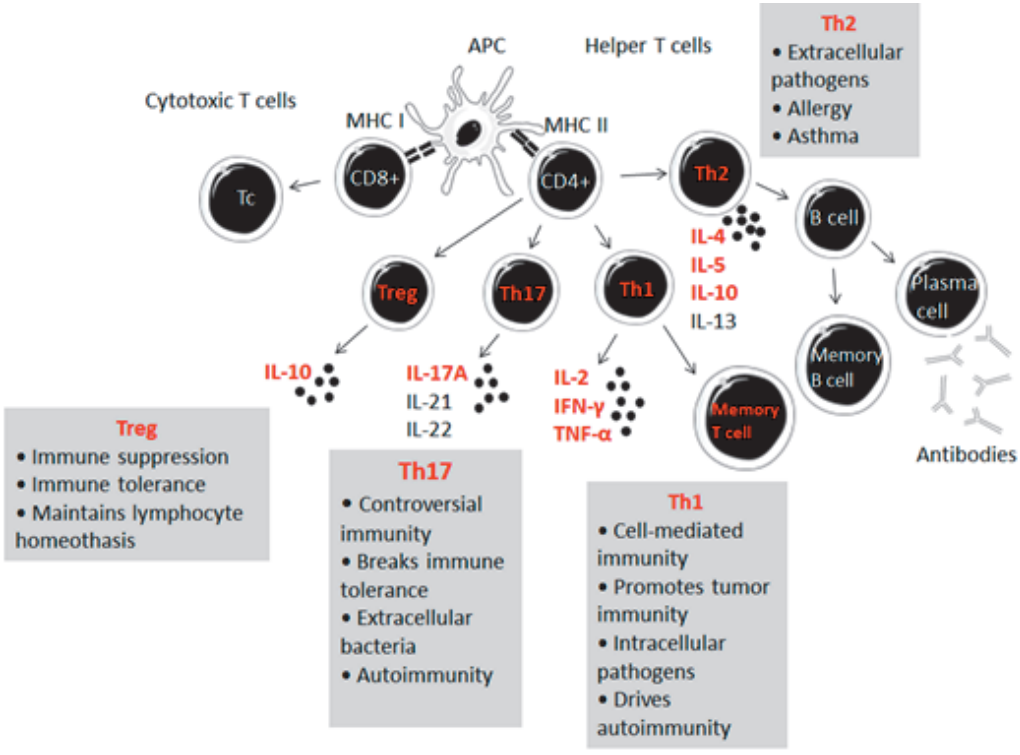

Figure 9. T cell differentiation after antigen presentation by APC. The molecules studied in this thesis are marked in red.

CD8+ CTLs recognize the antigens with the assistance of MHC I molecules. HPV has also developed defense mechanisms against CTLs. The HPV E7 oncoprotein is able to downregulate the expression of TAP-1, which is a central molecule in mounting MHC class I with the viral antigen (Deligeoroglou et al., 2013). This results in the suppression of HPV antigen presentation to CD8+ CTLs and allows HPV to evade the host's immune system. The HPV16 E5 protein also has the ability to downregulate the MHC/HLA I complex (Campo et al., 2010, Suprynowicz et al., 2010, Straight et al., 1995).

The IL-17 (also known as IL-17A) family has six members (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F) (Zou and Restifo, 2010). Th17 cells and their effector

cytokines, like IL-21, are both key players in inflammation, autoimmunity and allergic reactions (Zhang et al., 2011). The exact roles of Th17 cells and other IL-17A-expressing cells in cancer tissue are still unclear. IL-17A is known to upregulate the production of many pro-angiogenic factors of importance for tumor angiogenesis (Numasaki et al., 2005). Th17 cells are common in many cancer types and are located in the cancer tissue, while Th17 cells can be found in the peripheral blood of only a minority of patients (Zou and Restifo, 2010).

T reg cells are important for the maintenance of the immune homeostasis and have the capacity to suppress immune responses to antigens (Shevach, 2009, Tang and Bluestone, 2008). T regs express CD4 and CD25, in addition to the transcription factor Foxp3, which is necessary in the induction of tolerance in a normal situation. The increased number of T regs may hinder the immune response and simultaneously also inhibit cytokines, resulting in the suppression of antitumor defenses. T regs can recognize self-antigens and prevent autoimmunity, but are also involved in chronic and viral infections (Rouse and Suvas, 2004). It has been shown that the levels of TGF-β1 and TGF-β2 known to induce T reg activation are increased in lesions progressing from low-grade squamous intraepithelial lesion (LSIL) to invasive CC. Simultaneously, the levels of IL-12 and TNF-α are declined while IL-10 levels produced by T reg are increased (Mocellin et al., 2005, Peghini et al., 2012). The higher levels of IL-10 decrease CD8+ T cell infiltration and simultaneously increase the number of Foxp3+ T reg cells (Bhairavabhotla et al., 2007). Thus, IL-10 creates an immune-tolerant microenvironment which allows HPV-induced lesions to progress (Adurthi et al., 2008).

The balance between Th17 and T reg cells controls the immune response, and there is some evidence that the Th1/Th2 shift has a role in autoimmune diseases. However, it is not known whether the balance between Th17 and T reg cells has a role in cancer development (Zhang et al., 2011, Afzali et al., 2007). There are contrary results about the role of Th17 cells and IL-17 in the outcomes of patients with different types of cancers (Kryczek et al., 2009, Wu et al., 2009). Higher levels of Th17 cells have correlated with improved survival, while some studies have associated a high number of Th17 cells with poor prognosis for patients with CC and CIN (Punt et al., 2015, Zhang et al., 2015).

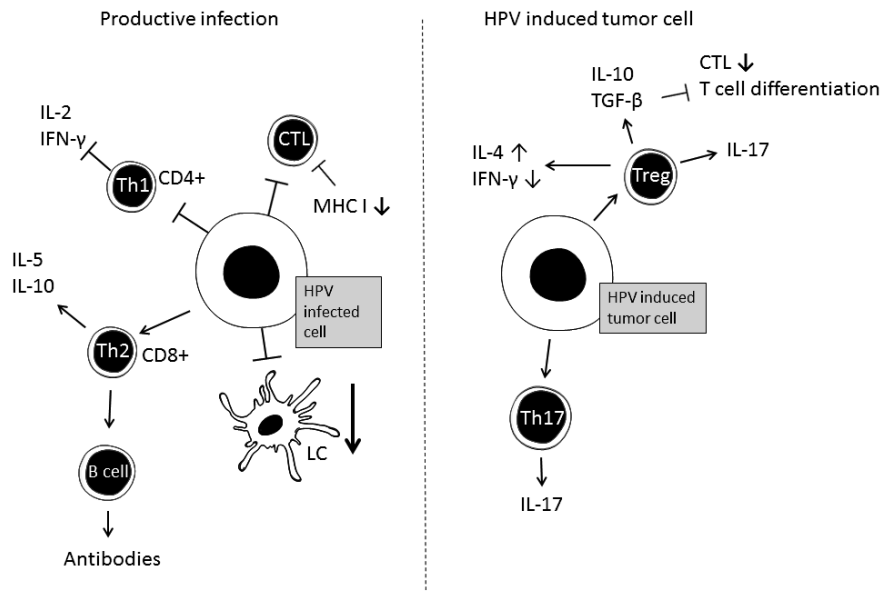

Figure 10. Central reactions in cell-mediated immunity in productive HPV infection and HPV-induced tumor cell. CD4+ T cells play a central role in initiating and maintaining anticancer immune responses. Regulatory CD4+CD25+ T cells (T regs) which express Foxp3 inhibit the antitumor functions of the effector T cells.

2.5.2 Antibody responses

B cells, which belong to the adaptive immune system, are able to act as APCs and present antigens in addition to secreting cytokines. CD4+ T cells assist B cells to mature and produce antibodies against a specific epitope. The majority of HPV-specific antibodies target the L1 capsid protein, and HPV antibodies against E2, E6, E7 and L2 have been shown to be of less importance and/or weaker (Viscidi et al., 2004). Approximately only half of cervical HPV DNA-positive women are HPV-seropositive (Clifford et al., 2007, Castro et al., 2014, Kirnbauer et al., 1994, Carter et al., 1996). Thus, HPV infection does not always lead to HPV seropositivity. Studies showing the poor concordance between HPV serology and the presence of HPV DNA have been based on analyses of simultaneously taken blood and genital samples (Ho et al., 2004, Skjeldestad et al., 2008). Therefore it is difficult to determine whether the HPV antibodies detected are due to a past, persistent or current HPV infection (Andersson et al., 2008, Vaccarella et al., 2010). Most of the studies have focused on HPV-specific IgG antibodies; IgA antibodies are also detectable in HPV-infected women, but they might be of shorter duration than IgG antibodies (Hagensee et al., 2000, af Geijersstam et al., 1998). There are contradictory results on the stability of IgG antibodies. The majority of studies have reported stable antibody levels over time, but some have found the decay of IgG antibodies within 36 months (Villa et al., 2006, Lehtinen et al., 2006, Ho et al., 2004, Syrjänen et al., 2009).

2.6 HPV VACCINES

2.6.1 Prophylactic vaccines

Not all HPV-induced cancers are able to be effectively treated, and therefore the therapeutic HPV vaccine might be a valuable tool for preventing HPV-induced cancers and perhaps other lesions, too (Gök et al., 2007, Kocken et al., 2011). There are three prophylactic HPV vaccines to prevent HPV infection available on the market, all based on virus-like particles (VLP) made of L1 protein: the bivalent vaccine is against HPV16 and HPV18 (Cervarix™, GlaxoSmithKline), the quadrivalent vaccine against HPV6, HPV11, HPV16 and HPV18 (GardsilTM, Merck), and the newest 9-valent vaccine is against HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58 (Gardasil®9, Merck). The VLPs in all vaccines are produced in either yeast or insect cells. The vaccines have been reported safe and effective even though their long-term efficiency needs further studies (Rey-Ares et al., 2012, La Torre et al., 2007, Lu et al., 2011, Dillner et al., 2010, Malagón et al., 2012). It was shown recently that the new 9vHPV vaccine is effective in preventing cervical, vulvar, and vaginal disease in addition to the persistent infections associated with the HPV-31, 33, 45, 52, and 58 genotypes. However, the effect of vaccination against the burden of the cancers cannot yet be determined (Joura et al., 2015).

HPV VLPs induce higher concentrations of neutralizing antibodies to L1 than the natural HPV infection and also generate cross-reactive and cross-neutralizing antibodies (Harro et al., 2001, Paavonen et al., 2007). VLPs are highly immunogenic because of the structure of the capsomers across the VPL's surface, which make it able to activate both innate and adaptive immune responses (Yan et al., 2005). VLP vaccines result in rapid access to the local lymph nodes and then to circulative immunity. These vaccines are highly immunogenic and they are rapidly bound by myeloid DCs and B lymphocytes, creating an effective antibody response to HPV (Harro et al., 2001, Yan et al., 2005).

2.6.2 Therapeutic vaccines

Several therapeutic HPV vaccines are under development to treat existing precancers or cancers (Davidson et al., 2004, Ohlschläger et al., 2006, Ferrara et al., 2003). Therapeutic vaccine strategies against HPV-driven cancers are focused on generating robust cytotoxic T lymphocyte (CTL) responses against the viral proteins E6 and E7 to clear infected cells. Unfortunately, most of the therapeutic HPV vaccines developed so far have failed at the clinical stage, even though the results in experimental models have been promising. HPV-specific CTLs are not efficient enough to eliminate cancer cells because HPV-infected cancer cells have several mechanisms for evading the immune system. The latest innovations concentrate more on modifying the microenvironment, eliminating T reg cells, inhibiting the immune attenuation via checkpoint inhibitors and stimulating the immune responses with immunostimulants (Skeate et al., 2016, van der Sluis et al., 2015).

3 AIMS OF THE STUDY

The Finnish Family HPV Study initiated in 1998 was originally designed to elucidate the dynamics of HPV infection in families, with a special focus on HPV transmission modes, early infections and the natural history of oral and genital HPV infections. The focus of my PhD studies was to elucidate the HPV-specific cell-mediated immune response in women with different HPV infection outcomes during the follow-up. More specifically, we determined the quantity and the function of the HPV 16-specific T memory cells and Th1 and Th2 cytokines in subgroups of women with different HPV infection outcomes during the FU. The leading hypothesis was that women with persistent HPV infection in the cervix or oral mucosa (or both) have impaired cell-mediated immune response to HPV.

The specific aims were:

1. to determine the concordance between genotype-specific HPV serology and HPV infection in the cervix and oral mucosa of the women during a 3-year follow-up.

2. to demonstrate HPV16-specific cell-mediated immunity in women with CIN and their children during a 14-year follow-up.

3. to compare HPV16-specific cell-mediated immunity in women who developed CIN with that of women who remained constantly negative for HPV-induced disease during a 10-year follow-up.

4. to analyze the differences in HPV16-specific cell-mediated immunity between women 1) with no HPV16 infection in oral mucosa, but HPV-seropositive or -negative during a 6-year follow-up, and 2) women with persistent oral HPV16 infection and testing either HPV-seropositive or -negative.

4 SUBJECTS, MATERIALS AND METHODS

4.1 SUBJECTS

4.1.1 Finnish Family HPV Study

The Finnish Family HPV Study is a longitudinal cohort study conducted at the University of Turku and Turku University Hospital, Finland. The main aim was to study the dynamics of HPV infections in mothers, fathers and their offspring. In total, 329 families were enrolled between 1998 and 2002, comprising 329 mothers enrolled in their 3rd trimester of pregnancy, 131 fathers and 331 newborns (includes two sets of twins). The Research Ethics Committee of Turku University Hospital approved the study protocol and its amendment (#2/1998, #2/2006, 45/180/2010 and TO7/008/2014). A total of 46 women were included in the cell-mediated immune studies.

4.1.2 Demographic data collected by questionnaire

At study onset and during the last visit (6-year time point), all women filled in a standardized questionnaire recording data on their socioeconomic status, general health, sexual and reproductive behavior, smoking and alcohol consumption, medication and history of sexually transmitted diseases (STDs) (Rintala et al., 2005, Louvanto et al., 2010a). Altogether, 69 items were included in the first questionnaire.

4.1.3 Classification of subjects

Written informed consent was obtained from all women included in this study (Papers I-IV).

Table 2. FU information from Papers I-IV.

Paper	Selection criteria	Number of subjects	Mean age (years)	Samples during the 6-year FU (paper I: during the 3-year FU)	Samples at 14-year timepoint
I	HPV DNA and antbody positive during FU	323 women	25.5 (18-38)	cervical and oral scrapings for HPV DNA analysis and genotyping blood sample for HPV antibody measurement	---
II, III	Cervical intraepithelial neoplasia (CIN)	10 women	37.0 (33.2-40.4)	oral and cervical scrapings for HPV DNA analysis PAP smear blood sample for HPV antibody measurement	blood sample for HPV16-specific CMI
III	HPV negative in genitals	22 women	40.0 (35.2-46.2)	oral scraping for HPV DNA analysis blood sample for HPV antibody measurement	blood sample for HPV16-specific CMI
IV	Oral HPV16 DNA+/ab+	4 women	39.5 (36.1-41.1)	oral and cervical scrapings for HPV DNA analysis blood sample for HPV antibody measurement	blood sample for HPV16-specific CMI genital, anal and oral scrapings for HPV DNA analysis PAP smear oral examination by dentist
IV	Oral HPV16 DNA+/ab-	6 women	38.5 (34.4-42.1)		
IV	Oral HPV16 DNA-/ab+	4 women	37.3 (35.4-39.9)		
IV	Oral HPV16 DNA-/ab-	7 women	39.8 (36.7-45.6)		
II	Children of CIN women	10 children	12.2 (11.2-13.2)	oral scraping for HPV DNA analysis blood sample for HPV antibody measurement	blood sample for HPV16-specific CMI

4.2 SAMPLE COLLECTIONS

4.2.1 Blood samples

(Paper I) Blood samples for serology analyses were taken at baseline and at 12, 24 and 36 months of FU. After collection, the blood samples were centrifuged at 2400 rpm for 10 min (Sorvall GLC-2; DuPont Instruments); the serum was divided into three 1 mL aliquots and stored first at −20 °C for no longer than 1 week and then at −70 °C until they were sent for analysis at the German Cancer Research Center (DKFZ), Heidelberg, Germany.

(Papers II-IV) Venous blood samples for the short-term lymphocyte stimulation test (LST) were collected from mothers (74 mL) and children (54 mL) in sodium-heparin collection tubes. The peripheral blood mononuclear cells (PBMCs) were isolated

within three hours by centrifugation over a Ficoll-Hypaque gradient (GE Healthcare Life Sciences, Uppsala, Sweden) and washed with PBS at the University of Turku, in the Department of Dentistry. Viable and dead cells were counted using trypan blue staining. To determine the proliferative capacity of HPV16-specific T cells by a short-term lymphocyte stimulation test, ~10×10^6 PBMCs were used and the remaining cells were frozen in 80% Fetal Bovine Serum (FBS, Biowest, EU quality) and 20% in DMSO (Merck, Darmstadt, Germany) (10 million PBMCs per mL per vial) using a NalgeneTM Cryo 1°C freezing container, which ensures a −1°C/min rate of cooling at −70°C, and after 1–2 days the cryopreserved PBMCs were stored in liquid nitrogen until further use. The median time from sample collection until the beginning of LST or cryopreservation was 3 hours. The serum from 9 mL of blood collected into a clotting tube was isolated by centrifugation for 7 minutes at 1000 g. The obtained autologous serum was used for short-term T cell proliferation assay.

4.2.2 Gynecological samples

(Papers I-IV) Cervical scrapings for HPV testing were taken at baseline and at 2, 12, 24 and 36 months FU visits. In Paper III, the cervical scrapings were taken at baseline, 2, 6, 12, 24, 36 months and 6.5 years. Cervical scrapings were taken with a cytobrush (Cytobrush; MedScand, Sweden) using 0.05 M PBS with 100 µg/mL gentamycin as a sampling medium. The samples were immediately frozen at −20 °C and stored at −70 °C.

(Paper II) Pap smears were taken at baseline, 6, 12, 24, 36 months, 6.5 years and after that at varying time points during the rest of the 14-year FU (Paper II, Additional File 1). Pap smears were taken by using a conventional three-sample technique with a wooden spatula and cytobrush (MedScand, Malmö, Sweden). The slides were fixed with a preservative (Spray-cyte, Sparks, Maryland, USA) and analyzed according to the Bethesda System (National Cancer Institute Workshop, 1988).

4.2.3 Anal samples

(Paper IV) Brush samples were taken from the anal canals of the women (Cytobrush®) by the gynecologist during a gynecological examination at the 14-year FU visit. Before sampling, Xylocain gel (AstraZeneca AB, Södertälje, Sweden) was spread on the anal area to make the sampling more pleasant for the woman. The samples were processed similarly to the gynecological samples.

4.2.4 Oral samples

(Papers I-IV) Scrapings from the oral cavity were taken from the buccal mucosa of both cheeks as well as from the upper and lower vestibular area using a small

cytobrush (Cytobrush; MedScan). The brush was immersed in 80% ethanol, frozen and stored at −70 °C until use.

4.3 HPV TESTING

4.3.1 HPV DNA testing

(Paper I) In the FU study visits, the HPV DNA was extracted from the scrapings using the high salt method (Miller et al., 1988). HPV testing was performed by using nested polymerase chain reaction (PCR) with MY09/MY11 as external primers and GP05+/GP06+ as internal primers (Snijders et al., 1990). The PCR products were hybridized with a digoxigenin-labeled HR-HPVoligoprobe cocktail (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56 and 58) (Anttila et al., 1999).

4.3.2 HPV genotyping

(Papers I, III, IV) HPV genotyping was performed using a Multimetrix kit (Progen Biotechnik), which detects the following 24 LR- and HR-HPV-genotypes: LR-HPV: 6, 11, 42, 43, 44 and 70 and HR-HPV: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82 (Schmitt et al., 2006). The test was performed as described in the protocol, except that we reamplified the nested PCR products for biotinylation with GP05+/bioGP06+-primers. The hybrids were analyzed using a Luminex analyzer (Bio-Plex 200 System; Bio-Rad Laboratories). The median fluorescence intensity (MFI) of at least 100 beads was computed for each bead set in the sample. The cut-off value for each run and HPV type was calculated as 1.5 times background MFI (negative control) + 5 MFI. All HPV16-positive samples were retested using the original sample, nested PCR and in-house bead-based HPV-genotyping method to exclude potential contamination.

4.3.3 HPV serology testing

(Papers I-IV) Antibodies to the major capsid protein L1 of HPV types 6, 11, 16, 18 and 45 were analyzed using multiplex HPV serology based on glutathione S-transferase fusion-protein capture on fluorescent beads, as described previously (Waterboer et al., 2005). Sera were scored as positive when the antigen-specific MFI values were greater than the cut-off level of 200 or 400 MFI (stringent) for the L1 antigen of individual HPV types (Michael et al., 2008). Seroconversion was defined by two conditions: (i) an MFI value below 200 in the first and above 200 in the second sample, and (ii) at least a twofold increase from the previous serum value. Similarly, in Paper I, antibody decay was defined by two conditions: (i) an MFI value above 200 in the first and below 200 in the second sample, and (ii) at least a twofold decrease from the previous serum value.

4.4 ASSAYS FOR CELL-MEDIATED IMMUNITY (II-IV)

4.4.1 HPV16-specific peptides

Panels of overlapping 30-35-mer peptides with HPV16 E2, E6, and E7 protein sequences were synthesized using the solid phase peptide synthesis (SPPS) method (ChinaPeptides Co. Shanghai, China), with a 14 or 15 amino acid overlap. Two pools of E2 peptides (E2.1 and E2.2) consisted of 12 or 11 (30-mer) peptides, respectively. Four pools of E6 and two pools of E7 peptides (E6.1-E6.4 and E7.1 and E7.2) consisted of two 32-mer or 35-mer peptides, respectively. Peptide quality was tested using mass spectrum and high-performance liquid chromatography (HPLC). Lyophilized peptides were dissolved in distilled H_2O or 10-20 µl DMSO, and then diluted in distilled H_2O to a final concentration of 62.5 µg/mL and stored at −20°C. These eight peptide pools were used to determine the proliferative capacity of HPV16-specific T cells, for cytokine polarization analysis and detecting the HPV16-specific Foxp3+ regulatory T cells. Memory response mix (MRM), consisting of tetanus toxoid, 0.75 fL/mL (Statens Serum Institut, Copenhagen, Denmark), Tuberculin PPD, 5 µg/mL (Statens Serum Institut), and Candida albicans, 155 µg/mL (Greer Laboratories, Lenoir, USA) was used as a positive control for the proliferation assays and cytokine production capacity of the PBMCs.

Table 3. HPV16 peptides divided into 8 peptide pools used in the lymphocyte stimulation test.

	HPV16					
	Pool	**Peptide**	**Amino acids**	**Pool**	**Peptide**	**Amino acids**
E2	E2.1	E2-1	1-30	E2.2	E2-13	181-210
		E2-2	16-45		E2-14	196-225
		E2-3	31-60		E2-15	211-240
		E2-4	46-75		E2-16	226-255
		E2-5	61-90		E2-17	241-270
		E2-6	76-105		E2-18	256-285
		E2-7	91-120		E2-19	271-300
		E2-8	106-135		E2-20	286-315
		E2-9	121-150		E2-21	301-330
		E2-10	136-165		E2-22	316-345
		E2-11	151-180		E2-23	331-365
		E2-12	166-195			
	Pool	**Peptide**	**Amino acids**	**Pool**	**Peptide**	**Amino acids**
E6	E6.1	E6-1	1-32	E6.3	E6-5	73-104
		E6-2	19-50		E6-6	91-122
	E6.2	E6-3	37-68	E6.4	E6-7	109-140
		E6-4	55-86		E6-8	127-158
	Pool	**Peptide**	**Amino acid**	**Pool**	**Peptide**	**Amino acid**
E7	E7.1	E7-1	1-35	E7.2	E7-3	43-77
		E7-2	22-56		E7-4	64-98

4.4.2 Lymphocyte stimulation test (LST)

The freshly isolated PBMCs with a density of 1.5×10^5 cells per well were seeded into U-bottomed 96-microtiter plates (Nunc, Roskilde, Denmark). Eight replicative wells were used for each peptide pool. The PBMCs were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Life Technologies, Belgium) containing 10% autologous serum and the given peptide pool at a final concentration of 5 µg/mL per peptide. PBMCs cultured with MRM were used as a positive control and with no antigen (medium-only) as a background control. After 6 days of culturing, the supernatants of each replicative well were collected for cytokine analysis, and a compensatory amount of IMDM supplied with 0.5µCi [3H]-Thymidine (PerkinElmer, Turku, Finland) per well was added. After 18 hours of incubation, the cells were harvested into Unifilter plates (PerkinElmer) using the FilterMateTM Cell Harvester (PerkinElmer). Subsequently, the filter plates were dried and counted on the 1450 MicroBeta+ counter (PerkinElmer). The cut-off value for counts per minute (CPM) values was determined by the average and 3×SD of the eight medium-only control wells. The stimulation index (SI) was calculated as the average of the eight tested wells divided by the average of the medium-only control wells. The proliferative response was defined as positive if the CPM values of at least six of the eight wells were above the cut-off value and the SI was ≥3.

4.4.3 Cytokine polarization analysis

The supernatants collected from LST at day 6 were analyzed using the BD cytometric bead array (CBA) Human Enhanced Sensitivity Flex Set System (BD Biosciences, Temse, Belgium) according to the manufacturer's instructions. Briefly, the flow cytometry-based CBA includes capture beads which have a distinct fluorescence, and these are coated with a capture antibody specific to a soluble protein. In our array, the levels of IFN-γ, TNF-α, IL-2, IL-5, IL-10, and IL-17A were determined. The capture beads and detection reagents were incubated with standards and unknown samples which contained the recognized analytes. During the incubation, sandwich complexes were formed that included the capture bead, analyte and detection reagent. These complexes can be analyzed with flow cytometry to identify particles with beads of known size and fluorescence. The detection limits for all cytokines were based on standard curves complying with the limits (274 fg/mL-823 fg/mL) suggested by the manufacturer. Positive antigen-induced cytokine production was defined as a cytokine concentration > 2× the concentration of the medium-only control.

4.4.4 HPV16- specific CD25+ CD4+ Foxp3+ regulatory T cell analysis

The frozen PBMCs were thawed and seeded into a 24-well plate (1.0×10^6 cells). The cells were cultured in IMDM containing 10% Human AB serum (Sigma-Aldrich, San Louis, USA), and the given peptide pools at a final concentration of 5 µg/mL per

peptide. PBMCs cultured without antigen (medium-only) were used as a background control. After 7 days of culturing, the cells were harvested, washed and stained first with surface markers CD25 (1:25) (Anti-CD25 FITC, clone M-A251, BD PharMingen, San Diego, CA), CD4 (1:100) (Anti-CD4-APC, clone RPA-T4, BD PharMingen), CD8 (1:30) (Anti-CD8 PerCP-Cy5.5; clone SK1, BD PharMingen). Subsequently, the cells were fixed and permeabilized using an intra-nuclear staining buffer set (FOXP3 Fix/Perm buffer set, Biolegend, San Diego, CA) according to the manufacturer's instructions. Before intracellular staining with Foxp3 (PE anti-human FOXP3, Biolegend) or isotype control (PE Mouse IgG1, κ Isotype Ctrl, Biolegend), blocking was done with 2% Fetal Calf Serum (FCS). After staining, the cells were washed and analyzed on the BD FACS Calibur cytometer (BD Bioscience). The fluorescent intensity of MRM-stimulated cells was used to set the gates for the other samples. An antigen-induced alteration in the population percentage was defined as a change of at least 2× the corresponding percentage in the medium-only control. In addition, after subtracting the background (medium-only) the cumulative percentage of antigen-stimulated Foxp3+ CD25+ CD4+ and Foxp3-CD25+ CD4+ cells from all wells were counted.

4.5 STATISTICAL ANALYSES (I-IV)

(I) All statistical analyses were performed using the SPSS (IBM, Inc., NY, USA, PASW Statistics version 18.0.3) and STATA (Stata Corp., College Station, TX, USA, version SE/11.1) software packages. Frequency tables were analyzed using the $\chi 2$ test or Fisher's exact test for categorical variables. Differences in the means of continuous variables (i.e. log-transformed HPV antibody titers) were analyzed using ANOVA (analysis of variance) after controlling for their normal distribution. The genotype-specific concordance was calculated between HPV antibodies and HPV DNA detection (in cervical and oral samples) at each time point during the FU period. We used regular (non-weighted) Cohen's Kappa, with 95% confidence intervals (CI) calculated using the exact method. The Kappa values were interpreted as follows: 0.00-0.20, poor agreement; 0.21-0.40, fair agreement; 0.41-0.60, moderate agreement; 0.61–0.80, substantial agreement; 0.81-1.00, almost perfect agreement. All tests were two-sided, and results with $p=<0.05$ were interpreted as statistically significant.

(II, III) All statistical analyses were run using an IBM SPSS® (IBM, Inc., NY, USA) software package (IBM SPSS Statistics for Windows, versions 20.0.0.1, 22.0.0.2 or 23.0.0.2). Frequency tables were analyzed using the $\chi 2$-test, with the likelihood ratio or Fisher's exact test for categorical variables. Differences in the means of continuous variables were analyzed using non-parametric (Mann-Whitney or Kruskal-Wallis) tests for two and multiple independent samples, respectively. A paired-samples test (Wilcoxon) was used to analyze the response levels in mother-child pairs. All statistical tests were two-sided and declared significant at p-value <0.05 level. The

Bonferroni correction was used to control for multiple comparisons between the cytokine results of the groups.

(IV) The means of secreted cytokine concentrations and proliferative responses of all groups were analyzed using a one-way ANOVA. The Bonferroni correction was used to control for multiple comparisons between the groups. The $\chi 2$ test was used to analyze the differences between the percentages of positive LST responses.

5 RESULTS

5.1 HPV6, 11, 16, 18 AND 45 SEROLOGY AND DNA CORRELATION (I)

5.1.1 No concordance between HPV serology and DNA

The serological response to the L1 proteins of HPV6, 11, 16, 18 and 45 was assessed in 323 women on four visits during the 36-month FU. In the pair-wise comparison of the HPV serology with the HPV DNA testing data from blood and genital samples taken simultaneously, no HPV genotype specific concordance was found (Paper I, Table 1). To account for the time lag in developing serological response after HPV infection, the same data were also analyzed by comparing the HPV status of samples taken 12 months apart (i.e. baseline HPV DNA compared with 12 month serology, 12 month DNA with 24 month serology, etc.) (Paper I, Table 2). Again, no concordance between HPV serology and HPV DNA was found.

5.1.2 Type-specific outcomes related to longitudinal HPV antibody levels

The women were divided into six different outcomes of their cervical HPV infections: (i) always negative, (ii) incident HPV infection (women who were HPV-negative at baseline and acquired new HPV infection during the FU), (iii) type-specific persistence, (iv) any HPV (non-type-specific) persistence, (v) fluctuation (intermittently HPV+ and HPV-), and (vi) clearance (transient) (Louvanto et al., 2010b, Louvanto et al., 2010a, Louvanto et al., 2011). The mean HPV antibody titers at each FU visit of the women according to these six outcomes of their cervical HPV16 infection are shown in Figure 11. The antibody titers of HPV genotype 18 at the 24 month visit are also shown. There were no differences (at any of the FU visits) in the titers of HPV 6, 11 and 45 antibodies between the six outcome patterns. Contradictory to that, the HPV16 antibody levels were significantly different between these HPV outcomes at all time points. The same was true for the HPV18 antibody titers measured at the 24-month FU visit.

Of the six outcome patterns, the highest (mean) levels of HPV16 antibodies were recorded among women who had cleared their HPV16 infection, whereas the lowest levels were detected among those who had acquired incident HPV16 infections (Figure 11).

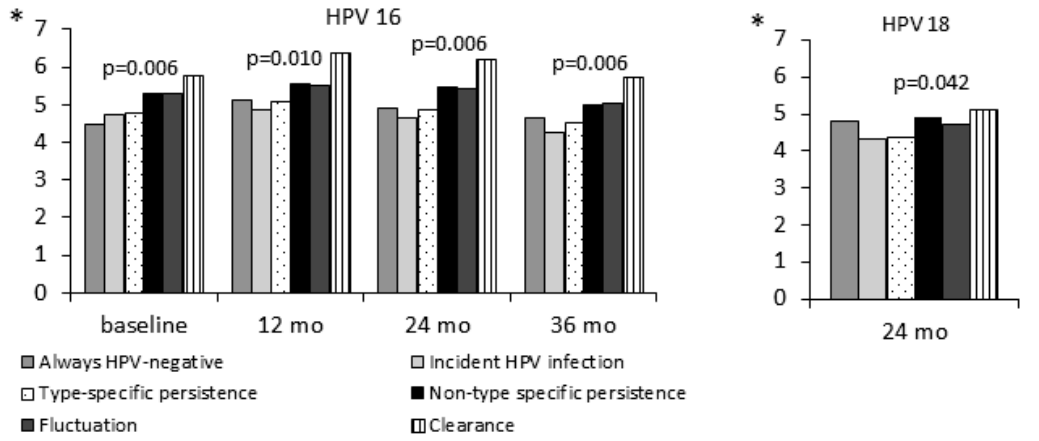

Figure 11. Mean antibody titers of HPV 16 and 18 according to the visit and the six different outcomes of their cervical HPV infection. The differences in HPV16 antibody titers between women with an incident and cleared infection were significant at all visits: p=0.024, p=0.002, p=0.007 and p=0.005, for baseline, 12, 24 and 36 months, respectively (ANOVA post-hoc analysis (LSD)). *Log-transformed mean titers (95% CI).

It was of interest to compare the dynamics of the genotype-specific HPV serology in two pairs of mothers: (i) those with incident HPV versus those remaining HPV-negative throughout the FU, and (ii) those with genotype-specific persistence versus those who cleared their infection. In the first pair (incident HPV vs. always HPV-negative), no differences in HPV antibody titers at any of the FU visits could be established (Paper I, Table 4). The same was true with the rates and times of type-specific seroconversion (positive or negative). In the second pair (persistent infections vs. clearance), a significant difference was found in HPV18 antibody titers, which were markedly higher among women who had cleared their HPV18 infections than in those with persistent HPV18 (Figure 12). However, the rate of seroconversion and the time for seroconversion were the same for all tested HPV antibodies, i.e. HPV6, 11, 16, 18 and 45.

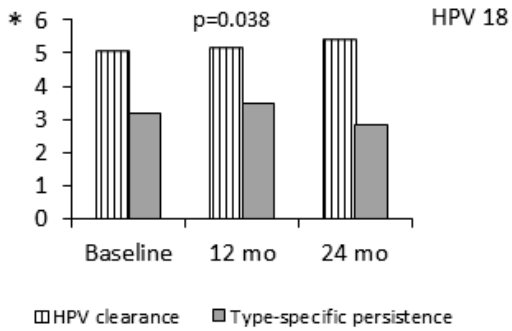

Figure 12. Mean antibody titers for women with persistent HPV18 infection and those who had cleared their genital HPV18 infection. *Log-transformed mean titers (95% CI).

Table 4. Paper II: Medical history of the mothers' cervical lesions, oral and genital HPV status and HPV serology of their children, followed since birth until the 14-year FU visit. The long-term persistence of HPV16 preceded all incident CIN2 and CIN3 lesions. ND = no data, NA = not applicable. * Time in months between the diagnosis of CIN and subsequent blood sampling for cell-mediated immune studies. The yellow and red square indicates when CIN was diagnosed and when the lesion was treated, respectively. All CIN2 and CIN3 lesions were treated with conization and followed by Pap tests according to routine. Three of the children were girls and seven were boys. None of these children had had any sexual contact or received prophylactic HPV vaccination before or during the cell-mediated immune study onset. Table modified from original publication II.

			baseline	3 d	1 mo	2 mo	6 mo	12 mo	18 mo	24 mo	30 mo	36 mo	42 mo	48 mo	54 mo	60 mo	66 mo	72 mo	78 mo	84 mo	90 mo	96 mo	102mo	108	120	124	Time of blood sample *
ID1	A	Cervical HPV	-			-		16,31		-		16,56															
		Pap/Colp	NSIL					NSIL	NSIL	NSIL		NSIL								NSIL		LSIL	CIN3	CIN1	NSIL		
		Antibodies	6,11,16,1					6,11,16,18		6,11,16		16															57
	B	oral HPV	-	-	-	-	-	-		-		-															
		Antibodies				-	-	-		-		-															
ID2	A	Cervical HPV	16,31,42			16,18		31		58	-	16,58									16						
		Pap/Colp	ASCUS			NSIL		ASCUS	NSIL	NSIL	NSIL/CI	NSIL/NCIN	NSIL/NCIN	NSIL/NCIN							NSIL	NSIL					
		Antibodies	6,16					16,11,18		6,16		6,18															129
	B	oral HPV	31	-	-	-	-	-		-		70						18					31				
		Antibodies								16,18																	
ID3	A	Cervical HPV	31			31		16, 70		6, 82		16															
		Pap/Colp	ASCUS					NSIL		NSIL		NSIL									LSIL	CIN2	CIN2	NSIL			
		Antibodies	6					6, 16, 18		6, 18																	62
	B	oral HPV	31	6	33	-	-	-		-								31									
		Antibodies				6	-	-		-		6															
ID4	A	Cervical HPV	-			-	-	58		18, 31		16, 39						-							31		
		Pap/Colp	NSIL					NSIL		NSIL		NSIL							NSIL		NSIL			HSIL	CIN2	CIN3	
		Antibodies	16					6, 16, 18		6, 16		6															31
	B	oral HPV	-	-	-	-	-	-		-		-															
		Antibodies			16			-		-		6															
ID5	A	Cervical HPV	-			-		16, 59		16		16								-	16	16					
		Pap/Colp	NSIL					NSIL		NSIL		NSIL						ASCUS	ASCUS		CIN3	CIN3	NCIN	NSIL			
		Antibodies	-					-		-		-															58
	B	oral HPV	6																								
		Antibodies				-	-	6		6, 18		16															
ID6	A	Cervical HPV	43			18,31		18			51	51							-					58	58		
		Pap/Colp	NSIL					ASCUS	LSIL, NCIN	NSIL, NCIN	ASCUS	ASCUS	NSIL/NCIN	LSIL/NCIN	ASCUS/NCIN		ASCUS		NSIL	NSIL		NSIL	HSIL	CIN3	CIN3	NCIN	
		Antibodies	6,16,18,4					6,16,18,45,			6,16,18	6,16,18															39
	B	oral HPV	-	-	18	-	-	-		-																	
		Antibodies					6	6		6		6															
ID7	A	Cervical HPV	16			-		16		16								16			16	16					
		Pap/Colp	NSIL					NSIL		NSIL								ASCUS	ASCUS		CIN3	CIN3	NSIL	NSIL			
		Antibodies	16					16		16																	51
	B	oral HPV	-	-	-	-	-	-		-		-															
		Antibodies			-	-	-	6		6																	
ID8	A	Cervical HPV	16			16		16		16		16					16	16									
		Pap/Colp	NSIL					NSIL		NSIL		NSIL				CIN2	NCIN	NCIN/ NSIL	NSIL	NSIL			CIN2				
		Antibodies	-					-		-		-															78
	B	oral HPV	16		16	-	-	-		-		-						-									
		Antibodies			-	-	6	6		-		-						-									
ID9	A	Cervical HPV	16,59			16,59												16	16			-					
		Pap/Colp																ASCUS	CIN2	CIN2	CIN1	CIN2	NSIL				
		Antibodies	6																								59
	B	oral HPV	-	-	-		-											-									
		Antibodies			6		-																				
ID10	A	Cervical HPV	-			-		-			-	16							16, 56				16, 66	16, 66			
		Pap/Colp	NSIL					NSIL			NSIL	NSIL						NSIL	LSIL	SIL, NCIN			LSIL/	NCIN			
		Antibodies	-					16			-																34
	B	oral HPV	16		16	-	-	-			-	-						-									
		Antibodies			-	-		6			-	-															
			baseline	3 d	1 mo	2 mo	6 mo	12 mo	18 mo	24 mo	30 mo	36 mo	42 mo	48 mo	54 mo	60 mo	66 mo	72 mo	78 mo	84 mo	90 mo	96 mo	102mo	108 mo	120 mo	124 mo	

Table 5, Paper III: HPV statuses of the 22 women who tested HPV-negative in their cervical samples and who remained HPV-negative in their cervix during FU. ND=no data. Table modified from original publication III.

	ID	Subject	baseline	2 mo	6 mo	12 mo	24 mo	36 mo	42 mo	48 mo	54 mo	60 mo	66 mo	72 mo	78 mo	84 mo	90 mo	96 mo	102 mo	108 mo	120 mo	124 mo
Cervical HPV disease free women	11	Oral HPV				70		16,18														
		Antibodies	11		ND	11	11	11														
	12	Oral HPV			ND			ND						ND								
		Antibodies			ND	ND	ND															
	13	Oral HPV																				
		Antibodies	6,11,16,18		ND	6,11,16,18,45	6,11,16,18	6,11,16														
	14	Oral HPV				58																
		Antibodies	18		ND	6,18	6,18	18														
	15	Oral HPV			ND	ND		ND						ND								
		Antibodies	6,11,16		ND	6	ND	ND														
	16	Oral HPV			16		16	16						ND								
		Antibodies	6		ND	6	6	6														
	17	Oral HPV	6											ND								
		Antibodies			ND	6,11,16,18,45	6,11,16,18,45	6,18														
	18	Oral HPV																				
		Antibodies	18		ND	18	18	18														
	19	Oral HPV	16	16			16															
		Antibodies	6,11,16		ND	6,11,16	6,11,16	6,16														
	20	Oral HPV												ND								
		Antibodies	6		ND	6	6,11	6,11,18														
	21	Oral HPV			70									ND								
		Antibodies			ND																	
	22	Oral HPV		16	16									ND								
		Antibodies			ND	6	6	16														
	23	Oral HPV												ND								
		Antibodies	6		ND	6,11,16,18,45	6,16,18	16,18														
	24	Oral HPV												ND								
		Antibodies	6		ND																	
	25	Oral HPV					16	16						ND								
		Antibodies	16,18		ND	6,16,18	6,16,18	16,18,45														
	26	Oral HPV																				
		Antibodies			ND																	
	27	Oral HPV		16				ND														
		Antibodies	6		ND	6	6	ND														
	28	Oral HPV		16		16		ND						ND								
		Antibodies	6		ND	6,11	ND	ND														
	29	Oral HPV																				
		Antibodies	6		ND	6,11,16,18,45	6,16,18	6,11,18														
	30	Oral HPV						16						ND								
		Antibodies	6		ND	6	6	6														
	31	Oral HPV		16	16		multiple types															
		Antibodies	6		ND	6	6	6														
	32	Oral HPV												ND								
		Antibodies	6,11,16,18,45		ND	6,11,16,18,45	ND	ND														

5.2 HPV16-SPECIFIC PROLIFERATIVE T CELL RESPONSE

5.2.1 Follow-up information

The structured questionnaire for recording the demographic data and the potential risk factors were collected at the baseline visit, and repeated at the 36-month and 6-year visits (Louvanto et al., 2010a, Rintala et al., 2005, Rautava et al., 2012, Syrjänen et al., 2009). The 10 women who developed HPV16-induced CIN lesions had longer FU from the hospital records (Rintala et al., 2012).

Table 6. Paper IV: HPV-specific genital, oral and serology data for the women during the 7-year FU. At the 14-year FU visit, samplings for genital, anal and oral HPV testing were performed and the results are summarized here together with the results of the Pap test. Oral examinations were performed by a dentist. Table modified from original publication IV.

												14 years				
Group	Mean age	ID		Baseline	2-mo	6-mo	12-mo	24-mo	36-mo	72-mo	7 years	HPV DNA genital	PAP smear	HPV DNA anal	HPV DNA oral	Oral examination
Oral HPV16 +/Ab+	39.5	40	HPV DNA gen	neg	neg		neg	neg	neg		na	neg	pap 1	neg	neg	healthy mucosa
			HPV DNA oral	neg	neg	neg	neg	16	16	na						
			Serology	6,11,16,18,45			6,11,18,45	6,11,16,18,45	6,11,16,18,45							
		41	HPV DNA gen	neg	neg		neg	neg	neg		neg	neg	pap 1	neg	16	healthy mucosa, lingual tori
			HPV DNA oral	16	16	neg	neg	16	neg	neg						
			Serology	6,11,16			6,11,16	6,11,16	6,16							
		42	HPV DNA gen	neg	neg		neg	neg	neg		na	11	pap 1	11	neg	healthy mucosa
			HPV DNA oral	neg	neg	neg	neg	16	16	na						
			Serology	16,18,45			6,16,18,45	6,16,18,45	16,18,45							
		43	HPV DNA gen	neg	neg		neg	neg	16		16, 18	11	pap 1	neg	6	healthy mucosa
			HPV DNA oral	66	16	neg	neg	neg	neg	neg						
			Serology	16			16	6,11	6,11,16							
Oral HPV16+/Ab–	38.4	44	HPV DNA gen	neg	neg		neg	neg	neg		na	73	pap 1	53	16	Mechanical trauma on lip
			HPV DNA oral	neg	neg	16	neg	16	16	na						
			Serology	6			6	6	6							
		45	HPV DNA gen	neg	16		neg	16	16		16	neg	pap 1	neg	6, 16	linea alba on both buccal mucosa
			HPV DNA oral	16	66	16	neg	16	neg	na						
			Serology	neg			neg	neg	neg							
		46	HPV DNA gen	neg	neg		16	16	16		neg	11	asc-us	neg	neg	healthy mucosa
			HPV DNA oral	16	16	na	6	neg	neg	neg						
			Serology	neg			neg	neg	neg							
		47	HPV DNA gen	neg	neg		na	na	na		na	na	na	na	neg	healthy mucosa
			HPV DNA oral	16	16	na	na	na	na	na						
			Serology	6			na	na	na							
		48	HPV DNA gen	neg	neg		neg	neg	neg		na	neg	pap 1	neg	neg	healthy mucosa, manibular tori
			HPV DNA oral	neg	16	16	neg	neg	neg	na						
			Serology	neg			6	6	16							
		49	HPV DNA gen	neg	neg		neg	neg	neg		neg	11	pap 1	neg	neg	healthy mucosa
			HPV DNA oral	neg	16	16	neg	16,18	neg	neg						
			Serology	6			6	6	6							
Oral HPV16–/Ab+	37.5	50	HPV DNA gen	neg	neg		16,82	neg	neg		16	neg	pap 1	neg	6	tiny hyperplasia of mucosa
			HPV DNA oral	neg	neg	neg	neg	neg	neg	neg						
			Serology	neg			6,11,16,18,45	6	na							
		51	HPV DNA gen	neg	neg		neg	neg	neg		na	neg	pap 1	39	neg	healthy mucosa
			HPV DNA oral	neg	neg	neg	neg	neg	neg	na						
			Serology	6			6,11,16,18,45	6,16,18	16,18							
		52	HPV DNA gen	neg	neg		16,33,35,58,59	33,59	33,59		neg	neg	pap 1	neg	neg	mandibular torus, left side
			HPV DNA oral	neg	neg	neg	neg	neg	neg	neg						
			Serology	6			6,18	neg	6,16,18							
		53	HPV DNA gen	neg	neg		neg	neg	neg		neg	neg	pap 1	neg	6	hyperplasia on leftbuccal mucosa, palatine torus
			HPV DNA oral	neg	neg	neg	neg	neg	neg	neg						
			Serology	6			6,11,16,18,45	6,16,18,45	6,11,18,45							
Oral HPV16–/Ab–	39.2	54	HPV DNA gen	neg	neg		na	na	na		na	na	na	na	neg	healthy mucosa, herpes simplex infection in lower lip
			HPV DNA oral	neg	neg	na	na	na	na	na						
			Serology	neg			na	na	na							
		55	HPV DNA gen	neg	neg		na	na	na		na	na	pap 1	na	6	healthy mucosa
			HPV DNA oral	neg	neg	na	na	na	na	na						
			Serology	6			na	na	na							
		56	HPV DNA gen	neg	neg		16	16	neg		neg	neg	pap 1	neg	neg	healthy mucosa
			HPV DNA oral	neg	neg	neg	neg	neg	neg	neg						
			Serology	6			6	6	6							
		57	HPV DNA gen	neg	neg		neg	neg	neg		neg	neg	pap 1	neg	neg	heangioma (3mm), upper lip
			HPV DNA oral	neg	neg	neg	neg	neg	neg	neg						
			Serology	neg			neg	neg	neg							
		58	HPV DNA gen	neg	neg		neg	16	6		16	neg	pap 1	neg	11	linea alba in both side off buccal mucosa
			HPV DNA oral	neg	neg	na	neg	neg	neg	na						
			Serology	6			6	na	6							
		59	HPV DNA gen	neg	neg		na	na	na		neg	neg	pap 1	neg	neg	small hyperplasia in frenulum
			HPV DNA oral	neg	neg	neg	na	na	na	neg						
			Serology	neg			na	na	na							
		60	HPV DNA gen	neg	neg		16,18	16	na		na	neg	pap 1	neg	neg	linea alba in both sides of buccal mucosa
			HPV DNA oral	neg	neg	neg	neg	neg	na	na						
			Serology	6			neg	neg	na							

5.2.2 Summary of LST, cytokine and Foxp3 results for all individuals

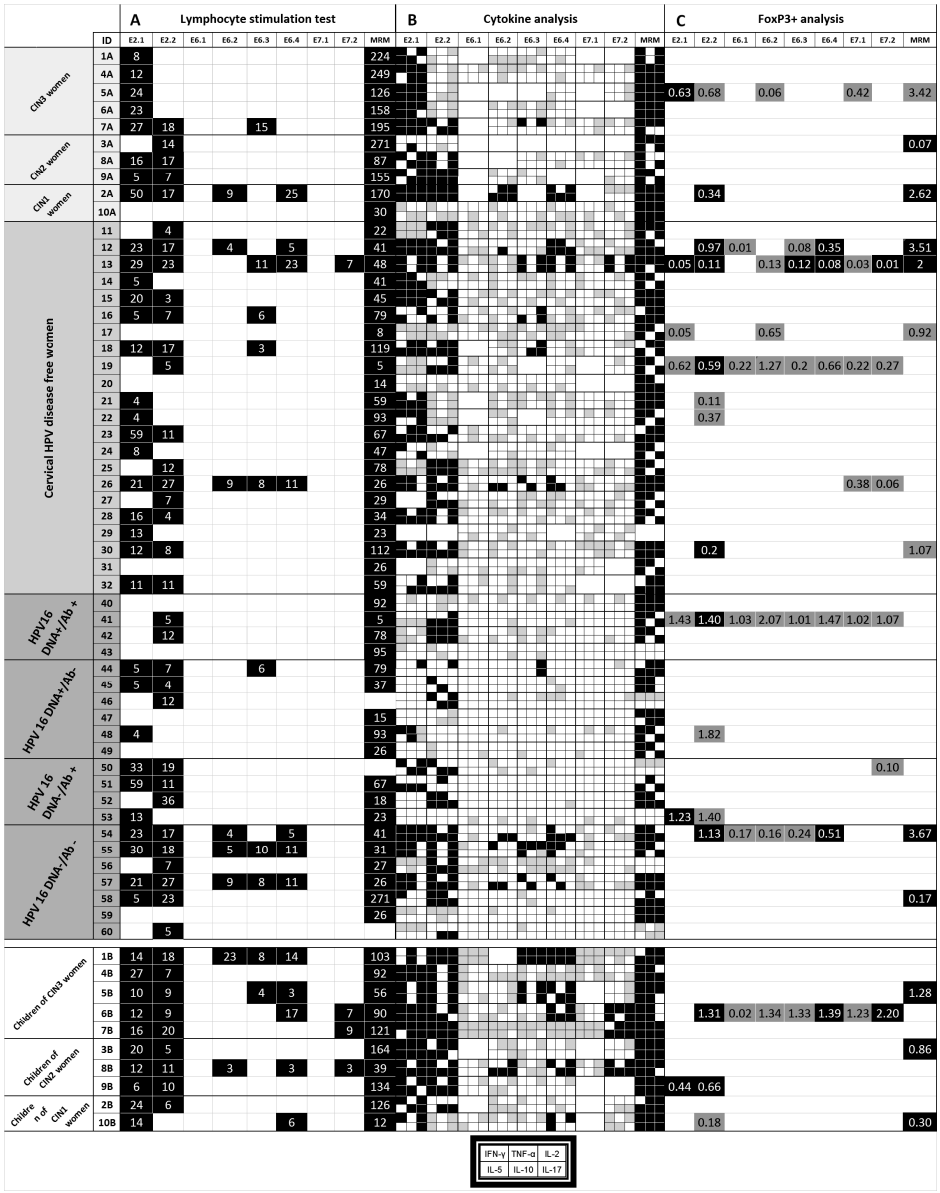

Figure 13. **Results of LST, cytokine and Foxp3 analysis of all subgroups.** Memory response mix (MRM) was used as a positive control. A) Only the positive results are presented with *black boxes* and the SI value is mentioned in the box in LST. B) Supernatants from the LST were analyzed for the presence of cytokines IFN-γ, TNF-α, IL-2, IL-5, IL-10, and IL-17A. One square in a box with 6 squares represents the production of the cytokine type as given in the index box lowest in the figure. Cytokines found in the supernatants taken from the wells with positive proliferative responses are marked with *dark colors*. *Lighter colors* are used when the corresponding LST result was negative. C) Positive (upregulation of T regs) responses are presented in the black boxes with the value measured. A *colored box* indicates the coincidence positive response in LST test. Upregulation of T regs is defined as at least twice as high as the percentage of T regs in the medium-only control.

5.2.3 All tested subgroups showed positive responses in the lymphocyte stimulation test

The PBMC derived from the women who tested oral HPV16-negative but HPV16-seropositive most commonly proliferated into E2-specific peptides among all women. The children analyzed in Paper II had even more common HPV16 E2-specific responses than any group of women described here. On the other hand, the response to HPV16 E2 peptides was infrequent in women who had oral HPV16 infection, regardless of the presence of HPV16 antibodies. The HPV16E6-specific response was prevalent in women who remained oral HPV16DNA- and HPV16-seronegative. In contrast, the PBMCs of women with oral HPV16 DNA and without any HPV antibodies less commonly showed proliferation after stimulation with the E6 peptides. E7-specific proliferation was found in only two groups: in women who were HPV-DNA-negative in their cervix during the entire FU and in the children of mothers who developed incident CIN (Figure 14).

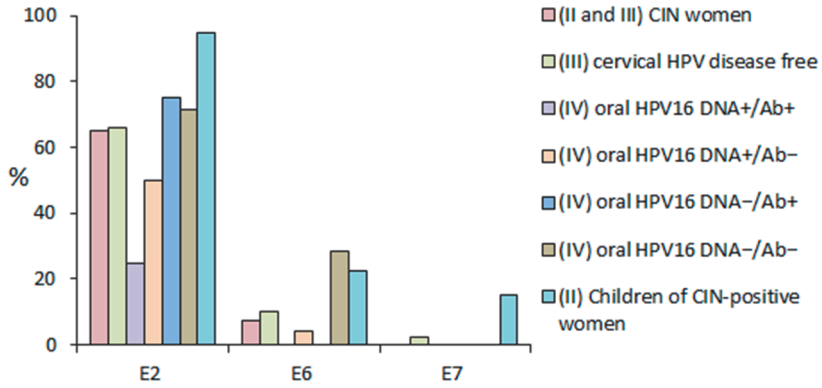

Figure 14. Proportional percentages of women with LST-positive responses after stimulation of PBMCs with HPV16-specific E2 , E6 and E7 peptides in all subgroups included in the present doctoral thesis.

Considering the median levels of the LST, the E2-specific peptides showed the highest SI in all groups and the range was also widest. The E6-specific responses were fairly equal between the study groups. Only one woman had a response to E7 peptides with an SI value of 7. The PBMC derived from three children responded to HPV16 E7 peptides (Figure 15).

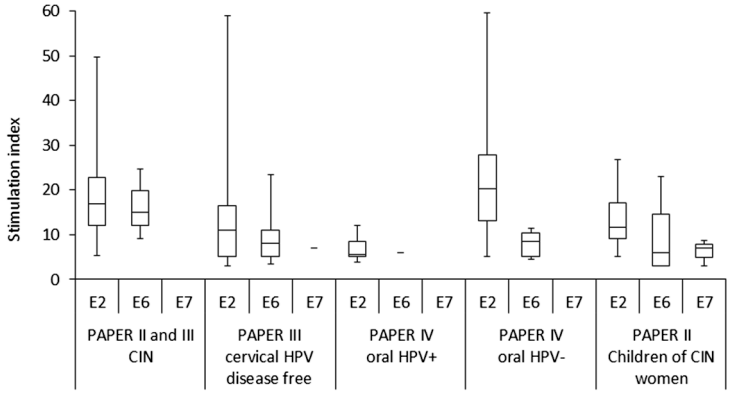

Figure 15. Median values of LST-positive responses after HPV16 peptide stimulation in different subgroups of women included in this doctoral thesis. The bars indicate the minimum and maximum values.

5.2.4 Women with CIN lesions might provide a HPV-specific protective immunity to their offspring (II)

(II) The lymphocyte proliferation capacities of PBMCs were analyzed for 10 women and their 10 offspring after stimulation with HPV16 E2, E6, and E7 peptides. The offspring had more HPV16 E2-specific LST-positivity than their mothers (p=0.018) (Figure 16). None of the mothers had any response to E7 peptides, but in three of their offspring the median SI for E7 peptides was 6.93 (range 3.00-8.63).

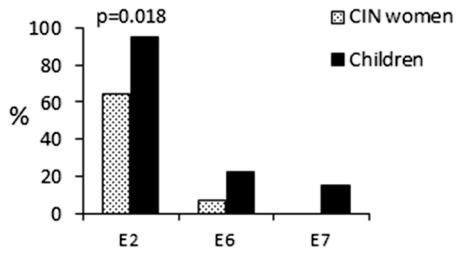

Figure 16. Percentage of CIN mothers and their offspring with LST-positive responses. The responses to two E2 peptide pools were summed together, as was done for the four E6 peptide pools and the two E7 peptide pools. The stimulation response of PBMC with E2 peptide pools in mothers and their offspring were statistically significantly different. (p=0.018).

5.2.5 LST results for women with past CIN lesions and HPV disease-free women were fairly similar

(III) The PBMC responses against the HPV16-specific E2, E6 and E7 peptides were analyzed in the CIN women and their controls who remained HPV-negative in their cervical samples during the entire FU.

HPV16 E2-, E6-, and E7-specific LST-positivity was nearly identical in the case and control women (Figure 17).

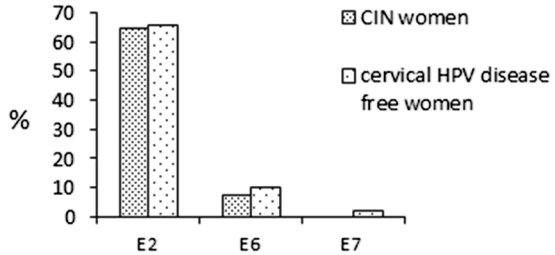

Figure 17. Percentage of LST-positive responses in CIN women and their HPV-negative controls. The results achieved with different peptide pools of HPV16 E2, E6 and E7 were summed together.

Risk factors of HPV infection as related to the LST results of the women

Based on the questionnaire, 70.0% (7/10) of the CIN women and 38.1% of the HPV-negative control women (8/21, data missing for one woman) were smokers. Previous genital warts were recorded by the cases in 10.0% (1/10) and in 40.0% (8/20) of the controls, the differences being not statistically significant. No significant differences were seen between the groups in terms of medication, number of sex partners, sexual habits, alcohol consumption and STDs. Smokers and users of alcohol had significantly higher LST MRM responses than the nonsmokers or nondrinkers (p=0.032 and 0.045, respectively), but no differences were found in cell-mediated immune responses against HPV16 E2, E6 and E7 peptides.

Women with a higher number (>10) of previous sex partners had a higher response to HPV16 E6-3 than women with fewer sex partners (p=0.047).

Based on oral HPV DNA and HPV serostatus, the following subgroups of women were included in the cell-mediated immune studies: Group 1, women with cervical HPV16 and a CIN lesion caused by HPV16 (n=5); Group 2, women with cervical HPV16, but a CIN lesion associated with another or unknown HPV genotype (n=5); Group 3, women with oral HPV16 and HPV16 serology, without any cervical HPV infection (n=14); Group 4, women with oral HPV16, but neither HPV16 serology nor cervical HPV16 infection (n=8). The only statistically significant difference among the groups was found in SI values after stimulation with MRM, which resulted in significantly higher SI values in Group 1 than in Groups 3 and 4 (p=0.023 and p=0.021) while the highest SI values were found in Group 2 (p=0.0001) (Paper III, table I).

5.2.6 Women with persistent oral HPV infection and women without oral HPV infection (IV)

The HPV16-specific E2, E6 and E7 peptides were used to assess the T lymphocyte proliferation capacity of the women, who were divided into four subgroups: 1) HPV16 DNA+/Ab+, 2) HPV16 DNA+/Ab–, 3) HPV16 DNA–/Ab+, 4) HPV16 DNA–/Ab–.

There were statistically significant differences in E2-specific positive responses among the different groups, as shown in Figure 18. The E6-specific response was also statistically significantly higher in the HPV16–/Ab+ group than in the HPV16+/Ab+ group (p=0.020). Women who were oral HPV16 DNA-negative and either HPV-seronegative or -positive had more LST-positivity after stimulation with E2 or E6 peptides (pooled data) than the other two groups. Women who were oral HPV16-DNA-positive and HPV-seropositive responded less to E2 peptides than the women from the other subgroups. There were statistically significant differences in responses for both the E2 and E6 peptide pools after grouping all women into two groups according to their oral HPV16 DNA status (Figure 19).

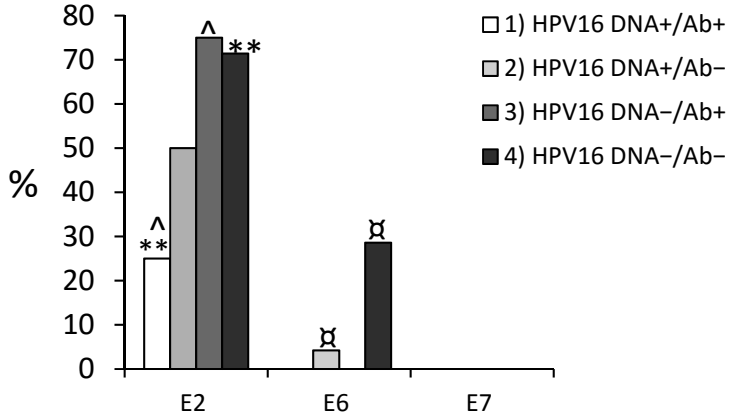

Figure 18. Proportional percentage of LST-positive responses in four different subgroups after stimulation of PBMCs with HPV16-specific E2, E6 and E7 peptides. There were statistically significant E2-specific responses between Groups 1 and 4 (**p=0.046) and between Groups 1 and 3 (^p=0.035). E6-specific responses were different between Groups 2 and 4 (¤p= 0.020). Figure modified from original publication IV.

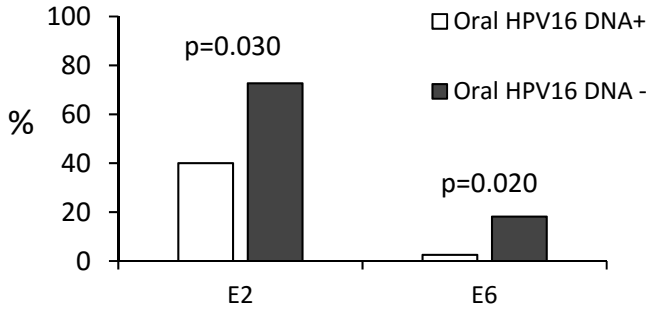

Figure 19. LST-positivity in women grouped according to their oral HPV status. Groups 1 and 2 formed a group of oral HPV16 DNA-positive women and Groups 3 and 4 a group of oral HPV16 DNA-negative women. Women who were oral HPV16 DNA-negative had more LST-positivity for both E2- and E6-specific peptide pools than women who were oral HPV16 DNA-positive (p< 0.030 and 0.020, respectively). Figure modified from original publication IV.

5.3 CYTOKINE PRODUCTION

5.3.1 Th2-type cytokines were more prominent in HPV-positive women than HPV-negative ones

The cytokine levels of IFN-γ, TNF-α, IL-2, IL-5, IL-10, and IL-17A were analyzed after a stimulation of HPV16-specific E2, E6 and E7 peptide pools. The cytokines were calculated only from the LST-positive test after seven days of incubation with HPV16 peptides.

When comparing the cytokine polarization among all groups, the most explicit difference between HPV-positive and HPV-negative women was the absence of IL-5 in the HPV-positive women. Only CIN women secreted IL-5, regardless of being HPV-positive during the FU. The proportion of IFN-γ among all secreted cytokines was greater in HPV-negative women than HPV-positive women (Figure 20). Overall, the Th1-type cytokines (IFN-γ, TNF-α, IL-2) were secreted more with a higher proportion in HPV-negative women than in HPV-positive women, whereas Th2-type cytokines (IL-5, IL-10) were more prominent in HPV-positive women. The cytokine profile of the children was fairly similar to that of HPV-negative women.

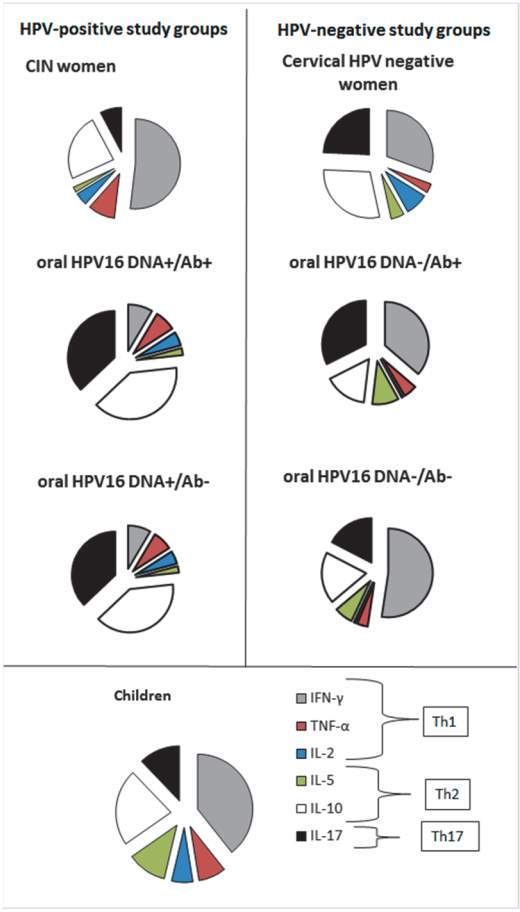

Figure 20. Proportion of LST-positive cytokine production after HPV16-stimulation. The proportional part of the cytokine was calculated from LST-positive cytokine responses of HPV16-specific E2-, E6- and E7-stimulated samples, which were summed together and divided by the sum of all cytokines secreted of the individual studied. The mean value was calculated from the ratios of all individuals.

The Th1-type cytokine IFN-γ was the most common and was secreted at the highest levels in all subgroups studied. Its concentration declined in the following order in the subgroups: oral HPV16-DNA-negative women, cervical HPV-negative women, children, CIN women and oral HPV16-positive women. Other Th1-type cytokines such as TNF-α and IL-2 were secreted only at low levels. The IL-10 was one of the Th2-type cytokines which were secreted at high level. The oral HPV16-DNA-positive women had the highest median level of IL-10, followed by oral HPV16-DNA-negative women, HPV disease-free women, children of CIN women and CIN women. The levels of IL-17A also differed among the subgroups. Oral HPV16-DNA-positive women had the highest levels of IL-17A (Figure 21).

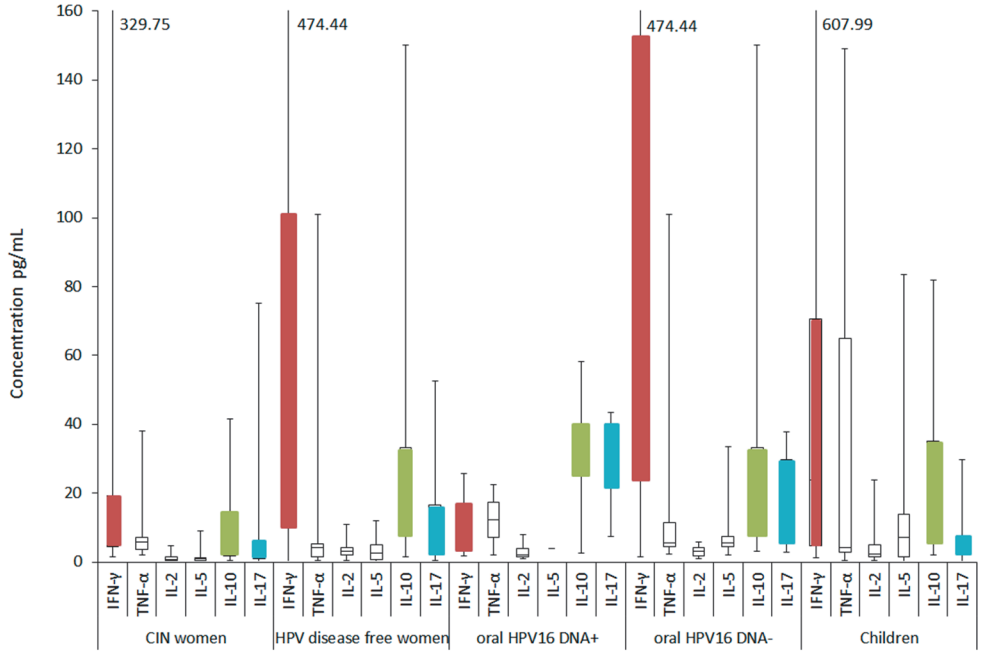

Figure 21. Cytokine secretion according to the subgroups after stimulation with HPV16-spesific peptides (E2.1, E2.2, E6.1, E6.2, E6.3, E6.4, E7.1 and E7.2). Only the values from LST-positive wells are included. The box is bounded on the top by the third quartile and the bottom by the first quartile, and divided by the median. The minimum and maximum are indicated by the whiskers.

5.3.2 The offspring of women with the past CIN neoplasia showed higher cytokine levels than their mothers (II)

Cytokines were measured in women with CIN lesions and their sexually unexperienced offspring. When comparing the mean values of mothers and their offspring, the cytokine levels of IL-2 (p=0.023), and IL-5 (p=0.028), were higher in offspring than in their mothers for all peptide pools, irrespective of the proliferative status. The offspring of mothers who had developed CIN3, showed significantly

higher IFN-γ (p=0.032) and TNF-α (p=0.008) levels than the other children. In the pair-wise comparison of mother-child pairs, the levels of IL-2 and IL-5 were statistically significantly different between the mother and offspring, with p-values of 0.013 and 0.009, respectively.

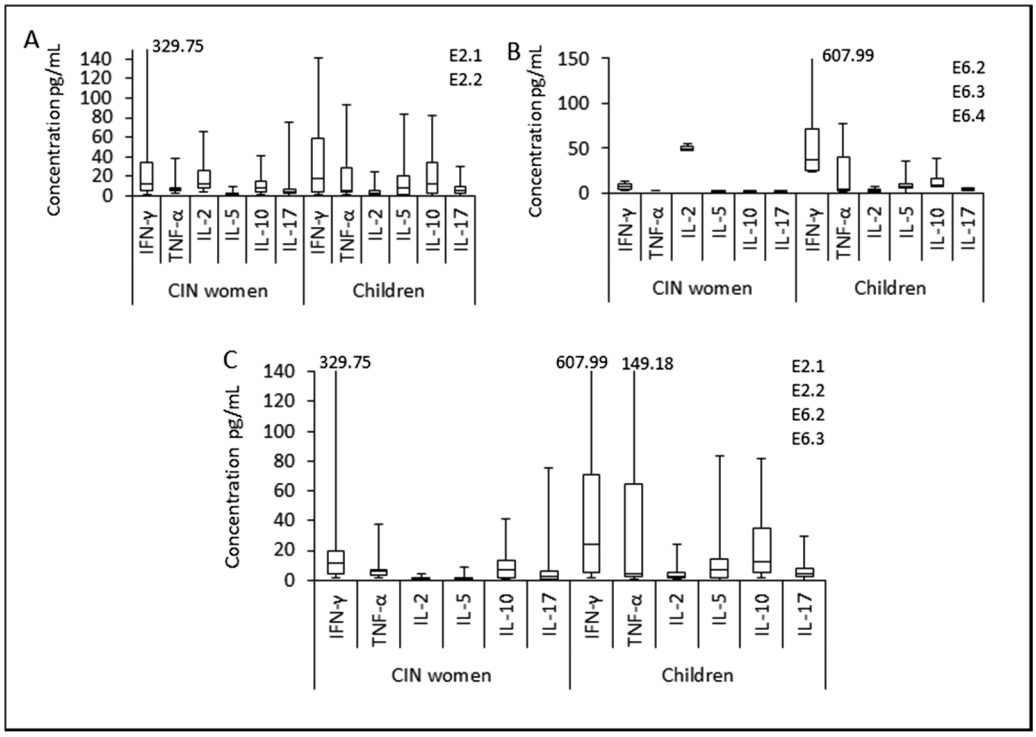

Figure 22. IFN-γ, TNF-α, IL-2, IL-5, IL-10, and IL-17A concentrations after stimulation with A) E2.1 and E2.2 peptides, B) E6.2, E6.3 and E6.4 peptides and C) all HPV16-specific peptides from LST-positive wells. The box is bounded on the top by the third quartile, the bottom by the first quartile, and divided by the median. The minimum and maximum are indicated by the whiskers.

5.3.3 No remarkable differences in cytokine analysis of women with past CIN lesions and cervical HPV disease-free women (III)

No significant differences were found between the CIN women and their controls except for IL-17A. IL-17A levels were statistically significantly higher in the controls (p=0.035) after HPV16 E6 peptide stimulation than in the CIN women. Figure 23 presents the median values of the measured cytokines from the LST-positive samples.

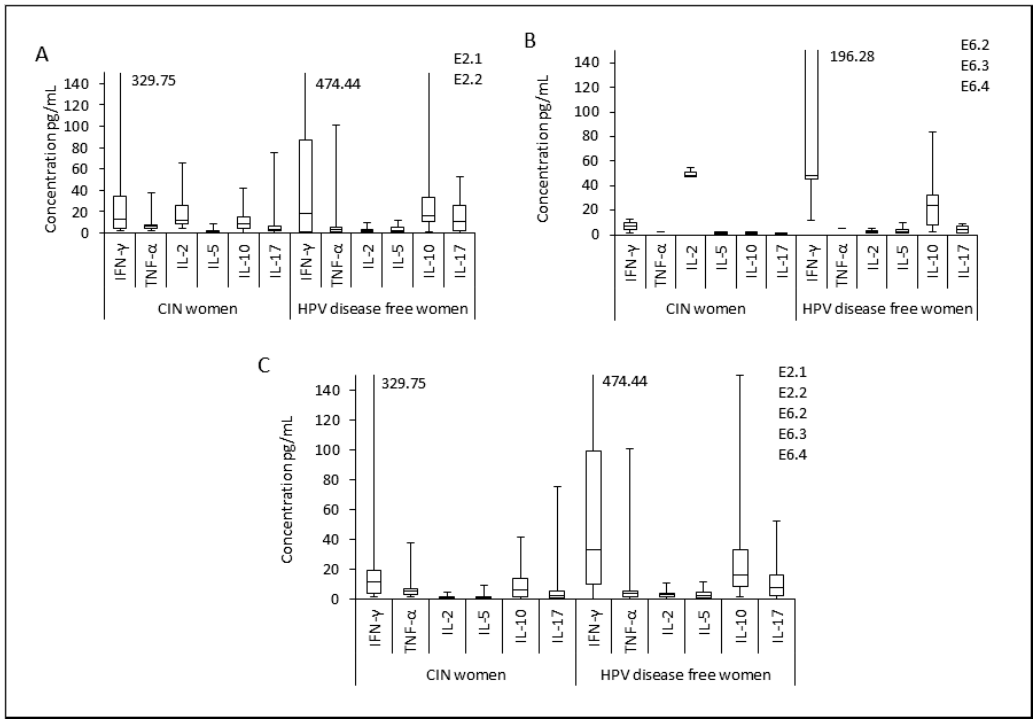

Figure 23. IFN-γ, TNF-α, IL-2, IL-5, IL-10, and IL-17A concentrations after stimulation with A) E2.1 and E2.2 peptides, B) E6.2, E6.3 and E6.4 peptides and C) all HPV16-specific peptides from LST-positive wells. The box is bounded on the top by the third quartile, the bottom by the first quartile, and divided by the median. The minimum and maximum are indicated by the whiskers.

Based on their oral HPV and HPV serostatus, women were classified into four groups as follows: Group 1, women with cervical HPV16 and a CIN lesion caused by HPV16 (n=5); Group 2, women with cervical HPV16, but a CIN lesion associated with another or unknown HPV genotype (n=5); Group 3, women with oral HPV16 and HPV16-specific antibodies, but no cervical HPV infection (n=14); Group 4, women with oral HPV16, but neither HPV16-specific antibodies nor cervical HPV16 infection (n=8). Group 4 had the highest IFN-γ secretion levels when stimulated by the HPV16 E6-4 peptide pool (p=0.034) and also the highest IFN-γ levels when the responses for all HPV16 E6 peptide pools were pooled together (p=0.050).

5.3.4 Women with persistent oral HPV infection showed more Th2-type cytokines (IV)

Women were divided into four subgroups: 1) HPV16 DNA+/Ab+, 2) HPV16 DNA+/Ab–, 3) HPV16 DNA–/Ab+, 4) HPV16 DNA–/Ab–. When all cytokine secretions irrespective of the HPV 16 peptides resulting in positive LST were analyzed, IFN-γ, IL-10 and IL-5 were the most commonly secreted in the group of women with

HPV16 DNA–/Ab– (Group 4) 1, IL-10, IL-17A and IFN-γ in Group 3 (HPV16 DNA–/Ab+) and IL-2, IL-10 and IFN-γ in Group 2 (HPV16 DNA+/Ab–).

There were only two LST-positive samples in Group 1, and therefore the cytokine results were not comparable to the other groups. IL-2, IL-5, IL-10 and IL-17A were all secreted similarly in both cases. There was no IL-4 secretion in any of the groups (Figure 13). Because there was no E7-specific LST-positivity, no E7-specific cytokine secretion was analyzed.

The proportional ratios of each cytokines were analyzed. When the four groups were combined to create two groups (HPV16-DNA-negative women and HPV16-DNA-positive women), there was a statistically significant difference in the ratios of IFN-γ and IL-5 (Figure 24).

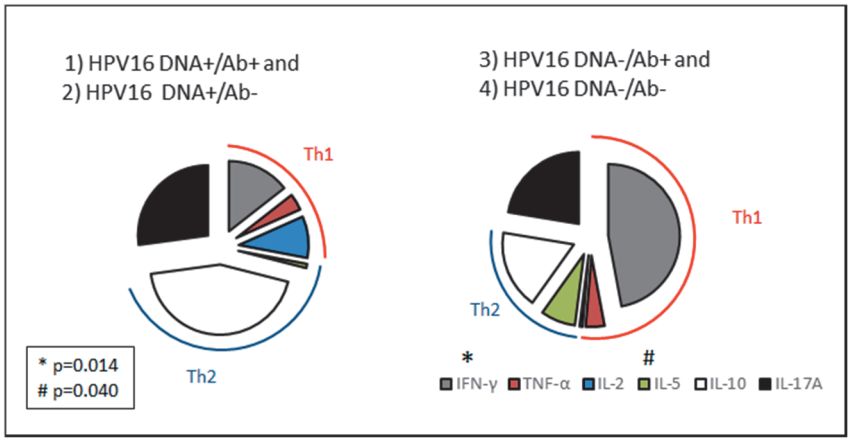

Figure 24. Proportional ratios of cytokines measured from LST-positive samples as related to the total cytokine secretion. The smaller subgroups were combined according to HPV16 status. The cytokines were summed from the LST-positive samples from each woman studied. The mean values of both the LST+ and LST– samples were also calculated. Then the LST+ cytokines were divided by cytokine values of the LST+ and LST– samples. Finally, the mean values of the given ratios were calculated for each subgroup. Figure modified from original publication IV.

Women who were HPV16-DNA-negative during the FU secreted more IFN-γ after E2.1 and E2.2 peptide stimulation than women who were HPV16-DNA-positive. In contrast, IL-10 and IL-17A levels were higher in the HPV16-DNA-positive women (Figure 25). The E6-specific (E6.2, E6.3, E6.4) cytokine levels were low in total, especially in the HPV16-DNA- positive group, because there was only one woman positive for E6 peptide (E6.3) in the LST. Women in Group 4 (HPV16 DNA-/Ab-) showed the highest cytokine levels compared to the other three groups when all cytokines secreted from E2.1, E2.2, E6.2, E6.3 and E.64 peptide pools were summed together. The concentrations of IFN-γ and IL-10 were highest in Group 4, while IFN-γ and IL-17A levels were highest in Group 3. IL-10 was the dominant cytokine in both Groups 2 (HPV16 DNA+/Ab-) and 1 (HPV16 DNA+/Ab+) (Figure 25).

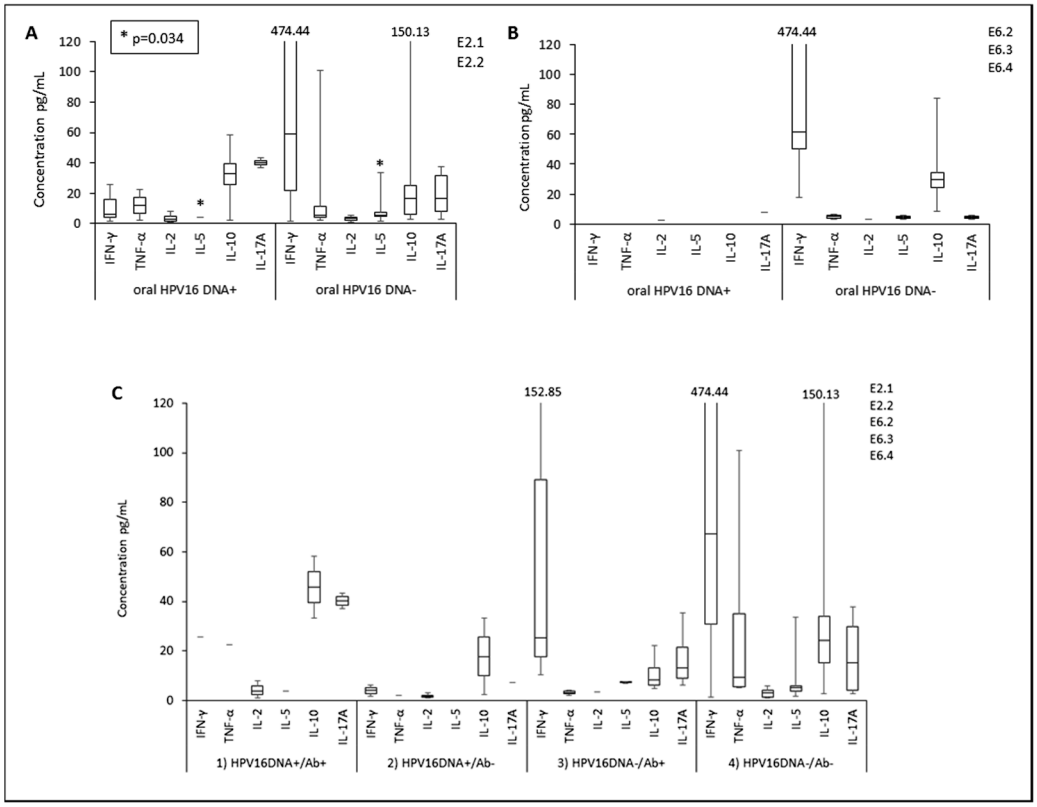

Figure 25. IFN-γ, TNF-α, IL-2, IL-5, IL-10, and IL-17A concentrations after stimulation with A) E2.1 and E2.2 peptides, B) E6.2, E6.3 and E6.4 peptides and C) all HPV16-specific peptides from LST-positive wells. The box is bounded on the top by the third quartile, the bottom by the first quartile, and divided by the median. The minimum and maximum are indicated by the whiskers. Figure adapted from original publication IV.

5.4 HPV16 –SPECIFIC CD4+CD25+FOXP3+ REGULATORY T CELLS (II-IV)

The frequency of CD4+CD25+Foxp3+ cells after a 7-day stimulation was analyzed and only a low amount of positive cells were detected as described in detail in Papers II, III and IV. Figure 13 summarizes the results of the CD4+CD25+FoxP3+ cells in different subgroups. No differences between the groups were observed.

6 DISCUSSION

The persistence of HR-HPV infection is known to be the major risk factor for HPV-induced precancerous lesions or cancer in women. The cell-mediated immunity has a central role in the regression of HPV lesions, but the exact mechanism is still partly unknown. Cell-mediated immune responses were studied in women who had been regularly followed up for six years before donating blood samples for cell-mediated immune analyses at the 14-year visit. Women were divided into six groups based on the outcome of their HPV infection during the FU: 1) women with persistent genital HPV infections which progressed to CIN lesions, 2) women who remained HPV-negative in their genital samples during the FU, 3) women who were oral HPV16-DNA- and -seropositive, 4) women who were oral HPV16-DNA-positive but HPV-seronegative, 5) women who were oral HPV16-DNA-negative but HPV-seropositive and 6) women who were oral HPV16-DNA-negative and also HPV-seronegative. Children of women with CIN lesions were also analyzed.

6.1 Lack of HPV16 E2 and E6-specific memory T cells is associated with persistent HPV16 infection

HPV16 E2- and E6-specific proliferation was less common in women with persistent HPV16 infections than in women who were HPV-negative during the FU. E7-specific proliferation was detected only in one HPV-negative woman and three children of mothers with a history of CIN lesions.

The proliferation of HPV16 E2- and E6-specific CD4+ T cells seems to protect against persistent genital HPV16 infection in women. Previously it has been shown that reactivity against E2 and E6 is typical for healthy HPV-negative individuals. Accordingly, circulating CD4+ and CD8+ T cells specific for HPV16 E2 and E6 antigens are frequently both found in subjects who have successfully cleared an HPV infection (de Jong et al., 2002, Welters et al., 2003, Woo et al., 2010, de Jong et al., 2004) and infrequently in CIN patients (Nakagawa et al., 1996). In contrast, HPV16 E6- and E7-specific T cell proliferation has been detected in patients suffering from oropharyngeal or CC (de Jong et al., 2004, Piersma et al., 2008). CD4+ T cells have a crucial role for humoral and cellular immunity evoked after viral infections. Thus, these impaired T cell proliferations might be considered as an undesirable event against protection for cancer development. Our results confirmed the previous studies where low frequencies of CD4+ T cells have been shown to allow the persistence of HPV16 infection (de Jong et al., 2004, Welters et al., 2008, Nakagawa et al., 1996). On the other hand, previous studies on women with LSIL revealed that HPV16 E2-specific

CD4+ T cell proliferation does not alone correlate with the regression of HPV-induced lesions, because HPV16-persistent women also display HPV16 E2-specific proliferation (Woo et al., 2010). Our results were in line with the previous presumption that the difference in IFN-γ-producing E2- or E6-specific T cells might affect the clinical outcome of HPV infection. Women who tested HPV-negative in cervical or in oral samples secreted IFN-γ at higher concentrations than women who had persistent HPV16 infection. IFN-γ response has been found to be an important prognostic marker for the clearance of high-risk HPV (Farhat et al., 2009, Welters et al., 2003).

The lack of E7-specific T cells might be due to the small sample size and more probably due to the fact that our study did not include any patients with CC. All women included were clinically healthy for HPV-induced lesions when the blood sampling was done for cell-mediated immune analysis. Only one woman belonging to the HPV-negative subgroup had both HPV16 E7-specific memory T cells and T reg cells. The HPV-specific FU data revealed that this woman had been HPV16-seropositive at several visits, which might indicate a past HPV16 infection or even an undetectable current HPV infection. However, HPV16 E7-specific CD4+ T cell immunity has rarely been detected in healthy individuals (Welters et al., 2003, van der Burg et al., 2001). The E7-specific expression is typical in CIN2/CIN3 lesions, because the protein decreases the levels of MHC I molecules, thereby diminishing the presentation of the viral antigens (Øvestad et al., 2010). The presentation of E7-specific T cells has also been associated with HNSCC (Albers et al., 2005). That event might lead to an imbalance of the tissue homeostasis favoring immune escape and cancer development similarly to the imbalance between cytotoxic T lymphocytes and suppressor T cells. In contrast, HPV16 E7-specific CD4+ T cells have been found to correlate with the regression of HPV16-induced HSIL (Peng et al., 2007). The persistence of HPV16 infection has also been associated with E7-specific immunity (de Gruijl et al., 1998), but our study did not confirm this. Our results are in accordance with earlier studies demonstrating that HPV16 E2-specific CD4+ T cells are a sign of HPV infection and E7-specific T cells do not express at the same time with E2-specific CD4+ T cells (Xue et al., 2010).

We found that some women with persistent cervical or oral HPV16 infection totally failed to produce HPV16-specific CD4+ memory T cells. These women might be at increased risk of developing some HPV-induced lesion or cancer after several years because of the inadequate activation of the cell-mediated immunity (de Jong et al., 2004). In addition some women who were either cervical or oral HPV-DNA-negative during the FU unsuccessfully developed HPV-specific memory T cells. Only one woman was totally negative for HPV during the entire FU. The others had either HPV16-specific antibodies or were oral HPV16-positive. This one totally HPV-negative woman might belong to a small minority of the population who have never acquired an HPV infection. To conclude, in reality it is challenging to create a control group of HPV-negative women, because HPV infects nearly all individuals during their

lives. HPV transmission can also occur in early life or even during the fetal time via the placenta (Koskimaa et al., 2012, Sarkola et al., 2008).

To our surprise, the proliferation of PBMC after HPV16 peptide exposure was surprisingly similar between women with previous CIN lesions and women who remained HPV-DNA-negative in the cervix during the FU. However, it must be noted that some of the women in the control group had tested oral HPV-DNA-positive during the FU. One would expect to find some differences in the HPV-specific immunity in the women who developed a severe HPV-induced cervical disease as compared to those who were HPV-negative for the cervix. One explanation could be that some of the control women could also have had earlier infections with CIN1 or CIN2 lesions which had cleared on their own before they entered into the study (Trimble et al., 2005, Moscicki et al., 2004, Syrjänen et al., 1992, Castle et al., 2009, Insinga et al., 2009). In addition, all patients were treated for their CIN before entering this cell-mediated immunity study, where the blood samples were taken 31-129 months after the diagnosis of the cervical disease. Thus, HPV16-specific cell-mediated immunity might have changed during the FU after the CIN treatment. One would even expect to see a better HPV-specific cell-mediated immunity in these women after cone treatment. However, we found no evidence of impaired or improved HPV-specific cell-mediated immunity in these women as compared with the cervical HPV-negative controls. It has been shown that the number of HPV16 E2-specific T cells are high regardless of the outcome of HPV infection after treatment (Jacobelli et al., 2012).

Oral HPV infection might be one plausible activator for HPV-specific cell-mediated immunity. Some studies on HPV-induced HNSCC have shown CD4+ T cells to be associated with good prognosis for the disease, while elevated numbers of Foxp3+ T reg cells indicated tumor progression (Badoual et al., 2006, Heusinkveld et al., 2012). In our study, we found that the HPV16 E2-specific proliferation was most common among women who were oral HPV16-DNA-negative (Groups 3 and 4). Similarly, E6-specific responsiveness was predominantly found in Group 4 (HPV16 DNA-/ab-). The lack of E7-specific proliferation might be related to the fact that none of the women in any subgroups had any serious oral lesions. None of the women in the oral control group were HPV16-positive at the 14-year study point, but some women had tested HPV16-DNA cervix or antibody-positive earlier during the FU. This difference in immune reactivity suggests that the HPV16-DNA and antibody-negative women (Group 4) might have the best protective cell-mediated immunity against progressive oral HPV16 infection. Despite the fact that these oral HPV16-DNA-negative women had never been oral HPV-DNA-positive during the whole 14-year FU, they might have had a subclinical oral HPV16 infection at some point in their life which had effectively activated their HPV16-specific cell-mediated immunity. The only difference between the HPV16-DNA-negative Groups 3 and 4 was the presence of HPV antibodies and absence of E6 reactivity in Group 3. However, the number of women in the subgroups is too small for further conclusions. The role of E2- and E6-specific T cells in resolving

the early stages of HPV infection has been studied for genital infections, but not in oral asymptomatic HPV16 infection (Farhat et al., 2009, Dillon et al., 2007, Woo et al., 2010).

The HPV16-specific proliferation of PBMC after E2, E6 and E7 peptides proved that HPV16-specific memory T cells were also common among children whose mothers had acquired an HPV-induced CIN lesion. All 10 children of these mothers had E2-specific proliferation and half of them had E6-specific proliferation. Some children also had E7-specific HPV16-specific memory T cells, which was an interesting observation, and probably a sign of a protective immunity. All children were sexually inexperienced, which indicates that these children must have had exposure to HPV16 infection at some body sites, probably through oral mucosa. These results reinforce the concept that some HPV infections are acquired as early as in childhood by nonsexual transmission routes and suggest that HPV immunity could be evoked early in life (Merckx et al., 2012, Syrjänen, 2010a). The important question is the age at which one experiences the first exposure to HPV, in which body site and how.

To sum up, we could identify differences in HPV16 E2-, E6- and E7-specific responses between women with persistent oral or cervical HPV16 infection and those who had only occasionally detectable HPV DNA or HPV serology in either site. The cell-mediated immunity caused by oral asymptomatic HPV16 infection has not been studied earlier. Our results indicate that oral HPV infection can also induce effective cell-mediated immune responses.

6.2 Th2 cytokines might achieve persistent HPV16 infection

Assessing the results of the HPV16-specific cytokine assay from all women studied in this thesis, we found that IFN-γ and IL-5 were most common in women without any persistent HPV infection. Furthermore, IFN-γ and IL-5 concentrations were highest in those women. In contrast, women with persistent oral HPV16 infection secreted more IL-10 than the controls. There is some previous evidence suggesting that IFN-γ is one possible prognostic marker for the clearance of HR-HPV (Welters et al., 2003, Scott et al., 1999). In our study, women with persistent HPV infection secreted mostly IL-10 and IL-17A. Similar cytokine distribution has also been observed in many other studies (de Jong et al., 2004, Bais et al., 2005, Bais et al., 2007). Increased levels of IL-10 have been detected in CIN lesions to inhibit T cell activation and Th1 cell differentiation (Alcocer-González et al., 2006, Prata et al., 2015, de Jong et al., 2004). At an early stage of HPV-induced disease, the IL-10 production may suppress the immune recognition of HPV by downregulating the HLA class I molecules (Mota et al., 1999, Rodríguez et al., 2012).

The proportional parts of IFN-α and IL-5 cytokines were also more dominant among all HPV16-DNA-negative women than among HPV16-positive women. Overall,

HPV16-DNA-positive women showed more Th type 2 cytokine patterns, especially with IL-10 secretion. There was, however, one exception: the group of women who had previous CIN lesions. Their cytokine panel simulated the panel of HPV16 negative ones, which was in line with their results for lymphocyte proliferation and FU history as discussed earlier in the text.

Oral HPV16-DNA-negative and Ab-negative women (Group 4) seemed to have the most protective cytokine combination among the subgroups studied. Although the number of subjects was fairly small, we were able to demonstrate that the presence of an HPV16-specific Th1-like response is associated with the absence of a detectable oral HPV16 infection. The results showed IFN-γ and IL-5 cytokine secretion by the oral HPV16-DNA-negative women, which was also present among women without any cervical HPV16 infection. Women with a persistent oral HPV16 infection who were also HPV16-seropositive (Group 1) secreted the highest concentration of IL-10. There are no previous studies on specific cytokine responses in oral HPV infection. We were able to show that Th type 2 cytokines might predispose for persistent oral HPV16 infection similarly to what is found in persistent cervical infections.

The only statistically significant difference between cervical HPV-negative control women and women with previous CIN lesions was the higher secretion of IL-17A by T cells in the control women ($p = 0.035$). The role of IL-17A in cancer development is controversial (Derhovanessian et al., 2009, Yang et al., 2014). A high proportion of IL-17A-producing CD4+ T cells in CIN women has been reported (Zhang et al., 2011), while Souza and coworkers found a higher amount of IL-17A in women with low-grade SIL than high-grade SIL (Souza et al., 2013). The role of Th17 cells in the progression of cervical cancer is still unknown. Whereas IL-17A with T regs is associated with more severe disease, Th17 cells (producing IL17 and IFN-γ) seem to be protective (Gosmann et al., 2014). On the other hand, patients with an advanced stage of cancer have been associated with increased IL-17-producing cells found in both peripheral blood and tumor tissues (Maruyama et al., 2010). One can speculate that the segregation of IL-17A has changed at some stage of the viral infection and tumor development. Our results presented here show that the control women (i.e. HPV-negative in cervix) had higher levels of IL-17A and IFN-γ in LST+ samples and also in HPV16 peptide-specific responses than women with past CIN, indicating that Th17 might play an important protective role in HPV-induced cervical diseases.

The cytokine polarization results of children were fairly similar to those of their mothers. When analyzing the proportional frequency of the individual cytokines, the portion of IL-5 was larger in children than in their mothers. Regardless of the severity of the mother's CIN lesion, the children seemed to have a more protective cytokine profile against HPV infection which was characterized by elevated IFN-γ and IL-5 secretions. We can conclude that based on our results on HPV16-specific proliferation and cytokine assays, and earlier results on the presence of HPV in the placenta and cord blood, the mother can infect her newborn or even the fetus, which aids in the

development of HPV-specific cell-mediated immunity in children naïve for sexual habits.

6.3 Reduced number of T reg cells

In the present study we were able to identify only a low number of T reg cells. Only a few women had a low amount of T reg cells in their LST-positive samples and there were no differences between the study groups. A total of 2 of 10 children were identified with measurable levels of HPV16-specific T reg cells, which might indicate that they are at risk of developing HPV-associated diseases in the future. In the literature, increased levels of CD4+CD25+ Foxp3+ cells have been found in women with CIN2/CIN3 and also in cervical cancer patients, both at the tumor site and in draining lymph nodes (Molling et al., 2007, Visser et al., 2007, Adurthi et al., 2008, Øvestad et al., 2010, Scott et al., 2009). We could not confirm this, because the women had either an asymptomatic HPV infection or a past CIN lesion or were HPV-negative. It is not known whether a persistent HPV infection leads to increased numbers of T reg cells, or whether increased numbers of T regs lead to persistence. T regs are able to suppress the proliferation of other T cells in the microenvironment through contact-dependent mechanisms or IL-10 and TGF-β secretion (Strauss et al., 2007, Bergmann et al., 2007).

6.4 Relation of HPV-specific antibodies to the presence of HPV DNA in the cervix and the outcome of HPV infection

We were interested in analyzing the concordance of HPV serology and the presence of HPV DNA in the genital and oral tracts of the women participating in the Finnish Family HPV Study. Furthermore, the prognostic value of HPV serology on the outcome of HPV infection was determined. Our results showed no HPV genotype-specific concordance between HPV serology and HPV DNA at any visit, regardless of the anatomical site of sampling. However, we were able to show that higher antibody levels correlated with the clearance of HPV16 and HPV18 infections, while lower antibody levels were related to incident cervical HPV16 infections as well as the persistence of HPV18 infections in the cervix. No association was found between HPV serology and oral HPV infections. Thus, our results support the concept that cervical mucosa might be the most important site of HPV infection from the serological point of view.

One of the major disappointments in HPV serology studies has been the missing (or at least low) temporal correlation between HPV antibody response and HPV DNA detection. Approximately only a half of all women tested HPV16-DNA-positive in the cervix have also tested HPV-16-seropositive (Skjeldestad et al., 2008, Ho et al., 2004,

Viscidi et al., 2004). One reason for this discordance could be that HPV serology also detects past exposures to HPV. The lack of antibodies might also be due to a transient HPV infection, because there is evidence that transient HPV infections do not always elicit seroconversion. In addition, some women with a persistent infection also fail to seroconvert (Carter et al., 2000).

The women in our cohort were all in their 3rd trimester of pregnancy at their baseline visit, which might have influenced their capacity to seroconvert to HPV. The effect of pregnancy on HPV detection and particularly HPV serology has not been studied in detail, but the protective role of a second pregnancy against the next HPV infection have been studied (Sarkola et al., 2009, Louvanto et al., 2010a). There is a chance that an HPV infection during pregnancy might be undetectable by the usual HPV testing, and after delivery, the virus can reactivate, leading to a serological response. Another possibility is that after pregnancy, an exposure to a new HPV genotype takes place, resulting in the same response.

The higher HPV16- and HPV18-specific antibody levels found in our study might possibly assist women to clear their HPV infections. The vaccination studies have revealed much higher antibody levels after vaccination than after natural HPV infections, and the high antibody levels have been related to the effect of vaccines in protecting against HPV (Ho et al., 2004, Harper, 2009, Stanley et al., 2006). However, the antibody levels needed for sustained, type-specific HPV protection after vaccination are unknown. It is most likely that antibodies that are elicited by a natural infection might protect the host against HPV reinfection with the same or related HPV type, or aid in quick clearance.

In addition to studying the HPV16-specific cell-mediated immunity responses of women, we also found that seronegativity might in some cases have protective aspects in oral HPV infections. In the cell-mediated immunity studies, the women with oral HPV16 DNA but no seropositivity had the highest IFN-γ HPV16 E6.4 peptide-specific levels, and also the women who were HPV16-DNA-negative but had HPV-specific antibodies had no HPV16 E6-specific memory T cells. These results attract attention to the role of HPV-specific antibodies and their relevance in resolving HPV infection.

6.5 Study strengths and limitations

This study design allowed the examination of the relation of HPV-specific cell-mediated immunity responses at the 14-years visit to the natural history of HPV16 infections in women. In this series of studies, we have tried to clarify the relationship between cell-mediated immunity markers such as HPV-specific T memory cells, Th1/Th2 cytokines and T regs and the outcomes of HPV16-specific infections in women.

The most obvious strength of this study is the long FU with diverse HPV-specific results during the 14-year FU. Women were carefully followed up at 8 visits to estimate the outcome of the HPV infection of an individual woman. The information we gained from mother-child pairs is unique in the world. No other study has managed to collect data on HPV16-specific cell-mediated immunity among family members. This information brings valuable new data on cell-mediated immunity as it is related to the natural history of HPV. However, it must be remembered that the immune response is a dynamic and rapidly changing process. In this thesis, cell-mediated immune responses were analyzed only at one study point, and therefore we were able to see only one flash of the cell-mediated immune response due to HPV16 infection. The Finnish Family HPV FU study was not originally planned to characterize HPV-specific cell-mediated immunity, and therefore the longitudinal data on cell-mediated immunty is unfortunately lacking. We also selected only HPV16 E2, E6 and E7 peptides for stimulating the PBMCs, because all experiments had to be run with unfrozen cells. Reactivity to E1 and E5 peptides in addition to L1 would have given more important information. In addition, the analysis of additional cytokines such as IL-21, IL-22 and IL-13 would have strengthened the study to provide a more accurate understanding of the role of cell-mediated immunity in the natural course of HPV infection.

Based on the FU data, we were able to create several subgroups of women to clarify the HPV-specific cell-mediated immunity in the natural history of HPV infection. The grouping was performed after careful planning. However, there are no absolute means to detect HPV infection or to determine the infection as latent or chronic based on a specific viral expression pattern, such as have been used for hepatitis B or herpes simplex virus infections. Thus, we cannot totally exclude any misclassification in the subgroups. Furthermore, the number of women in the individual groups was limited. However, the long FU with frequent sampling provides data on the profiles of HPV infections, whether persistent, cleared or totally HPV-negative, and thus the results can be considered to be reliable. The only way to strengthen the results is to analyze more women with a similar grouping, as done in this thesis. The study was partly descriptive and the statistical strength remained insufficient due to the limited number of women. However, despite the limitations of this study, we were able to find important and statistically significant differences in HPV-specific cell-mediated immunity between certain subgroups.

7 CONCLUSIONS

1. The lack of HPV16 E2- and E6-specific T memory cells and Th type 2 cytokines predisposed women for persistent oral HPV16 infection.

2. HPV16 infection in either the oral mucosa or cervix was associated with similar HPV-specific responses. Importantly, women who remained HPV16-negative during the FU responded more for HPV16 E2 and E6 peptides and secreted more Th type 1 cytokines than women with persistent HPV16 infections.

3. HPV16 can also be transmitted via nonsexual routes. The early maternal transmission of HPV might also result in HPV-specific cell-mediated immunity in children naïve for sexual contacts.

4. HPV serology does not predict current HPV infection, as there was no concordance between genotype-specific HPV serology and HPV infection in the cervix or oral mucosa. Titers of HPV antibodies at high levels might indicate the clearance of HPV16 or 18 infections. In contrast, low HPV16 antibody levels might be a sign of incident HPV16 infection, while low HPV18-specific antibody levels might allow the persistence of HPV18 infection.

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