Mucosal immunity of the female genital tract after HPV infection and vaccination

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Introduction - Mucosal immunity

Mucosal immunity of the female genital tract

The mucosal tissue of the human body is a key factor for our health and immunity. Since it is not protected by skin, it offers a point of entry which is favored by many pathogens. To ensure a healthy microenvironment while still allowing the mucosal tissue to fulfill its role, the mucosal immune system has developed a remarkable balance. A good example of this balance between tolerance and immune responses is the female genital tract (FGT). The FGT has to be tolerant to sperm and the implantation of the fetus to ensure pregnancy [Höglund 2003], while there are many STDs targeting the FGT. The outcome of STDs varies from possible infertility in the case of syphilis, to death in the case of HIV and cervical cancer.

The FGT is composed of the Fallopian tubes, exo- and endocervix, uterus and vagina, and can be subdivided into the lower and upper genital tract. The vagina and ectocervix are located in the lower genital tract, and like the GI tract they both house commensal bacteria which play a role in the defense against pathogens [Hillier 1999]. Another important aspect of mucosal immunity is the production of mucus by epithelial cells. Mucus is a thick, slimy gel which protects the mucosa against pathogens by encapsulating them and subsequently exporting them away from the mucosa [Lamont 1992]. It also helps immune molecules, e.g. antibodies, reach their target antigens [Saltzman 1994].

Keratinocytes, the predominant cell type of mucosal epithelia, divide rapidly at so-called metaplastic zones. The keratinocytes differentiate into either columnar or squamous epithelial cells, forming a squamo-columnar junction (also referred to as transitional zone or transformation zone) [Chow 2010]. In the FGT, this junction separates the columnar cells of the endocervix from the squamous epithelial cells of the exocervix, and these different cell substrates are target of different pathogens. *C. albicans* and *T. vaginalis* can infect the vagina, and *C. trachomatis* and *N. gonorrhea* colonize the cervix. Human papillomavirus (HPV) mainly targets the keratinocytes located at the squamo-columnar junction [Pudney 2005]. The different regions of the lower genital tract also display different immune cell concentrations. The ectocervix and squamo-columnar junction are more biased towards cytotoxic T lymphocyte responses whereas the endocervical tissue mediates humoral responses due to a high amount of plasma cells [Pudney 2005]. These plasma cells are located subepithelially in lamina propria and excrete secretory IgA and IgG, although most of the cervical IgG is transudated from serum [Kutteh 1999, Russel & Mestecky 2010]. The fact that the ratio of albumin/IgG in serum is similar to that in the cervical excretions, and that monomeric/polymeric IgA ratios are almost inversely related between serum and cervical excretions is in agreement with this theory [Kutteh 1996].

The polymeric Ig receptor, or pIgR, is present on the epithelial cells of the upper genital tract as well as on ectocervical glands [Russel & Mestecky 2002]. The pIgR transports polymeric IgA (pIgA) from the basolateral side of epithelial cells to the apical site where they are readily excreted into the lumen as secretory IgA (sIgA). This sIgA finds its main function in excluding harmful agents like pathogens or toxins and enhancing their capture by mucus, without causing inflammation. Antigens that evade these antibodies and enter the mucosa encounter IgGs which do elicit inflammation [Brandtzaeg 1997]. While IgA is the predominant antibody found in almost all mucosal secretions, cervicovaginal secretions contain mostly IgG [Kutteh 2005, Mestecky 2005, Wright 2002]. How IgG enters cervicovaginal secretions is currently not fully understood, however it is clear they primarily come from the serum via transudation. The most notable evidence for transudation comes from vaccine studies, where systematically administered vaccines like the HPV vaccine induce protective

immunity in the FGT through neutralizing antibodies [Schwarz 2010, Kemp 2008, Scherpenisse 2012]. In short, IgG is the predominant antibody in female genital secretions, in contrast to other mucosal secretions. Mucosal IgG is produced both locally and derived from serum through transudation.

Common mucosal immune system

Most mucosal tissues have specialized tissue areas which are the inductive sites for mucosal immune responses. These are referred to as mucosal-associated lymphoid tissues (MALT). The best characterized is the gut-associated lymphoid tissue (GALT), but others include nasal- (NALT) and bronchus- associated lymphoid tissue (BALT). The Peyer's patches and solitary B-cell follicles (GALT) are located mainly in the small and large intestine, respectively, although the lymphoid cells of GALT are also able to affect immunity in the urogenital tract and mammary glands. BALT and NALT also contribute to mucosal tissues other than their own, and taken together this is referred to as the common mucosal immune system (CMIS) [Brandtzaeg 1997].

While the mucosal system is a very immunogenic environment, most vaccines currently on the market are administered intramuscularly, intradermally or subcutaneously, even for pathogens targeting mucosa. Examples include the MMR vaccine and the more recently approved HPV vaccine. There is an increasing interest in the development of mucosal vaccines. However, a vaccine that would have to be applied in the genital tract would probably not be accepted as easily by the general public. The existence of the common mucosal immune system would suggest that a vaccine applied in a more practical mucosal tissue, e.g. sublingual tissue, could also induce immunity in the genital tract. There is sufficient evidence to support the induction of genital immunity through intranasal immunization in mice as well as in humans [Brandtzaeg 1997; Wu 2000; Bergquist 1997; Gallichan 1995; Johansson 1998; Pal 1996 ; Wu 1996; Kozlowski 2002]. In contrast, sublingual immunization was found to induce only modest cervical/vaginal immune responses in humans [Huo 2012]. Furthermore, the evidence for the existence CMIS in humans is presented in several other studies [Lyons 2008, Kozlowski 2002, Kozlowski 1999]. However, it is still not fully understood how this can be translated to vaccine design.

The FGT, although part of the mucosal immune system, has features which are not shared with other mucosal compartments. Most notable is the dominant mucosal antibody. In the FGT this is IgG coming primarily from serum, whereas in other mocusa it is IgA and IgM. While most mucosa contains MALT, no analogues structures can be found in the FGT [Naz 2012].

Another difference between the FGT and other mocusa is, not surprisingly, the menstrual cycle. Taking the menstruation as the starting point of the cycle, the subsequent phases are the follicular, ovulatory and luteal phases. These phases show variation in the amount of antibodies present in cervical, but not in serum, saliva or rectal secretions. Antibodies reach their maximum level prior to ovulation, and lowest concentration during ovulation attributed to dilution of cervical mucus [Kutteh 1996, Ma 1999, Nardelli-Haefliger 2003]. In women taking oral contraceptives, a more stable level of total as well as vaccine-induced immunoglobulins is found, and it is therefore suggested that endogenous sex hormones are primarily responsible for the antibody concentration in the FGT [Nardelli-Haefliger 2003]. This is important in the concept of vaccination, as a vaccine might not result in similar immunity in women on contraceptives or women in different phases of menstruation.

Recently, two vaccines have become available on the market. These vaccines induce antibodies against several HPV subtypes, which are thought to be the main mechanism of protection. However, information about the role of the mucosal immune system after infection or vaccination is

limited. In this review we will focus on the immune response against HPV after infection or vaccination.

Human Papillomavirus

HPV subtypes

Human papillomavirus (HPV) is the most prominent pathogen in the female genital tract. It is highly prevalent in young men and women who recently became sexually active. In a vast majority of cases, the immune system is perfectly capable of tracking down and removing the virus from the body. In some cases it remains as an asymptomatic latent infection. In some cases though, the infection persist long enough to develop pathology in the form of uninhibited cell growth. There are more than a hundred different genotypes, of which approximately 40 can infect the genital tract. HPV can be classified into two groups, based on their clinical outcome: High-risk HPVs that can cause cancer and low-risk HPVs that form benign warts. Nearly 100% of all cervical cancers is caused by high-risk HPVs, and of all the high-risk HPVs, HPV-16 and HPV-18 together account for over 70% of all cervical cancers worldwide [Walboomers, 1999; Bosch 2008]. Of all the low-risk HPVs the two most prominent types are HPV-6 and HPV-11 which together cause over 90% of all genital warts. It is for this reason that the currently two available vaccines contain these HPV serotypes, the bivalent vaccine targeting HPV-16 and -18 and the quadrivalent vaccine targeting HPV-16, -18, -6 and -11. The properties of the vaccines will be discussed later.

Papillomaviruses have been classified into many different genera, species, types and variants. The classification most commonly referred to is the HPV type (e.g. HPV-16 or HPV-9). The classification is based on the diversity of the L1 protein, which is also a good indication for total genomic diversity. Different types are approximately 80-90% identical in their DNA sequence. Almost all the human papillomaviruses belong to the genera alpha- or beta-papillomaviruses [de Villiers, 2004]. Papillomaviruses have been found to infect almost all vertebrae except fish, however specific virus types are unable to infect host-related species [Chow 2010]. HPV is therefore not only tissue-specific, as will be discussed later, but also host-specific.

Biology of HP

HPV has an icosahedral form, and lacks an envelope. It has a small, double-stranded DNA genome of approximately 8000 base pairs in size that contains eight open reading frames. The genes are classified as early (E1, E2, E4, E5, E6 and E7) and late (L1 and L2), depending on their time of expression. E3 and E8 are only present in a few HPV types, and information about these genes is scarce [Syrjänen 2012]. The early gene products contribute to infection, immune evasion and reproduction, while the late proteins form the viral capsid and are involved in assembly. The genome organization as well as the genes and the proteins they encode are well-documented [Chow 2010, Syrjänen 2012; Hebner 2006; Stanley 2012].

The E1 and E2 proteins are associated with replication of viral DNA. E1 is responsible for aligning cell and viral genome replication, and also plays a role in the episome maintenance, because viruses lacking this protein are readily integrated into the host genome [Frattini 1996]. E2 is also a transcription factor, illustrated by the existence of four E2-binding sites in the DNA at the upstream regulatory region (URR) of the HPV genome. This region also contains binding sites for several cellular transcription factors that influence HPV transcription [Hebner 2006].

E4 is present primarily as a fusion protein between E1 and E4, commonly referred to as E1^E4. While being the most abundant HPV protein, it is not fully characterized and its function is not understood. It is probably related to the release of virions, as it modifies cornified cell envelopes, and degrades cytokeratins [Hebner 2006, Syrjänen 2012]. Although called early protein 4, E4 is actually active in the later stages of the viral life cycle [Nakahara 2005].

E5, E6 and E7 are the oncoproteins of HPV. E5 carries out this role mainly through recycling of EGFR to the cell surface and inhibiting p27/p21 [Syrjänen 2012], although only 60% of HPV-16 tumors express E5 suggesting this protein is not a necessity for cancer but does aid in oncogenic progression [Venuti 2011]. Also, it has been shown that E5 does not increase overall disease severity in mice that express both E6 and E7 [Maufort, 2010]. The fact that E5 is not expressed after viral DNA integration into the host genome, and that this happens in progressive CIN, the function of E5 might be limited to early oncogenesis [Syrjänen 2012]. While E5 is no longer expressed after genome integration, E6 and E7 become overexpressed. In addition, while E5 is not necessary for malignancy, both E6 and E7 need to be expressed for a long period of time. The main mechanism by which E6 exerts its oncogenic potential is well known. The protein binds to the ubiquitin ligase E6-associated protein (E6-AP). This complex is able to degrade p53, which subsequently inhibits p21 [Song 2000]. E7 degrades the retinoblastoma tumor suppressor pRB, resulting in activation of E2F-dependent transcription. This causes a continued S-phase in already differentiated cells [Mclaughlin-drubin 2010]. Recently it became clear that a protein complex called p130-DREAM is targeted by E6 and E7, and the repression of this complex is necessary to prevent a stop in cell division [Nor Rashid 2011]. Moreover, there are many other host proteins interacting with E6 and E7 that have a role in cell division and regulation, which are summarized by Syrjänen *et al*.

The viral major capsid protein L1 and minor capsid protein L2 are expressed late in the viral life cycle, as they are required for assembly and release of virions. The capsid consists of 360 monomers of the L1 protein, assembled into 72 pentamers that also contain one L2 protein located on the inside of the capsid. The L2 protein, able to bind to L1 and viral DNA, has a more regulatory role in the process of virion assembly, recruiting viral genomes for encapsidation [Hebner, 2006]. While both L1 and L2 are required for viral assembly, L1 alone can form virus-like particles (VLPs), which will be discussed later as they are the main component of HPV vaccines [Giannini 2006]. The cell-binding abilities of L1 and viral entry will be discussed below.

Natural infection route of HPV

The natural infection route of HPV is well documented [Letian 2010, Chow 2010, Doorbar 2005, Stanley 2008, Stanley 2012, Hebner 2006]. HPV comes into contact with their target cell, the basal keratinocyte, through microabrasions or wounds, probably obtained through sexual intercourse in the case of the FGT or rectum. While most HPV types infect only the skin or the mucosa, some types can infect both [Chow 2010]. After infection has been established, HPV is present at a low copy number of approximately 10 to 100 copies per cell [Howley 1996]. The low copy number has resulted in the inability to culture HPV efficiently. However, the group of Wang *et al*, developed a system that produced HPV-18 genomes in primary human keratinocytes, which subsequently provided high titers of infectious virions that could infect naïve cultures [Wang 2009]. HPVs are very restricted in their host tissue specificity as they are only able to infect and replicate in fully differentiating keratinocytes. The squamo-columnar junction is particularly susceptible to HPV infection, as it is not protected by non-replicating cells. After infection of this zone, the columnar epithelium can be infected by HPV, but HPV cannot reproduce there, although the formation of carcinoma can still

occur [Chow 2010]. Columnar epithelial cells seem to be preferentially infected by HPV-18, because HPV-18 is more frequently found in the endocervix, and in women with cervical ectopy, a condition where columnar cells extend beyond the endocervix onto the vagina, HPV-18 is more frequently found [Monroy 2010].

As mentioned earlier, the cell cycle of the basal keratinocyte and the virus life cycle are tightly linked. When these cells migrate outwards towards the epithelium, they differentiate into epithelial cells themselves, and perform their role as a protective cell-layer. The process of differentiation is inhibited by HPV E6 and E7 proteins, but the cells still migrate outwards [Doorbar 2005]. When the cells proliferate, the viral DNA proliferates along up to copy numbers of 1000 HPV genomes. This triggers the expression of the late genes and subsequently particle formation and virion release [Syrjänen 2012].

There are multiple cellular receptors that interact with HPV, as is summarized by Letian *et al* [Letian 2010]. The α6 integrin subunit is upregulated upon wounding and helps to initiate DNA replication in keratinocytes, both of which are favorable conditions for HPV. Also antibodies to α6 integrin reduce HPV VLP binding, further confirming the role of α6 integrin as a receptor for HPV. Heparan sulfate proteoglycan (HSPG) is a target receptor for many viruses, and several studies strongly suggest it binds HPV as well. HPV infection is inhibited by heparin and by removing HSPG. Furthermore, the HSPG syndecan-1 is also upregulated in wounded tissue [Letian 2010]. After virus-host cell receptor binding, HPV is internalized either through clathrin-mediated endocytosis, caveolar endocytosis, or via tetraspanin-enriched microdomains [Letian 2010].

While HPV only infects epithelial tissue, HPV DNA has been found in peripheral blood from CIN and cervical cancer patients, as well as newborns and pediatric patients. This suggests a possible transport mechanism via blood, but how this occurs is currently unclear [de Freitas 2012].

FGT immune response to natural infection with HPV

Humoral responses to HPV

To mediate protection against cervical cancer, a good immune response is critical. It has been suggested that Ig-mediated immunity is especially crucial in the earlier stages of infection while cytotoxic T lymphocytes are located in cervical lesions and more important later on [Sheu 2007]. HPV infection is non-lytic, and is mostly confined to warts and lesions. This shelters them from the immune system, and the time it takes for systemic antibodies to be produced against HPV is therefore quite long and only 60% of women seroconvert. Data about the local antibody response to HPV are limited. As was mentioned earlier, mucosal IgA and IgG are produced locally, but most of the mucosal IgG is derived from serum.

Cervical mucosal antibodies

Is the presence of cervical IgA or IgG an indication for HPV infection?

All available studies (nine full-length, two abstracts) with data on cervical HPV-specific antibodies in HPV-positive and -negative women were collected (Table 1). The majority of these studies were limited to cervical IgA targeted against HPV-16. About half of these also included cervical IgG responses. Two studies included HPV-18-specific IgA, and only one study included HPV-18-specific IgG.

The results regarding any correlation between the presence of HPV-DNA and cervical antibodies against the corresponding HPV-types are inconsistent. E.g., cervical IgA antibodies to HPV-16 were found in 7-70% of HPV-16 positive and in 4-65% of HPV-16 negative individuals. All studies combined (except for Dreyfus *et al*., since their abstract only mentions the percentage), 28% of HPV-16 DNA positive women had cervical IgA anti-HPV-16 antibodies versus 14% of HPV-16 DNA negative women. This suggests that on average, women with cervical IgA anti-HPV-16 antibodies have twice as much chance of being infected with HPV-16 than women without these antibodies. This indicates that cervical IgA antibodies to HPV are not protective, since protective antibodies would be more present in women without detectable HPV infection. Similar results were found for IgG, and also for HPV-18 IgA and IgG, although for HPV-18 this was based on relatively few studies. Progressively lower differences in chance are seen in IgG anti-HPV-16, IgA anti-HPV-18 and IgG anti-HPV-18 data. From these studies, it is difficult to assess whether mucosal cervical antibodies are indicative of current HPV infection. The huge amount of variation might be due to different study populations, as some studies enrolled women from a gynecology clinic while another used students between 18-24 years [Hagensee 2000]. Latent infections could be responsible for some of the relatively high percentages of HPV negative but Ig positive women. Cross-reactivity is hard to account for, since most studies only looked for DNA of the corresponding HPV-subtype. However, in a recent study, cervical IgA anti-HPV-18 was found in patients who were positive for HPV-18 DNA as well as DNA from non16/18 HPV-types, suggesting cross-reactivity [Monroy 2010]. The menstruation cycle was corrected for by measuring total IgG/IgA. All studies except one used an enzyme-linked immunosorbent assay (ELISA) to measure antibodies, and most used HPV-VLP as the target antigen. The study done by Hagensee used a luminescence immunoassay (LIA), which they found to be more sensitive than the ELISA. Despite this higher sensitivity, their percentages of HPV negative but Ig positive women were among the lowest.

The group of Hagensee also looked at HPV-DNA status 4-12 months prior to antibody measurement. Women who were HPV-DNA positive 4-12 months prior to antibody measurement

were significantly more likely to have cervical anti-HPV antibodies, compared to both HPV-DNA negative women and women who were HPV-DNA positive only during antibody measurement time [Hagensee 2000].

	HPV-16 DNA detected Cervical IgA anti-HPV-16 antibodies detected / total (%)	No HPV-16 DNA detected Cervical IgA anti-HPV-16 antibodies detected / total (%)	Increased chance of HPV-16 DNA positivity if IgA-positive
Dillner 1993	5/8 (63%)	31/48 (65%)	0.97
Veress 1994 (ao)	12/35 (34%)	22/128 (17%)	2
Dreyfus 1995 (ao)	49%	15%	3.26
Wang 1996	17/127 (13%)	21/484 (4%)	3.25
Bontkes 1999	13/65 (20%)	9/111 (8%)	2.5
Hagensee 2000	2/27 (7%)	34/576 (7%)	1.16
Onda 2003	23/67 (34%)	16/118 (14%)	2.43
Sasagawa 2003	35/57 (61%)	148/546 (27%)	2.26
Yescas 2003	65/311 (21%)	9/171 (5%)	4.2
Passmore 2007	21/30 (70%)	22/73 (30%)	2.33
Mbulawa 2008	4/16 (25%)	3/22 (11%)	2.27
Total	208/743 (28%)*	315/2277 (14%)*	2.02

	HPV-16 DNA detected Cervical IgG anti-HPV-16 antibodies detected / total (%)	No HPV-16 DNA detected Cervical IgG anti-HPV-16 antibodies detected / total (%)	Increased chance of HPV-16 DNA positivity if IgG-positive
Dillner 1993	-	-	-
Veress 1994 (ao)	-	-	-
Dreyfus 1995 (ao)	-	-	-
Wang 1996	-	-	-
Bontkes 1999	9/65 (14%)	9/111 (8%)	1.75
Hagensee 2000	6/27 (22%)	68/576 (12%)	1.83
Onda 2003	-	-	-
Sasagawa 2003	12/57 (21%)	140/546 (26%)	0.81
Yescas 2003	93/311 (30%)	0/171 (0%)	-
Passmore 2007	-	-	-
Mbulawa 2008	9/16 (56%)	19/32 (59%)	0.95
Total	129/476 (27%)	236/1436 (16%)	1.65

	HPV-18 DNA detected Cervical IgA anti-HPV-18 antibodies detected / total (%)	No HPV-18 DNA detected Cervical IgA anti-HPV-18 antibodies detected / total (%)	Increased chance of HPV-18 DNA positivity if IgA-positive
Dillner 1993	-	-	-
Veress 1994 (ao)	-	-	-
Dreyfus 1995 (ao)	-	-	-
Wang 1996	5/41 (12%)	21/570 (4%)	3
Bontkes 1999	-	-	-
Hagensee 2000	-	-	-
Onda 2003	-	-	-
Sasagawa 2003	12/28 (43%)	179/575 (31%)	1.39
Yescas 2003	-	-	-
Passmore 2007	-	-	-
Mbulawa 2008	-	-	-
Total	17/69 (25%)	200/1045 (19%)	1.29

	HPV-18 DNA detected Cervical IgG anti-HPV-18 antibodies detected / total (%)	No HPV-18 DNA detected Cervical IgG anti-HPV-18 antibodies detected / total (%)	Increased chance of HPV-18 DNA positivity if IgG-positive
Dillner 1993	-	-	-
Veress 1994 (ao)	-	-	-
Dreyfus 1995 (ao)	-	-	-
Wang 1996	-	-	-
Bontkes 1999	-	-	-
Hagensee 2000	-	-	-
Onda 2003	-	-	-
Sasagawa 2003	8/28 (29%)	147/575 (26%)	1.12
Yescas 2003	-	-	-
Passmore 2007	-	-	-
Mbulawa 2008	-	-	-

Table 1. Presence of cervical IgA and IgG specific to HPV-16 or HPV-18 in women with or without corresponding HPV infection.

* = Data from Dreyfus *et al*. not included

ao = Abstract only

Is the presence of cervical IgA or IgG an indication for HPV-induced disease and disease progression?

The presence of mucosal IgG is not an indication for further HPV-induced disease progression (Table 2). The data suggests that cervical IgA is not protective, as it is increased in higher grade cervical disease, although not all studies report this finding. In one study [Monroy 2010], mucosal antibody responses to HPV-16 were significantly stronger in cervical ectopy patients compared to healthy controls, but significantly lower compared to CIN1 patients. Responses to HPV-18 were significantly higher in the cervical ectopy patients compared to both healthy controls and CIN1 patients, which makes sense in the light that cervical ectopy is characterized by an increase of columnar epithelial cells, the preferred host cell for HPV-18.

	No disease	**CIN1 or LSIL**	**CIN2/3 or HSIL**	**Condyloma**
Dillner 1993 HPV-16 IgA [$]	- 17.8% (5/28)			58% (17/29)*
Wang 1996 HPV-16 IgA HPV-18 IgA	 9.4% (39/414) 5.8% (24/414)	 27.9% (12/43)* 9.3% (4/43)	 16.4% (21/128)* 7.8% (10/128)	 11.5% (3/26) 7.7% (2/26)
Bontkes 1999 HPV-16 IgA HPV-16 IgG		 16.5% (13/79)[#] 7.6% (6/79)[#]	 18.4% (9/49) 16.3% (8/49)	
Sasagawa 2003 HPV IgA HPV IgG	 46% (231/501) 36% (181/501)	 58% (26/45)* 44% (20/45)	 75% (21/28)*/** 64% (18/28)	
Passmore 2007 HPV-16 IgA HPV-16 IgG	 44.4% (12/27) 61.5% (8/13)	 22.6% (7/31) 28.6% (4/14)	 53.3% (24/45)** 45% (9/20)	
Mbulawa 2008 HPV-16 IgA HPV-16 IgG	 50% (10/20) 50% (10/20)	 30% (8/27) 52% (14/27)	 38% (14/37) 57% (21/37)	

Table 2. Presence of cervical IgA and IgG in relation to disease status.
$ = Antigen was E7. E2 gave similar results, L1 and L2 gave no difference between healthy control and disease
LSIL = low-grade squamous intraepithelial lesions, comparable to CIN1
HSIL = high-grade squamous intraepithelial lesions, comparable to CIN2/3
[#]= CIN 0 and CIN 1 were grouped
*= significantly higher than control (*) or lesser disease status (**)

In a study on HPV-16 specific antibody responses in women with CIN [Passmore 2007], there was a significant increase in the prevalence and magnitude of cervical HPV-16 IgA responses in women with CIN2/3 compared to CIN1, suggesting again that local cervical antibodies are not associated with better disease outcome. A remarkable finding was that although only 1% of women (1/91) had detectable oral HPV-16 DNA, oral HPV-16 IgA was most prevalent (61.2%), and significantly more prevalent than cervical IgA (41.7%) and serum IgA antibodies (36.8%). Overall, their data show no

evidence for immunological linkage between oral and genital mucosal compartments or between either of the mucosa and systemic compartments.

Similar to HPV-infection, there seems to be a delay in the presence of measurable cervical IgG and secretory IgA after the detection of lesions caused by HPV. For cervical IgG this delay was 4 months after detection of lesions, while for secretory IgA it was found to be 4-8 months [Hagensee 2000].

Cellular response to natural infection

Cell-mediated immunity is important in controlling HPV infections. Penn *et al* reported that renal transplant recipients, who have dampened cellular immune responses, had a much higher risk (up to 100-fold) of obtaining HPV-related cancers [Penn, 1986]. Also, AIDS patients have much higher chances of being infected with HPV and these infections generally last longer [Critchlow 1998]. This points to a role of cellular immunity in the control of HPV infection [Hagensee 2000]. Langerhans cells are especially important, as they are first to interact with HPV-infected cells.

Langerhans cells

Langerhans cells (LCs) are the primary antigen-presenting cells in the FGT, and therefore play a crucial role during the local mucosal immune response to HPV. In a normal immune response to HPV, infected keratinocytes secrete cytokines, activating LCs which subsequently recruit lymphocytes. There is also some evidence for a direct cytotoxic effect of LCs toward infected keratinocytes [Le Poole 2008]. The evidence that Langerhans cells are depleted in non-inflammatory HPV-related lesions and warts is strong [Brawan 1986, Hughes 1988, Leong 2010, Jimenez-Flores 2006, Nakayama 2011, Sperling 2012, Matthews 2003]. In inflammatory warts, however, a high number of LCs are present. A range of lymphoid cell subspecies, predominantly CD8+ T cells, which seem to be targeting the virus-infected keratinocytes [Nakayama 2011], accompanies them. Macrophage Inflammatory Protein 3 alpha (also referred to as MIP-3α or CCL20) and E-cadherin play a pivotal role in the mechanism behind the reduced LC migration, as they are both downregulated in HPV-infected keratinocytes [Nakayama 2011, Sperling 2012, Guess 2005, Matthews 2003, Barcelos 2009]. This is in line with the function of these proteins. MIP3α is the strongest LC-recruiting chemokine [Dieu-Nosjean 2000] and E-cadherin is an adhesion molecule that binds LCs to keratinocytes [Tang 1993]. The molecular mechanism behind MIP-3α downregulation has been recently suggested as the direct interaction between oncoprotein E7 and the transcription factor of MIP-3α [Sperling 2012], while the expression of E6 [Matthews 2003] and E7 [Caberg 2008] in cells reduces cell surface E-cadherin. E6 and E7 silencing induces the chemotaxis and infiltration of LCs further confirming their role [Caberg 2009]. In contrast, another study shows that E7 reduces E-cadherin levels by epigenetically repressing transcription, while E6 seemed to have no effect on the expression of E-cadherin [Laurson 2010]. The minor capsid protein L2 was recently found to possess similar immune evasion properties, inhibiting the maturation of LCs [Fahey 2009].

There seems to be a discrepancy between observations of the number of LCs infiltrating HPV-infected tissue. It has been suggested that this might be due to several reasons: the location of biopsies taken, different disease stages, different cell markers, and a different methodological approach [Jimenez-Flores 2006]. Some studies report that HPV-induced LC depletion only affects the immediate microenvironment [Leong 2010, Matthews 2003], supporting the notion that the location

of biopsy is crucial. In lesions infected with the beta genus of HPV normal LC densities and an increase in the amount of Tregs were found, suggesting a different mechanism of immune evasion [Leong 2010].

Lymphocytes

From studies on HIV-positive patients, it has become clear that lymphocytes are associated with protection against HPV and subsequent cervical cancer [Scott 2001]. Especially the $CD8^+$/regulatory T cell ratio within the tumor is critical, as an increase in this ratio in the favor of $CD8^+$ T cells favors tumor rejection, while the regulatory T cells lower local immunity. A high infiltration of $CD8^+$ T cells into the tumor inhibits metastases into the lymph node, thereby increasing clinical outcome. This is especially true for patients who have circulating anti-HPV-specific circulating lymphocytes [Piersma 2007]. Cytotoxic T cells are present in tumors. However, their presence in an already further progressed stage of cancer suggests that they are inadequately activated [Zehbe 2007]. There seems to be some evidence that NK cells are less prominent and their function is inhibited in cervical cancer tissue [Patel 2009]. NK cells have been found in the squamo-columnar junction of HPV-infected women [Syrjänen 1986]

Immune evasion strategies

HPV has a range of mechanisms by which it can evade detection by the immune system. These mechanisms have been reviewed by Kanodia *et al.* [Kanodia 2007]. First of all, the virus keeps a low profile in several ways. It has a non-lytic cycle, which limits pro-inflammatory signals and cytokines from triggering dendritic cells. The gene products of HPV are not secreted, and the E-proteins are expressed mainly in the nucleus at low levels. Also, shedded virions which are highly immunogenic are present only at very low copy numbers and are exported from the epithelium. Furthermore, molecular mimicry, skewing cytokine profiles, inhibition of APC migration and prevention of apoptosis are mentioned as ways by which HPV prevents detection and clearance by the immune system [Kanodia 2007].

Secondly, HPV manipulates the innate immune system. The innate immune system is a fast acting, non-specific defense which is the first line of protection against pathogens. Pattern recognition receptors (PRRs) play a key role in the innate immunity. Toll-like receptor 3 (TLR3), melanoma-differentiation antigen 5 (MDA5) and retinoic acid-inducible gene 1 (RIG-I) which recognize dsRNA and TLR9 which recognizes dsDNA are the main receptors recognizing virus-related patterns. In an extensive genome-wide expression profiling assay, HPV was found to dampen inflammatory responses in undifferentiated keratinocytes. The expression of TLR3, PKR, MDA5 and RIG-I was not affected by HPV, however, their downstream signaling pathways are inhibited. Moreover, genes in the antigen presenting pathway, the inflammasome, antiviral cytokines, pro-inflammatory and chemotactic cytokines were downregulated [Karim *et al*, 2011]. TLR9 recognizes dsDNA-derived CpG motifs [Lund 2003]. In normal keratinocytes, the addition of CpG oligonucleotides leads to secretion of high concentrations of IL-8. When these cells express HPV16 E6 and E7, this effect is completely abolished. HPV18 E6 and E7 downregulate TLR9 to a lesser degree, whereas HPV6 does not downregulate TLR9 at all [Hasan 2007]. The NF-kB pathway seems to play a key role in the suppression of TLR9 expression [Hirsch 2010]. Interferon-kappa (IFN-k) is a type I interferon, which is expressed in keratinocytes [LaFleur 2001]. Downregulation of IFN-k can be detected in cells expressing E6 or both E6 and E7, but not E7 alone. In normal mucosa IFN-k is constitutively expressed, whereas in CIN 1 this is already reduced, and in HPV16/18 cervical

carcinoma cell lines IFN-k is completely undetectable [Rincon-Orozco 2009]. This suggests an early role for IFN-k in the progression into CIN and cervical cancer.

Lastly, another way to modulate immune responses is through regulatory T cells (Tregs). Tregs are usually identified as positive for CD25, CD4 and FoxP3. Tregs dampen immune responses before they do too much damage to the body, and provide tolerance to self-antigens. Since HPV relies on suppression of immune responses over a long period of time to ensure a latent infection, a possible role exists for Tregs in acquiring tolerance to the virus. An accumulation of Tregs was indeed found in CIN and cervical cancer patients, and depletion of these cells led to an increased T cell response against HPV-16 [Patel 2009]. Another group found functional Tregs in large genital warts, and T cells (non-Tregs) in those warts were hyporesponsive [Cao 2012]. In HPV-6 positive genital warts, an accumulation of Tregs was found which suppressed anti-HPV immune responses [Cao, 2012]. How exactly HPV influences Tregs to induce tolerance is currently not known.

Latent infection and disease progression

While in many cases the immune system can clear the virus from host cells, albeit after 1-2 years [Richardson 2003], latent infection sometimes occur. It is difficult to distinguish between viral clearance and latent infection, as the latently existing virus may not be detectable via PCR. It is also unclear whether clearance of natural infection leads to immunity, and if so, how specific and long-lasting this immunity is. This is illustrated by Gravitt [Gravitt, 2011], who divides women who are HPV DNA negative, but previously HPV DNA positive, into three groups: Uninfected immune, uninfected susceptible and infected women. It is currently not possible to distinguish between these three groups. Therefore, there is great difficulty in assessing the risk of reinfection or the possibility of latent infections. Evidence exists for latent HPV infection in rabbits [Amella 1994], but for humans it is less clear. There is, however, some evidence for the existence of latent HPV infection in humans. Most notably, in immunocompromised individuals, e.g. SCID-patients [Laffort 2004] or HIV-positive women [Nowak 2011], HPV infection is increased. The fact that the same is true for HIV-positive women who are sexually abstinent [Strickler 2005] supports the theory of latent infection. Yet, conclusive evidence remains to be found.

Cervical cancer afflicts about 500.000 women around the world every year. HPV has been found in nearly 100% of cervical cancers, and the two subtypes HPV-16 and HPV-18 together are responsible for approximately 70% of all cervical cancers. Other risk factors of cervical cancer include smoking, number of live births, oral contraceptives, early pregnancy and early age of first sexual activity [Castellsague 2002, Louie 1997, Vaccarella 2006]. Cervical cancer is preceded by several stages of cervical intraepithelial neoplasia, or CIN. When infected with high-risk HPV for a prolonged period of time, CIN1 progresses to CIN2 and subsequently to cervical cancer precursor lesions (CIN3), which if left untreated will develop into cancer in more than 33% of cases [Gravitt 2011]. The time from first infection with a high-risk HPV to CIN3 takes about 2-3 years, after which it can take 10 to 30 years for invasive cervical cancer to develop [Schiffman 2011]. Papanicolaou (Pap) smear is a way to detect the presence of CIN3, and since women can take the sample at home and send it to a lab by mail, appliance is high [Singer 1995]. CIN3 can be treated relatively easily and safely. Although this has reduced cervical cancer dramatically in the developed world, the limitations of this cytological screening test are now clear and HPV DNA testing is becoming more popular. A positive high-risk HPV DNA test in women older than 30 provides a high positive predictive value of risk of CIN3 and cervical cancer, since at this age the probability of the infection being a persistent one is high [Boone 2012]. The Bethesda classification system uses the abbreviations ASCUS (atypical squamous cells of

undetermined significance), LSIL (low-grade squamous intraepithelial lesions) and HSIL (high-grade SIL) [Solomon 2002]. Despite increasingly better screening methods, HPV is still a persistent problem in both developed and undeveloped countries. Besides cervical cancer, HPV has also been associated with head, neck, penile, anal, breast, lung, vulvar and vaginal cancers [Carlos 2012, Syrjänen 2012].

A higher viral load of high-risk HPV has been suggested to indicate higher risk of developing precursors for cervical cancers, namely CIN2 and CIN 3, especially for HPV-16 [Gravitt 2007], however, this effect is not so clear in other studies [Lorincz 2002, Hesselink 2009]. A higher viral load might indicate an increased chance of immune detection and clearance, while a lower viral load might indicate immune evasion and persistent infection. Therefore, the suggestion that higher viral load correlates to higher chance on progressive CIN seems contra-indicative. Several correlations have been found between host factors and disease progression. E6-specific cytotoxic T-lymphocytes (CTLs) are inversely correlated to disease progression [Scott 2001], suggesting a protective role of immune cell responses. HLA-G, an immunotolerant protein, seems to be upregulated in further progressed stages of CIN and cervical cancer, possibly allowing immune evasion [Li 2012]. Also the presence of both IgA and IgG cervical anti-HPV antibodies has been reported as an indication for reduced cervical disease in some studies, [Hagensee 2000] but some report no correlation [Passmore 2007].

HPV can take advantage of host cell transcription and translation machinery resulting in alternative transcription dependent on starting points of the DNA codon, alternative splicing and post-translation modification of proteins. The E6 protein is a good example of this. In high-risk HPVs E6 can be translated into three different proteins via alternative splicing. The ability of high-risk HPVs to cause cancer and the inability of low-risk HPVs to do the same is directly linked to the oncogenic properties of the E6/E7 proteins [Syrjänen 2012]. It can be hypothesized that DNA sequence variation within e.g. HPV-16, resulting in different mutations in E6 (or E7) can display differential oncogenic properties.

HPV in menopausal women

The prevalence of HPV is highest in those women who recently became sexually active, and decreases with increasing age. The menopausal transition, however, comes with an increased risk of infection, as was found in a large study among Taiwanese women. In this study HPV prevalence was 10% (944/9430) vs. 12.9% (683/5294) for non- and post-menopausal women, respectively ($p < 0.0001$) [Lai 2012]. A similar prevalence has been found in other studies [Smith 2004, Smith 2008], while a prevalence of around 30% has also been documented [Gyllensten 2012, Garcia-Pineres 2006]. Several factors that may account for the increase in HPV prevalence are: increased sexual risk behavior, physiological changes that increase detectability of HPV, reactivation of latent infections due to immunological senescence and a longer persistence of HPV infections in older women [González 2010]. Also, the prevalence of HPV is heavily influenced by geographical and regional differences [Lai 2012], so comparing different study populations has to be done carefully.

In a large Taiwanese cohort study, it was found that menopausal women have twice as much risk of having cervical cancer compared to pre-menopausal women [Liao 2012]. However, the study does not mention any correction for age. Therefore, this finding is not surprising as it takes a long time for CIN to progress into cervical cancer so menopausal women who are in general older than pre-menopausal women will have more chance of having cervical cancer. In contrast, cytologic regression of abnormal squamous cells does not seem to be influenced by menopausal status or age [Wang 2009].

An early age at which women go into menopause might be an important risk factor for the development of cervical cancer [Franceschi 2003], and for HPV infection [Smith 2002], but results on this are inconsistent [Smith 2004].

Entering the menopause comes with hormonal fluctuations and in post-menopausal women, estrogen and progesterone levels are lower compared to pre-menopausal women. Some women undergo hormone replacement therapy (HRT) to reduce some of the symptoms associated with lower hormone levels due to menopause. It has been postulated that, because estrogen and progesterone receptors are present in HPV-related cervical lesions, HRT might play a role in HPV infection and cervical cancer [Lacey 2000]. Interestingly, Smith *et al.* found that past and current users of HRT had an increased risk of current HPV infection compared to never-users. They did not, however, have an increased risk of an abnormal Pap smear or past HPV-related disease [Smith 2003]. Other studies are both confirming and in contrast to these results. The correlation between HRT use and HPV prevalence was found to be of marginal significance in one study [Althoff 2009], not significant in another [Ba 2005], and yet another study found a large increase in risk of HPV infection for women on HRT [Burke 2009]. It is mentioned that although current HRT users are likely to have annual Pap smears taken, past HRT users, who are also at an increased risk according to some studies, may not get Pap smears taken and are therefore more at risk to develop cervical cancer [Smith 2002].

HPV-positive menopausal women might have altered immune responses. Marks *et al* studied the cervical concentrations of pro- and anti-inflammatory cytokines and chemokines in menopausal women in the context of high risk-HPV infection [Marks 2011]. In HPV-negative menopausal women, they found a correlation between IL-2 and T-cell specific effector molecules such as IL-5, IL-7, IL-9, IL-12 and IL-15. In high risk-HPV positive menopausal women, they noticed a shift from IL-2 to EOTAXIN (an eosinophil-selective chemokine). They state that their data supports a hypothesis for a potential role of granulocyte-like cells and other non-antigen specific immune cells in mediating HPV host response in older women [Marks 2011].

Most studies that investigate the relation between HPV and menopause only classify women as pre- or postmenopausal. The Stages of Reproductive Aging Workshop (STRAW) system of classification categorizes women into pre- and postmenopausal, but also into “early transition” and “late transition”. Women going through these transition stages are known as “perimenopausal”. One study that distinguished between pre-, peri, and postmenopausal phases found a significant increase in HPV detection only among the perimenopausal phase [Althoff 2009]. Another shortcoming is that most studies classify into the pre- and postmenopausal stages only by age. The study of Althoff *et al*. found no strong correlation between age and menopausal stage. Measuring hormone levels and other biomarkers may prove to be a more accurate approach in defining menopausal stage, but this has been difficult due to intra-individual variability [Althoff 2009].

The prevalence of serum neutralizing antibodies was found to decline with age in women infected with high risk-HPVs [Scherpenisse 2012(seroprevalence)]. This is possibly due to less shedding of HPV and different hormonal productions [Marais 1997].

FGT immune response to vaccines against HPV

Cervarix and Gardasil

Until recently, there were hardly any vaccines available against sexually transmitted diseases, namely only for hepatitis A and B. In 2006, Gardasil and Cervarix became available on the market. These vaccines contain HPV-16 and -18 (and also HPV-6 and HPV-11 in the case of Gardasil) VLPs with recombinant L1 protein. Cervarix is produced by GlaxoSmithKline, using a baculovirus expression system and formulated with AS04 adjuvant. It is licensed for administration at 0, 1 and 6 months. Gardasil is produced by Merck, in yeast and formulated with amorphous aluminum hydroxyphosphate sulfate salt. It is administered at 0, 2 and 6 months [Stanley 2010]. The vaccines are prophylactic, and it is therefore adviced to be only administered to women who have not yet become sexually active, as they are the only ones who are guaranteed free of HPV. Although women who have a current infection can also be vaccinated, it is currently not clear if this will induce a protective immune response. While both vaccines have proven to be safe and protective, a serological correlate for protection has yet to be found, although evidence suggests that the prevalence of neutralizing antibodies is vital in protection against HPV infection [Harper 2004, Paavonen 2009, Romanowski 2009, De Carvalho 2010, Wheeler 2012].

Humoral response

Cellular responses are very important in the induction of immunity to the intracellular pathogen HPV. However, it seems that antibodies confer (long)-lasting immunity against papillomavirus infection. The first study displaying protective immunity through antibodies dates back to 1937, when the group of Shope *et al* injected papillomavirus intramuscularly or intravenously into rabbits. This generated neutralizing serum antibodies, and the rabbits were subsequently challenged with the virus and were found to be immune [Shope 1937]. In humans the HPV vaccines induce serum antibodies as well. However, in order to block viral entry into the basal keratinocytes, virus-neutralizing antibodies need to be present in cervicovaginal secretions (CVS). Data on vaccine-induced HPV-specific antibodies in CVS are scarce, with only a few studies having assessed the presence of these antibodies [Kemp 2008, Einstein 2009, Schwarz 2009, Petäjä 2011, Scherpenisse 2012]. Schwarz also did an analysis on four studies investigating HPV vaccine induced antibodies in the CVS [Schwarz 2010]. However, two are included in the above mentioned studies [Einstein 2009, Schwarz 2009], while one does not actually contain any data on cervical antibodies [Pedersen 2007], and one is a clinical trial funded by GSK of which results are currently not available.

These 5 studies [Kemp 2008, Einstein 2009, Schwarz 2009, Petäjä 2011, Scherpenisse 2012] determined only IgG levels except for Scherpenisse *et al.* who included IgA. All studies related to the bivalent vaccine except for Einstein *et al*. who studied the quadrivalent vaccine. Scherpenisse *et al* used a multiplex-immunoassay (MIA) to measure the HPV-specific antibody levels. Kemp *et al* used two different assays for the determination of antibodies, ELISA and secreted alkaline phosphatase neutralization assay (SEAP-NA). Since the ELISA and SEAP provided highly similar results [Kemp 2008], and the ELISA has higher reproducibility and is also used in most other studies, we will focus here on the ELISA results.

Presence of cervical antibodies after vaccination

The presence of cervical antibodies is thought to be critical in protective immunity against HPV infection. Kemp *et al.* studied the prevalence of IgG antibodies in the CVS found before (n= 5) and after vaccination (n=50, 12 months after first dose). Samples taken before vaccination were IgG negative for both HPV-16 and HPV-18, and after vaccination all CVS samples were positive. Einstein *et al.* measured positivity in CVS 7 months after the first vaccination. The bivalent vaccine induced cervical HPV-16 and HPV-18 antibodies in 95.8% (46/48) and in 89.6% (43/48) of immunized women respectively, compared to 0% (0/24) and 4.2% (1/24) before vaccination. The quadrivalent vaccine induced CVS antibody concentrations in a similar percentage of individuals: 89.5% (51/57) for HPV-16 and 70.2% (40/57) for HPV-18 [Einstein 2009]. Scherpenisse *et al* detected 88% and 70% positivity for HPV-16 and HPV-18 antibodies in CVS up to 24 months after vaccination [Scherpenisse 2012]. Similarly, the group of Petäjä found anti-HPV-16 and anti-HPV-18 antibodies in the CVS in 84.1% (58/69) and 69.7% (46/66) of their patients 48 months after vaccination, and similar results were found 24 and 36 months after vaccinations. The subjects thus remained seropositive for at least this amount of time. This suggests a consistent transudation over the course of at least 4 years [Petäjä 2011]. To sum up, the bivalent and quadrivalent induce an antibody response against HPV-16 and HPV-18 which probably transudates into the CVS in about 85-100% and 70-100% of vaccinated women.

Correlations between serum and cervical anti-HPV antibodies

A correlation between serum and cervical anti-HPV antibodies points towards possible transudation of antibodies. The correlations between serum and cervical secretion antibodies found by Kemp *et al.,* amounted to R= 0.73 and 0.75 for HPV-16 and HPV-18, respectively. Two other groups measured similar correlations of R=0.73–0.90 for HPV-16 and R=0.82–0.93 for HPV-18 [Schwarz 2009], R=0.84-0.93 for HPV-16 and R=0.90-0.93 for HPV-18 [Petäjä 2011]. Scherpenisse *et al*. found substantially lower correlations: R= 0.58 for HPV-16 IgG and R=0.50 for HPV-18 IgG and R=0.54 for HPV-16 and HPV-18 IgA.

Subjects with a detectable level of anti-HPV-16 or anti-HPV-18 antibodies in their CVS had higher serum concentrations of these antibodies compared to those without cervical antibodies. This difference in serum concentrations increased with time [Petäjä 2011]. This might indicate that transudation is dependent on a high level of serum antibodies. Overall, the high correlations between serum and cervical anti-HPV antibodies in all studies indicate transudation. A high correlation between serum and cervical IgG antibodies against tetanus and diphtheria toxoid was also found, and since these antibodies are not locally produced, they could be used as a transudation marker [Scherpenisse 2012].

Crossreactivity

Good correlations between HPV-16 and HPV-18 antibodies in serum (R=0.75) and (R=0.81) in CVS were found, meaning that antibody responses to one antigen indicate a high change of good antibody response towards the other [Kemp 2008]. Some cross-reactivity between HPV-16/18 and other HPV types was also found in CVS [Scherpenisse 2012] as well as in serum [Smith 2007], with antibodies towards HPV-45 and HPV-18 showing the highest degree of correlation.

Influence of the menstrual cycle and oral contraceptives

Another study described vaccine-induced HPV-specific antibodies in CVS in a group of women vaccinated with an experimental HPV-16 vaccine based on HPV-16 VLPs [Nardelli-Haefliger 2003]. They mainly focused on the effect of the menstrual cycle and oral contraceptives. The women taking oral contraceptives had a relatively stable level of vaccine-induced IgG antibodies (and also of total Igs) in CVS. In contrast, during ovulation a significantly lower amount of vaccine-induced cervical IgG was detected. The correlation between serum and cervical antibodies also seems to be reduced during ovulation [Kemp 2008], although the influence of oral contraceptives is debated [Kemp 2008, Scherpenisse 2012].

Cellular response

No information currently exists on cellular mucosal immune responses to HPV after immunization with the bi- or quadrivalent vaccine in humans. At the most, some studies have investigated the interaction between HPV VLPs and mucosal immune cells. These VLPs are experimental vaccine candidates.

One such VLP, the chimeric VLP (cVLP), consists of a fusion between the E7 oncoprotein and L1 or L2 capsid proteins of HPV-16. It is currently a candidate for a therapeutic vaccine, as it was found to protect mice against E7-containing tumors [Greenstone 1998]. The mechanism behind this immunization is thought to be due to strong interaction between DCs and cVLPs, and the subsequent activation of $CD8^+$ T-cells [Rudolf 2001].

LCs can bind and take up HPV VLPs, but unlike DCs they do not initiate an immune response [Fausch 2003]. A key difference in the binding of VLPs to LCs and DCs is the cell surface receptor on these cells. LCs are the only cells expressing Langerin, a receptor which internalizes its ligand and delivers it to so-called Birbeck granules [Valladeau 1999]. Bousarghin et al found HPV-16 VLPs to colocalize with langerin on LCs, while on DCs heparan sulfates are the receptors used for entry [Bousarghin 2005]. Some evidence exists that heparan sulfate acts as a costimulatory signal to T-cells, which provides a possible explanation for the difference in immune response following DC or LC binding [Boursarghin 2005]. Fausch et al investigated several pathways downstream of virus receptors on DCs and LCs after HPV VLP stimulation. They found that the MAPK, NF-kB and PI-3K pathways were all activated in DCs, but not in LCs after HPV VLP interaction [Fausch 2005].

Oral infection and oral immunity to HPV

Prevalence of oral HPV and related cancers

Oral prevalence of HPV was found to increase with age up to about 4.5% in healthy adults [D'Souza 2011]. In contrast, another study has showed that oral HPV infection was highest in children, and decreased with age, with HPV-13 as the most prevalent type [Marais 2006]. In a study including 70 women with and 70 women without HPV genital lesions, oral HPV was found in 26 (37%) and 3 (4%) of them, respectively [Gonçalves 2006]. In another study including 100 women with a clinical history of genital HPV infection, 81% (81/100) had detectable HPV-DNA in oral mucosal cells, and alcohol consumption was found to be a risk factor [Peixoto 2011]. In women with CIN, oral infection with HPV is increased similarly to cervical infection with HPV, indicating deficient immunity at both

cervical and oral mucosa. The most prevalent oral HPV types were HPV-11, -28, -33 and -72 [Passmore 2007]. In contrast to cervical cancer, only half of oral cancers are positive for HPV-DNA.

Oral immune responses

Oral secretory IgA levels in the saliva are much lower in women who are HPV positive in the oral or genital mucosa [Gonçalves 2006] compared to healthy controls, suggesting a protective role for this antibody in saliva. In contrast, oral IgG and IgA are present much more often in women who display CIN compared to healthy controls [Marais 2001]. In that study, oral anti-HPV IgA was predominant over IgG, which is in agreement to other mucosal areas except for the IgG dominant FGT. The correlation of CIN and oral humoral responses could indicate a functional common mucosal immune system, however the group of Marais did not determine whether the CIN patients also had detectable oral HPV. Therefore, it cannot be established if the antibody response was induced by CIN or by an undetected oral infection. In a later study by Marais, oral HPV-specific antibody prevalence was found to be low in children and increasing with age in adults, up to 41% and 38% for HPV-16 IgA and IgG, respectively. The largest increase was observed between children and adolescents, and it is postulated that this is due to (the start of) sexual activity [Marais 2006]. In addition, no significant difference in oral IgA or IgG levels could be found between cervical HPV positive and negative women. Oral IgA HPV-16 antibodies were the most prevalent (antibodies) in that study (61.2%), although only 1 patient in the study was HPV-16 oral positive. Passmore *et al* showed a poor correlation between the HPV-16-specific IgG and IgA antibody responses in the FGT and oral compartments [Passmore 2007].

Using indirect immunofluorescence (IFI), oral anti-HPV IgA was detected in a group of 100 women with a clinical history of genital HPV infection [Peixoto 2011]. No correlation between the presence of oral HPV and oral anti-HPV IgA could be established, but it was suggested this might be due to the technique used. The specificity of IFI was 43.2% and the sensitivity 52.6%. Peixoto *et al* also indicated an association between recurrent genital HPV lesions and oral anti-HPV IgA.

HPV-6 and HPV-11, the most prevalent subtypes found in benign genital warts, can infect the larynx and subsequently cause Recurrent Respiratory Papillomatosis (RRP) [Bonagura 2011]. While benign, these growths can eventually block the airways up to the point of mortality. The disease is most prevalent in children aged 2-3 years of age (transmitted from mother to infant) [Armstrong 2000] and adults, and the growths can be very recurrent, sometimes requiring monthly removal. Bonagura *et al* [Bonagura 2011] reviewed the cellular responses in RPP patients. They propose that in the context of RPP, HPV proteins induce an inhibitory cycle which affects and gets driven by regulatory T-cells, memory Th2-like T-cells and immature DCs, causing a suppression of anti-HPV Th1-like immune function. Both PBMCs and T-cells found in patients with RRP show an increased and constitutive expression of IL-4. T-cell alloreactivity is suppressed by E6, and NK cytotoxicity is defective [Bonagura 2011]. In short, in RPP patients HPV-6 and HPV-11 are capable of inducing tolerance and inhibit T-cell function, much like HPV-16 and -18 in genital infections.

No oral antibody measurements have been performed following immunization with the bivalent or quadrivalent HPV vaccines.

HPV infections and immune responses in males

The information on HPV infection in men is scarce compared to the information on HPV infection in women. HPV infection in men portrays similar clinical outcomes: cancer (anal, penile, prostate, oral) caused by high-risk HPVs, and genital warts and RPP caused by low-risk HPVs. RPP is a lot more common in adult men than in adult women (4:1 ratio) [Bonagura 2011]. In line with cervical cancer, HPV-16 is the predominant type in penile squamous cell carcinomas (SCCs), however, HPV DNA seems to be less prevalent in penile SSCs compared to cervical cancers [Heideman 2007]. HPV DNA has been detected in the urine of men with urethral condylomata, suggesting a possible transmission route for HPV, but also an easy screening method since the HPV in urine and condylomata were of the same subtype [Iwasawa 1996]. This could be useful, as in men it is unclear what the best sampling technique is or which genital site to sample from [Partridge 2006].]. In a study performed on young Mexican soldiers, HPV DNA was prevalent in 46.4% of samples taken of the external genitalia, much higher than in the urethra (20.8%) or the meatus (12.1%).The distribution of HPV types in different anatomical sites was equal, and the clustering of multiple HPV types occured at random similar to infections in women [Vaccarella 2011]. As for transmission, a possibility of HPV transmitted via sperm exists as the vas deferens has been found to be HPV-positive [Rintala 2002].

Partridge et al reviewed 12 studies on HPV prevalence, and found a prevalence varying from 3.5% to 40% [Partridge 2006]. The most frequently detected high risk HPV was in almost all studies HPV-16. A more recent study which looked that investigated clustering of HPV infections in men found HPV-16 to be most prevalent in single infections, but HPV-62 was most prevalent in men infected with multiple HPV subtypes [Vaccarella 2011]. Nonetheless, HPV clustering happened at random.

The prevalence of HPV antibodes is similar in men and women. Scherpenisse et al found similar seroprevalence between males and females of middle aged and adult cohorts. At a younger age, women had a higher seroprevalence than men, although it is stated that the seroprevalence found in men in that study is lower compared to other studies, while for females it is similar [Scherpenisse 2012 prevalence]. Concordance between HPV subtypes in husband and wife was not found by Franceschi [Franceschi 2002]. In contrast, in another study performed in Brazilian couples, concordance of at least one HPV subtype was found in 56% of these couples, and 84% of the men had the same high risk-HPV as their female partner, suggesting an increased efficacy for vaccination if men are included [de Lima Rocha 2012].

Summary / Future perspectives

HPV is a very common sexually transmitted pathogen. Most men and women will get infected soon after the onset of sexual activity. In most cases, this infection will be asymptomatic and the virus will be cleared within the next year. In some cases, though, infection persists. Virtually all cases of cervical cancer are linked to a long, persistent HPV infection. Recently, two vaccines became available on the market, which induce protective antibodies against HPV. HPV is a strictly intraepithelial pathogen, there is no viremia, and the immune system has poor access to the infected tissues. HPV targets the keratinocytes of the female genital tract, and therefore the mucosal immune system is the first line of defense. The protective antibodies induced by the vaccines are thought to transudate from serum into cervicovaginal secretions. However, information about the role of the mucosal immune system after HPV infection is scarce. In this literature study, we focused on the genital mucosal immune responses following HPV infection and vaccination.

There exists a lot of variation in the studies that investigated the correlations between cervical anti-HPV IgG/IgA and HPV-DNA. However, on average, the chance of having a current HPV-16 infection was twice as high when cervical IgA antibodies to HPV-16 were present. This suggests that these antibodies are non-protective. In contrast to these non-protective antibodies are the results from vaccine studies, where it has been proven that vaccine induced serum antibodies are the protective factor. One study showed that cervical antibodies were detectable in more women 4-12 months after infection compared to women with current infection. Perhaps the increased prevalence of HPV infection among cervical anti-HPV IgA/IgG positive women is the result of a slow-working immune response, and in those women HPV is in the process of being cleared from the system. Studies that investigate HPV infection (and possible HPV-related disease progression) and cervical antibody status in women throughout their lives could help elucidate this subject.

Much research is currently being focussed on mucosal vaccines. However, because HPV is specialized in avoiding immune responses in the FGT, a vaccine administered in the FGT might not be very effective. Even though HPV VLPs, which are the antigens used in the vaccines, do not contain most of the HPV proteins necessary for immune evasion, the fact that they elicit an immune response in DCs but not in LCs favors a vaccine administration close to DCs. The current HPV vaccines emphasize this, as they target DCs, and induce strong antibody responses. A therapeutic vaccine against HPV, the candidate for which is a chimeric VLP, also supposedly works through interactions with DCs.

Despite working prophylactic vaccines, more research into the interaction between the immune system and HPV is warranted. Studies on the cellular response after HPV vaccination could shed some light on the interactions between the cellular mucosal immune system and HPV, and perhaps further help develop a therapeutic vaccine.

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