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Dissecting the Influence of Human Immunodeficiency Virus Type 1 on Human Papilloma Virus Infection, Disease and Immunity

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I hereby declare, that the submitted thesis entitled:

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Mbeya, 26.08.2022 — Wilbert Laurence Mbuya

place, date — Signature doctoral candidate

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List of abbreviations

AIDS – Acquired Immunodeficiency Syndrome

ART – Antiretroviral Therapy

CC – Cervical Cancer

CCR5 – Chemokine Receptor 5

DFG – Deutsche Forschungsgemeinschaft

ELISpot – Enzyme Linked ImmunoSpot Assay

FBS – Fetal Bovine Serum

HIV-1 – Human Immunodeficiency Virus type1

HLA-DR - Human Leukocyte Antigen – DR isotype

HPV – Human Papillomavirus

HSIL – High grade Squamous Intraepithelial Lesion

IFNγ – Interferon Gamma

LSIL – Low grade Squamous Intraepithelial Lesion

PBMCs – Peripheral Blood Mononuclear Cells

PD-1 – Programmed Cell Death Protein-1

PPD - Purified Proteins Derivative

SCC – Squamous Cell Carcinoma

SFC/10^6 – Spot Forming Cells per Million PBMCs

VL – Viral Load

WHO – World Health Organization

List of publications

1. **Mbuya W,** Held K, Mcharo RD, Haule A, Mhizde J, Mnkai J, et al. Depletion of Human Papilloma Virus E6- and E7-Oncoprotein-Specific T-Cell Responses in Women Living With HIV. Frontiers in Immunology. 2021;12:4473.

2. **Mbuya W**, Mcharo R, Mhizde J, Mnkai J, Mahenge A, Mwakatima M, et al. Depletion and activation of mucosal CD4 T cells in HIV infected women with HPV-associated lesions of the cervix uteri. PLOS ONE. 2020 Oct 2;15(10):e0240154.

3. Mcharo R, Lennemann T, France J, Torres L, Garí M, **Mbuya W,** et al. HPV Type Distribution in HIV Positive and Negative Women With or Without Cervical Dysplasia or Cancer in East Africa. Frontiers in Oncology. 2021;11:4679.

Contribution to the publications

The work presented in the thesis is part of the Deutsche Forschungsgemeinschaft (DFG) funded 2H study, which dissects Human immunodeficiency virus and Human papilloma virus (HIV-HPV) interaction in Tanzanian women. Within this study, I contributed to overall research coordination, laboratory analyses, statistical analyses of generated data, visualization of research data, drafting figures and manuscript preparation, scientific output as outlined in the three statements below:

1.1 Contribution to article I (Mbuya *et al*. 2020)

This publication assessed the effects of HIV-1 and cervical HPV infections on cervical T cells and expression of the HIV co-receptor CCR5, T-cell gut homing receptor and HIV facilitator/co-receptor $\alpha_4\beta_7$, and the activation marker HLA-DR. This publication reported that HIV, HPV infection, and cervical lesions are associated with increased proportion of activated cervical T cells, thereby possibly hindering effective HPV clearance and promoting cervical cancer. However, HPV infection was not associated with a change in proportions of cervical T cells expressing HIV co-receptors CCR5 and $\alpha_4\beta_7$. This implies that HPV does not increase the risk for HIV acquisition by recruitment of CD4 T cells expressing the HIV co-receptors CCR5 and $\alpha_4\beta_7$. My contribution to this publication was through performing flow cytometry immunophenotyping laboratory procedures and analysis using BD Facs Canto II and FlowJo respectively, statistical data analysis, preparing all figures for this manuscript and manuscript preparation. Furthermore, after submission of the manuscript to PLOS One, I contributed by responding to the reviewers' comments.

1.2 Contribution to article II (Mbuya *et al*. 2021)

This publication dissected the effects of HIV-1 and HPV infection on the frequency of response and magnitude of HPV-specific T-cell reactivity to identify a possible mechanism how HIV weakens HPV-specific immunity. This article reported that HPV-specific T cells from HIV+ women are depleted or dysfunctional, particularly in women with severe immunosuppression, detectable viral load and those with precancerous lesions and cancer. Furthermore, the article showed HPV16 oncoproteins are significantly less immunogenic when compared to oncoproteins from HPV18 and HPV45. This observation sheds more light on the exceptional potential of HPV16 to cause cancer because it's oncoproteins are least recognized by T cells and therefore foster prolonged HPV16 viral persistence.I contributed to this publication by conducting the Enzyme linked Immunospot assays (ELISPOT), statistical data analysis, preparing figures for publication and manuscript drafting as the first author and corresponding author. Furthermore, I submitted the manuscript to Frontiers Immunology and contributed to responding to the reviewers' comments.

1.3 Contribution to article III (Mcharo *et al*. 2021)

This publication examined the effects of HIV-1 infection on the HPV type-specific prevalence, with the primary focus to determine whether HIV infection alters the distribution of HPV genotypes causing cancer in HIV+ women. This article reported that regardless of HIV infection, HPV16, 18, and 45 were associated with the vast majority of cancer cases. These findings should guide national cervical cancer screening programs and national vaccination programs to focus on HPV16, 18 and 45, regardless of HIV infection. Furthermore, findings of this publication guided the analysis and focus on HPV16, 18 and 45 and interpretation of article II (Mbuya et al. 2021). For this publication I contributed to HPV genotyping analysis, overall coordination of the project, writing and reviewing the manuscript.

2. Introduction

Human papillomaviruses (HPV) are a group of more than 200 viral genotypes classified under the *Papillomaviridae* family [1–3]. The majority of these viruses are harmless while a few can cause disease - in both men and women – and are associated with skin warts, conjunctival papillomas, respiratory papillomatosis, and cancers of the base of the tongue and tonsils, as well as anal cancer. In men, HPV is associated with penile cancer, while in women, the virus is associated with cancers of the vagina, vulva and cervix uteri [4].

2.1 HPV genome and viral structure

All HPVs are non-enveloped, with an icosahedral capsid of approximately 55nm in diameter [5]. Genetically, the virus has 8 genes, packed into 8 kilobase pairs (kbs) of circular double stranded DNA genome (**Fig 1)**. Functionally, the HPV genome is divided into three parts [6,7]. The first is a long control region (LCR), a non-coding part of the HPV genome. It contains regulatory sequences and is associated with control of viral synthesis. The second is the early coding region; this open reading frame (ORF) codes for early proteins E1 to E7. The early proteins are synthesized early in the viral infection and primarily function to regulate transcription and translation of viral proteins. Finally, the late coding region contains genes for the late proteins L1 and L2. The late genes L1 and L2 code for structural proteins L1 and L2. These proteins self–assemble to form a viral capsid that binds and encapsulates the viral genetic material [8–10]

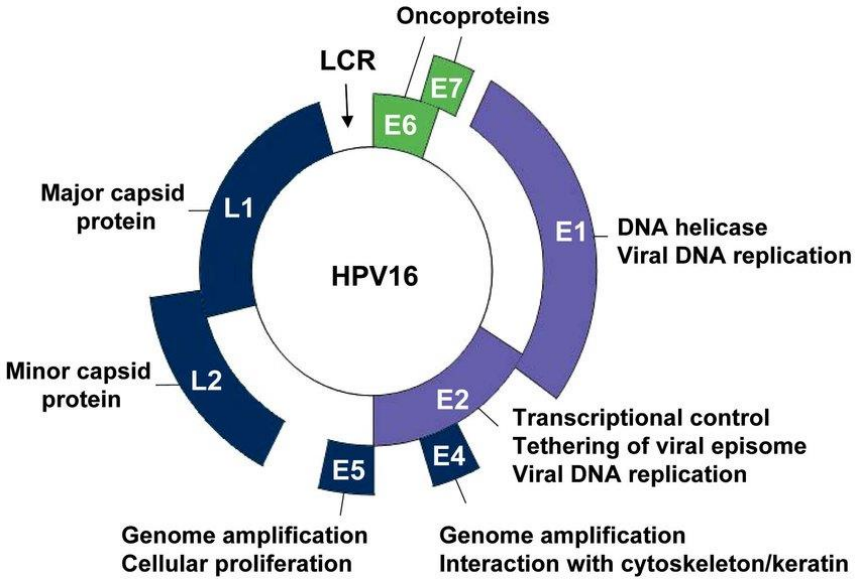

Figure 1: HPV genome and associated proteins [10] .

2.2 HPV Classification

HPV can be classified by their oncogenic potential or genetic variation of the L1 gene [1,2,11].

Classification by oncogenic potential

With respect to the ability to cause cancer, HPV are classified into 3 groups [7]: First, the low risk (LR)-HPVs. These viruses are unlikely to cause cancer, they are associated with skin and genital warts. Examples include HPV6 and 11. The second group contains putative high risk-HPV (PHR-HPV). These viruses are rarely associated with cancers and mostly in individuals who are immunocompromised. Examples include HPV26, 53 and 66. The last group are the high-risk (HR)-HPVs, these are associated with squamous intraepithelial lesions of anogenital and oropharyngeal regions. HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and HPV68 are regarded as high risk HPV [12]. In women, HPV16 and 18 together account for about 70% of all cervical cancer (CC) cases [13–15].

Classification by molecular sequencing

All HPVs are classified under the *papillomaviridae* family and then divided into 5 genera (alpha, beta, gamma, mu and nu) [1,2,6,11]. The classification is based on the nucleotide sequence of the L1 gene, the most conserved gene in papillomaviruses. HPV whose L1 gene nucleotide sequence differs between 2% and 10% are termed as different subtypes while those whose nucleotide sequence differs by greater than 10% are grouped as different HPV genotypes [2,11]. The HPV genotypes HPV16 and 18, as well as other HR HPVs, belong to the alpha genus [16].

2.3 HPV life cycle and oncogenesis

2.3.1 HPV life cycle

HPVs are epithelial-tropic viruses that perpetuate their life cycle by infecting cells of the basal epithelium [9]. They enter the basal epithelium through micro-wounds resulting from skin trauma [17]. Once at the basal epithelium, the viral capsid protein L1 attaches to heparan sulfate proteoglycans (HSPG) found on the extracellular matrix and on the cell surface membrane [18,19]. The exact unique cell surface entry receptor to which HPV binds to prior to endocytosis is controversial. Some researchers have even suggested that HPV does not have one unique cell surface receptor, and rather might bind to a number of different cell membranes receptors [20,21]. Generally, it is accepted that L1 capsid protein binds to adhesion alpha-4 beta-6 integrin on the surface of epithelial cells [22], syndecan-1 [20,23] and laminin-5 [24,25]. The initial binding of L1 to HSPG induces conformational changes on the capsid that exposes the previously inaccessible furin cleavage sites within the L2 proteins for proteolytic cleavage furin [26]. The conformational change serves to facilitate binding of L1 to cell membrane receptors or to facilitate binding of HPV to a so far unknown secondary receptor with greater affinity. This triggers endocytosis of the virus particle by either clathrin- or caveolae-dependent pathways. The virus then travels to the nucleus of the cell through cellular microtubules and microfilaments. Once inside the nucleus of

keratinocytes, the virus starts viral gene expression and replication to form new viral particles. This process is tightly regulated and synchronized with cellular transcription and translation processes. Assembly of viral particles occurs in mature terminally differentiated epidermal keratinocytes. Newly synthesized and assembled viruses are shed through desquamation. It is worth reiterating that the HPV lifecycle is non-cytopathic and does not have a viremic phase (**Fig 3**) [9,27–29].

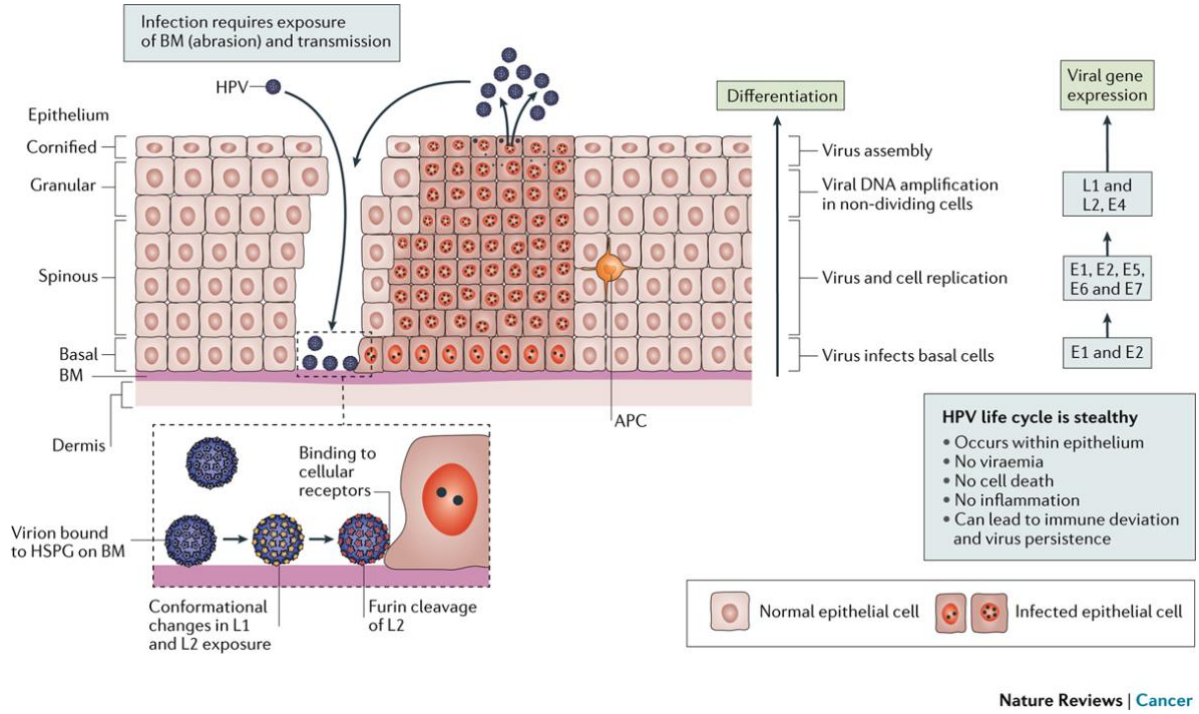

Figure 3: HPV life cycle from Roden and Stern [30]

2.3.2 Oncogenesis

Cervical cancer arises from epithelial cells of squamo-columnar junction of the cervix infected with any of HR-HPV genotypes [31]. HPV does not set out to cause cancer, rather the cancer is a byproduct of dysregulation of transcription and translation of HPV oncogenes E6 and E7. Normally, synthesis of E6 and E7 is tightly regulated by the transcription factor HPV protein E2 and by synchronization with host cellular factors [32,33]. At times, for so far unresolved reasons, HPV DNA may integrate with cellular DNA [34]. HPV DNA integration usually results in disruption/breakage of the E2 gene leading to loss of functional of E2 proteins [35]. In the absence of E2, E6 and E7 proteins are constitutively expressed, resulting in uncontrolled cell division [36]. HPV proteins E6 and E7 can then lead to cancer by interacting with the host tumor suppressor protein p53, and with transcription factor retinoblastoma family of proteins (pRB) respectively. [37,38]. Primarily, p53 is a transcription factor that binds to human genome at multiple sites [39] to inhibit cancer by cell cycle regulation, promoting DNA repair, and apoptosis in metabolically stressed or damaged cells [40]. The HPV E6 protein binds to p53 protein tagging it for

ubiquitination and subsequently proteolytic cleavage, thereby abolishing its anti-tumor activity and inducing carcinogenesis [41]. pRB is another anti-tumor protein in human cells that inhibits cellular division by maintaining the cell in the G-phase of the cell cycle by binding and inhibiting the functionality of E2F [42], a transcription factor that promotes cells division and synthesis [43]. The HPV E7 oncoprotein binds to pRB, releasing E2F, and the released E2F locks the cell in the S-phase of the cell cycle [44]. The uncontrolled cell division interrupts the life cycle of HPV, resulting in a halt of the production of new HPV viral particles.

In summary, metabolic stress, host/viral genetic instability, along with abrogation of cellular mechanism controlling cancer and DNA repair, lead to loss of cell cycle regulation and breakage of DNA resulting in genetic mutations, precancerous lesions and subsequently cancer. It is worth stating that lesion progression is not unidirectional; in immunocompetent individuals, about 50% - 70% of the lesions can regress to normal cervix [45,46] but once the lesions reach cancer stage, regression is not possible. For immunocompromised women, especially those with HIV, HPV clearance and lesion regression rates are low [47,48].

2.4 HPV and Cervical Cancer

2.4.1 HPV and cervical cancer Epidemiology

HPV is the most common sexual transmitted virus in sub-Saharan Africa and throughout the world [49,50]. The burden of HPV and associated cervical cancer is highest in regions where HIV is co-prevalent [51]. In 2020, Tanzania had an HIV prevalence of 4.8% [52] and a 9.3% cumulative risk for cervical cancer by the age of 75 [53]. This makes east African women at great risk of HPV infection and associated cervical cancer, **Fig 2**.

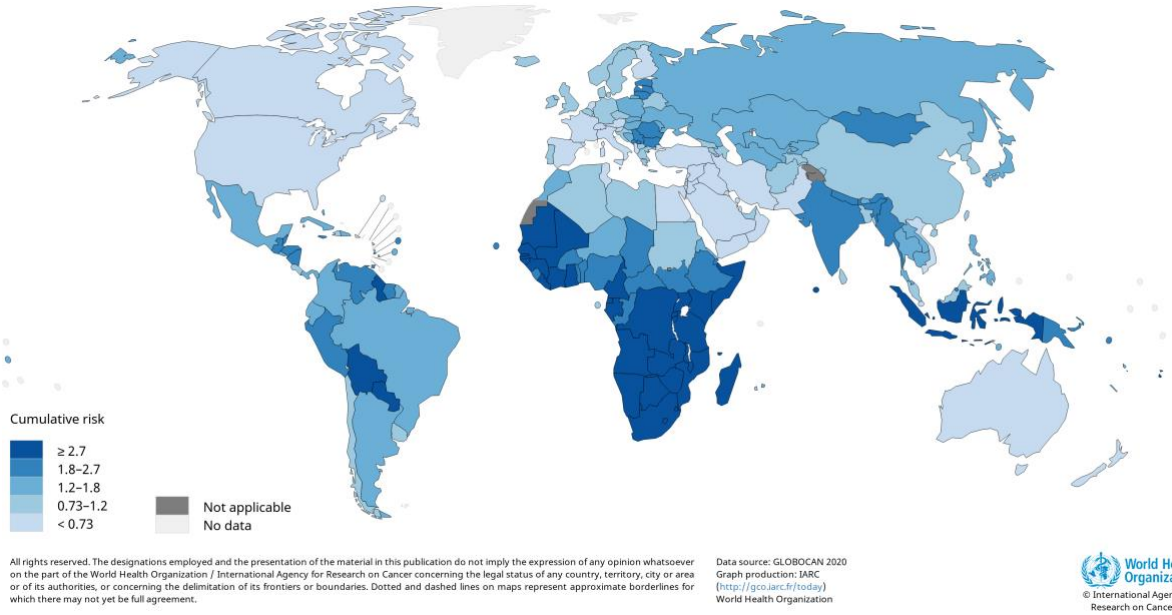

Figure 2: Heatmap showing estimated cumulative risk of incidence for cervical cancer in 2020 for women up to 75 years.

2.4.2 Diagnosis, treatment, and vaccination

2.4.2.1 Diagnosis

HPV diagnosis involves the detection of HPV or HPV proteins in cervical tissue by either polymerase chain reaction (PCR) or immunohistochemistry. To prevent cervical cancer, the World health organization (WHO) recommends molecular detection of HR HPV DNA as a screening tool. HIV negative women, 30 years of age and above should be screened every 5 to 10 years while women living with HIV should be screened from the age of 25 and at a frequency of 3 to 5 years [54].

Cervical lesions can be diagnosed by either:

(I) Visual inspection by acetic acid or visual inspection by Lugol's iodine. When 3 – 5% acetic acid is applied to abnormal cells in the cervix, the solution turns from clear to whitish [55,56] while Lugol's Iodine when applied to abnormal cells turns from brown – black to yellow *in situ* [55].

(II) Papanicolaou's (PAP) smear, is a cytology based method used to diagnose abnormal cervical cells. Here, cells are collected from the cervix by an Ayre's spatula and cytobrush, fixed and transferred onto a microscope slide. In the lab, the slides are stained with Papanicolaou's stain and analyzed for abnormal cells by a pathologist. Results from the pap smear can be either no lesions, low grade squamous intra-epithelial lesions (LSIL), high grade squamous intra epithelial lesion (HSIL) and squamous cell carcinoma (SCC) [57].

(III) Histology based techniques can be used to diagnose and stage suspicious cervical cancer tissues, as confirmation after visual inspection and/or pap smear. Here biopsies are collected, formalin fixed, paraffin embedded, transferred on a slide and stained with Hematoxylin and Eosin (H & E) dyes [58]. Results are reported according to the Bethesda system of classifying and reporting cervical pathology results. Federation Internationale de Gynecologie et d'Obstetrique (FIGO) staging is used to group stages of cervical cancer. There are 4 FIGO stages (I - IV): Stage I, cancer is within the cervix. Stage II, cancer has evolved beyond cervix but has not reached pelvic wall. In FIGO stage III, the cancer has reached the pelvic wall while for FIGO stage IV, the cancer has evolved beyond pelvic walls [59].

2.4.2.2 Treatment

There is no medical treatment for HPV infection, the HPV viruses are usually cleared from the body within two years by the immune system [60]. However, once a cervical lesions develops,

the lesion can be treated depending on the age of the patient, stage of the lesion i.e. the cervical intraepithelial neoplasia (CIN) stage, and any other co-morbidities the patient may have. Pre-cancerous lesions are treated according to WHO guidelines. No intervention is done for CIN-I, here the expectation is that the lesions will spontaneously regress to normal. The patient should be followed-up for three years on an annual basis. Those with CIN-II are usually treated with cryotherapy and laser vaporization of the lesions. Once successful, the patient should be followed up annually. Patients with CIN-III undergo loop electrosurgical excision procedure (LEEP) of lesion and should similarly be followed up every three months. Once the lesions have advanced to cervical cancer, treatment options are surgical removal of tumor including hysterectomy, chemoradiation and brachytherapy. When these options are not possible because the cancer has progressed too far, the last resort is palliative care [61].

2.4.2.3 Immunization

Vaccination for many infections, including HPVs, can be divided into either prophylactic or therapeutic vaccines. Currently, 3 prophylactic vaccines are approved by the United States Food and Drug Administration (FDA): Cervarix against HPV16 and 18, Gardasil against HPV6, 11, 16 and 18, and Gardasil-9 against HPV6, 11, 16, 18 as well as HPV types 31, 33, 45, 52 and 58 [62]. These vaccines are based on self-assembling virus like particles (VLP) of the L1 viral capsid [63]. Once vaccinated, individuals develop antibodies against the viral capsid of HPV. These antibodies bind to virus particles inhibiting infection of keratinocytes.

Vaccines are recommended for pre-adolescent sexually naive girls, and in some countries for boys. These vaccines are expensive and so mostly available in western countries and few low and middle income countries. As a result, HPV infection and disease remain a burden in many Asian and sub-Saharan African countries [64].

In Tanzania, HPV prophylactic vaccine is available and can be offered to those willing at no cost. Data on the uptake of the vaccine is difficult to get, however there are indications that many girls do not get the vaccine because of various anti-vaccine fears.

2.5 Immune Response to HPV

2.5.1 Innate Immunity against HPV

Intact skin, acting as a physical barrier, is the first obstacle to pathogens - including HPVs - have to overcome [65]. Without access to the basal membrane of the epithelium, HPVs are not able to access and bind to heparan sulphate proteoglycans (HSPGs) on the basal layer of the skin. Moreover, the skin along with associated glands secrete antimicrobial agents such as defensins, lactoferrin and dermcidin, compounds that are important in defending the body against HPV and other pathogens [66].

Once HPV has successfully infected epithelial cells, the initial immune response to the virus is initiated by infected keratinocytes themselves and subsequently skin-resident immune cells take over [67]. Infected keratinocytes can detect viral DNA through Toll like receptors (TLR) and respond by secreting TNF-alpha, IL-8 and type 1 interferons (IFN), i.e. IFN-alpha and IFN-beta [68], these cytokines function in an autocrine and paracrine manner to induce antiviral and anti-proliferative cellular states [67,69]. Furthermore, the secreted type -1 interferon attracts and activates immune cells in the skin [70]. HPV infected keratinocytes secrete monocyte chemotactic protein-1 (MCP-1) that will attract macrophages to infected cells [67]. Langerhans cells are the primary professional antigen presenting cells located in the epithelium [65]. These cells can engulf viral infected cells, process antigens, and move to the nearest lymph nodes for antigen presentation to naïve CD4 and CD8 T cells [71]. This process will lead to priming of HPV-specific B and T lymphocytes [72]. The role of Langerhans cells in HPV infection is a subject of investigation. A number of studies have reported a reduction in the quantity and functionality of Langerhans cells following HPV infection. The mechanism by which HPV hampers the functionality of these cells is still being investigated.

2.5.2 Adaptive Immunity against HPV

HPV antigen presentation to naive T cells in lymphoid nodes leads to the induction of HPV-specific CD4 and CD8 T lymphocyte response as well as B cells that work together to clear virus infected epithelial cells [73].

HPV-specific T cells have a crucial role in HPV clearance and in the control of HPV associated cervical warts and lesions [74–78]. The evidence for this stems from reports of regressing warts and cervical intraepithelial neoplasia being infiltrated with T-cells [79,80]. Data shows that the frequency of HPV-specific T-cell responses is reduced in women with advanced lesions compared to those without cervical lesions [81]. Furthermore, HIV+ women are disproportionality infected with HPV and have greater cervical cancer incidence [82].

The role of B cells in HPV-immunity has been studied in detail in HPV prophylactic vaccine trials. In this case, the protective effect of vaccines is attributed to antibodies against L1 capsid proteins of the HPV virion [83]. Antibodies against L1 and L2 capsid proteins of HPV will bind to the virion and cause steric hindrance to block HPV attaching to HSPGs on the surface of basal membrane epithelial cells [84–86]. Approximately only half of HPV+ women develop weak IgG and/or IgA antibodies six to eighteen months post infection [87]. Furthermore, anti-HPV antibodies have been found in vaginal secretion of HPV+ women. When present, these antibodies are said to have a protective role against auto-infection and re-infection rather than neutralizing an active HPV infection [88]. It is therefore plausible that cell mediated immunity (CMI) rather than antibody mediated immunity is essential in clearance of HPV and lesion regression. Together these studies highlight the importance of T-cells in the control of HPV and associated diseases. Since HIV infection is associated with a depletion of pathogen-specific CD4 T cells [89,90], we hypothesized

that HIV-induced depletion and/or dysfunction of HPV-specific T-cell immunity is responsible for the high propensity of HIV+ women to get HPV infection and develop cervical cancer.

2.5.3 HPV Immune Evasion

HPVs have successfully co-evolved with humans and other vertebrates for millions of years [91]. This co-evolution and adaptation to the human host has equipped HPV with numerous mechanisms to effectively infect humans and establish a productive infection. Listed below are some select strategies employed by this virus:

I. Location of Infection

HPV infects keratinocytes in the epidermis, this is far from the reach of most circulating immune cells [9]. Langerhans cells are the only professional antigen presenting cells (APC) that are relatively more abundant in the skin [67,65]. However, HPV hampers Langerhans cells trafficking to infected sites [92,93] and dampens their ability to process antigens [94]. Fully formed virions are only assembled after synthesis of L1 and L2 capsid proteins that occur in terminally differentiated cells on the uppermost layer of the skin [9]. This makes it difficult for professional APC to encounter, process HPV virions (that are naturally immunogenic) and present HPV peptides to immune cells. Early proteins E1-E7 are synthesized early in the HPV life cycle but in very minute quantities and moreover remain within the nucleus of the infected cells [9].

II. Non-cytopathic life cycle

Cell lysis exposes viral particles and attracts immune cells to the site of infection. However, HPV life cycle does not involve a cell lysis stage, rather newly made virions are released as terminally differentiated keratinocytes are shed off from the epidermis [28,9]. If the life cycle involved cell death, it is likely that this would activate the immune system and recruit various immune cells to fight the pathogen causing cell lysis.

III. No viremia

Pathogens whose life cycle involves a viremic phase are typically more immunogenic and can be cleared more effectively [95]. As HPV is an epithelial-tropic virus causing only local infections, it's lifecycle does not have a systemic phase at any point [9,27]. Therefore, HPV keeps itself well hidden from immune cells at all times. It worth noting that some viruses such as HIV and Hepatitis viruses are associated with long lasting chronic infections even though they can be detected in the blood [96,97].

IV. Induction of Th2 immune response

A proinflammatory cytokine state is essential in the clearance of various pathogens [95]. HPV, through its early viral proteins, can interfere with the Th1/Th2 balance to induce a

Th2 immune state marked by reduced IFN-gamma secretion and elevated pro-tumorigenic cytokines such as IL-6, IL-8, and IL-10 by modulating the expression of cellular pathways associated with pathogen immune responses [98,99]. For example, HPV E2 protein inhibits interferon transcription in keratinocytes [100] while E6 proteins is known to inhibit interferon secretions by interfering with DNA methylation [101] and IFN-k TING pathway [102]. An anti-inflammatory state is associated with immune tolerance rather than viral clearance, leading to cervical cancer disease progression [103,104].

V. Downregulation of MHC Class-I and CD1d

Cellular proteins MHC class-I and CD1d are essential for antigen presentation to T cells and pathogen clearance [105]. Viral proteins E5 and E6 inhibit the expression of MHC class-I [106,107]. Downregulation of MHC class-I will hamper presentation of viral peptides in HPV infected keratinocytes to CD8 T cells. Furthermore, HPV E5 protein has been associated with the downregulation of CD1d [108], thereby escaping from natural killer T cells [109,110].

VI. Inhibit the expression of Toll Like Receptor 9 (TLR-9)

The protein Toll like receptor – 9 (TLR-9), located in the cytoplasm of immune cells, can detect double stranded intracellular foreign DNA [111]. Foreign nucleic acids within the cells, including those from HPV virus can be detected by TLR-9 to set in motion the secretion of chemokines and cytokines initiating pathogen clearance [112]. Through a series of event and molecular complexes, HPV-oncoprotein E7 inhibits transcription of the *TLR-9* gene, thereby quenching the HPV infected keratinocyte of TLR-9 [113,114].

VII. Blocking secretion of type I interferons

Secretion of type I interferons by virus infected cells, as well as immune cells, serves to halt viral propagation to uninfected cells by inducing an antiviral state [115]. E6 proteins from HPV16 and 18 are known to block the activation of NF-kB, which in turn inhibits the production of IFN-alpha and IFN-beta [116,117]. The inhibition of type I interferons will therefore hinder the control of HPV.

VIII. Hampering macrophage functionality

Macrophages phagocytize, process and present foreign peptides to naive T cells in order to induce the proliferation of HPV-specific CD8 and CD4 T cells [73]. Monocyte chemoattractant protein -1 (MCP-1) is an important cytokine in attracting monocytes and macrophages from the systemic circulation [118]. Production of MCP-1 is enhanced by p53 , but since p53 is degraded by HR-HPV E6 proteins [119], there is diminished availability of MCP-1 and, in turn, migration of macrophages to HPV infected sites. Likewise, HR-HPV oncoproteins inhibit the secretion of macrophage inflammatory protein

3 alpha [92]. This is another potent chemoattractant of macrophages including skin resident macrophages.

IX. Codon usage bias

Codons refers to a set of three nucleic acids directing proteins synthesis [120]. HPV has a codon usage bias that is different from that of humans. Having a different codon usage bias ensures that viral protein synthesis is kept at a minimum level [121,122].

2.6 HIV-HPV Co-Pathogenesis

Both HIV and HPV infections are transmitted sexually [9,123]. Various studies link HIV infection with the increase in HPV infection and cervical cancers among HIV+ women [82,51,124]. Conversely, some studies report an increased risk of HIV infection amongst HPV+ women [125–127]. It is therefore important to study how these two virus infections affect each other.

2.6.1 Role of HIV on HPV

HIV+ women have a multi-fold increased risk of HPV infection and disease when compared to HIV negative women [82,124]. HIV increases the risk for HPV infection and progression to cervical cancer at the molecular and immunological level [82,128,129]. In fact, HPV associated cervical cancer is termed as an AIDS-defining illness [130]. At the molecular level, the HIV viral protein tat has been shown to disrupt epithelial tight junctions [131,132]. Disruption of the intact physical structure of the epithelium, may increase the probability for HPV virions to access its target cells at the basal layer of the skin. Furthermore, since HIV depletes CD4 T cells, this weakens both T and B cell mediated immunity and gives rise to opportunistic infection such as tuberculosis and Kaposi's sarcoma [133]. It is plausible that HIV induces depletion and/or dysfunction of HPV-specific T cells hindering HPV clearance. Therefore, HIV+ women have been reported to have longer HPV viral persistence and faster rates of lesion progression [134–136]. Antiretroviral therapy (ART) inhibits HIV viral replication, and in turn lowers plasma HIV viral load (VL) and reconstitutes circulating CD4 T-cell counts [137]. Treatment of HIV infections with ART has been linked to a decrease in the incidence of HIV-associated opportunistic infections such as Kaposi's sarcoma, cryptosporidiosis, and tuberculosis [138]. Conversely, there has been some controversy on the beneficial impact of ART in reducing the impact of HIV on HPV infection and disease. Some studies report no beneficial impact of ART on HPV infection and cervical cancer [139–141] while some studies report a decrease in the incidence of cervical cancer among HIV+ women on ART [142–145]. The inconclusive impact of ART on HPV-associated diseases may arise from differences in these studies, such as the population studied, duration and adherence to ART usage, as well as nadir CD4 T-cell counts at ART initiation. Furthermore, HPV does not evoke a strong immune response [146,147] and even in immunocompetent individuals, B and T cell immune reactivity is variable, of low magnitude and often not detectable [148]. Since HIV+ women on ART live longer and, on average, are infected with HPV at a younger age than HIV- women, their cumulative risk for development of cervical cancer may not drop significantly. Furthermore,

absence of vigorous cervical cancer screening campaigns in sub-Saharan Africa makes it hard to dissect the role of ART in reducing the risk of cervical cancer amongst HIV+ women.

2.6.2 Role of HPV on HIV infection risk.

The risk to acquire HIV has been linked to increased frequency and/or upregulation of the cell surface proteins C-C Chemokine receptor – 5 (CCR5) [149,150], alpha 4 beta 7 ($\alpha_4\beta_7$) [123,151] and Human Leukocyte Antigen – DR (HLA-DR) on CD4 T cells [152,153] .

CCR5, a receptor for MIP 1 beta/alpha and RANTES, is upregulated upon activation of T cells [154]. This implies that any condition that causes either upregulation of CCR5 or an increase in the proportion of CCR5+ CD4 T cells will increase the risk of HIV infection. Alpha 4 beta 7 ($\alpha_4\beta_7$) integrin is a protein found on various lymphocytes [155]. It functions as a gut homing receptor and its ligand is mucosal addressin cell adhesion molecule (MAdCAM). This integrin is essential in the migration of lymphocytes from the systemic circulation to the gut mucosa [156]. During HIV infection, this integrin functions as an entry co-receptor for the virus. HIV gp140 will bind to $\alpha_4\beta_7$ before binding to CCR5 and CD4 T-cell receptors. When HIV viral particles bud out of CD4 T cells, the viral particles carry with them $\alpha_4\beta_7$ integrin, previously anchored on the surface on the CD4 T cell [157]. The integrin will traffic HIV virus particles to the gut. This is speculated to be one of the reasons why early viral infection is associated with depletion of CD4 T cells in the gut mucosa. This implies that an up- or down-regulation and/or alteration in the proportion of cervical $\alpha_4\beta_7$+ T cells may influence the risk of acquiring and control of HIV infection.

Human Leukocyte Antigen – DR (HLA-DR) is an MHC class II protein found on the surface of immune cells [158]. HLA-DR functions to bind and present endogenous processed antigens to immune cells, therefore the molecule is upregulated during infections [158]. Activated immune cells rapidly divide, secrete cytokines and upregulate HLA-DR [159]. In the context of HIV infection, immune activation (upregulation of HLA-DR) is marker of disease progression [160]. Activated CD4 T cells are at risk of HIV infection and when infected, activation drives HIV viral particle synthesis and propagation [96] because activated cells divide at a greater rate and have relatively upregulated expression CCR5 and $\alpha_4\beta_7$. Continued immune activation resulting from chronic infections leads to immune exhaustion, characterized by upregulation of immune check point inhibitor such as PD-1, CTLA-4 and TIM-3 [161]. Exhausted immune cells are less efficient in clearing pathogens [162].

A number of studies have shown that HPV+ compared to HPV- individuals are at greater risk of contracting HIV [125,163–165] .The exact mechanism is yet to be described. Possibly, cervical HPV infection induces recruitment of CCR5+ and $\alpha_4\beta_7$+ CD4 T cells to the cervix. Such cells are at great risk for HIV infection because CCR5 and $\alpha_4\beta_7$ makes these CD4 T cells a prime target for HIV. Another mechanism could be that HPV infection and associated lesions induce T cell activation, which could increase the risk of HIV acquisition in cervical T cells

2.7. Objectives

The main objective of this work was to study the effect of HIV infection on HPV infection and HPV-specific T cell immunity to understand why HIV+ women are disproportionately burdened by HPV infection and disease. In addition, we studied whether HPV infection and associated lesions increase the proportions of cervical T cells expressing receptors CCR5, $\alpha_4\beta_7$ and HLA-DR, thereby increasing the risk for HIV infection among HIV- women.

Article I

Depletion and Activation of Mucosal CD4 T Cells in HIV Infected Women With HPV-associated Lesions of the Cervix Uteri

HIV infection alters the proportion and phenotype of systemic T cells [166]. To dissect the effect of HIV infection on cervical immunity, I characterized cervical T-cell populations, compared the cervical T-cell phenotype to systemic blood, and subsequently determined the effect of HIV on the proportions and phenotype of cervical T cells. Additionally, I assessed the effect of cervical HPV infection on the proportions of cervical T cells expressing cellular receptors CCR5, $\alpha_4\beta_7$ and HLA-DR, thereby potentially increasing the risk for HIV acquisition.

Article II

Depletion of Human Papilloma Virus E6- and E7-Oncoprotein-Specific T-Cell Responses in Women Living With HIV

It has been shown that HIV depletes and induces dysfunction of pathogen-specific T cells, such as those of tuberculosis and EBV [133]. It is therefore plausible that HIV infection is associated with impaired systemic HPV-specific T-cell functionality, thereby reducing HPV clearance and in effect promoting HPV infection persistence and disease progression. Using a cohort of women with well characterized HIV status, HIV plasma viral load, CD4 counts, ART data, and cervical pathology diagnosis, I compared systemic HPV-oncoprotein-specific T-cell responses and reactivity between HIV+ and HIV- women.

Article III

HPV type distribution in HIV positive and negative women with or without cervical dysplasia or cancer in East Africa

While it has been shown that HIV+ women have a multifold increased risk for cervical HPV infections and cancer [82], it is not completely clear whether HPV genotypes other than HPV16 and 18 cause a greater proportion of cancers in these women. This article therefore assessed the effect of HIV on the distribution of HPV genotypes in women with and without cancer.

3. Discussion

HR HPV infections are a major cause of cervical cancer [4]. The risk for cancer is elevated in women living with HIV since they have been shown have a multifold increased risk for HPV infection and cervical cancer [82,141,167]. It therefore important to investigate the role of HIV on HPV infection diversity and the effect of HIV on HPV immunity to better understand HIV-HPV co-pathogenesis. Conversely, a number of publications link HPV infection to an increased risk for HIV [125,127,165]. The mechanisms by which HIV increases risk for HPV infection and cervical cancer disease are not completely clear. Similarly, increased risk for HIV amongst HPV+ women is not well understood. This dissertation therefore focused on the effect of HIV and HPV on the cervical T-cell phenotype and systemic HPV-oncoprotein T-cell functionality by characterizing the proportions of cervical T-cells and quantifying the magnitude of systemic HPV-oncoprotein T-cell reactivity in HIV and HPV infected women.

Taken together, the three publications in this thesis show that HPV16, 18 and 45 are the most oncogenic HPV types regardless of HIV infection. Cervical HPV infection was associated with an increase in HLA-DR+ but not CCR5 and $\alpha_4\beta_7$ cervical T cells, and that HPV-oncoprotein systemic T-cell responses are rare and of low magnitude. Furthermore, only HPV18 and HPV45 (not HPV16) infections could be linked to HPV type specific immune responses. More importantly, the publications show evidence for HIV associated depletion and activation of cervical CD4 T cells as well as impaired systemic HPV-oncoprotein specific T cell responses. Similarly, cervical HPV-associated lesions were associated with an increase in HLA-DR+ cervical T cells and lower systemic HPV-specific T-cell reactivity. Additionally, advanced HIV disease (low CD4 counts and detectable viremia) was linked to more cervical HPV infections.

Effect HIV infection on the HPV genotype diversity

Even though HIV is associated with a multifold increased risk for HPV infection [82,141,145], there was no difference in HPV genotype diversity associated with cancer. The genotypes HPV16, HPV18 and HPV45 were associated with the majority (roughly 85%) of cervical cancer cases, regardless of HIV status. The genotypes HPV35 and HPV58 were more prevalent in HIV+ with HSIL; nonetheless as the lesions progressed, the genotype diversity constricted and HPV16, 18 and 45 accounted for the vast majority of cancers regardless of HIV status. HPV35 infections were frequent in HIV+ women with HSIL with above 25% occurance rate and also cancer with 11% occurance rate, but typically HPV35 occurred together with other coinfecting HR HPV types, making it difficult to pinpoint the type that caused the HSIL. These results are in line with those from a meta-analysis published by Clifford et al [167]. Therefore, regardless of HIV infection, cervical cancer screening programs and national vaccination programs should focus on HPV types HPV16, HPV18 and HPV45.

Effect of cervical HPV infection on cervical T-cell phenotype and systemic HPV-specific T-cell immunity

Cervical HPV infection did not affect the proportions of neither CD4 or CD8 cervical T-cell proportions. Similarly, the proportions of HIV co-receptors $\alpha_4\beta_7$ and CCR5 were similar between HPV+ and HPV- women. These findings imply that HPV does not increase the risk for HIV acquisition by increasing the proportions of cervical CD4 T cells bearing HIV co-receptors $\alpha_4\beta_7$ and CCR5. However, HPV infection was associated with an increase in HLA-DR+ cervical CD4 T cells co-expressing $\alpha_4\beta_7$ and CCR5 HIV co-receptors. Since immune activation as measured by HLA-DR and CD38 has been shown to increase the risk for HIV infection [168] and that gp120 of HIV preferentially infect CD4 T cells expressing CCR5 [150], it is therefore plausible that a cervical HPV infection may increase the risk for HIV infection by recruiting to the cervix activated CD4 T cells co-expressing HIV co-receptors. T-cell activation may then promote reverse transcription of the HIV genome post entry in these cells and therefore increase chances of successful, productive cellular HIV infection.

Cervical HPV infections induce weak and infrequent systemic HPV-oncoprotein systemic T-cell responses [147]. This is likely attributed to the a-viremic nature of the HPV life cycles [9] and various other immune evasion mechanisms associated with HPV [110]. Amongst the three most relevant HPV types - HPV16, HPV18 and HPV45 - HPV16 had the lowest magnitude of T-cell reactivity and frequency of T-cell response. The low inherent immunogenicity of HPV16 therefore potentially contributes to its high potential in establishing persistent infections leading to cancer. The low immunogenic nature of HPV16 has been highlighted in a similar way by other studies [81,169,170].

Reduced systemic HPV-oncoprotein specific T-cell responses and activated cervical T cells in HIV+ women

HIV infection was associated with a reduction of cervical CD4 T-cells, implying that HIV induced CD4 depletion observed by other studies in the gut mucosa [171] and the blood [172] is mirrored in the cervix. These results complement those of Mckinnon et al., reporting a rapid depletion of Th17 CD4 T cells in the cervix of HIV+ female sex workers in Kenya [173]. CD4 T cells appear to be important in the clearance of HPV infections: Steele et al., reported CD4 T-cell (not CD8 T-cell) responses were more frequent in women without cervical lesion when compared to those with lesions [81] while Kenter et al., reported that regression of vulvar intraepithelial neoplasia was associated with infiltration CD4 T cells [174]. Additionally, multiple studies have further underpinned the importance of CD4 T cells by publishing that higher odds of HPV infection were associated with reduced CD4 counts [129,175,176].

In terms of T-cell functionality, article II of this dissertation reported that HIV infection was associated with reduced HPV18 and HPV45 oncoprotein-specific T-cell responses. Nicol et al., published that T-cells infiltrating the cervix of HIV-HPV co-infected women had reduced expressions of the cytokines IL-6, TNF-alpha, and interferon-gamma [177]. These results imply

that HIV infection induces dysfunction of HPV-specific T-cells, hindering HPV clearance, and in turn promoting cervical cancer in HIV positive women. HIV induced pathogen-specific T-cell dysfunction is not unique to HPV. Geldmacher et al., [90,89] and Jacobson et al., [178] showed reduced functionality of *M.tb*- and CMV-specific T cells in HIV co-infected individuals. Pathogen-specific T-cell dysfunction in HIV+ individuals is likely to be linked with chronic systemic inflammation and immune activation that leads to senescence and exhaustion of T-cells. Indeed, article I has shown that HIV infection is associated with increased proportions of HLA-DR+ cervical CD4 T cells and that increase of these cells is inversely proportional to a decrease in CD4 T-cell counts. Furthermore, advanced HIV disease (low CD4 counts and detectable HIV viral load) have been linked to further reduced HPV-specific T-cell reactivity. Taken together, articles I and II show that in the cervix, HIV infection depletes cervical CD4 T-cells and promotes recruitment of activated CD4 T-cells, while systemically, HIV induces depletion of HPV-specific T-cell responses.

Effect of HPV-associated lesions on systemic HPV-oncoprotein specific T-cell responses and activation of cervical T cells

The published articles I and II show that independent of HIV status, HPV-associated lesions are associated with depleted systemic HPV-oncoprotein specific T-cell responses and increased proportion of HLA-DR+ cervical T cells. Chronic immune activation, especially with persistent long lasting infections are associated with an upregulation of negative check point inhibitors such as PD-1, CTLA-4, and TIM-3, a profile distinct for exhausted immune cells [162]. Exhausted immune cells are less functional and therefore ineffective in clearing infections [179]. As published in article I, HPV associated lesions are linked with increased proportion of HLA-DR+ (activated) CD4 and CD8 T cells. It is therefore plausible that HPV-specific cervical T cells are exhausted as a result of chronic immune activation, thereby inhibiting HPV clearance and promoting cervical cancer oncogenesis. In the article II, we have published that magnitude of T-cell reactivity is reduced in women with higher grade lesions and cancer when compared to those without lesion, especially among women living with HIV. Therefore lesion progression is associated with cervical T cell immune activation and reduced systemic HPV-specific T cell immunity. To better understand HIV-HPV co-pathogenesis, I am currently investigating the effect of HPV and HIV infections on systemic and cervical T cells immune exhaustion phenotype.

4. Future prospects

Future research efforts will focus on interrogating the effects of HIV on HPV viral activity, specifically oncogene mRNA quantification and HPV viral load. Linking HPV viral activity to HPV immunity will build a more complete picture on the effect of HIV on HPV infection and immunity. Additionally, through a collaboration with Dr. Janine Kimpel of the Medical University of Innsbruck, we are developing a therapeutic VSV-GP-HPV16 vaccine to promote clearance of persistent HPV16 infections, especially among women living with HIV. HPV16 viral clearance should avert a significant proportion of cervical cancer cases.

5. Summary (in English)

HIV positive women have a multifold increased risk for HPV infection and are therefore disproportionately burdened by cervical cancer. Since HIV induces dysfunction of different pathogen-specific T cells, We hypothesized that HIV induces depletion and/or dysfunction of cervical and systemic HPV-specific immunity. HIV+ and HIV-, women with and without cervical lesions were recruited into a case control study conducted from 2013 to 2021 in Mbeya, Tanzania. Clinical parameters such as HPV genotyping, cervical lesion diagnosis, HIV viral load, CD4 counts and ART usage was collected. The immunophenotype of cervical T cell was determined by flow cytometry and systemic HPV-oncoprotein specific T-cell immunity was quantified by an ELISpot assay. We reported that HPV16, 18, and 45 are responsible for majority of cervical cancer cases regardless of HIV infection status. HIV infection was associated with depletion of HPV-oncoprotein T-cell immunity, and with depletion and activation of cervical T cells. When combined and discussed, these articles shed more light on the adverse effects of HIV on HPV infection and immunity. Since women living HIV are at increased risk of persistent HPV infection and cervical cancer, more effort should be directed at HR HPV screening and treatment of cervical cancer in these women.

Zusammenfassung (Deutsch)

HIV-positive Frauen haben ein vielfach erhöhtes Risiko für krebserregende hochrisiko HPV Infektionen und sind daher unverhältnismäßig häufig von Gebärmutterhalskrebs betroffen. Da eine HIV-Infektion erregerspezifische T-Zellantworten beeinträchtigen kann, haben wir die Hypothese aufgestellt, dass eine HIV-Infektion trotz antiretroviraler Therapie (ART) eine Funktionsstörung der HPV-spezifischen Immunität auslöst, welche wiederum die Immunkontrolle bzw. Eliminierung von HPV Infektionen beeinträchtigt und so die Krankheitsprogression und Krebsentstehung begünstigt. Von 2013 bis 2021 wurden in Mbeya, Tansania HIV-positive und negative Frauen mit und ohne Gebärmutterhalsläsionen, sowie Gebärmutterhalskarzinomen in eine Case-Control Studie rekrutiert und klinische Parameter wie infizierender HPV-Genotyp, Gebärmutterhalsläsionen, HIV-Viruslast, CD4-T Zellzahlen und ART-Daten erhoben. Immunologische Untersuchungen beinhalteten die Charakterisierung von T-Zellen aus Zervixabstrichen mittels Durchflusszytometrie, sowie Quantifizierung von systemischen HPV-Onkoprotein-spezifische T-Zellen für HPV16, 18 und weiteren HR HPV Genotypen mittels ELISpot-Assay auf frisch Isolierten PBMZs. In den im Rahmen dieser Doktorarbeit veröffentlichten Artikeln konnte gezeigt werden, dass die allermeisten Gebärmutterhalskrebsfälle durch HPV16, 18 und 45 Infektionen erklärt werden können, unabhängig vom HIV Status. Dies ist Bemerkenswert, da in hochgradigen „präkanzerösen“ Gebärmutterhalsläsionen eine ganze Reihe weiterer HR HPV Typen, wie HPV35, häufig detektiert wurden, diese aber auch in HIV+ Frauen nur vergleichsweise selten zur Krebsentstehung beitragen. In HPV18 bzw HPV45 positiven Frauen, ging eine HIV-Infektion mit einer Depletion der HPV-Onkoprotein-spezifischen T-Zellen einher und diese war besonders ausgeprägt in HIV+ Frauen mit geringer CD4 T Zellanzahl, solchen mit HIV Viremie und solchen, welche bereits hochgradige Lesionen oder Krebs des Gebärmutterhalses hatten. Die Aktivierung von CD4 T-Zellen des Gebärmutterhalses in HIV+ Frauen mit HPV Läsionen könnte diese Zellen möglicherweise auch für eine direkte HIV Infektion prädisponieren. Zusammen legen diese Daten einen kausalen Zusammenhang zwischen HIV-induzierter Depletion von HPV-spezifischen T Zellantworten, erhöhter HPV Persistenz und beschleunigter Karzinogenese nahe.

PLOS ONE

RESEARCH ARTICLE

Depletion and activation of mucosal CD4 T cells in HIV infected women with HPV-associated lesions of the cervix uteri

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Abstract

Background

The burden of HPV-associated premalignant and malignant cervical lesions remains high in HIV+ women even under ART treatment. In order to identify possible underlying pathophysiologic mechanisms, we studied activation and HIV co-receptor expression in cervical T-cell populations in relation to HIV, HPV and cervical lesion status.

Methods

Cervical cytobrush (n = 468: 253 HIV- and 215 HIV+; 71% on ART) and blood (in a subset of 39 women) was collected from women in Mbeya, Tanzania. Clinical data on HIV and HPV infection, as well as ART status was collected. T cell populations were characterized using multiparametric flow cytometry-based on their expression of markers for cellular activation (HLA-DR), and memory (CD45RO), as well as HIV co-receptors (CCR5, $\alpha_4\beta_7$).

Results

Cervical and blood T cells differed significantly, with higher frequencies of T cells expressing CD45RO, as well as the HIV co-receptors CCR5 and $\alpha_4\beta_7$ in the cervical mucosa. The skewed CD4/CD8 T cell ratio in blood of HIV+ women was mirrored in the cervical mucosa and HPV co-infection was linked to lower levels of mucosal CD4 T cells in HIV+ women (% median: 22 vs 32; p = 0.04). In addition, HIV and HPV infection, and especially HPV-associated cervical lesions were linked to significantly higher frequencies of HLA-DR+ CD4 and CD8 T cells (p-values < 0.05). Interestingly, HPV infection did not significantly alter frequencies of CCR5+ or $\alpha_4\beta_7$+ CD4 T cells.

Abbreviations: AIDS, Acquired Immunodeficiency Syndrome; ART, Antiretroviral therapy; CC, Cervical Cancer; CCR5, Chemokine receptor 5; FBS, Fetal Bovine Serum; HIV, Human Immunodeficiency Virus; HLA-DR, Human Leukocyte Antigen–DR isotype; HPV, Human Papillomavirus; HSIL, High-grade Squamous Intraepithelial Lesion; LSIL, Low-grade Squamous Intraepithelial Lesion.

Conclusion

The increased proportion of activated cervical T cells associated with HPV and HIV infection, as well as HPV-associated lesions, together with the HIV-induced depletion of cervical CD4 T cells, may increase the risk for HPV infection, associated premalignant lesions and cancer in HIV+ women. Further, high levels of activated CD4 T cells associated with HPV and HPV-associated lesions could contribute to a higher susceptibility to HIV in HPV infected women.

Introduction

Human Immunodeficiency Virus (HIV) and Human Papilloma Viruses (HPV) are both sexually transmitted viruses that cause chronic infections and disease [1,2]. HPVs are a family of about 200 types of small, non-enveloped double-stranded DNA viruses which infect epithelial cells [3]. Depending on their oncogenic propensity, HPV group into either low-risk HPV (LR-HPV) or high-risk HPV (HR-HPV).HR-HPV types HPV16 and HPV18 together are linked to over 70% of all cervical cancer (CC) cases worldwide [4]. Persistent HR-HPV genital infections increase the risk of CC [5]. Most infections with HPV are subclinical; however, immunocompromised individuals, such as HIV+ women, have high incidence and persistent HPV infection and progress rapidly from HPV-associated lesions to invasive CC [6–8]. Antiretroviral therapy (ART) inhibits HIV viral replication, reconstitutes CD4 T-cell counts and immunity, thereby decreasing the risk for opportunistic infections such as Kaposi's sarcoma, candidiasis and tuberculosis [9,10]. In contrast to most AIDS-defining diseases, the burden of HPV-associated premalignant and malignant lesions remains high in HIV infected individuals despite the initiation of ART [7].

It has been shown that the frequency of immune cells and inflammatory cytokines within HPV-associated premalignant cervical lesions are suppressed in HIV infected women [10–12], demonstrating a direct effect of HIV on the cervical immune response. Furthermore, chronic HIV-induced immune activation, marked by an increased proportion of CD38+ and HLA-DR + systemic T cells [13], is associated with immune dysfunction and damage of mucosa [14–18], resulting in an increased persistence of HPV infections in HIV+ individuals [19,20]. Upregulation of HLA-DR has also been reported on epithelial cells in pre-cancerous lesions and genital warts [21].

Sexually transmitted infections such as gonorrhoea, syphilis, and chlamydia are known to cause genital ulcers and trigger an inflammatory response that is associated with recruitment of immune cells, including CD4 T cells, to the genital area, thereby increasing the risk for HIV acquisition [22,23]. An increased risk for HIV acquisition has also been reported in HPV infected women, especially for those with multiple HR-HPV infections [24–28]. This might be due to mucosal and cytokine milieu changes induced by HPV infection or recruitment of activated CD4 T cells to the cervix; as such cells provide suitable targets for HIV infection and replication. The inflammation associated with lesions has been shown to increase the HIV acquisition risk [28–32].

To elucidate the mechanism by which HIV increases the risk for HPV-infection or progression of associated lesions and vice versa, we analysed the frequency of T-cell lineage, memory, and activation markers, as well as the HIV co-receptors CCR5 and $\alpha_4\beta_7$ within peripheral and mucosal T cells by flow cytometry. Characterisation of T cells in the cervical mucosal is crucial to understand the impact of HPV, an epitheliotropic virus without a systemic phase, on the

cervical immune response [33–35]. Specifically, we studied phenotypic differences in systemic and mucosal T-cell populations and the effect of HPV and HIV infection on the T-cell composition in the cervix. We further sought to understand, whether HPV infection, as well as disease, is associated with cervical immune activation and alterations in the expression of HIV co-receptors, thereby rendering HPV infected women at risk higher of acquiring HIV.

Material and methods

Study population

Volunteers were recruited as part of an HIV-HPV (2H) study that started in 2013 and is currently ongoing in Mbeya, Tanzania. The 2H study is a prospective, longitudinal case-control study which aims to assess the effect of HIV infection and ART treatment on HPV infection and disease. For the data presented herein, 468 HIV+ and HIV- women from 18 years of age and above attending the cervical cancer screening clinics in Mbeya were recruited. Only cytobrush samples with complete laboratory documentation that could be linked to clinical data, no visible blood contamination, more than 150 T cells events in the CD3 gate, and satisfactory staining quality were included in the statistical analysis. Of these 468 samples, cervical pathology and HPV genotyping data were available for 440 and 213 women respectively, while data for ART usage was available for 201 of the 215 HIV+ women.

Ethical consideration

All study participants were fully briefed on the study, and written informed consent was obtained prior to enrolment/participation. The Mbeya Medical Research and Ethics Review Committee reference: MRH/R.10/8/Vol. VI/107, the Tanzanian National Health Research Ethics Committee reference: NIMR/HQ/R.8a/Vol. IX/1422 and the Ethics Committee of the medical faculty of the University of Munich (Project ID: 308–11) provided specific approval for this study before commencement of the study.

Specimen collection

Cervical cells were obtained from the endocervix by inserting a cytobrush (Solann) into the endocervical wall and gently rotating the brush 360˚. Part of the specimen was used for cytological examination by Papanicolaou testing with the remainder being collected in 5 mL complete media (10% FBS (Sigma) in RPMI-1640 (Gibco), 50U/mL Penicillin, 50μg/mL Streptomycin (Gibco) and 1x antibiotic-antimycotic solution (Sigma)). A second cytobrush sample was obtained for HPV genotyping and stored in 5 mL PreservCyt cell collection media (Roche). Cells were thoroughly flushed from the cytobrush using a Pasteur pipette and complete media. Cervical cell suspension from the first cytobrush was then transferred to a 5 mL falcon tube, pelleted by centrifugation at 570*g* for 10 minutes, washed with 2ml wash buffer (PBS with 1% bovine serum albumin and 0.05% sodium azide) and centrifuged (1600rm for 6 minutes) twice before flow cytometric analysis. Cervical cells from the second cytobrush were aliquoted and short-term (-20˚C) or long-term (-80˚C) stored for HPV genotyping.

EDTA anti-coagulated peripheral blood was collected by venepuncture, and whole blood used for *ex vivo* analysis. Both specimen types, cervical cells and whole blood, were processed immediately after specimen collection.

Flow cytometric ex vivo characterisation of cervical and peripheral T-cell populations. The following anti-human antibodies were used for staining of cytobrush and whole blood samples: CD3-Pacific Blue (clone UCHT1, BD), CD4-PerCP Cy5.5 (clone OKT4, eBioscience), CD8-Horizon-V500 (clone RPA-T, BD), CD45-APC-H7 (clone 2D1, BD),

CD45RO-PE (clone UCHL1, BD), HLA-DR-APC (cloneG46-6 BD-Pharmingen) (only for cytobrush samples), α4β7-FITC (clone FIB504, Biolegend), and CCR5-PEcy7 (clone 2D7/CCR5, BD). Cells were incubated with the antibody cocktail at 4˚C for 30 minutes in the dark, washed with FACS wash buffer (PBS with 1% bovine serum albumin and 0.05% sodium azide), and acquired on a FACS Canto II (BD) after fixation with 2% paraformaldehyde in water. Only cervical cytobrush samples without visible red blood cells, satisfactory staining quality, and T-cell counts above 150 cells were included in the analysis. For whole blood, a cut-off of at least 10,000 lymphocyte events was applied. Fluorescent spill over compensation was conducted with antibody capture beads (BD) stained separately with the individual antibodies used in the test samples. Gating was guided for both sample types (peripheral blood and cytobrush) separately by fluorescence minus one (FMO) controls. Flow cytometry data was processed using FlowJo version 10.4 (Tree Star Inc.).

Genotyping of cervical HPV infection

To identify the infecting HPV genotype, DNA was extracted from cervical cells stored at -20˚C in PreservCyt Solution (Roche) using QIAamp DNA mini kit (Qiagen), followed by HPV genotyping using the LINEAR ARRAY® HPV Genotyping Test (Roche) as per manufacturer's instructions. This assay qualitatively detects and identifies thirty-seven HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108). Women who tested positive for any of the 37 HPV types detected by the Roche linear array genotyping test were considered to have an ongoing cervical HPV infection. Only the presence or absence of an ongoing HPV infection, irrespective of infecting subtype, was considered for the following analysis.

Evaluation of histological and cytological pathology

Routine cytology by Papanicolaou testing and, if biopsied were collected, histology based on Haematoxylin & Eosin staining were performed at the pathology department of the Mbeya Zonal Referral Hospital (MZRH) Pathology department. The lesions were diagnosed and reported as per the Bethesda system for cytopathological classification.

CD4 cell counts

Absolute CD4 T-cell counts were analysed from blood samples as part of routine patient management using BD Trucount tubes (BD) and were acquired on a BD FACSCalibur (BD).

Statistical analysis

Baseline characteristics, as well as clinical and HIV related information were extracted from data collection forms that were entered and quality controlled in a study tailored SQL database. Statistical testing included the following tests; Mann-Whitney U test was employed to assess the effect of HIV and/or HPV infection on the proportion of CD4+ and CD8+ T cells, as well as the frequency of $\alpha_4\beta_7$, CCR5 and HLA-DR expression on T cells with respect to HIV and or HPV infection. Wilcoxon matched-pairs signed ranks test was used to evaluate the difference in proportions of CD4, CD8, HIV receptors CCR5 and $\alpha_4\beta_7$ and the memory marker CD45RO between peripheral blood and cervical mucosal sample from the same participant. Spearman's rank order correlation test was used to evaluate the association between HLA-DR on CD4+ T cells with the corresponding CD4 count of the same participant. The sample sizes for different analysis may differ because not all data for all parameters was available for each patient. The exact number is indicated in the respective figure legends. For all tests a two-tailed

p-value of < 0.05 was regarded as significant. Stata version 14 (StataCorp, USA) and GraphPad Prism software version 7 (GraphPad Software Inc, USA) were used for statistical analysis.

Results

Description of the cohort

All women included in this study were attending the cervical cancer screening clinics in Mbeya, Tanzania and were enrolled into the 2H study between 2013 and 2017. Table 1 provides clinical details of the study volunteers. Overall, 468 women were included in this analysis, of which 215 (46%) were HIV+. ART status was recorded for 201 of these HIV+ women with 153 (71%) being on ART treatment. A definitive pathological diagnosis was available for 440 women; 15 women had cytologically confirmed Low-grade Squamous Intraepithelial Lesions (LSIL), 13 were classified as High-grade Squamous Intraepithelial Lesions (HSIL), 32 were diagnosed with cervical cancer, and 380 had no lesions. HPV genotyping data was available for 213 women of which 57% (n = 122) had HPV infection(s).

Cervical T cells differ from peripheral blood T cells

To assess compositional and phenotypic differences between T cells in the cervical mucosal and the peripheral blood compartment, we first compared CD4 and CD8 T cells from both anatomical compartments in a subgroup of 39 women (22 HIV- & 17 HIV+). As memory CD4 T cells are the primary target for HIV infection and replication we also compared the percentage of memory T cells as defined by CD45R0 and HIV co-receptor (CCR5 and $\alpha_4\beta_7$ integrin) expression. Representative flow cytometry dot plots from one participant for these markers are shown in Fig 1 and the overall results are presented in Table 2. The median percentage of CD3+CD45+ cells of total events was much lower in the cervical cells than in peripheral blood (0.33% vs 11.71%; $p < 0.001$) (S1A Fig). A skewed CD4/CD8 ratio that was observed in blood following HIV infection was mirrored in the mucosal samples. Interestingly, CD4 median fluorescence intensity (MFI) was 1.8-fold higher in peripheral blood than in cervical cells, while CD4+CCR5 and CD8 MFI were 1.2 and 2.6 fold higher in cervical cells than

Table 1. Clinical details of study participants included in the cohort stratified by HIV status. The number of study participants (n) is given for each strata.

Clinical parameter	Total N = 468	HIV—N = 253	HIV + N = 215
Median age [IQR]	38 [31–45]	38 [29–48]	38 [31–43]
HIV+ on ART	n/a	n/a	153 (71%)
HIV+ not on ART			48 (22%)
Missing ART information			14 (7%)
Median CD4 counts [IQR (cells/μl)]	n/a	n/a	422 [245–614]
Pathological diagnosis†	**N = 440**	**N = 240**	**N = 200**
CC	32 (7%)	16 (7%)	16 (8%)
HSIL	13 (3%)	6 (3%)	7 (4%)
LSIL	15 (3%)	5 (2%)	10 (5%)
No lesion	380 (86%)	213 (89%)	167 (84%)
Cervical HPV diagnosis	**N = 213**	**N = 113**	**N = 100**
HPV infected	122 (57%)	47 (42%)	75 (75%)

† pathology diagnosis based on cytology and confirmed by histology

CC = cervical cancer, HSIL = high grade intraepithelial lesion, LSIL = low grade intraepithelial lesion, ART = antiretroviral therapy

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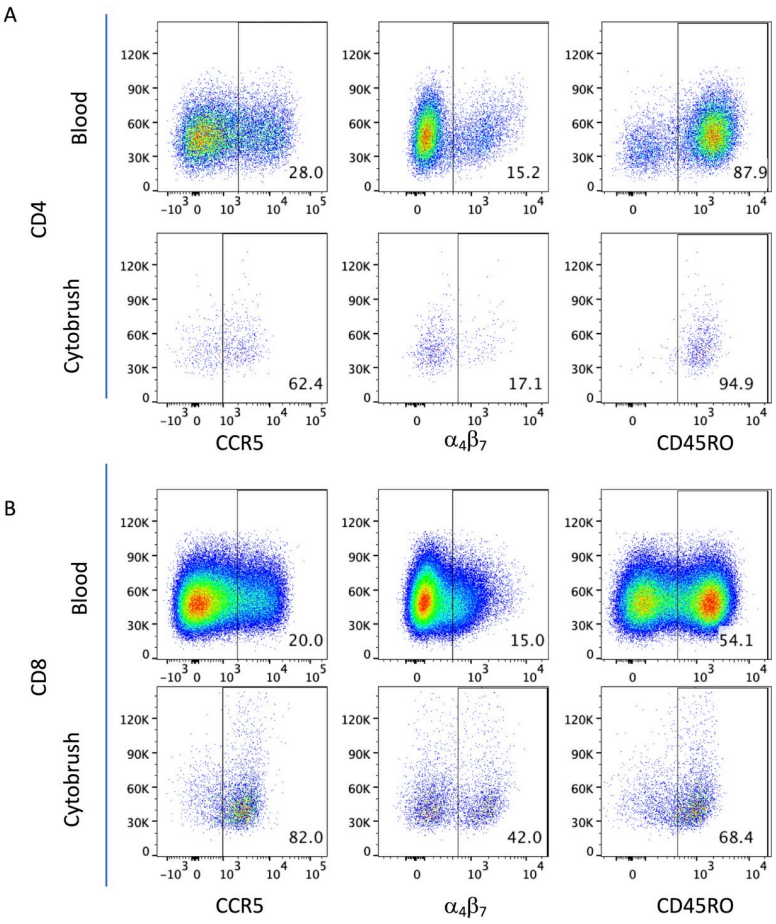

Fig 1. Phenotypic characterization of T cells from cervical mucosa and peripheral blood. Representative pseudocolour dot plots of peripheral whole blood and cervical cells showing the expression of CCR5 (left panels), $\alpha_4\beta_7$ (middle panels) and CD45RO (right panels) on CD4 and CD8 T cells (**A** and **B**, respectively). Samples were first gated on CD3+CD45+ T cells and then analysed for the expression of phenotypic markers of interest on CD4+ and CD8+ T cells. FMO controls were used to set gates for each sample type (peripheral blood or cytobrush) separately.

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blood, respectively (both $p<0.001$). CD45RO+ memory T cell frequencies were 1.21 and 1.74 fold higher in cervical than in peripheral blood CD4 and CD8 T cells ($p = 0.011$ and $p < 0.001$, respectively). Percentages of cells positive for the HIV co-receptor CCR5 were over 2-fold increase in the cervical mucosa compared to peripheral blood ($p<0.001$), both, on total and memory CD4 T cells. However, the percentage of $\alpha_4\beta_7$-integrin positive cells within total and memory CD4 T cells was comparable between the two compartments. Median frequencies of CCR5+ or $\alpha_4\beta_7$+ CD8 T cells were higher in the mucosa than in the blood ($p < 0.001$ and $p = 0.002$, respectively). Stratification of data by HIV status did not alter the observed difference in relative proportions of mucosal and peripheral CD45RO+ and CCR5+ T cells. Stratification by HIV status did not reveal an apparent effect of HIV on the relative proportion of CD45RO+ and CCR5+ mucosal and peripheral T cells. However, among HIV+ subjects, mucosal samples tended to have reduced frequencies of $\alpha_4\beta_7$+ CD4 and CD8 T cells compared to blood (% median: 21.50 vs 16.30 and % median: 39.10 vs 30.80, $p = 0.525$ and respectively, **S1A and S1B Fig**).

Overall, these results show that frequencies of HIV co-receptor positive T cells differ significantly between peripheral blood and cervical mucosa T cells.

Table 2. Comparison of HIV co-receptors (CCR5 and $\alpha_4\beta_7$) and memory marker (CD45RO) expression on T cells in mucosal cytobrush samples vs peripheral blood from HIV positive and negative women (n = 39; 22 HIV- & 17 HIV+).

parameter	parent population	cervical mucosa [median]	peripheral blood [median]	p-value
% CD4+	CD45+CD3+ T cells	41.9	50.3	0.178
% CD4+ (HIV-)	CD45+CD3+ T cells	54.1	60.3	0.147
% CD4+ (HIV+)	CD45+CD3+ T cells	23.9	24.2	0.384
CD4/CD8 ratio (HIV-)	CD45+CD3+ T cells	1.6	2	0.384
CD4/CD8 ratio (HIV+)	CD45+CD3+ T cells	0.4	0.4	0.525
CD4 MFI	CD45+CD3+ T cells	1788	3332	<0.001
% $\alpha_4\beta_7$+	CD45+CD3+CD4+ T cells	20.6	17.8	0.646
% $\alpha_4\beta_7$ MFI	CD45+CD3+CD4+ T cells	1204	1152	0.686
% CCR5+	CD45+CD3+CD4+ T cells	46.3	18.2	<0.001
CCR5+ MFI	CD45+CD3+CD4+ T cells	1957	2442	<0.001
% CD45RO+	CD45+CD3+CD4+ T cells	78.8	64.6	0.011
% $\alpha_4\beta_7$+	CD45+CD3+CD4+CD45RO+T cells	20.4	19.1	0.618
% CCR5+	CD45+CD3+CD4+CD45RO+ T cells	49.1	23.9	<0.001
% CD8+	CD45+CD3+ T cells	44.8	38.6	0.666
CD8+ MFI	CD45+CD3+ T cells	5313	1994	<0.001
% $\alpha_4\beta_7$+	CD45+CD3+CD8+ T cells	46.8	32.7	0.002
$\alpha_4\beta_7$+ MFI	CD45+CD3+CD8+ T cells	1292	849	<0.001
% CCR5+	CD45+CD3+CD8+ T cells	65.6	28.8	<0.001
CCR5+ MFI	CD45+CD3+CD8+ T cells	2288	2283	0.7562
% CD45RO+	CD45+CD3+CD8+ T cells	43.6	25	<0.001

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HPV infection is associated with decreased frequencies of CD4 T cells in the cervix of HIV+ women

Next, we assessed the effect of an infection with HIV, HPV, or a co-infection with both viruses on mucosal T-cell populations of all women with available HPV genotyping results (n = 213). As expected, HIV infection was significantly associated with lower CD4 and higher CD8 T cell

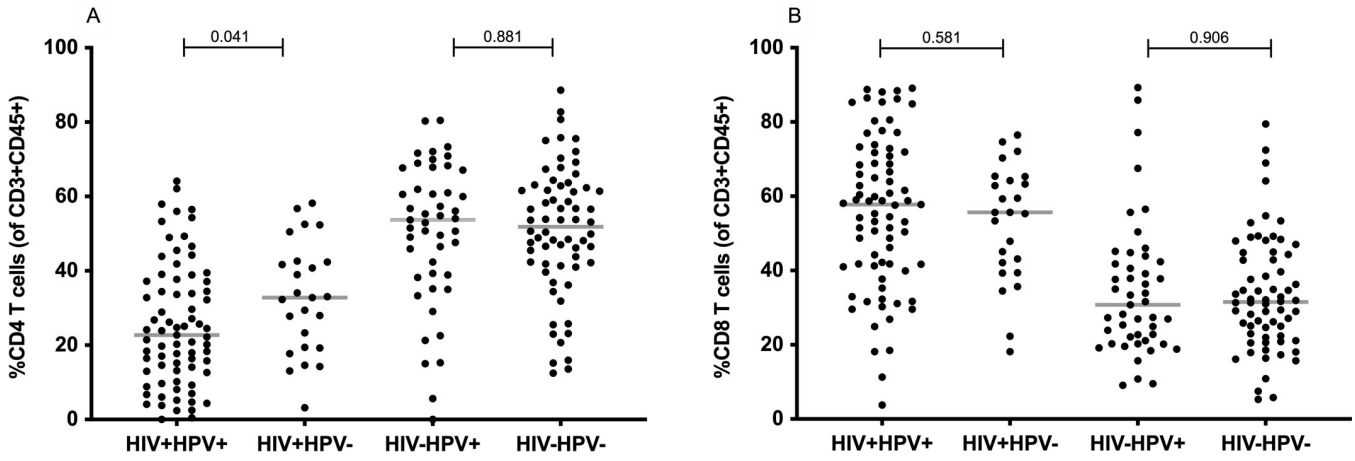

Fig 2. Cervical CD4 and CD8 T-cell frequencies stratified by HIV and HPV infection status (n = 213; HIV+ HPV+ = 75, HIV+ HPV- = 25, HIV- HPV+ = 47, HIV- HPV- = 66). Frequency of cervical CD4 (**A**) and CD8 (**B**) T cells is shown as percent of CD3+CD45+ lymphocytes. Each dot represents one patient. HPV and HIV infections status are indicated on the x-axis. P-values were calculated using the Mann-Whitney U-test. For this graph and the subsequent graphs, the median is indicated by a horizontal line within the data points while the p-values are indicated on top of the data points.

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frequencies in the cervix (median % in the total cohort (HIV- vs HIV+): 50.5 vs 25.1 for CD4 and 32.1 vs 55.7 for CD8; p-value for both < 0.001, (S2A and S2B Fig)), similar to what is commonly described in peripheral blood. Antiretroviral treatment increased CD4 T cell frequencies in the cervix, but not to levels observed in HIV- individuals (median % (ART- vs ART+): 16.3 vs 26.8, p = 0.001, S3A Fig). When stratified by both viral infections, a further moderate decline in median frequency of cervical CD4 T cells was observed in HIV+HPV + women compared to HIV+HPV- women (median %: 22.7 vs 32.8, p = 0.041). In contrast, amongst HIV- women, HPV infection had no apparent influence on CD4 or CD8 T cell frequencies (Fig 2A). No difference can be seen in the proportion of CD8 T cells between HIV + women with or without HPV (Fig 2B).

HPV infection does not alter the frequency of CD4 T cells expressing CCR5 and $\alpha_4\beta_7$ in the cervix

Since CCR5 and $\alpha_4\beta_7$ are essential for HIV to infect CD4 T cells, we assessed whether HPV infection alters the expression of these receptors on cervical T cells, thereby rendering HPV infected individuals more prone to HIV infection. HPV infection was not associated with a significant change in frequencies of $\alpha_4\beta_7$+ CD4 (p = 0.852) and CCR5+ CD4 (p = 0.166) cervical T cells (Fig 3). The frequency of these receptors on CD4 T cells was not significantly different when the data was further stratified into different permutations of HIV and HPV co-infections (S4A and S4B Fig). On the other hand, in HIV+ women we observed significantly lower frequencies of CD4 T cells expressing $\alpha_4\beta_7$ (p = 0.005) or CCR5 (p < 0.001) as compared to HIV- women, irrespective of HPV infection (Fig 3). Among these HIV+ women, frequencies of CCR5 and $\alpha_4\beta_7$ on CD4 T cells remained at similar levels despite ART treatment (S3B and S3C Fig, Fig 3)

HPV infection, HPV-associated lesions and HIV are linked to an increased frequency of HLA-DR+ T cells in the cervix

To determine the effect of HPV infection and HPV-associated lesions (HSIL, LSIL, and CC) on immune activation in cervical T cells, we determined the frequency of T cells expressing the activation marker HLA-DR. The proportion of HLA-DR+ cells on total cervical CD4 T

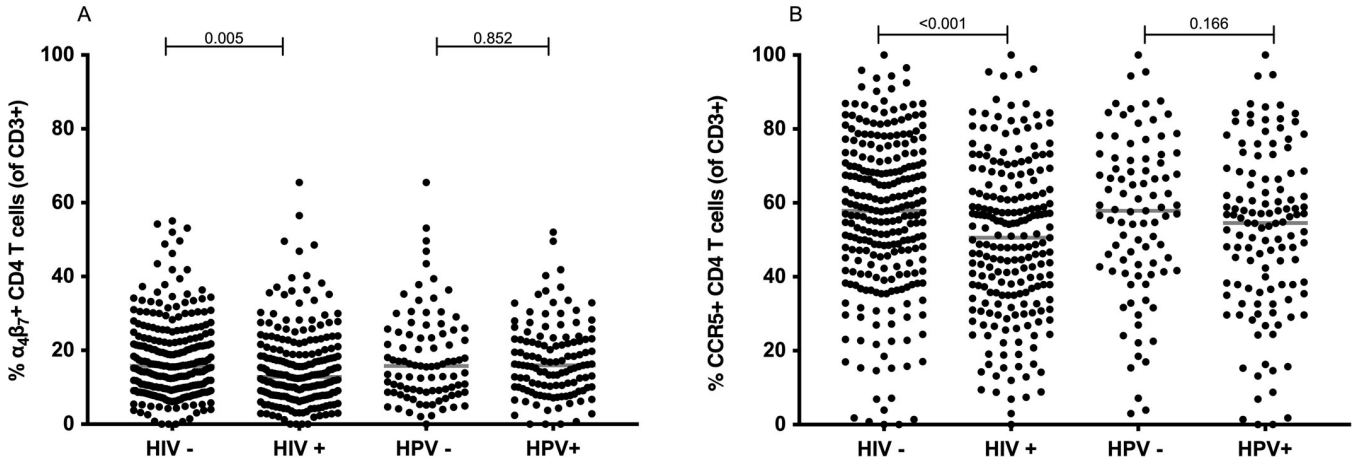

Fig 3. $\alpha_4\beta_7$ and CCR5 frequencies on cervical CD4 T cells stratified by HIV and HPV infection status (HIV- = 215, HIV+ = 253, HPV— = 91, HPV+ = 122). The frequency of $\alpha_4\beta_7$+CD4 (**A**) and CCR5+CD4 (**B**) T cells is shown as a proportion of CD3 T cells for each sample. HIV and HPV infections status is indicated on the x-axis. Median frequencies are indicated. Statistical analysis was performed using the Mann-Whitney U-test.

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cells and CCR5+ $\alpha_4\beta_7$+ CD4 T cells was higher in HPV+ compared to HPV- individuals (median % for HLA-DR+ CD4 T cells: 36.20 vs 25.00, p = 0.016; Fig 4A; median % CCR5+ $\alpha_4\beta_7$+ HLA-DR+ CD4 T cells: 43.65 vs 30.20, p = 0.014; Fig 4B). However, when the data was stratified by HIV and HPV infections, no statistical difference was observed in the frequency of HLA-DR+ CD4 or HLA-DR+ CD8 T cells between the groups (S4C and S4D Fig). Furthermore, HPV-associated lesions were significantly associated with higher percentages of HLA-DR+ T cells, in both CD4 (Fig 4D) and CD8 T cells (Fig 4E). For CD4 T cells, HIV + women with lesions had high median percentages of HLA-DR+ cells as compared to HIV + women without lesions (median %: 39 vs 23, p = <0.001; Fig 4C). Similarly, HIV- women with lesions had a significantly higher frequency of HLA-DR+ CD4 T cells (median %:26 vs 19, p = 0.044) than those without lesions (Fig 4D). The same effect of lesions on the frequency of HLA-DR+ cells is seen for CD8 T cells, where women with lesions had a higher percentage of cells expressing HLA-DR as compared to those without lesions (HIV+: median %: 36 vs 59, p < 0.001, HIV-: median %: 32 vs 54, p = 0.009; Fig 4E).

HIV+ women had moderately higher frequencies of HLA-DR+ CD4 T cells and CCR5+ $\alpha_4\beta_7$+ HLA-DR+ CD4 T cells when compared to HIV- women (median % for HLA-DR+ CD4 + T cells: 26 vs 20, p = 0.017; median % for CCR5+ $\alpha_4\beta_7$+ HLA-DR+ CD4 T cells: 36 vs 29, p = 0.001; Fig 4A and 4B respectively). No significant effect of an HIV infection on the frequencies of HLA-DR+ CD8 T cells was observed (%median 35 vs 40, p = 0.271; Fig 4C). Amongst HIV+ women, no significant difference in the frequency of HLA-DR+ CD4 T cells was observed between women on and off ART (S3D Fig). A negative correlation of the

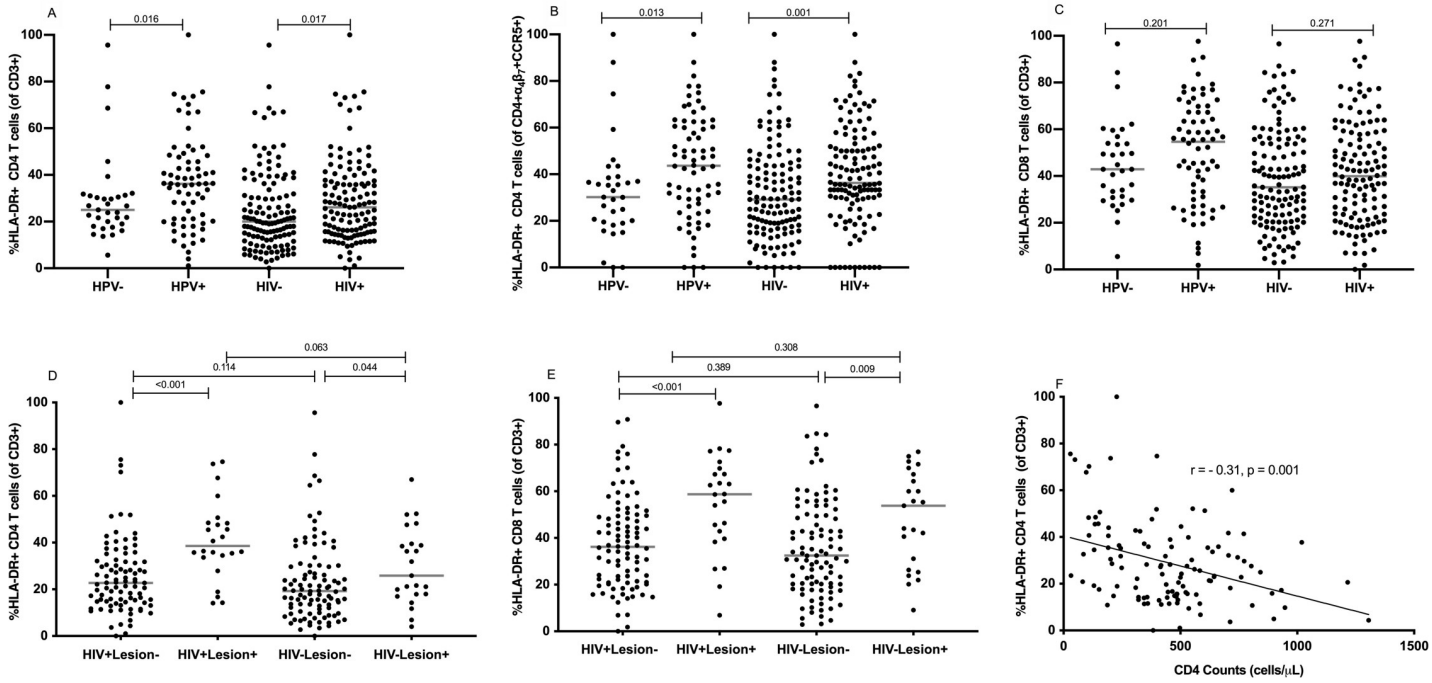

Fig 4. Effect of HIV, HPV and HPV-associated pathology on frequencies of HLA-DR+ T cells. A, B, C: Comparison of the frequencies of HLA-DR+ CD4 (**A**), HLA-DR+CCR5+ $\alpha_4\beta_7$+ CD4 (**B**) and HLA-DR+ CD8 (**C**) T cells on HIV- (n = 129) vs HIV+ (n = 126) women and HPV- (n = 33) vs HPV+ (n = 70). The frequency of HLA-DR+ cells is shown as percent of CD4, CCR5+ $\alpha_4\beta_7$+ CD4 or CD8 T cells in relation to HIV and HPV status. **D**,**E**: Comparison of the percentage of HLA-DR + cells in CD4 (**D**) and CD8 (**E**) T cells in relation to different permutations of HIV and cervical lesion (n = 237; HIV+ Lesion- = 92, HIV+ Lesion+ = 24, HIV- Lesion- = 98, HIV- Lesion+ = 23). **F**: Negative correlation of HLA-DR+CD4+ cervical T cell frequencies with peripheral blood absolute CD4 T-cell counts in HIV+ women (n = 113, r = -0.3111, p < 0.001). Median frequencies, correlation coefficient, and p-values are indicated in the graphs. Statistical analyses were performed using Mann-Whitney U-test (**Fig 4A–4E**) and Spearman correlation test (Fig 4F).

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percentage of HLA-DR+ CD4+ cervical T cells to peripheral blood absolute CD4 counts of the same individual could be observed in HIV+ women (r = 0.31, p = 0.001; **Fig 4F**).

Discussion

Women living with HIV, even under ART-treatment, have an increased risk for incident and persistent HPV infection and progress more rapidly to HPV-associated lesions and eventually invasive cervical cancer [6–8]. Immune cells residing in the cervical mucosa are important in the control of HIV and HPV infections [36] and have been shown to be dysregulated in HIV + women [11,12,37,38]. To analyse whether the high risk for HPV infection and fast progression to HPV-associated cervical cancer in HIV+ women [39] might be associated with HIV-induced changes in cervical T cells, we characterised the expression of HIV co-receptors and activation markers on cervical and peripheral T cells in relation to HIV and HPV infection, and disease, in a cohort of 468 HIV+ and HIV- women attending the cervical cancer screening clinics in Mbeya, Tanzania. We report that the percentage of T cells expressing the memory marker CD45RO as well as the HIV co-receptor CCR5 and $\alpha_4\beta_7$ in the cervix significantly differs from peripheral blood, underlining the importance of studying the local immune response at the site of HPV-infection. Generally, In healthy individuals, T cells comprise 15–30% of all immune cells in whole blood, CD4 T cells being the majority with 60–70% while CD8 T cell are 20–30% of all T cells [40,41]. The proportion of cervical T cells and associated subsets in health individuals are unknown since the majority of studies report proportions and functionality in these cells during infections and diseases [42–45]. Herein we report a skewed CD4/CD8 T-cell ratio in the blood of HIV+ women was mirrored in the cervical mucosa, and the frequency of CD4 T cells was further decreased in HIV+ women with a co-existing HPV-infection. Low CD4 T-cell frequencies in the cervical mucosa may, therefore, contribute to increased HPV infection and persistence rates in HIV+ women. HIV-infection was also associated with a marked decrease in CCR5+ and $\alpha_4\beta_7$+ cervical CD4 T cells and an increase in HLA DR^+ CD4 T cells, demonstrating an apparent effect of HIV infection on the homing properties and activation of cervical T cells. It can, therefore, be speculated that these alterations contribute to a less effective HPV-clearance in HIV+ women.

HPV infection and HPV-associated lesions were also linked to elevated levels of immune activation thus may contribute to immune dysregulation through over-activation in the cervix. Human T cells show a marked differential distribution of cell subsets and phenotypical characteristics in different body compartments [46]. Assessing differences in T-cell marker and HIV co-receptor expression on peripheral and cervical T cells, we observed that the proportion of T cells expressing the HIV co-receptor CCR5 and $\alpha_4\beta_7$ as well as the memory marker CD45RO is higher in cervical T cells than in peripheral blood T cells. The higher frequency of CCR5 expressing cells in the cervix is in line with the findings of McKinnon et al., [44] who reported a higher frequency of CCR5+ Th17 cells in the cervix compared to blood. Additionally, cervical T cells when compared to peripheral T cells showed higher CD4 molecule expression per cell, as demonstrated by an increase in the MFI. We, therefore, hypothesize that high proportion of CD45RO+CCR5+CD4+ cells together with high cellular CD4 expression density increases HIV susceptibility of mucosal cervical as compared to peripheral blood T cells.

HIV induced systemic loss of CD4 T cells increases susceptibility to different opportunistic infections, including HPV [47,48].Similarly, depletion of CD4 T cells at mucosal sites [49,50] and within the genital tract during HIV infection has previously been demonstrated [45]. In line with these earlier findings, we report a low frequency of cervical CD4 T cells in HIV + women and further lower CD4 T cell frequencies in HIV+HPV+ compared to HIV+HPV- women. However, among HIV- women, HPV infection was not associated with a change in

CD4 T-cell frequencies which implies that the low percentage of CD4 T cells in HIV+HPV + women is an effect of the HIV rather than the HPV infection. Our results are in line with a study by Palesfsky *et al*., demonstrating HIV+ women with low CD4 T-cell counts were more likely to be HPV infected [48]. A low frequency of cervical CD4 T cells is likely to be associated with immune dysfunction and elevates the risk of acquiring an HPV infection.

Both CCR5 and $\alpha_4\beta_7$ are essential for HIV attachment to CD4 T cells. It is well established that HIV preferentially infects CD4 T cells expressing CCR5 [51,52] or $\alpha_4\beta_7$ [53,54]. McKinnon *et al*., observed an enhanced in-vitro HIV susceptibility in cervical CD4 T cells of HIV -women was associated with co-expression of CCR5 and $\alpha_4\beta_7$ [55,56]. However, their study did not address whether these markers are altered in HPV-infected individuals. Our results show that HPV infection has no effect on the percentage of cervical CD4 T cells expressing CCR5 or $\alpha_4\beta_7$, implying that HPV infection does not increase the risk of HIV acquisition via upregulation of CCR5 and $\alpha_4\beta_7$ on cervical CD4 T cells. On the other hand, activated T cells are susceptible to HIV infection [57–59]; therefore activation of T cells as a result of HPV-associated lesions could increase the risk of acquiring HIV amongst HIV- women with HPV associated lesions. In contrast to HPV, we observed that HIV infection was associated with an inverted CD4/CD8 ratio and a decreased percentage of cervical T cells expressing CCR5 or $\alpha_4\beta_7$. This suggests an HIV-driven depletion of these T cells from this anatomical compartment. Similar results have been reported for the gastrointestinal mucosa during acute and treated HIV infection, where CCR5+, as well as β_7^{Hi} CD4 T cells were strongly and persistently depleted in HIV+ compared to HIV- individuals [60,61]. The exact mechanism by which these cells are depleted, whether by a direct cytopathic effect of HIV infection or bystander killing, is still unknown.

Chronic immune activation has been associated with exhaustion, senescence, and death of immune cells [62,63]. It has also been linked to a high risk of acquiring HIV infection [30,57], as well as to fast HIV disease progression [17]. Using a Rhesus monkey (*Macaca mulata*) Simian immunodeficiency virus (SIV) intravaginal infection model, Zhang et al., [49] have shown that HLA-DR+ cervical T cells are among the earliest cellular targets for SIV infection. In this study, these cells had a 4-fold increased levels of SIV RNA as compared to HLA-DR- cervical T cells, suggesting a critical role of immune activation of cervical T cells during early virus propagation that precedes systemic dissemination of AIDS virus infection. A phenotype of increased activation in HIV+ subjects has also been demonstrated for peripheral blood CD4 lymphocytes [64].

Our results show that HPV infection was associated with an increase in the proportion of HLA-DR+ total CD4 and CD4 T cells expressing HIV co-receptors CCR5 and $\alpha_4\beta_7$. However, stratification by different permutations of HIV-HPV co-infection (HIV-HPV-, HIV-HPV+, HIV+HPV-, HIV+HPV+) did not show any statistical significance, possibly due to the current small sample size. Even though HPV infection was not associated with altered frequency of HIV co-receptors, the increase the in proportion of activated cervical CD4 T cells expressing HIV co-receptors could increase the risk of HIV acquisition among HIV- individuals [25,28, 55].

Furthermore, we observed an HIV-status independent increase in the percentage of HLA-DR+ T cells in women with HPV infections and especially cervical lesions. This indicates that both HPV infection and the associated lesions alter the activation of T cells in the cervical mucosa, and that such alterations could contribute to increased HIV susceptibility in HIV— women. The exact mechanisms by which HPV increases HIV acquisition are yet unknown since most studies examining this phenomenon are observational [25]. Liebenberg et al., [28], however, have shown that HPV infection is associated with a distinct cytokine profile, associated an elevated risk to acquire HIV. Plausibly, CD4 T cells are recruited to the cervix by these

cytokines as a result of HPV infection, and associated pre-cancerous lesions become prime targets for HIV, thereby increasing the risk of HIV acquisition. In addition, our results suggest that HPV-associated lesions are by no means "immunological quiescent". This is furthered supported by Papasavvas *et al*., who reported an increased frequency of activated CD8 T cells in peripheral blood as a result of a HR-HPV infection [65]. Future studies focussing on HPV-specific T cells could improve our understanding on the effect of HPV on HIV acquisition and shed more light on the impact of HIV on the HPV-specific immune response. Furthermore, exploring the influence of immune exhaustion on cervical T cells as a consequence of persistent HPV infection may lead to a better understanding of how cervical immune cells contribute to the combat of pathogens that infect the body via the cervical mucosa.

Similar to peripheral blood, we report elevated immune activation in cervical CD4 T cells from HIV+ when compared to HIV- women. Even though ART treatment partially restored CD4 T cell frequencies in HIV+ women, the level of immune activation in cervical T cells remained high despite ART, indicating ART might not fully reverse HIV-induced immune dysfunction. Moreover, the level of immune activation in cervical T cells was inversely correlated to peripheral blood absolute CD4 counts of the same individual. These results are in line with Jaspan *et al*., where HIV infection was associated with a high percentage of T cells expressing HLA-DR, CD38 and Ki67 in both cervical and peripheral T cells [66].

In conclusion, we show that HPV infection was not associated with an upregulation of HIV co-receptors on cervical CD4 T cells, therefore implying a different mechanism for a possible increased risk of HIV acquisition in HPV-infected women. Furthermore, the low frequency of cervical CD4 T cells associated with a higher state of immune activation in cervical T cells of HIV+ women despite ART treatment, compounded with the immune activation resulting from HPV and HPV induced lesions, might hinder efficient HPV clearance from the cervical mucosa and pave the way for cervical cancer.

Supporting information

S1 Table. Demographic and flow cytometric data. Study participant information: HIV status, ART status, HPV infection status and cervical lesion diagnosis. Flow cytometric data: Cell counts and percentages of cellular subsets expressing T cell memory and activation markers as well as HIV co-receptor molecules.
(XLSX)

S1 Fig. Proportions of T cells and $\alpha_4\beta_7$+ T cells in blood and cervix. A: Percentage of CD3+CD45+ cervical and peripheral T cells stratified by sample source. (n = 39). The frequency of CD3+CD45+ T cells is shown as a proportion off all events collected for each sample. Sample source is indicated on the x-axis. Statistical analysis was performed using the Wilcoxon matched-pairs signed ranks test. **B,C**: Percentage of $\alpha_4\beta_7$+CD4+ and $\alpha_4\beta_7$+CD8+ cervical and peripheral T cells stratified by sample source and HIV status. (n = 39, HIV- = 22 and HIV+ = 17). The frequency of $\alpha_4\beta_7$+CD4+ (**B**) and $\alpha_4\beta_7$+C8+ (**C**) T cells is shown as a proportion of CD3+CD45+ T cells for each sample. HIV status and sample source is indicated on the x-axis. Statistical analysis was performed using the Wilcoxon matched-pairs signed ranks test.
(TIF)

S2 Fig. Percentage of CD4+ and CD8+ cervical T cells stratified by HIV status. (n = 468; HIV- = 253 and HIV+ = 215). The frequency of CD4+ (**A**) and C8+ (**B**) T cells is shown as a proportion of CD3+CD45+ T cells for each sample. HIV status is indicated on the x-axis. The median frequencies are indicated. Statistical analysis was performed using the Mann-Whitney U-test.
(TIF)

S3 Fig. Frequencies of CCR5+, $\alpha_4\beta_7$+, or HLA-DR+ T cells stratified by HIV and ART. A: Cervical CD4 T cells proportions stratified by HIV and ART usage status (n = 454; HIV- = 253, HIV+ART+ = 153 and HIV+ART- = 48). The frequency of cervical CD4 T cells is shown as a percent of CD3+CD45+. Each dot represents one patient. HIV status and ART usage is indicated on the X axis. The median percentages are indicated. Statistical analysis was performed using the Mann-Whitney U-test. **B,C**: Percentage of CCR5+ and $\alpha_4\beta_7$+ cervical CD4 T cells stratified by HIV and ART status (n = 454; HIV- = 253, HIV+ART+ = 153, HIV+ART- = 48) The frequency of CCR5+CD4+ (B) and $\alpha_4\beta_7$+CD4+ (C) T cells is shown as a proportion of CD3+CD45+ T cells for each sample. HIV and ART status is indicated on the x-axis. The median frequencies are indicated. Statistical analysis was performed using the Mann-Whitney U-test. **D:** Percentage of HLA-DR+ CD4+ cervical T cells stratified by HIV and ART status. (n = 241; HIV- = 129, HIV+ART+ = 84, HIV+ART- = 28)The percentage of HLA-DR+ CD4 + T cells is shown as a proportion of CD3+CD45+ T cells for each sample. HIV and ART status is indicated on the x-axis. The median frequencies are indicated. Statistical analysis was performed using the Mann-Whitney U-test.
(TIF)

S4 Fig. Frequencies of CCR5+, $\alpha_4\beta_7$+, or HLA-DR+ T cells stratified by HIV and HPV infection status. A,B: $\alpha_4\beta_7$ and CCR5 frequencies on cervical CD4 T cells stratified by HIV and HPV infection status (n = 215; HIV+HPV+ = 75, HIV+HPV- = 25, HIV-HPV+ = 47, HIV-HPV- = 66). The frequency of $\alpha_4\beta_7$+CD4+ (A) and CCR5+CD4+ (B) T cells is shown as a proportion of CD3+CD45+ T cells for each sample. HIV and HPV infections status is indicated on the x-axis. The median frequencies are indicated. Statistical analysis was performed using the Mann-Whitney U-test. **C,D:** Percentage of HLA-DR+ CD4 and CD8 cervical T cells stratified by HIV and HPV infection status. (n = 103; HIV+HPV+ = 40, HIV+HPV- = 6, HIV-HPV+ = 30, HIV-HPV- = 27). The percentage of HLA-DR+ CD4+ (C) and HLA-DR + CD8+ T cells (D) is shown as a proportion of CD3+CD45+ T cells for each sample. HIV and HPV infections status is indicated on the x-axis. The median frequencies are indicated. Statistical analysis was performed using the Mann-Whitney U-test.
(TIF)

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Depletion of Human Papilloma Virus E6- and E7-Oncoprotein-Specific T-Cell Responses in Women Living With HIV

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Background: Cervical cancer - caused by persistent High Risk Human Papilloma Virus (HR HPV) infections - is the second most common cancer affecting women globally. HIV infection increases the risk for HPV persistence, associated disease progression and malignant cell transformation. We therefore hypothesized that this risk increase is directly linked to HIV infection associated dysfunction or depletion of HPV-oncoprotein-specific T-cell responses.

Methods: The 2H study specifically included HIV+ and HIV- women with and without cervical lesions and cancer to analyze HPV oncogene-specific T cell responses in relation to HPV infection, cervical lesion status and HIV status. Oncoprotein E6 and E7 specific T-cell responses were quantified for the most relevant types HPV16, 18 and 45 and control antigens (CMV-pp65) and *M.tb*-PPD in 373 women, using fresh peripheral blood mononuclear cells in an IFN-γ release ELISpot assay.

Results: Overall, systemic E6- and E7-oncoprotein-specific T-cell responses were infrequent and of low magnitude, when compared to CMV-pp65 and *M.tb*-PPD ($p < 0.001$ for all HR HPV types). Within HIV negative women infected with either HPV16, 18 or 45, HPV16 infected women had lowest frequency of autologous-type-E6/E7-specific T-cell responses (33%, 16/49), as compared to HPV18 (46% (6/13), $p = 0.516$) and HPV45 (69% (9/13), $p = 0.026$) infected women. Prevalent HPV18 and 45, but not HPV16 infections were linked to detectable oncoprotein-specific T-cell responses, and for these infections, HIV infection significantly diminished T-cell responses targeting the autologous infecting genotype. Within women living with HIV, low CD4 T-cell counts, detectable HIV

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viremia as well as cancerous and precancerous lesions were significantly associated with depletion of HPV oncoprotein-specific T-cell responses.

Discussion: Depletion of HPV-oncoprotein-specific T-cell responses likely contributes to the increased risk for HR HPV persistence and associated cancerogenesis in women living with HIV. The low inherent immunogenicity of HPV16 oncoproteins may contribute to the exceptional potential for cancerogenesis associated with HPV16 infections.

Keywords: HPV, HIV, T-cell response, oncoprotein, cervical cancer

INTRODUCTION

Cervical cancer, typically caused by persistent high risk human papilloma virus (HR HPV) infections, is the second most frequent cancer affecting women worldwide, with 570,000 new cases and 311,000 deaths per year (1). Eighty percent of worldwide cervical cancer cases occur in low-income countries, with sub-Saharan African (SSA) countries being amongst the most heavily affected (2). HIV infection dramatically increases the risk for HPV infection, persistence and rapid progression to cervical cancer (3–7), resulting in elevated cervical cancer incidences in regions with high HIV prevalence rates.

HR HPV infections are common and typically cause transient infections that are cleared within two years (8). However, HR HPVs can also establish persistent infections that cause cervical dysplasia which can subsequently progress to squamous cell carcinoma (SCC) (9, 10). HPV16, as well as the types HPV18 and 45, globally and within East Africa cause the vast majority of SCC (11; Mcharo et al., 2021, in press).

T cells are thought to play a key role in clearing HPV infection and disease. T-cell infiltration into HPV-associated skin and genital warts has been linked to regression of the warts (12, 13). Similarly, infiltrating T cells are predominant in cervical intraepithelial lesions and cancers (14–16). HPV16 E6- or E7-specific T-cell responses have been detected more frequently in women clearing HPV16 infections than in those with persistent infection (17–19). Furthermore, a number of studies report a decrease in the frequency of systemic HPV-E6/E7-specific CD4 and CD8 T-cell responses as cervical lesions progress to cancer (20–22). However, overall, systemic HPV-specific T-cell responses are infrequent and difficult to measure (23) and hence difficult to study.

While the precise mechanisms of HIV pathogenesis remain subject of intense investigations, the depletion of pathogen-specific T-cell responses has been linked to the risk for disease by opportunistic infections such as *Mycobacterium tuberculosis* (*M.tb*), cytomegalovirus (CMV) and HR HPVs in people living with HIV (22, 24, 25). For instance, *M.tb*-specific Th1 cells are preferentially depleted early during the course of HIV infection, and tuberculosis is often the first opportunistic infection affecting HIV+ patients (26–28). Likewise, eventual loss of CMV- and Epstein-Bar Virus (EBV)-specific CD4 T cells precede CMV end organ disease and development of AIDS related EBV-associated lymphomas, respectively (29, 30). Antiretroviral therapy (ART) interferes with HIV replication, reverses CD4 T-cell depletion and improves memory T-cell responses against most common opportunistic pathogens (31–33). This ART-induced immune reconstitution protects against most AIDS-defining diseases and as a consequence, prolongs the life of HIV+ individuals (34). The incidence of malignancies caused by HR HPV, however, has not decreased during the era of ART - a phenomenon that is still not fully understood (35). Amongst other factors, the level of immune reconstitution is influenced by differences in ART adherence between individual patients and the stage of HIV disease progression at which ART had been initiated. Guidelines on the use of antiretroviral drugs for treating and preventing HIV infection by WHO define immunological failure as CD4 T-cell counts of 250 cells/mm^3 or less proceeded by clinical failure, and virological failure is defined as two sequential viral loads (VL) levels of 1000 or more copies/mL within 3 months (36).

In this study, we addressed the hypothesis that HIV infection depletes HR HPV-specific T-cell responses as a possible underlying mechanism for increased HPV persistence and accelerated cancerogenesis in women living with HIV. We therefore examined the frequency and magnitude of HR HPV-specific T-cell responses determined by IFN-γ release ELISpot assay in relation to HR HPV and HIV infection, ART status, systemic HIV VL levels and CD4 T-cell count.

MATERIAL AND METHODS

Study Population

The women studied herein were part of the prospective, longitudinal case-control 2H study. This study was designed to dissect the influence of HIV on HPV infection and carcinogenesis and was conducted from 2013 to 2020 in Mbeya, South-West Tanzania. HIV+ and HIV- women above the age of 18 attending the Cervical Carcinoma Screening (CCS) at the Mbeya Zonal Referral Hospital HIV Care and Treatment Centre, at the META Gynecological Outpatient Department of the Mbeya Zonal Referral Hospital, the Matema Lutheran Hospital as well as health care facilities in the greater Mbeya urban and regional area with CCS services were recruited and screened for cervical lesions, cancer and for HIV infection. Selected volunteers were then fully enrolled within 2 month after the screening visit to allow for immunological analyses and collection of peripheral blood mononuclear cells (PBMC) for specific study groups defined by HIV infection and the presence or absence of high and low grade intraepithelial lesions and

cancer of the cervix. The primary focus of the immunological analyses was to determine frequency and magnitude of systemic HPV-oncoprotein-specific T-cells responses in relation to HIV infection and cervical lesion status. Therefore, IFN-γ release ELISpot assay results from 373 well characterized 2H study participants were analysed cross-sectionally. A detailed description of the clinical parameters of these study volunteers is shown in **Table 1**. The quantification of HPV-specific T-cell responses by IFN-γ release ELISpot is described in detail below.

Ethical Consideration

Ethical clearance was obtained from the Mbeya Medical Research and Ethics review Committee (MRH/R.10/8/Vol. VI/107), the Tanzanian National Health Research Ethics Committee (NIMR/HQ/R.8a/Vol. IX/1422) and the Ethics Committee of the Medical Faculty of University of Munich (project ID: 308-11) before commencement of the 2H study. All study participants were fully briefed on study procedures, and signed informed consent was obtained from all study participants before enrolment. All procedures pertaining to clinical examination of the volunteers and sample collection for laboratory assays were performed by certified clinicians and in adherence to the Tanzanian National Guidelines.

Specimen Collection for Clinical and Immunological Assessments

Peripheral blood for absolute CD4 T-cell counts and HIV VL quantification was collected into EDTA tubes (BD) and peripheral blood mononuclear cells (PBMCs) for the quantification HPV-specific T-cell response by the IFN-γ release ELISpot assay were isolated by Ficoll density gradient centrifugation of whole blood collected into ACD tubes (BD) using the manufacturer's protocol. Cervical cells for HPV genotyping were obtained from the endocervix by gently rotating a cytobrush (Solann) 360 degrees around the endocervical wall. The brush was immediately transferred into a falcon tube with 5 mL PreservCyt cell collection media (Roche). Pap smear for Papanicolaou testing was collected by gently rotating an Ayres spatula in the ectocervix. In cancer suspicious cases, a biopsy was taken for further histological diagnosis at the pathology department of the Mbeya Zonal Referral Hospital.

HIV Diagnosis, ART Status Determination, CD4 T-Cell Counts, and HIV VL Quantification

HIV testing was done at recruitment into the 2H study using two independent HIV-Rapid tests: First by Determine HIV1/2 (Abbott Laboratories, South Africa) and then confirmed with Uni-Gold HIV Rapid Test (Trinity Biotech, South Africa). For HIV+ women, absolute CD4 T-cell counts were analysed from peripheral blood samples using BD Trucount tubes (BD) and acquired on a BD FACSCalibur (BD), while HIV VL was quantified using COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test (Roche), both according to manufacturer's instructions. Information on ART status was obtained by interviewing the volunteer.

HPV Genotyping

Cervical cells were collected and stored as described above in PreservCyt® Solution (Roche) and subjected to HPV genotyping using Roche linear array genotyping kit following the manufacturer's instructions. This assay detects thirty-seven HPV (including all HR HPV) genotypes: HPV types 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108. The following analyses focused mainly on HPV types 16, 18 and 45.

TABLE 1 | Clinical data of study participants stratified by HIV status.

	Total	HIV negative	HIV positive	p value
Median age (interquartile range)	41 (34 – 51)	46 (37 – 60)	39 (32 – 45)	<0.001[a]
cytohistological diagnosis (n) †	n = 373	n = 171 (47%)	n = 202 (53%)	
SCC	126	75 (60%)	51 (40%)	<0.001[b]
HSIL/CIN2+	44	10 (23%)	34 (77%)	0.001 [b]
LSIL/CIN1	39	7 (18%)	32 (82%)	<0.001 [b]
no lesion	162	78 (48%)	84 (52%)	0.464 [b]
AGC	2	1 (50%)	1 (50%)	
Molecular HPV diagnosis (n)	n = 343	n = 149	n = 194	
HPV 16+	107	49 (46%)	58 (54%)	0.559 [b]
HPV 18+	49	13 (27%)	36 (73%)	0.012 [b]
HPV 45+	38	16 (42%)	22 (58%)	1.000 [b]
Any HR-HPV+	243	88 (36%)	155 (64%)	<0.001 [b]
HR-HPV-	100	61 (61%)	39 (39%)	<0.001 [b]
HPV-	89	56 (63%)	33 (37%)	<0.001 [b]
ART status, CD4 and HIV VL counts (n)	n = 373	n = 171	n = 202	
On ART	n/a.	n/a.	75% (139/185)	
HIV+ with ≤ 250 CD4 T cells/mm^3	n/a.	n/a.	34% (61/177)	
HIV+ with ≥ 1001 HIV RNA copies/ml	n/a.	n/a.	34% (61/179)	

†pathology diagnosis based on cytology and confirmed by histology. SCC, cervical cancer; HSIL, high grade intraepithelial lesion; CIN, cervical intraepithelial neoplasia; LSIL, low grade intraepithelial lesion; ART, antiretroviral therapy; AGC, Atypical glandular cells; n/a, not applicable. For statistical analysis: [a]students t-test; [b]Fisher's exact test.

Diagnosis of Cervical Pathology

Screening for cervical cancer and lesions was performed by visual inspection during the clinical assessment and by routine cytology using Papanicolaou testing. In cases where more detailed histologic diagnosis was required, biopsies were collected, and Hematoxylin & Eosin staining was performed at the pathology department of the Mbeya Zonal Referral Hospital (MZRH) Pathology department according to national guidelines. Cervical pathology results were reported as per the Bethesda system for reporting cervical or vaginal diagnoses (37).

Antigens for *In Vitro* Restimulation of PBMCs

The following synthetic peptides were used for *ex vivo* re-stimulation of PBMCs: 15mer peptide pools overlapping by 11 amino acids for oncoproteins E6 and E7 specific to HPV16, 18 and 45 (Peptides and Elephants) and purified protein derivative from *M.tb* (*M.tb*-PPD: Staten Serum Institute) as well as CMV phosphoprotein 65 (CMV-pp65) overlapping peptide pools (Peptides and Elephants) were used to assess *M.tb*- and CMV-specific systemic T-cell responses. Phytohemagglutinin (PHA) (Sigma) was used as a positive control while complete media only [RPMI-1640 with 0.5% penicillin-streptomycin, 1% HEPES and 10% inactivated fetal bovine serum (FBS)] was used as negative control to determine assay background signal.

Quantification of HPV-Oncoprotein-Specific T-Cell Responses by IFN-γ Release ELISpot

Pathogen-specific T cells were quantified by IFN-γ release ELISpot assay. 96 well plates (MultiScreen$_{HTS}$ IP filter, 0.45 μm, Millipore) were pre-wetted 4 times with 200 μl sterile phosphate buffer saline (PBS) and coated with 50μl of anti-human IFN-γ capture antibody (5 μg/ml, clone 1-D1K, Mabtech). To allow the antibody to bind, the plates were placed at 4°C overnight. Before seeding cells, the plates were washed 4 times with sterile PBS to remove any unbound capture antibody and then blocked for 30 minutes with 200 μl complete medium [RPMI-1640 with 0.5% penicillin-streptomycin, 1% HEPES and 10% inactivated fetal bovine serum (FBS)]. 200,000 freshly isolated PBMCs were plated per well in duplicates and pathogen-specific antigens added as follows: 2μg/ml for each overlapping peptide of E6 and E7 for HPV16, 18 and 45; 2μg/ml for each peptide of CMV-pp65; 10μg/ml of *M.tb*-PPD and 40μg/ml of phytohemagglutinin (PHA). Complete media added to cells served as negative control (background). The plate was then incubated at 37°C and 4.5% CO_2 for 20 hours. After incubation, the plate was washed 5 times with PBS, and finally 100 μl of biotinylated anti-IFN-γ monoclonal antibody (1μg/ml, clone 7-B6-1, Mabtech) in PBS containing 0.5% FBS was added. The plate was then incubated in the dark at room temperature for 2 hours. This was followed by 5 times washing with PBS and addition of 100μl of streptavidin - alkaline phosphatase conjugate (Mabtech) at a concentration of 1μg/ml in PBS with 0.5% FBS and then a further incubation for 1 hour. The plates were then washed 5 times to remove any unbound streptavidin - alkaline phosphatase. To develop the plates, 100μl BCIP/NBT substrate solution (Thermo Scientific) was added to all wells. Plates were incubated in the dark for 10 minutes. Plate development was stopped by rinsing the plate 3 times with distilled water. The plate was left to dry overnight by placing it in the dark at room temperature. Spots representing IFN-γ-secreting cells were quantified by an automated CTL ELISpot reader (Immunospot) followed by manual quality control. An image of ELISpot results of a representative subject is shown in **Figure 1A**. Valid results were defined by a count of less than 100 SFC/10^6 PBMCs in the negative control and more than 1000 SFC/10^6 PBMCs in the positive control. For statistical analysis, a positive response was defined by 25 or more SFC/10^6 PBMCs and by being greater than three-fold the background. T-cell reactivity was defined by the quantity of SFC/10^6 PBMCs after subtracting the quantity SFC/10^6 in the negative control wells (background signal).

Statistical Analysis

Stata version 14 (StataCorp, USA) and GraphPad Prism software version 9 (GraphPad Software Inc, USA) were used for statistical analysis. Two tailed Mann-Whitney U testing was performed to assess the difference in magnitude of T-cell reactivity in terms defined as of Spot Forming Cells per one million PBMCs (SFC/10^6 PBMC) between different permutations of HIV and cervical pathology status. Fisher's exact test was used to test associations between each E6 or E7 HPV type-specific response and HIV infection. Spearman's rank correlation test was used to determine the relationship between absolute CD4 counts and HIV viral load with HPV type specific T-cell reactivity. The definitions for a positive responses and T-cell reactivity have been provided in section above detailing IFN-γ release ELISpot assay

RESULTS

Description of the Cohort

A complete summary of cervical diagnosis, HPV genotyping results and HIV-associated clinical parameters of study volunteers stratified by HIV status is provided in **Table 1**. In total, data from 373 women with median age of 41 (34 – 51 years interquartile range), cytohistological diagnosis of cervical lesions, known HIV status and valid ELISpot results were included in the statistical analyses presented herein. Cervical pathology data was available for all women included; 34% (126/373) had SCC, 12% (44/373) had HSIL/CIN2+, 11% (39/373) had LSIL/CIN1, 0.5% (2/373) had atypical cells of undermined significance and 43% (162/373) did not have any cervical lesions. HPV genotyping data was available for 343 women included in the present analysis: 107 where HPV16+, 49 HPV18+ and 38 women were HPV45 positive. Seventy six women were infected with one or more of the remaining 11 HR HPV types. One hundred women were infected with non-HR HPV types and 89 did not have any HPV infection.

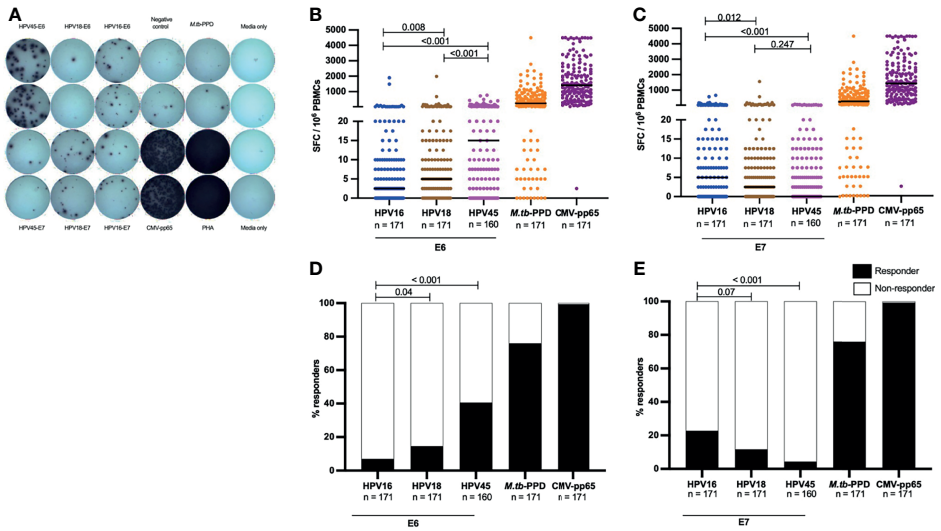

FIGURE 1 | HR HPV oncoproteins have a low inherent systemic immunogenicity in HIV- women. A representative image of an IFN-γ release ELISpot assay plate **(A)**, showing T-cell reactivity against E6 and E7 oncoproteins for HPV16, 18 and 45, and responses against CMV-pp65, Mtb-PPD, and control wells (negative control wells (complete media + PBMCs), media only wells and positive (PHA) control wells) is shown in **(A)**. The magnitude of T-cell reactivity against both HPV oncogenes, as well as *M.tb*-PPD and CMV-pp65 is given in SFC/10^6 PBMCs. PBMCs were stimulated with E6 **(B)** and E7 **(C)** HPV16, 18, 45 type specific oncoproteins, and control peptides *M.tb*-PPD and CMV-pp65 overnight and SFC/10^6 PBMCs were recorded for each sample. Each dot represents one study volunteer and total numbers of women analysed is stated in the x-axis legend. Median SFC/10^6 PBMCs is indicated by a black line. Statistical analysis was performed using the Mann-Whitney U-test. The proportion of responders for E6 **(D)** and E7 **(E)** as well as *M.tb*-PPD and CMV-pp65 are shown as percentage. The black bars indicate the proportion of individuals with a response while the white bar represent the proportion of individuals without a response, totaling to 100%. The n is given in the figure legend. Statistical analysis was performed using Fisher's exact test, respective p-values are shown in the graph.

Of the 202 women living with HIV, 75% were on ART, 34% had a CD4 count of 250 cells/μl or less and 34% of women had a HIV viral load of above 1000 RNA copies/ml (**Table 1**). In summary, the majority of women enrolled in this study were on ART and had a well-controlled HIV infection.

Low Inherent Systemic Immunogenicity of HR HPV Oncoproteins

We first determined the magnitude of HPV-specific T-cell reactivity and proportions of HPV-oncoprotein-specific T cell responders in HIV negative women alone prior to determining the effect of HIV on the aforementioned parameters.

Overall, amongst all HIV negative women, the E6- and E7-specific T-cell reactivity was of low magnitude. The highest median SFC/10^6 PBMCs for oncoprotein-specific T cells was 15 SFC/10^6 PBMCs against HPV45-E6 (**Figure 1B**), this was significantly lower than the median magnitude of *M.tb*-specific T-cell reactivity (245 SFC/10^6 PBMCs, p<0.001) and CMV-pp65-specific T-cell reactivity (median: 1412 SFC/10^6 PBMCs, p<0.001). Amongst E6-specific T-cell reactivities, HPV16-E6 was the least immunogenic with median of 2.5 SFC/10^6 PBMCs, this was half the median reactivity of HPV18-E6 (p = 0.008) and one sixth that of HPV45-E6 reactivity [p<0.001, (**Figure 1B**)]. An inverse pattern was observed for E7-specific T-cell reactivity. Here, HPV16-E7 had a low median reactivity of 5 SFC/10^6 PBMCs, which was nonetheless double that of HPV18-E7, p = 0.012 and 5 times higher than HPV45-E7, p <0.001 (**Figure 1C**), but still below threshold for a positive response as defined by our study.

We then analyzed the proportions of HIV negative women with a positive E6-/E7-oncoprotein T-cell response. Even though HPV16 was the most frequently detected HPV type within the study population (**Table 1**), T-cell responses to HPV16-E6 were significantly less frequent (7%, 12 of 171 women), compared to T-cell responses to HPV18-E6 (15%, 25 of 171 women, p=0.04) and HPV45-E6 (41%, 65 of 160 women, p <0.001) (**Figure 1D**). In contrast, HPV16-E7-specific T-cell responses were detected in 23% (39 of 171 women), and hence more frequently detected as compared to HPV18 (11%, 20 of 171 women, p=0.07) and HPV45 (4%, 7 of 160 women, p<0.001). T-cell responses to the control antigens CMV-pp65 and *M.tb*-PPD were detected in most women at 99% (170/171, p<0.001) and 76% (130/171, p<0.001) respectively (**Figure 1E**).

These data demonstrate a low T-cell reactivity for HPV oncoproteins in peripheral blood for the clinically most relevant HR HPV types in adult HIV negative women and show that significant differences in immunogenicity exist between the oncoproteins of the three most relevant HPV types.

HPV18 and 45 but Not HPV16 Infections Are Linked to Detectable E6- and E7-Specific T-Cell Responses Targeting the Infecting HPV Type

To determine the effect of prevalent HPV infection on the frequency of HPV-specific oncoprotein T-cell responses, we assessed whether HPV16, 18 or 45 infections in HIV- women were linked to detectable E6-/E7-specific T-cell responses against

the autologous infecting HPV genotype in comparison to women not infected with either HPV16, 18 or 45, respectively.

In HPV16 infected women only a non-significant increase in the frequency of HPV16-E6-specific responses was observed (**Figure 2**). 14% (7/49) of HPV16 infected women had HPV16-E6-specific T-cell responses compared to 5% of HPV16 negative women (5/100, p= 0.061). Women infected with HPV18 and 45 were significantly more likely to mount HPV type-E6-specific T-cell responses compared to those without these HPV infection (**Figure 2**); 38% (5/13) of HPV18 infected women responded to HPV18-E6 compared to 14% of HPV18 negative women (19/136, p = 0.037) and 69% of HPV45 infected women (9/13) responded to HPV45-E6 compared to 37% of HPV45 negative women (47/126, p = 0.036). A similar pattern was observed for E7-specific T-cell responses targeting autologous HPV type (**Figure 2**). Here the proportion of responders was 27% (13/49) for HPV16 infected women versus 23% (23/100) for HPV16 negative women (p = 0.686); 38% (5/13) for HPV18 infected women versus 10% (14/136) for HPV18 negative women (p = 0.013) and 23% (3/13) for HPV45 infected versus 3% (4/126) in HPV45 negative women (p = 0.018). When combining autologous-type-E6/E7-specific T-cell responses, the overall lowest frequency was detected in HPV16 infected women (33%, 16/49), as compared to women infected with HPV18 (46% (6/13), p = 0.516); and compared to women infected with HPV45 (69% (9/13), p = 0.026, data not shown). Taken together, these results show that prevalent HPV18 and 45, but not HPV16 infections, were linked to a significantly higher likelihood of mounting oncoprotein-specific T-cell responses against the infecting HPV type and suggest a particularly low inherent immunogenicity of HPV16 oncoproteins in direct comparison to the clinically relevant HR HPV types 18 and 45.

Depletion of Oncoprotein-Specific T-Cell Responses Targeting the Infecting HR HPV Type in Women Living With HIV

To assess the effect of HIV infection on systemic HPV T-cell responses, we stratified the data into HIV– and HIV+ groups and subsequently analyzed the frequency of response and the magnitude of HR HPV oncoprotein-specific T-cell reactivity within these two groups.

Taking into account women with and without HPV infection, the frequency of E6-specifc T cell responses was comparable between HIV- and HIV+ women for HPV16 and HPV18 specific responses, but significantly differed for HPV45 specific responses (p = 0.006, **Table 2**). HIV infection further had no measurable effect on the proportion of HR HPV-E7-specific T-cell responses Also, the magnitude of HPV16- and HPV18-E6-specific T-cell reactivity was similar between HIV- and HIV+ volunteers, irrespective of whether or not the women had an ongoing HPV infection. Conversely, HPV45-E6-specific T-cell reactivity was significantly decreased in HIV+ women, p = 0.012 (**Supplementary Figure 1**). For E7-specific T-cell reactivity, HIV infection had no measurable effect on the magnitude of T-cell reactivity.(**Supplementary Figure 1B**).

However, the effect HIV on the frequency of response and magnitude of HPV-specific T-cell reactivity was more apparent when the analysis focused on women with an ongoing HPV infection. Amongst HPV18 and HPV45 infected women, HIV infection was associated with diminished E6-specific T-cell responses targeting the infecting genotype. For HPV18-E6, only 11% (4/32) of HIV+HPV18+ women responded compared to 38% (5/13, p = 0.043) in HIV-HPV18+ women. Similarly, only 24% of HIV+HPV45+ women (5/21) responded to HPV45-E6 compared to 69% of HIV-HPV45+ women (9/13,

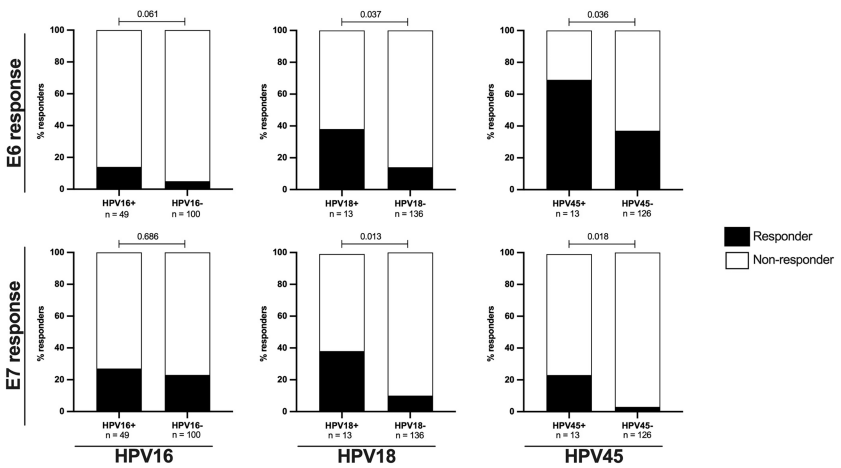

FIGURE 2 | The proportions of E6/E7 HPV type specific oncoprotein T-cell responses in are increased in HIV- women with ongoing HPV infection. The proportion of HIV- women with HPV type specific oncoprotein T-cell responses in relation to HPV16, 18 or 45 infection status is shown as percentage. The upper panels presents data for E6 HPV type specific responses while the lower panel presents data for E7 HPV type specific responses. The black bars represent the percentage of responders while the white bars represent the percentage of non-responders, totaling to 100%. The n is given in the figure legend. Statistical analysis was performed using Fisher's exact test, respective p-values are shown in the graph.

TABLE 2 | Proportions of E6- and E7-specific T-cell responses stratified by HIV status.

	E6-specific T cell response			E7-specific T cell response		
	% (n) HIV-	% (n) HIV+	p value	% (n) HIV-	% (n) HIV+	p value
HPV16 responders	7 (12/171)	8 (17/202)	0.700	23 (39/171)	24 (49/202)	0.807
HPV16 non-responders	93 (159/171)	92 (185/202)		77 (132/171)	76 (153/202)	
HPV18 responders	15 (25/171)	10 (21/202)	0.269	12 (20/171)	15 (31/202)	0.365
HPV18 non-responders	85 (146/171)	90 (181/202)		88 (151/171)	85 (171/202)	
HPV45 responders	41 (65/160)	26 (48/184)	0.006	4 (7/160)	8 (15/184)	0.187
HPV45 non-responders	59 (95/160)	74 (136/184)		96 (153/160)	92 (169/184)	

p = 0.014) (**Figure 3**). In HPV16 infected women, autologous HPV16-E6-specific T-cell responses were rarely detected regardless of HIV co-infection, with 14% (7/49, HIV-) vs 12% (7/58, HIV+, p = 0.780) recognition. For autologous E7-specific T-cell responses, the response rates were low in HPV16 and 45 infected women regardless of HIV co-infection. However, HIV infection was again associated with significantly lower HPV18-E7-specific responses with 11% (4/36) vs 38% (5/13, p = 0.043) in HIV+ vs HIV- women, respectively (**Figure 3**). Analyses of autologous E6-/E7- T cell reactivity showed that E6-specific autologous reactivity was comparable between HIV- and HIV+ for HPV16. Conversely, median magnitude of T-cell reactivity for HPV18-E6 was 5-fold higher in HIV- when compared to HIV+ (p = 0.050), while reactivity for HPV45-E6 was 4-fold higher in HIV- as compared to HIV+ (p = 0.011, **Figure 4A**). There was no difference in the median magnitude of autologous E7-specifc T-cell reactivity for the three HR HPV types included in this analyses (**Figure 4B).**

Depletion of HR HPV E6- and E7-Oncoprotein Specific T-Cell Responses in Women Living With HIV Is Linked to Low CD4 Counts and Detectable HIV Plasma Viremia

Since advanced HIV disease has been correlated with loss of pathogen-specific T cells, we analyzed HPV oncoprotein-specific T-cell reactivity in the context of HIV plasma viremia and CD4 T-cell depletion in HIV+ women with no cervical lesion stratified by HIV VL of less or greater than 1000 copies per ml and those with CD4 T-cells counts of less or greater than 250 cells/μl. These cut offs are aligned with WHO guidelines, which define immunological failure as CD4 T cell counts of 250 cells/mm^3 or less and virological failure is defined as two sequential viral loads (VL) levels of 1000 or more copies/mL within 3 months (36). HIV+ women with > 250 CD4 T cells had significantly higher HPV E6-specific T-cell reactivity compared to those with ≤ 250 CD4 T cell counts/μl for HPV16, 18 and 45 (**Figure 5A**),

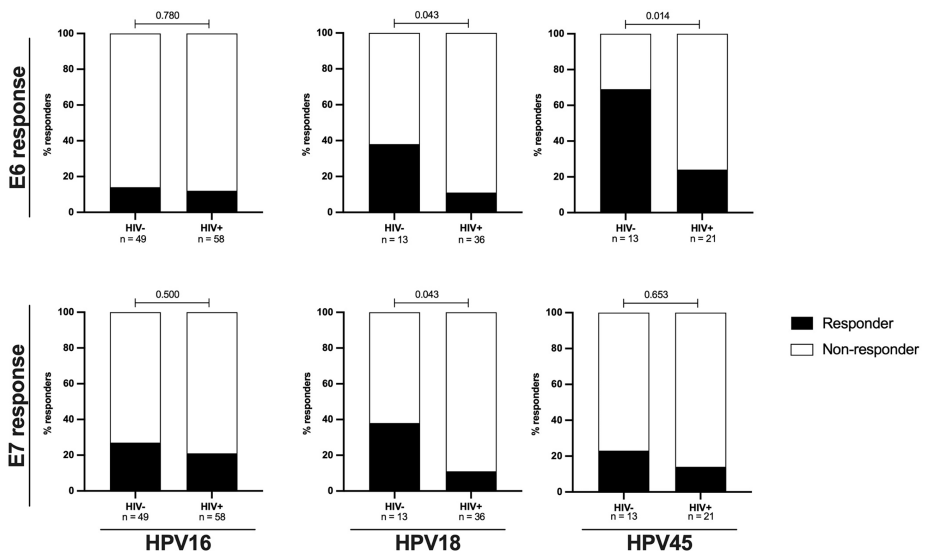

FIGURE 3 | The proportions of autologous E6/E7 HPV type specific oncoprotein T-cell responses are reduced in HIV+ women. The proportion of women with autologous HPV16, 18 or 45 type specific T-cell responses stratified by HIV status is shown as percentage. Statistical analysis was performed using Fisher's exact test, respective p-values are shown in the graph. The black bars represent the percentage of responders while the white bars represent the percentage of non-responders, totaling to 100%. The n is given in the figure legend. The upper panels presents data for E6 HPV type specific responses while the lower panel presents data for E7 HPV type specific responses.

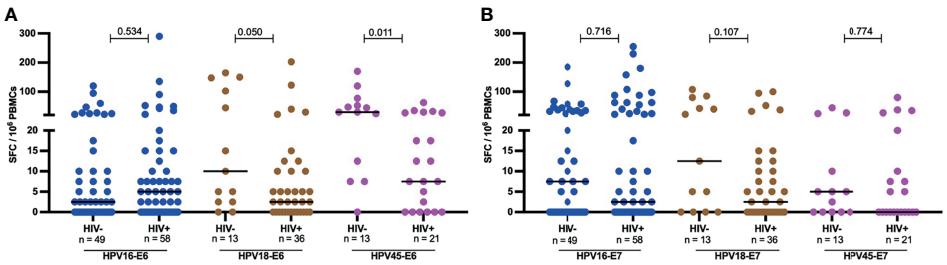

FIGURE 4 | Magnitude of autologous HPV oncoprotein type specific T-cell reactivity. The magnitude of autologous T-cell reactivity in SFC/10^6 against E6 **(A)** and E7 **(B)** HPV16, 18, 45 type specific oncoproteins stratified by HIV status. Each dot represents an individual study participant, median SFC/10^6 PBMCs is indicated with black line in the graph and the n is indicated in the figure legends. Statistical analysis was performed using the Mann-Whitney U-test, the respective p-value is shown in the figures.

with a 5-fold lower median for HPV16 (p = 0.037), 7-fold lower median for HPV18 (p = 0.009) and 3-fold lower median for HPV45 (p = 0.039). Similarly, E7-specific T-cell reactivity (**Figure 5B**), was higher in volunteers with > 250 CD4 T cells compared those with ≤ 250 CD4 T cells; 25-fold lower median for HPV16, p = 0.011 and 5-fold lower median for HPV18 reactivity, while no difference was observed for HPV45. Interestingly, no such differences were observed for *M.tb*-specific or CMV-specific T-cell reactivity (**Figure 5C**). In addition, we observed a positive correlation between absolute CD4 counts and HPV16 E7 as well as for HPV18 E6-specific T cell reactivity, r = 0.226 p = 0.044 and r = 0.333, p = 0.003, respectively (**Figure 5D, E**).

With respect to HIV viral load, HPV45 E6 T-cell reactivity was 2.3 times lower in samples which had ≥ 1001 HIV VL copies/mL compared to samples with ≤ 1000 (p = 0.029, **Supplementary Figure 2A**). There was no significant difference in E6-specific T-cell reactivity for HPV16 and 18 (**Supplementary Figure 2A**), and for E7-specific T-cell reactivity for HPV16, 18 and 45 (**Supplementary Figure 2B**). Interestingly, there was a weak negative correlation between HPV45 E6 T-cell reactivity and HIV RNA copies/ml, r = -0.273, p = 0.024 (**Supplementary Figure 2C**).

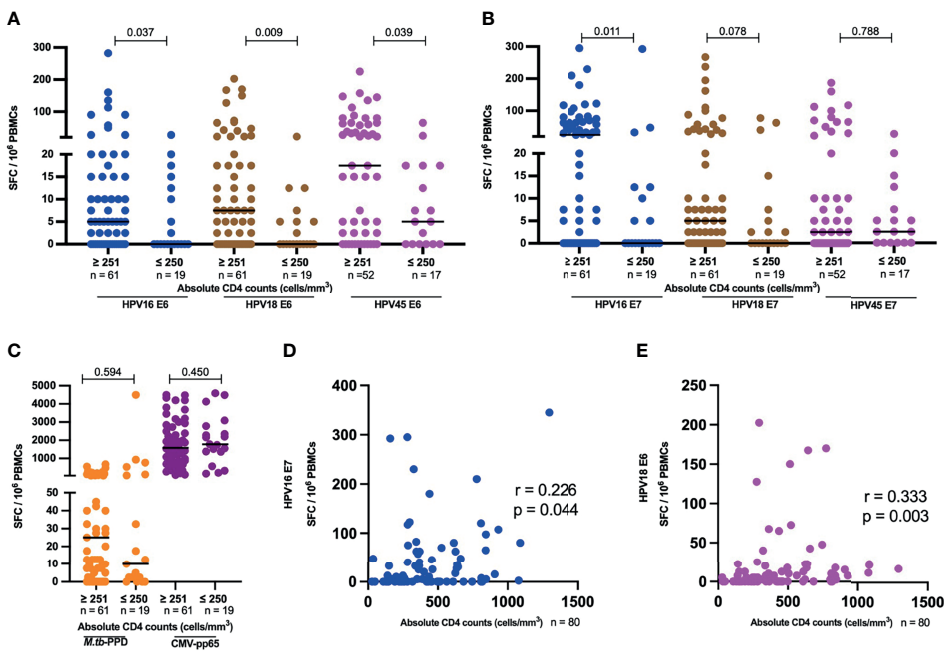

FIGURE 5 | HPV-specific oncoprotein T-cell reactivity is reduced in HIV+ women with advanced HIV disease. The magnitude of reactivity against E6 **(A)** and E7 **(B)** HPV16, 18, 45 type specific oncoproteins **(C)** *M.tb*-PPD and CMV-pp65 is shown as SFC/10^6 PBMCs and stratified by CD4 counts. Each dot represents an individual study participant, median SFC/10^6 PBMCs is indicated with black line in the graph and the n is indicated in the figure legends. Statistical analysis was performed using the Mann-Whitney U-test. Correlation between absolute CD4 counts and HPV16 E7 **(D)** as well for HPV18 E6 **(E)** T-cell reactivity in SFC/10^6 PBMCs is shown, correlation coefficients (r) and p-values are shown in the graphs. Statistical analysis was performed using the spearman's ranks correlation test.

Selective Depletion of HR HPV Oncogene-Specific T-Cell Responses in HIV+ Women With Precancerous Lesions or Cervical Cancer

To evaluate the possible link between precancerous and cancerous lesions with depleted HPV oncoprotein-specific T-cell reactivity in HIV+ women, we compared the magnitude of E6- and E7-specific T-cell reactivity in women with and without HSIL or SCC. HIV+ women with HSIL or SCC compared to those with no lesion had significantly lower median magnitudes of E6-specific T cells (**Figure 6A**); 2-fold less for HPV16- and 18-E6-specific T-cell responses ($p = 0.037$ and 0.031, respectively) and 3-fold less for HPV45-specific T cell responses, $p = 0.024$. No significant differences for E7-specific T-cell reactivity was observed for any of the three HPV types (**Figure 6B**) nor for control antigens *M.tb*-PPD and CMV-pp65 (**Figure 6C**).

DISCUSSION

In this study, we address the hypothesis that HIV-induced dysfunction or depletion of HR HPV-oncoprotein-specific T cells contributes to the increased risk for cervical cancer in HIV+ women. We analyzed T-cell responses targeting HPV types 16, 18 and 45, as these cause the vast majority of cervical cancer cases regardless of HIV infection or global region (11). We found that: a) HR HPV oncoproteins have a low inherent systemic immunogenicity with the lowest oncoprotein-specific T-cell reactivity observed for HPV16 - the most cancerogenic type; b) among HIV negative women, autologous HR HPV type-specific T-cell responses were significantly more frequently detected in women with a given HR HPV infection, compared to those without such an infection; c) HIV infection, advanced HIV disease progression and ongoing HIV viral replication were all linked to depletion of HR HPV-specific T-cell responses; d) Selective depletion of HR HPV-oncoprotein-specific T-cell reactivity was observed in HIV+ women with precancerous cervical lesions and cancer, whereas other pathogen-specific responses were preserved in these women. Together these results support the concept that depletion or dysfunction of HR HPV-specific T-cell responses predisposes HIV+ women to increased HPV persistence followed by the development of precancerous cervical lesions and cancer.

The very low inherent immunogenicity of HR HPV oncoproteins in our study confirms results from previous studies (38, 39). The non-cytopathic aviremic nature of the virus (40, 41) as well as the highly confined expression of the small E6 and E7 oncoproteins to the basal and parabasal cells of the cervical epithelium may all contribute to poor systemic immunogenicity of E6 and E7. It is noteworthy that HPV16 immunogenicity in the total cohort was particularly low when compared to HPV18 and HPV45. Indeed, in contrast to HPV18 and HPV45 infections, HPV16 infection was not linked to increased T-cell recognition of HPV16 E6 or E7. We therefore hypothesize that this particularly low inherent HPV16 oncoprotein immunogenicity plays a role for the remarkable persistence and cancerogenic potential of HPV16 infections as compared to other HR HPV types.

Systemic T-cell reactivity to the control antigens *M.tb*-PPD and CMV-pp65 was robust and as described before, HIV infection was associated with depletion of *M.tb*-specific, but not CMV-specific T-cell responses (26, 27). The latter were slightly increased in HIV infected women, confirming previous studies by our (26, 27) and other groups (42). The magnitude of E6 and E7 HR HPV type-specific systemic T-cell reactivity differed amongst HPV16, 18 and 45. For E6 peptides, the HPV16-E6 peptide was the least immunogenic while HPV45-E6 was the most immunogenic. Plausibly, the low immunogenicity of HPV16-E6 when compared to the HPV18-E6 and HPV45-E6 oncoproteins contributes to the cancerogenic potential of HPV16 cancers regardless of HIV infection. Interestingly, the converse was observed for E7 peptides, there, HPV16-E7 was the most immunogenic whereby HPV45-E7 was least immunogenic. It is worth reiterating that the median magnitude of these T-cell responses were below what is deemed a positive T-cell response. It is plausible that anti-E6 and E7- HPV systemic T-cell responses contribute differently to HPV clearance and cervical lesion regression. Tong et al., showed that E6, but not E7, systemic CD4 T-cell responses were associated with regression of anal high grade squamous

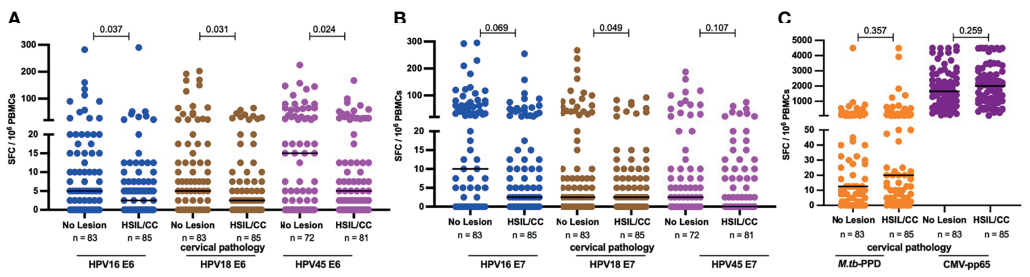

FIGURE 6 | HPV-specific oncoprotein T-cell reactivity is reduced in HIV+ women with high grade intraepithelial lesions or cancer. The magnitude of reactivity against E6 **(A)** and E7 **(B)** HPV16, 18, 45 type specific oncoproteins **(C)** Mtb-PPD and CMV-pp65 is shown as SFC/10^6 PBMCs and stratified by cervical pathology for these HIV+ women. Each dot represents an individual study participant, median SFC/10^6 PBMCs is indicated with black line in the graph, p values are indicated in the graphs and the n is indicated in the figure legends. Statistical analysis was performed using the Mann-Whitney U-test.

intraepithelial lesions (43). Contrary, Seresini et al., have shown that PBMCs when stimulated with HPV18-E7 produce different CD4 cytokine profiles depending on whether the PBMC donor was HPV18+ or HPV18-. The aforementioned difference was not observed when the cells were stimulated with HPV18-E6 peptide (44). These findings are in line with our observation that E6 and E7 oncoproteins from the same HPV type differ in immunogenicity and may therefore have differing roles in the anti-HPV immune response. Nonetheless, grouping either E6 or E7 HPV type specific response as a response to a particular HPV type still indicated HPV16 as the least immunogenic and HPV45 as the most immunogenic.

HIV infection was associated with reduced frequency and magnitude of autologous HPV18- and 45-specific T-cell reactivity in HIV+ women, even though most of these women were on efficient ART. This implies that ART initiation may often not fully restore HPV-specific T-cell function which likely contributes to decreased HPV clearance and increased rates of cervical lesions. Indeed, most women living with HIV and diagnosed with cervical cancer in this study also were on ART treatment, which is consistent with other studies (45). Nicol and colleagues showed that while HPV infection increased T-cell infiltration and the secretion of IL-6, TNF-α and IFN-γ in cervical biopsies of HPV+ individuals, HIV-HPV co-infected individuals had significantly reduced amounts of IFN-γ, IL-6 and TNF-α (16), suggesting that HPV-specific T-cell responses are depleted or dysfunctional also within the mucosa.

Even though we have not observed any difference between HIV+ and HIV- women in frequency or reactivity of autologous T-cell immunity to HPV16, we find that women with advanced HIV disease (less than 250 CD4 T cells) and with no cervical lesions, had significantly lower immune reactivity against HPV16 than those with higher CD4 T-cell counts. This implies that advanced immunodeficiency rather than HIV positivity alone affects HPV16-specific T-cell reactivity, regardless of ART status. Indeed, ART status alone does not represent an adequate measure of immune reconstitution. Better measures would be: HIV viral suppression and reconstitution of peripheral and mucosal CD4 T-cells counts (36). Possibly, the majority of women who started ART before the *test and treat era* might not fully and adequately restore their HPV-specific mucosal immunity because of starting HIV treatment at a low nadir CD4 T-cell count (46, 47). Our results show that women with more advanced HIV disease had significantly lower magnitudes of HPV-oncoprotein-specific T-cell reactivity compared to those with less advanced HIV progression. The combined data therefore links the increased risk for HR HPV infection and associated cervical disease caused by HIV infection – particularly evident in women with more advanced acquired immunodeficiency - to dysfunction and depletion of HPV-specific T cell immunity. Indeed, a large number of studies have reported an inverse relationship between absolute CD4 T-cell counts and HPV infection rates and subsequent development of pre-cancerous lesions and SCC (48–50). In this context, it will be important to clarify whether early ART treatment initiation, when CD4 T-cell counts are still high, may counteract HIV-induced T-cell dysfunction, reduce risk for HPV virus persistence thereby reducing the risk of cervical cancer. The importance of CD4 T cells with respect to HPV infection and disease has been strengthened by Steele et al., showing in their study that even though the frequency of systemic anti-HPV CD8 T-cell responses was twice that of CD4 T-cell responses, CD4 T-cell reactivity was reduced in patients with advanced lesions, implying that CD4 T cells are crucial in HPV clearance and cervical lesion regression (38). Similarly, Coleman et al., have shown that regressing genital warts are predominantly infiltrated by activated memory CD4 T cells, further underpinning the importance of CD4 T cells (12).

Furthermore, we report that women living with HIV and with high-grade intraepithelial cervical lesions or cancer (HSIL/SCC) had significantly lower systemic HR HPV oncoprotein T-cell reactivity compared to those without cervical lesions. Cervical lesions (especially cancer) are associated with T-cell exhaustion, T-cell activation and inflammation (51–53). Capacity for HPV-specific IFN-γ production by T cells may therefore continuously be reduced as cervical lesion progress (and more oncoproteins are being expressed). Together these data are consistent with the hypothesis that HIV infection and lesions act synergistically to enhance immune dysfunction of the host response to HPV infection, which in turn counteracts HPV clearance and accelerates cancerogenesis.

Quantifying systemic T-cell responses to HR HPV oncoproteins is challenging as the lack of a viremic phase and the numerous immune evasion mechanisms of HPV negatively impact systemic HPV-specific T-cell reactivity. To overcome this challenge, different studies have modified their ELISpot assays in a variety of ways: a) increasing the time to culture during the *ex vivo* re-stimulation (3 – 9 days) (21, 54, 55), b) co-culturing lymphocytes with IL-2 for 9 days (55), or c) pulsing autologous dendritic cells prior to long term stimulation (56). In different studies the final peptide concertation ranges from 2μg/ml to 10μg/ml. While these approaches may have their merits, our approach of direct short-term ex vivo re-stimulation of freshly isolated PBMC with HR HPV type specific peptides has the advantage of closely reflecting systemic T-cell reactivity *in vivo*. Due to the infrequent nature of systemic HPV responses, especially in HIV+ women, we could not analyze the effects of CD4 levels, HIV RNA plasma levels as well as cervical pathology on autologous systemic HPV-specific T-cell immunity. However, we believe that effects of the aforementioned parameters on HPV-specific immunity is consistent with the results presented regardless of presence of an ongoing HPV infection.

In conclusion, we observed that HIV-associated depletion of HR HPV oncoprotein-specific T-cell reactivity is particularly pronounced in patients with progressed, viremic HIV infection and in those with HPV associated premalignant and malignant cervical lesions. This low HR HPV-oncoprotein-specific T-cell reactivity likely contributes to the increased HR HPV persistence, cervical lesion progression and accelerated cancer development in HIV+ women.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Mbeya Medical Research and Ethics review Committee (MRH/R.10/8/Vol. VI/107), the Tanzanian National Health Research Ethics Committee (NIMR/HQ/R.8a/Vol. IX/1422) and the Ethics Committee of the Medical Faculty of University of Munich (project ID: 308-11). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, CG, AK, and RK. Formal analysis, WM, KH, MC, and CG. Clinical investigation, AK, RM, MS, FR, and LT. Laboratory investigation, WM, AH, JaM, JoM, AM, MM, WoM, RK, LT, MC, and CG. Resources, MH, LM, FR, LT, and RK. Data curation, PA and ES. Supervision, AK, KH, RM, MH, LM, OG, MC, and CG. Project administration, AK, RM, MH, LM, OG, and CG. Funding acquisition, CG, AK, MH, and LM. All authors contributed to writing and review of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.742861/full#supplementary-material

Supplementary Figure 1 | HIV status does not affect magnitude of HPV type specific oncoprotein T-cell reactivity. The magnitude of T cell reactivity against E6 **(A)** and E7 **(B)** HPV16, 18, 45 type specific oncoproteins is shown as SFC/10^6 PBMCs and stratified by HIV status. Each dot represents an individual study participant, median SFC/10^6 PBMCs is indicated with a black line in the graph, p values are indicated in the graphs and the n is indicated in the figure legends. Statistical analysis was performed using the Mann-Whitney U-test

Supplementary Figure 2 | HPV45 E6-specific oncoprotein T-cell reactivity is reduced in HIV+ with elevated HIV RNA copies/ml. The magnitude of reactivity against E6 **(A)** and E7 **(B)** HPV16, 18, 45 type specific oncoproteins among HIV+ women is shown as SFC/10^6 PBMCs and stratified by HIV viral load. Each dot represents an individual study participant, median SFC/10^6 PBMCs is indicated with a black line in the graph, p values are indicated in the graphs and the n is indicated in the figure legends. Statistical analysis was performed using the Mann-Whitney U-test. Correlation between HIV copies/ml and HPV45 E6 T cell reactivity in SFC/10^6 PBMCs **(C)** is shown, correlation coefficients and p values are shown in the graphs. Statistical analysis was performed using the spearman's ranks correlation test.

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HPV Type Distribution in HIV Positive and Negative Women With or Without Cervical Dysplasia or Cancer in East Africa

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Background: Women living with HIV in sub-Saharan Africa are at increased risk to develop cervical cancer (CC), which is caused by persistent infection with 13 oncogenic human papilloma viruses (HR-HPVs). It is important to accurately identify and target HIV-positive women at highest risk to develop CC for early therapeutic intervention.

Methods: A total of 2,134 HIV+ and HIV– women from South-West Tanzania were prospectively screened for cervical cancer and precancerous lesions. Women with cervical cancer (n=236), high- and low-grade squamous intraepithelial lesions (HSIL: n=68, LSIL: n=74), and without lesion (n=426) underwent high-resolution HPV genotyping.

Results: Eighty percent of women who were diagnosed with HSIL or LSIL were living with HIV. Any lesion, young age, HIV status, and depleted CD4 T cell counts were independent risk factors for HPV infections, which were predominantly caused by HR-HPV types. While multiple HR-HPV type infections were predominant in HIV+ women with HSIL, single-type infections predominated in HIV+ CC cases (p=0.0006). HPV16, 18, and 45 accounted for 85% (68/80) and 75% (82/110) of HIV+ and HIV– CC cases, respectively. Of note, HPV35, the most frequent HPV type in HSIL-positive women living with HIV, was rarely detected as a single-type infection in HSIL and cancer cases.

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Conclusion: HPV16, 18, and 45 should receive special attention for molecular diagnostic algorithms during CC prevention programs for HIV+ women from sub-Saharan Africa. HPV35 may have a high potential to induce HSIL in women living with HIV, but less potential to cause cervical cancer in single-type infections.

Keywords: human papilloma virus—HPV, human immunodeficiency virus—HIV, cervical cancer, cervical dysplasia, high-grade intraepithelial lesions, low-grade intraepithelial lesions, molecular diagnosis

INTRODUCTION

Cervical cancer (CC) is the fourth most frequent cancer in women globally with 570,000 new cases and 311,000 deaths in 2018. Eighty-five percent of these CC cases occur in low- and middle-income countries (1). Sub-Saharan Africa (SSA) is the most heavily affected region globally; women here have the highest cumulative lifetime risk to develop CC—above 5% for many countries—and CC is the leading cause of death from malignancy in many SSA countries (2). These "quasi epidemic" dimensions at least partially result from regional particularities, including a poor screening capacity for identification of women at risk for CC development and high prevalence of HIV infection.

Thirteen "high risk" (HR) HPV types can cause CC and precancerous cervical intraepithelial neoplasias (CIN) within the anogenital region and the oral cavity (3, 4) and therefore have been classified as direct carcinogens. E6 and E7 oncoproteins from high-risk HPVs contribute to this carcinogenic potential and are able to drive cell cycle entry in the upper epithelial layers and to stimulate cell proliferation in the basal and parabasal layers (5). HPV16 (>50%) and 18 (>10%) account for the majority of CC cases worldwide, followed by HPV45 and HPV31, while the rest is caused by other high-risk HPV types (6, 7). HIV infection is a major risk factor to develop CC and other HPV-associated cancers. Two large American population-based studies in HIV-infected women reported a 9- and 5-fold increased risk to develop CC compared to HIV negative women (8, 9). Newly acquired HIV infection immediately increases the risk for detectable HR-HPV infections (10, 11), whereas chronic HIV infection is linked to a high prevalence of diverse HR-HPV type infections, longer HPV persistence, high risk for precancerous lesions, and a more rapid disease progression to CC (9, 12–17). HIV^+ women from SSA are also affected by high prevalence rates of diverse oncogenic HR-HPV infections and associated reproductive tract cytologic abnormalities (16, 18–20). Sahasrabuddhe and colleagues found premalignant high-grade squamous intraepithelial lesions (HSIL) or squamous cell carcinoma (SCC) in more than half of HIV^+ Zambian women seeking HIV/AIDS treatment (16), and these diagnoses were often associated with HPV types other than 16 and 18. This study and other studies support the hypothesis that HR-HPV types other than 16 and 18 more frequently cause CC in HIV^+ compared to HIV^- women (15, 16, 21).

Diagnostic algorithms tailored to a specific SSA context can contribute to better guide CC prevention and therapeutic intervention strategies, such as cryotherapy and excision surgery, and guide design of therapeutic vaccines as a possible non-invasive alternative for women with persistent HR-HPV infection and premalignant lesions. Because of the apparent interaction of infections with HIV and carcinogenic HPVs, it is also important to accurately identify and target HIV+ women at highest risk to develop CC for early therapeutic intervention.

In order to identify risk factors for HR-HPV infection and to address the hypothesis that HR-HPV types other than HPV16 and 18 more frequently cause cancer in HIV+ *versus* HIV− women, we have studied HPV infection in 2H study participants with CC, high- and low-grade squamous intraepithelial lesions (HSIL and LSIL), and without cervical lesions in relation to HIV status, ART treatment, and disease progression. HSIL, CIN2 and 3 are referred to as HSIL in the manuscript. LSIL and CIN1 are referred to as LSIL in the manuscript.

MATERIAL AND METHODS

Study Settings and Participants

The 2H study is a prospective study in the Mbeya Region, South-West Tanzania, embedded within the Tanzanian cervical cancer screening program. Between March 2013 and August 2020, overall 2,146 women were included and screened for cytohistological diagnosis of precancerous and cancerous lesions. The majority of study participants were recruited in the cervical cancer screening program at the Department of Obstetrics and Gynecology and at the HIV care and treatment center (CTC) of the Mbeya Zonal Referral Hospital (MZRH). In addition, women were recruited from other CTC in the Mbeya region, during mobile CC screening activities using a Mobile Diagnostic and Testing Centre, and during CC mass community screenings conducted in Mbeya region. From these locations, women with positive visual inspection using acetic acid (VIA) findings or cancer-suspicious were specifically targeted for study inclusion. For study inclusion, women had to be 18 years and above. Exclusion criteria included current pregnancy, prisoners, mentally disturbed women, or women in a serious health condition for whom study participation or informed consent procedures would imply an undue burden. All study participants were fully briefed on the study procedures, and written informed consent was required prior to enrolment. Ethical clearance was obtained from the Mbeya Medical Research and Ethics Review Committee (MRH/R.10/8/Vol. VI/107), the Tanzanian National Health Research Ethics Committee (NIMR/HQ/R.8a/Vol. IX/1422), and the Ethics Committee of the Medical Faculty of Munich University before commencement of the study.

Clinical Procedures

CC screening was performed following the Tanzanian National Guidelines by trained and certified nurses. All women underwent gynecological examination including speculum examination of the naive vagina and cervix uteri. The cervix was described for the clinical presence of cervicitis, tumor, ulceration, or other significant lesions. In addition to routine procedures, a Papanicolaou (pap) smear was taken from all women from the ectocervix using an Ayres spatula and two endocervical brush samples, one for cytology and one for HPV genotyping. In cases suspicious for cancer, biopsies were collected. Women diagnosed with CC or precancerous lesions (CIN2/CIN3) were referred to the Mbeya Zonal Referral Hospital (MZRH) gynecological clinic for re-assessment of lesions, and if indicated for FIGO staging. Treatment recommendation including Loop Electrosurgical Excision Procedure (LEEP), hysterectomy, or referral to the Ocean Road Cancer Institute in Dar es Salaam for radio-chemotherapy were provided by gynecologists.

Evaluation of Histological and Cytological Pathology

Routine cytology by Pap testing was performed on all subjects for assessment of cervical pathology by two pathologists. If needed, further histologic characterizations were performed based on hematoxylin and eosin staining at the MZRH pathology department. The Bethesda system for reporting cervical or vaginal cytological diagnoses was used for reporting results. In addition, histological changes were described by degree of severity [No lesion, cervical intraepithelial neoplasia (CIN) 1, CIN 2, CIN 3, Cervical Cancer] if performed. For external quality control, a subset of cytology and histology slides was subjected to assessment by a third pathologist.

Assessment of HIV-Associated Parameters

HIV status was determined for those with unknown HIV status. HIV history information, including data on antiretroviral therapy, were obtained through patient interviews, patient's clinic cards, or extracted from hospital charts where applicable. For women with unknown or previously negative HIV status, HIV counseling and testing were performed based on the national HIV testing algorithm with a first screening rapid test (Determine HIV1/2, Abbott Laboratories, South Africa) followed by a second confirmatory rapid test (Uni-Gold HIV Rapid Test, Trinity Biotech, South Africa) if the first test result was positive. In the case of discordant rapid test results, the test was repeated with a different sample of the same patient. If the repeated test was still inconclusive, then an ELISA was performed (Bio-Rad GS HIV-1/HIV-2 PLUS O EIA, Bio-Rad laboratories GmbH, Munich, Germany). All tests were performed at the College of American Pathologists (CAP)-accredited MMRC laboratories.

Detection of Human Papilloma Virus Infection and HPV Genotyping Procedures

HPV genotyping was performed for selected participants with well-defined HIV status and cytohistological diagnosis as shown in **Figure 1**. Cervical cells collected by cytobrush sampling were transferred into medium (PreservCyt® Solution) and sent to the

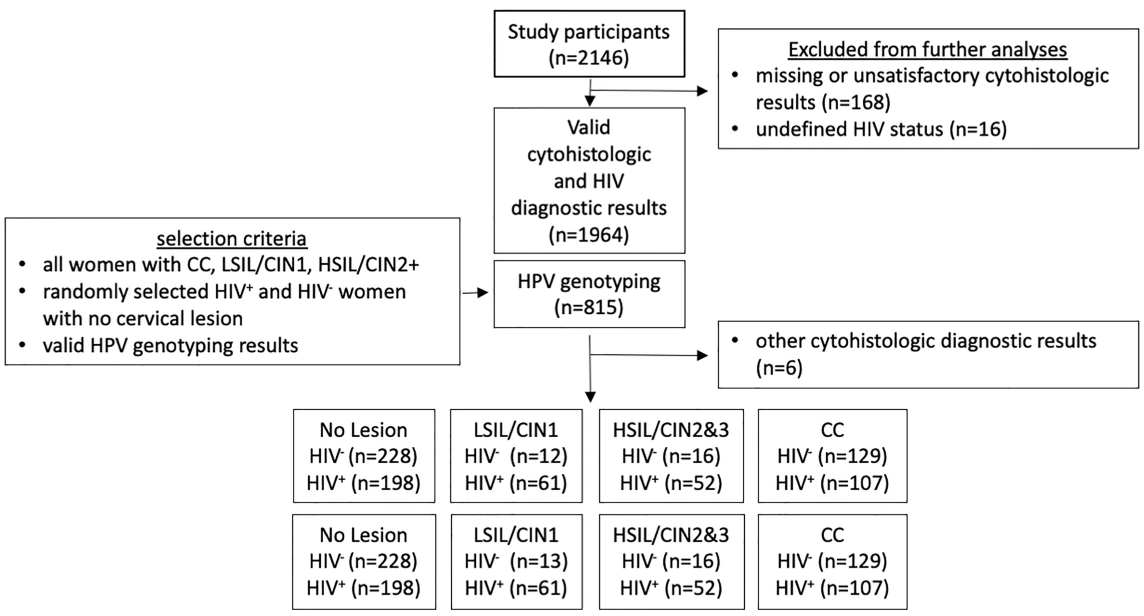

FIGURE 1 | Flow Diagram of subjects included for HPV genotyping analyses. A total of 2,146 women were screened during the 2H study, including patient referrals with cervical cancer and women with a positive visual inspection with acetic acid result during cervical cancer screening program. Valid cytohistologic diagnoses based on Pap smear and/or histology was obtained for 1,964 of these women. HPV genotyping was then performed for 815 women and to include all cervical cancer cases, all women with cervical intraepithelial lesions, as well as the similar number of women living with or without HIV and with no lesions.

molecular diagnostics laboratory at the NIMR-MMRC for HPV genotyping. HPV genotyping analysis was performed using the Linear Array® HPV Genotyping Test (Roche Molecular Systems) according to manufacturer instructions. The test identifies 37 different HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108). Samples without detectable cellular DNA were excluded from analyses. Please note that this assay cannot distinguish HPV52 infections in samples containing HPV33, HPV35, and/or HPV58. Since 2013, the NIMR-MMRC HPV genotyping laboratory has regularly participated in the WHO HPV genotyping proficiency testing.

Statistical Analyses

Data analysis and graphics were performed using the statistical software GraphPad prism 6.0 and R (R Development Core Team, 2021) and the ggplot R-package (22). For descriptive analyses, boxplots and scatterplots were used. Mann-Whitney (Wilcoxon) test was used to assess differences between groups in terms of age and cytohistological diagnosis stratified by HIV status. The two-sided Fisher's exact test was used to determine whether the frequency of occurrence of individual HPV types significantly differs between HIV+ women with LSIL, HSIL, and CC being compared to HIV+ women without cervical lesion. Different multivariate regression models were used in order to assess the relationship between the number of HPV infections and related risk factors. The first model was performed on all the study participants and was adjusted for age in years, HIV status, and cytohistological diagnosis. The second model was performed on HIV+ women and was adjusted for age and cytohistological diagnosis, as well as antiretroviral treatment (ARV) and number of CD4 cells counts (below or above 250). And finally, a third model was applied on HIV+ women and adjusted for age and type of lesion (cancer *vs.* no cancer). P-values <0.05 were considered statistically significant.

RESULTS

Cohort Characteristics

Between March 2013 and August 2020, 2,146 women from Mbeya region, South-West Tanzania, were enrolled into the 2H study. Of these women, 168 had missing diagnostic or unsatisfactory results. Other missing diagnostic included an undefined or inconclusive HIV status (n=16), as well as an undefined ART-status. All these women were excluded from analysis (**Figure 1**). Valid cytohistological and HIV diagnostic results were available for 1,964 of these women with a valid cytohistologic (Pap smear and/or histologic analyses). Other identified cervical pathologies included ASC-US (n=1), AGC Neoplasia (n=2), carcinoid (n=1), and tuberculosis of the cervix (n=2) and were also excluded from this analysis.

High-resolution HPV genotyping was performed on selected study participants to study characteristics of HPV infection in HIV+ and HIV− women in a case-control design, as shown in **Figure 1**. These analyses included valid results from 815 women; all cases of CC (n=236), all cases with HSIL or CIN2/CIN3 (HSIL, n=68), women with LSIL or CIN1 (LSIL, n=74) with available cytobrush samples, and a similar number of randomly assigned HIV− and HIV+ women without cervical lesions (n= 228 *vs* n=198, total 426). Of note, despite screening of more than 2,000 women during the study, only few HIV− women with HSIL or LSIL could be identified and included in these analyses. Indeed, 76% of HSIL cases and 84% of LSIL cases identified during the study period were women living with HIV. **Table 1** summarizes age and HIV disease progression parameters (ART status, HIV viral load and CD4 counts) for the study groups.

Risk Factors for HPV Infection

Among the study participants with complete data, we determined the number of HPV types and stratified the results by HIV status and cytohistological diagnosis (**Figure 2A**). HIV infection was associated with a significantly higher number of detected HPV types in women regardless of cervical lesion or cancer status ($p<0.005$ for all). The proportion of HIV+ women (of all women) with multiple concurrent HPV-type infections was always higher, as compared to HIV-negative women (**Supplementary Figure 1**). HIV infection was also significantly associated with a higher number of different HPV-type infections in age groups up to 50 years of age ($p<0.0001$ for all age groups, **Figure 2B**). Within women living with HIV, more advanced immunosuppression defined by CD4 T cell counts of below 250/ul correlated with higher numbers of detected HPV types, particularly for those without lesions and those with LSIL ($p<0.05$), but not for those with cervical cancer ($p=0.87$), (**Figure 2C**).

The multivariate regression models showed similar trends. When including all the study participants, age was found to be negatively associated with number of HPV infections (beta-coefficient: −0.26, 95% CI: −0.51; −0.010), while HIV infection

TABLE 1 | Summarizes the age and—for HIV-positive women—ART status, viremic suppression, and CD4 counts.

Diagnosis	HIV− women		HIV+ women				
	N	Age (median, IQR)	n	Age (median, IQR)	On ART (%)	HIV-RNA <1,000 copies/ml (%)*	CD4 count (median, IQR)
No lesion	228	38 (32–48)	198	37 (31–42)	74% (NA: n=13)	62% (48/77)	407 (262–608)
LSIL	13	38 (27–52)	61	34 (29–40)	73% (NA: n=6)	64% (14/22)	387 (185–548)
HSIL	16	40 (36–51)	52	38 (32–43)	81% (NA: n=3)	56% (18/32)	368 (232–494)
Cancer	129	58 (48–74)	107	37 (39–50)	77% (NA: n=15)	68% (45/66)	358 (195–579)

**The chosen viral load cutoff is aligned with WHO guidelines for virological failure defined as two sequential viral loads (VL) levels of 1,000 or more copies/ml within 3 months (Organization 2016).*

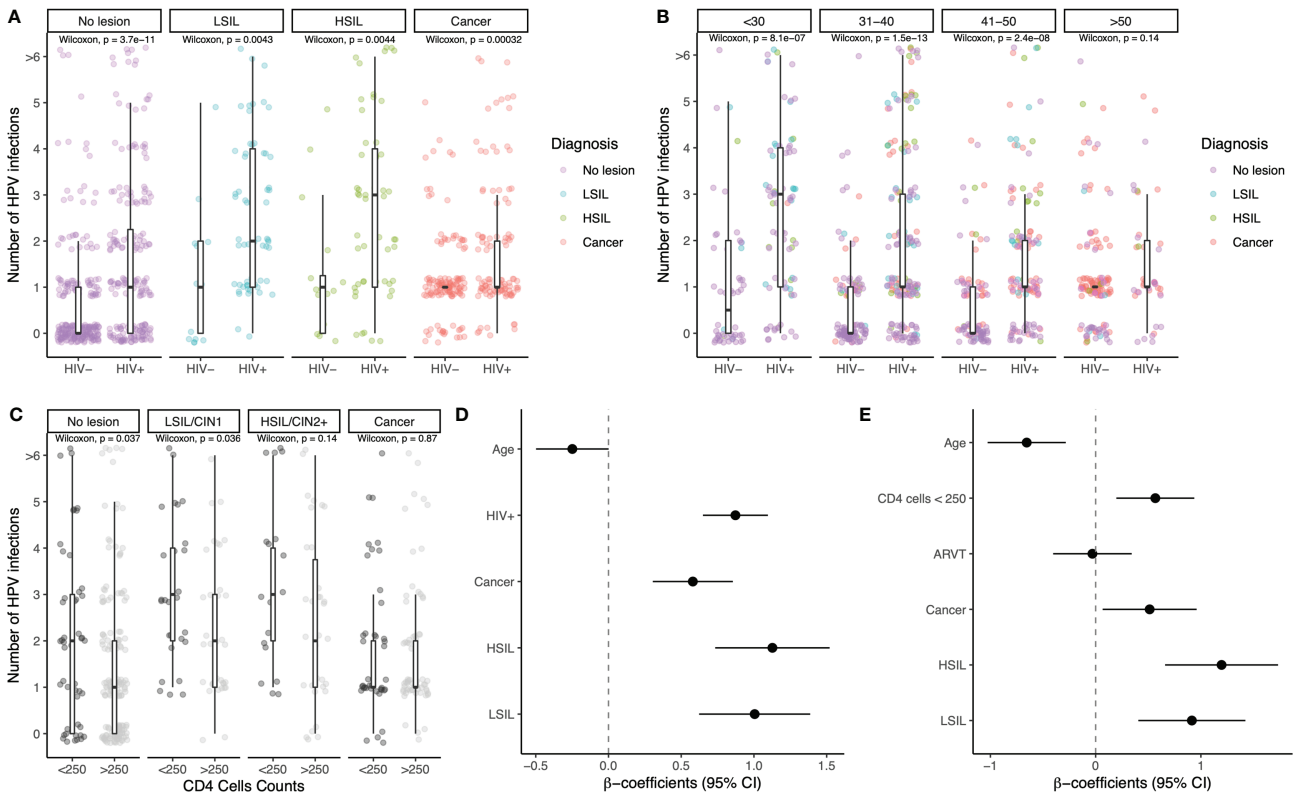

FIGURE 2 | Number of different HPV infections in relation to suspected risk factors. Number of HPV infections stratified by HIV status and cervical lesion status **(A)**, by HIV status and age groups **(B)**, and for women living with HIV stratified by CD4 T cell count (below and above 250) and lesion status **(C)**. Boxplots showing the median and interquartile range as well as outliers are indicated. Statistical analyses were performed using the Wilcoxon test. Multivariate linear regression results with number of HPV infections as dependent variable are shown in **(D)** for all women, adjusting by age, HIV status, and cytohistological diagnosis, and **(E)** for women living with HIV, adjusting by age in years, cytohistological diagnosis, number of CD4 T cell count (above *vs.* below 250), and antiretroviral treatment (no *vs.* yes). The individual risk factors are indicated on the y-axis; beta-coefficients and 95% confidence intervals are shown on the x-axis. In order to better visualize different data points that overlap, we used jitter, a graphical representation strategy in the ggplot R-package.

and the three cytohistological diagnoses of LSIL, HSIL, and Cancer were positively associated, with statistically significant results (**Figure 2D**). When the model was performed only on HIV+ women, the previous associations for age and cytohistological diagnosis remained, suggesting eventual clearance of most HPV infections over time even in HIV+ women. For participants with CD4 cell counts below 250, the association was found to be positive and statistically significant (beta-coefficient: 0.57, 95% CI: 0.20; 0.94), while ART was not found to be associated with the number of HPV infections (beta-coefficient: −0.031, 95% CI: −0.40; 0.34) (**Figure 2E**).

Characterization of HPV Genotypes in Groups Classified by HIV Status and Cytohistologic Diagnosis

HPV genotypes detected in the eight different groups as classified by HIV and cytohistologic diagnosis of cervical lesions and cancer are shown in **Figure 3**. Overall, infections with HR-HPV types and in particular HPV16 predominated regardless of cytohistological diagnoses and HIV status as compared to low-risk HPV types (**Figure 3**).

HPV Genotyping Analyses in Women Without Lesions

Two hundred twenty-eight HIV− and 198 HIV+ women without cervical lesions were randomly selected and subjected to HPV genotyping. Within these, HIV+ women were more frequently infected with HPV (64 *versus* 36%, p<0.0001) and—if HPV infected—had a higher proportion of multiple HPV types (64 *versus* 40%, p=0.0006) than HIV− women. Women up to 30 years of age had the highest HPV infection rates with 49 and 76% for HIV− (n=47) and HIV+ women (n=50), respectively, compared to women above 30 years (p<0.0001, chi-square test); of note, 23% (HIV−) and 63% (HIV+) women up to 30 years of age were infected with two or more HPV types. Rates of single- and multiple-type infections declined with age up to the age of 50 years; of all age groups, 41–50-year-old women had the lowest rates of HPV infections with only 31 and 54% of HIV− and HIV+ women being HPV infected, respectively. Similarly, the rate of HIV− and HIV+ women with two or more HPV types was decreased to 8 and 29% in this age group, respectively. There was a trend of higher rates of HPV infections in HIV− women above 50 years, compared to 31–40 and 41–50 year age groups

	No Lesion				LSIL/CIN1				LSIL v. NoLesion p-value	HSIL/CIN2+				HSIL v NoLesion p-value	Cervical Cancer				CC v NoLesion p-value
	HIV neg (n=228)		HIV+ (n=198)		HIV neg (n=13)		HIV+ (n=61)			HIV neg (n=16)		HIV+ (n=52)			HIV neg (n=129)		HIV+ (n=107)		
	%	N	%	N	%	N	%	N		%	N	%	N		%	N	%	N	
HPV6	0,0	0	2,5	5	0,0	0	4,9	3	0.09	6,3	1	7,7	4	**0.0046**	0,8	1	1,9	2	nd
HPV11	0,0	0	2,0	4	0,0	0	3,3	2	0.21	0,0	0	1,9	1	nd	0,0	0	2,8	3	nd
HPV16	14,0	32	18,7	37	30,8	4	41,0	25	**<0.0001**	25,0	4	28,8	15	**0.01**	41,9	54	52,3	56	**< 0.0001**
HPV18	2,6	6	7,1	14	7,7	1	11,5	7	**0.047**	6,3	1	17,3	9	**0.002**	20,2	26	22,4	24	**< 0.0001**
HPV26	1,8	4	1,5	3	7,7	1	6,6	4	**0.02**	0,0	0	1,9	1	nd	0,8	1	0,9	1	nd
HPV31	1,8	4	4,0	8	15,4	2	9,8	6	**0.004**	12,5	1	7,7	4	**0.055**	1,6	2	4,7	5	nd
HPV33	4,4	10	3,0	6	0,0	0	13,1	8	**0.02**	6,3	1	17,3	9	**0.0006**	1,6	2	1,9	2	nd
HPV35	4,8	11	8,6	17	23,1	3	14,8	9	**0.008**	6,3	1	26,9	14	**< 0.0001**	7,0	9	11,2	12	0.28
HPV39	1,3	3	4,0	8	0,0	0	6,6	4	0.25	0,0	0	9,6	5	**0.04**	0,8	1	0,9	1	nd
HPV40	0,0	0	1,0	2	0,0	0	1,6	1	nd	0,0	0	1,9	1	nd	0,0	0	0,0	0	nd
HPV42	0,4	1	1,5	3	0,0	0	0,0	0	nd	0,0	0	1,9	1	nd	0,0	0	1,9	2	nd
HPV45	4,8	11	9,1	18	7,7	1	9,8	6	0.33	18,8	3	15,4	8	**0.01**	14,0	18	14,0	15	**0.003**
HPV51	3,1	7	10,6	21	15,4	2	19,7	12	**0.001**	6,3	1	11,5	6	0.19	0,8	1	4,7	5	**0.03**
HPV52	0,0	0	7,6	15	0,0	0	9,8	6	nd	6,3	1	7,7	4	nd	8,5	11	6,5	7	nd
HPV53	1,8	4	3,5	7	0,0	0	4,9	3	0.43	6,3	1	11,5	6	**0.004**	3,1	4	5,6	6	0.25
HPV54	0,9	2	2,5	5	0,0	0	3,3	2	0.62	0,0	0	3,8	2	nd	0,0	0	4,7	5	nd
HPV55	0,9	2	2,5	5	0,0	0	9,8	6	**0.006**	0,0	0	1,9	1	nd	1,6	2	1,9	2	nd
HPV56	0,4	1	4,0	8	0,0	0	6,6	4	0.10	0,0	0	1,9	1	nd	0,8	1	0,0	0	nd
HPV58	2,6	6	7,6	15	0,0	0	16,4	10	**0.008**	6,3	1	15,4	8	**0.009**	0,8	1	3,7	4	0.09
HPV59	2,2	5	4,5	9	0,0	0	4,9	3	0.72	0,0	0	9,6	5	0.08	2,3	3	6,5	7	0.52
HPV61	3,5	8	5,1	10	0,0	0	0,0	0	nd	6,3	1	5,8	3	0.5	3,9	5	3,7	4	nd
HPV62	1,8	4	6,6	13	0,0	0	8,2	5	0.34	0,0	0	13,5	7	**0.02**	2,3	3	4,7	5	nd
HPV64	0,0	0	0,0	0	0,0	0	0,0	0	nd	0,0	0	0,0	0	nd	0,0	0	0,0	0	nd
HPV66	1,8	4	8,6	17	7,7	1	9,8	6	0.16	0,0	0	11,5	6	0.14	0,8	1	1,9	2	nd
HPV67	1,3	3	1,0	2	0,0	0	1,6	1	nd	0,0	0	1,9	1	nd	0,8	1	0,0	0	nd
HPV68	1,3	3	7,1	14	0,0	0	11,5	7	0.07	0,0	0	3,8	2	nd	0,0	0	0,9	1	nd
HPV69	0,0	0	2,5	5	0,0	0	3,3	2	nd	0,0	0	1,9	1	nd	1,6	2	2,8	3	nd
HPV70	1,3	3	3,0	6	0,0	0	1,6	1	nd	0,0	0	3,8	2	nd	1,6	2	5,6	6	0.32
HPV71	0,9	2	1,5	3	0,0	0	1,6	1	nd	0,0	0	1,9	1	nd	0,0	0	2,8	3	nd
HPV72	0,0	0	1,0	2	0,0	0	0,0	0	nd	0,0	0	1,9	1	nd	0,8	1	1,9	2	nd
HPV73	0,4	1	1,5	3	0,0	0	9,8	6	**0.001**	0,0	0	7,7	4	**0.01**	0,0	0	1,9	2	nd
HPV81	1,3	3	1,5	3	0,0	0	4,9	3	0.13	0,0	0	11,5	6	**0.002**	0,8	1	6,5	7	0.1
HPV82	0,0	0	3,0	6	0,0	0	4,9	3	0.13	0,0	0	0,0	0	nd	0,0	0	0,0	0	nd
HPV83	0,4	1	1,0	2	0,0	0	1,6	1	nd	0,0	0	3,8	2	nd	0,8	1	1,9	2	nd
HPV84	2,2	5	4,5	9	0,0	0	6,6	4	0.31	12,5	2	3,8	2	0.27	0,8	1	3,7	4	nd
HPVIS39	0,0	0	3,0	6	0,0	0	3,3	2	nd	0,0	0	0,0	0	nd	0,0	0	0,9	1	nd
CP6108	0,9	2	2,0	4	0,0	0	1,6	1	nd	6,3	1	7,7	4	**0.008**	0,8	1	0,9	1	nd
HPV16/18/45	15,2	43	27,8	55	30,8	4	52,5	32	**<0.0001**	43,8	7	50	26	**< 0.0001**	71,3	92	81,3	87	**<0.0001**

FIGURE 3 | Frequency of occurrence of HPV types in HIV+ and HIV− women stratified by cytohistological diagnosis. HR-HPV types are indicated in bold. Frequencies are color coded in different shades as follows: 0% (light green), 0.1 to 5.0% (light blue), 5.1 to 10.0% (light yellow), 10.1 to 15.0% (light orange), 15.1 to 20.0% (faint red), 20.1 to 25.0% (orange), 25.1 to 30.0% (red-orange), >30.0 (strong red). The two-sided Fisher's exact test was for significance testing and was calculated to compare all women with cervical cancer, HSIL, LSIL to women without lesion, regardless of HIV status. nd, not determined.

(p=0.22); 8 of 34 HIV− women above 50 years (24%) had prevalent infection with HPV16, compared with only 6 of 61 (10%) in the 41–50 year group (p=0.13). In summary, in this univariate analysis of women without cervical lesions, younger age and HIV infection were associated with higher frequencies of HPV infections. ART− HIV^+ women (n= 49) had slightly higher HPV infection rates compared to ART+ women (n=136, 73 *versus* 60% infected, p=0.09). There were no statistical differences in the number of HPV types detected per women between ART+ and ART− women without lesions.

HIV+ women without lesion were more frequently infected with a potentially carcinogenic HR-HPV type as compared to HIV− women (55 *vs* 31%, p<0.0001). HPV16 was detected in 14% of HIV− and 19% of HIV+ women without lesion. Infections with other HPV types were rarely detected (<5% prevalence per type) in HIV− women and were most often caused by the HPV45, 35, 33, each with prevalence rates of 4–5%, followed by HPV51 (3%). In HIV+ women without lesions, HR-HPV types 51, 45, 35, 66, 52, and 58 and 18 and the low-risk HPV types, HPV61, 62, and 68, all occurred at rates of 5 to 10%.

HPV Genotyping Analyses in Women With Low-Grade Intraepithelial Lesions and/or CIN1

HIV− (n=13) and HIV+ women (n=61) with LSIL were subjected to HPV genotyping analyses. Fifty-three percent of HIV− and 97% of HIV+ women with LSIL had detectable HPV infections, and almost all these women were infected with at least one HR-HPV type. HPV16 was the most frequently detected HPV genotype in these women with infection prevalence of 32 and 41% for HIV+ and HIV− women, respectively. Thirty-one percent and 52% of HIV− and HIV+ women were infected with either HPV16, 18, and/or 45, which were found to be the most carcinogenic HPV types in our study. Besides HPV16, HPV35 (n=3) and 51 (n=2) infections were most frequent. Five of seven subjects had infections with more than one HR-HPV type. LSIL-positive women living with HIV were characterized by a high diversity of infecting HPV types; HPV51 was detected with second highest infection prevalence (20%), followed by HPV58, 35 (both above 15%) and 18, 33, 45, as well as some low-risk types as shown in **Figure 3**. More than one HPV type

was detected in 70% of HIV+ women with LSIL regardless of ART status (p=1.0). When comparing women with LSIL to women without cervical lesions, the following HPV types were significantly associated with LSIL: HPV16 (p<0.0001), HPV51 and HPV73 (both p=0.001), HPV31 (p=0.004), HPV55 (p=0.006), HPV35 and HPV58 (p=0.008), HPV26 and HPV33 (p=0.02), and HPV18 (p=0.047).

In LSIL+ women living with HIV, the prevalence of clinically most relevant HR-HPV types differed by age groups; while women up to 30 years of age (n=18) had similar frequencies for HPV16, 18, 35, and 45 ranging from 18 to 28%, the relative frequency of $HPV16^{+}$ LSIL findings increased to 48% (14/29) and 46% (6 of 13) in the age groups 31–40 and 41–50 years, respectively, whereas the frequency of HPV18 (3 and 15%), HPV35 (10 and 8%), and HPV 45 (3 and 8%) decreased in these age groups, which is consistent with a higher persistence of HPV16 infections as compared to the other clinically relevant HR-HPVs.

HPV Genotyping Analyses in Women With High-Grade Intraepithelial Lesions and/or CIN2+

HPV infection patterns in women with HSIL were similar to what was observed in women with LSIL; 71% of HIV− (n=14) and 90% of HIV+ women (n=52) had detectable HPV infections. The vast majority of these infections were associated with one or more HR-HPV types. With 29% prevalence for both HIV infection strata, HPV16 was the most frequent type. Overall, 67% of HIV+ women with HSIL were infected with multiple HPV types, a higher proportion as compared to their HIV− counterparts (p=0.014). HSIL findings in HIV+ women were associated with a broad spectrum of HR-HPV types. Particularly high infection frequencies were found for HPV35 (26%), followed by HPV18, 33, 45, and 58 (both above 15%) and five additional HPV types occurring in >10% of women. There was no significant difference in the number of HPV types detected in ART+ (n=40) and ART− (n=9) HIV+ subjects with high-grade lesions (p=0.23). Within HSIL women living with HIV, HPV16 infection frequencies declined with age from 44% (4 of 9) in the <30-year-olds, to 27% (7 of 27) and 23% (3 of 13) in the 31–40 and 41–50 year age groups, respectively, whereas for other highly clinically relevant HR-HPV types, prevalence increased with age as follows in these same age groups: HPV18 occurred at 0% (0 of 9, <30 years), 19% (5 of 26, 31–40 years), and 23% (3 of 13, 41–50 years). HPV35 occurred at 22% (2 of 9), 23% (6 of 26), and 46% (6 of 13). HPV45 occurred at 11% (1 of 9), 7% (2 of 26), and 23% (3 of 13).

When comparing all women with HSIL to all women without cervical lesions, 13 HPV genotypes were associated with HSIL, with statistically significant results: HPV35 (p<0.0001), HPV33 (p=0.0006), HPV18 (p=0.002), HPV81 (p=0.002), HPV53 (p=0.004), HPV6 (p=0.005), HPV CP6108 (p=0.008), HPV58 (p=0.009), HPV16 (p=0.01), HPV45 (p=0.01), HPV73 (p=0.01), HPV62 (p=0.02), HPV39 (p=0.04).

Together our results so far show substantially increased prevalence rates of HPV and HR-HPV infection in HIV^{+} women regardless of lesion status, despite most of these being on efficient ART treatment. While ART suppressed HIV viremia in most of these women, it was not associated with a significant reduction in HR-HPV prevalence. LSILs and HSILs were often associated with diverse and multiple HR-HPV type infections in HIV+ women. However, the high prevalence of multiple HPV infections in these women did not allow to conclude whether detected HPV types caused HSIL with risk of further progression to cancer or were often just cohabitating. The decreasing prevalence of HPV16+ HSIL with age and high prevalence of HPV16+ cancers in women living with HIV, often at relatively young age, is consistent with the higher carcinogenic potential of HPV16, whereas HSILs associated with HPV35 may persist but not progress frequently, consistent with the high number of HV35+ HSILs in women living with HIV between 30 and 50 years of age.

HPV16, 18, and 45 Potentially Cause the Vast Majority Cervical Cancers Regardless of HIV Infection and ART Status

We originally hypothesized that higher proportions of none-16/18 HR-HPV types, which are frequently associated with squamous intraepithelial lesions in women living with HIV, also cause CC more frequently in HIV+ than in HIV− women. To address this hypothesis, 129 HIV− and 107 HIV+ women with CC were subjected to HPV genotyping analyses. HPV16 (52 *versus* 42%), 18 (22 *versus* 20%), and 45 (14% for both) occurred with similar prevalence rates in HIV+ and HIV− cancer cases, respectively (**Figure 3**). In contrast to our study hypothesis, the composition of HR-HPV types in cancers was overall comparable between HIV+ and HIV− women. When combining HPV16, 18, and 45, those accounted for the vast majority of HPV+ cancer cases regardless of HIV status, with 85% of HIV+ (87 of 99) and 84% of HIV− (92 of 110) (**Figure 4**). In contrast to HIV-associated cancer cases, only 54% (26 of 48) of HIV-associated HSIL cases were associated with 16/18/45-HPV type infections (p<0.0001, **Figure 4A**). Further multiple HR-HPV types were detected in 59 of 101 HIV+ women with intraepithelial cervical lesions, but in only 33 of 99 HIV+ cancer cases (p=0.0006, **Figure 4B**, HPV+ cases only). Similarly, in women living with HIV, the median number of HPV types was reduced in cases of cancer compared to women with cervical lesions (**Figure 4B**), and this may relate. Together these data show that the number of HR-HPV types of cancer cases was significantly contracted in HIV+ women with CC women with single HR-type infections causing the majority HIV+ cancer cases; HPV16 (n=36), HPV18 (n=14), and HPV45 (n=7) and HPV35 (n=4), HPV31 (n=2), HPV52 and 59 (both n=1) were detected as a single-genotype infection in HIV+ cancer cases.

While most cancers were squamous cervical cancers, 12 cases were diagnosed as adenocarcinomas. In five of these, no HR-HPV type was detected. HPV18 was found in five HIV− women and was the most frequently detected HR-HPV type in adenocarcinoma cases. HPV16 was found in two adenocarcinoma cases in combination with HPV18 or HPV39. HPV45 was detected as a single infection in one HIV+ adenocarcinoma case.

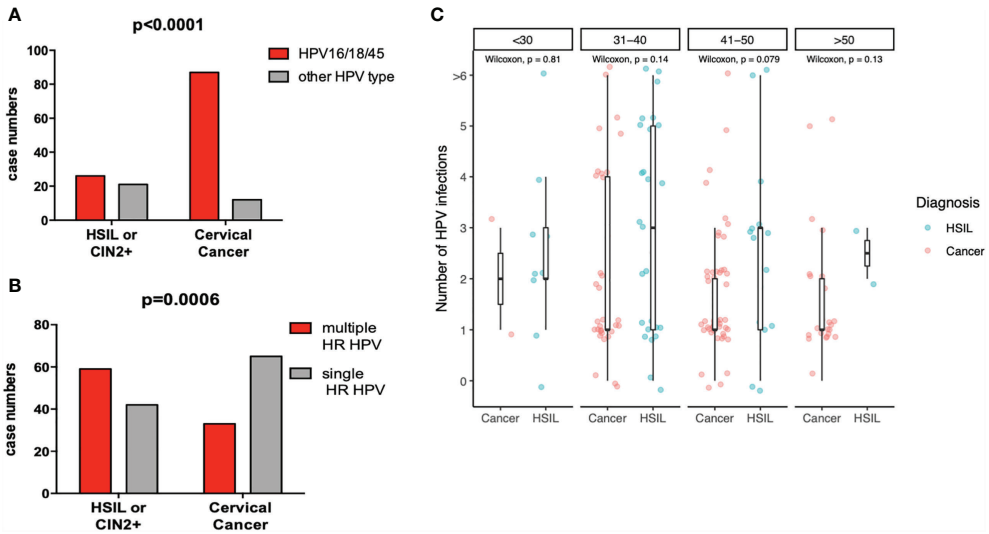

FIGURE 4 | Decreased HPV type diversity and shifting HPV genotype distribution in HIV+ women with cervical cancer. **(A)** shows the number of subjects with HSIL and cervical cancer cases that were associated with HPV16, 18, or HPV45 infection (red bars) *versus* those that were not (gray bar) and **(B)** those associated with multiple HR-HPV types *versus* single HR-HPV types. Indicated p-values were calculated using the two-sided Fisher's exact test. **(C)** shows the number of HPV types detected in HIV$^+$ women with cervical cancer or HSIL in different age groups. Statistical analyses were performed by Wilcoxon test.

In summary, HPV16 was by far the most frequent HPV type causing CC, followed by HPV18 and 45 regardless of HIV status. In HIV+ CC cases, HPV type diversity was reduced, and distribution pattern shifted towards few cancer-causing types when compared to high- and low-grade intraepithelial cervical lesions in women living with HIV. While other HR-HPV types, such as HPV35 and 58, were disproportionally increased in HSIL, these were infrequently detected in cancer cases regardless of HIV infection (see below). Hence, such types most probably have a high potential to cause "precancerous" lesions in women living with HIV, but with relatively little risk of progression. Instead, lesions caused by such types may persist for a very long time without progression or eventually be cleared even in many women living with HIV. Interestingly, there was no difference in the age distribution between HIV$^+$ women with CC associated with HPV16 (n=55) *versus* CC associated with non-HPV16 types, suggesting a comparable pace of cancerogenesis between HPV16 and HPV18/45+ precancerous lesions to cancer.

DISCUSSION

The 2H study is among the largest prospective studies to date with the aim to dissect mechanisms underlying the HIV-associated risk-increase for CC progression in a setting of high endemicity for both infections. The study screened more than 2,000 women for cervical cancer and precancerous lesions who were then classified into four groups delineated by HIV and cytohistological diagnosis. We found (1) that the vast majority of women who received a diagnosis of LSIL and HSIL were HIV positive; (2) that any diagnoses of cervical lesion and cancer, as well as HIV infection and low CD4 counts, were identified as independently associated with infection with one or more HPV types and with HR-HPVs, which accounted for the vast majority of these infections; and (3) that—against our original hypothesis—HPV16, 18, and also 45 infections accounted for roughly 85% of HPV+ cancers in women regardless of their HIV status, whereas more diverse HR-HPV type infections were associated with HSIL. Importantly, age was associated with a reduction of HPV types in women living with HIV, suggesting that most HR-HPV infections are eventually being cleared despite HIV.

There is a broad consensus that molecular diagnoses of HR-HPV infection should be incorporated into standard CC screening procedures to identify and monitor risk for HPV disease progression (23) (S3 Guidelines Germany). Women above 30 years with persistent HR-HPV infection have a high risk to develop high-grade precancerous cervical lesions (24, 25), and a high proportion of these develop cervical cancer. One major problem to incorporate molecular diagnoses in a meaningful way to CC screening procedures tailored to women living with HIV is the high prevalence and diversity of HR-HPV infections in these women with and without precancerous lesions (16, 19–21). Some of these reports implied that *less virulent*, non-HPV16/18 HR-HPV types are responsible for a greater proportion of CC cases as compared to HIV negative women (15, 16, 20, 21). Our results are consistent with these previously published data for women living with HIV showing a high diversity of HR-HPV types in these women but argue against the hypothesis that HIV substantially alters the relative carcinogenicity of less virulent HR-HPV types as reported previously (21). HIV-associated depletion of HR HPV oncogene-specific T cell responses in coinfected women may potentially contribute to the increased HPV prevalence and persistence rates (Mbuya et al., 2021, in revision).

HPV35, which is not included in the Gardasil9 vaccine and closely related to HPV16, is a genotype of specific interest. A recent study suggested a strong link between HPV35 and cervical carcinogenesis, particularly in women of African ancestry (26). In our study, HPV35 was the most prevalent in HSILs and detected in 11% (HIV+) and 7% (HIV−) cancer cases. However, only few of these cancers contained HPV35 alone. Instead, 67 and 56% of HPV35+ cancers in women living with and without HIV contained additional HR-HPV types, most often HPV types 16, 18, and/or 45 (as well as others). HPV35+ cancers may have been caused by these "very high risk" types, and we consider this likely in most of these cases. Indeed, roughly 80% of HPV35+ HSILs also contained other HR-HPV types in our study. Hence, the cancerogenic potential of HPV35 is well probably below that of HPV16, 18, and 45 also in the studied sub-Saharan African populations. One possible explanation for this frequent "co-habitation" in cancer and HSIL cases is that HPV35 may benefit from a precancerous or cancerous microenvironment caused by other HR-HPV infections.

Our results therefore suggest that infections with HPV16, 18, and 45, which cause the greatest risk for women in Africa, are by far the most dangerous types also in HIV+ women to develop CC. Indeed, the HR-HPV genotype distribution pattern in HIV+ women observed in our study was almost identical to those reported in a recent meta-analyses (27) that included data from above 700 CC and almost 400 HSIL cases. Further, in our study, HPV58 and 35 were significantly associated with and frequently detected in HSIL cases but comparatively infrequent in HIV+ CC cases, which again is consistent with the data reported previously (27). Other HR-HPV types were often detected in HIV+ CC cases as compared to HIV− cancer cases but were primarily accounted for by multiple HPV infections, similar to what we highlighted for HPV35 in our study. Together these studies provide solid evidence that infections with HR-HPV types other than HPV16/18 and 45 often do not progress to cancer in HIV+ women, despite their potential to induce HSIL or cohabitate with HR-HPV types. This finding is important because HPV16/18 vaccination provides some cross-protection also from HPV45 infection (as well as HPV31 and 33) and associated CC progression (28). It is therefore most important to address whether protective efficiency of HPV vaccination is reduced by subsequent HIV infection. If not, mass vaccinations even using standard HPV16/18 vaccination should end this CC epidemic regardless of HIV control measures.

CC screening algorithms should be optimized and, if necessary, tailored to the special needs of women in resource-poor settings. The paucity of trained pathologists makes diagnosis of precancerous lesions difficult to impossible in many resource-poor settings of SSA. Further, Pap smear and histology-based diagnosis of intraepithelial lesions is extremely time-consuming and basically impossible to implement and scale up in most settings in SSA. Molecular HR-HPV tests—particularly assays based on RT-PCR—have certain advantages over screening by cyto-histology; they are comparatively easy to implement, standardize, highly specific to detect HR-HPV infections, and can in principle be upscaled to high throughput at the point-of-care. The problem of this approach, particularly in HIV+ women, is many HR-HPV genotyping tests do not differentiate between the 13 HR-HPVs. As infection with diverse HR-HPV types is so common in HIV+ women, molecular diagnostic tests generally should better differentiate between HPV types with high, medium, and very low risk of carcinogenesis. Type-specific diagnosis will much more accurately link HPV diagnosis with risk of cancer progression regardless of HIV status. Further, only type-specific diagnostic methods can diagnose truly persistent HR-HPV infections as a cause of disease progression (24). In SSA settings, molecular diagnostic approaches should therefore particularly focus on HPV16, 18, or 45 women for advanced diagnostic and therapeutic workup, e.g., in centralized facilities.

One major limitation of this study was the numbers reported for HPV52 may be too low, because the method used did not allow accurate detection of this HPV type, particularly in our study characterized by many multiple HR-HPV infections. The study also did not include enough patients with a diagnosis of adenocarcinoma to draw any definite conclusions. However, the fact that HPV18 infections accounted for most of the HPV+ adenocarcinomas and that five out of 12 adenocarcinomas were HPV negative is of interest. Possibly, several of these patients did not have adenocarcinoma of the cervix but of adenocarcinoma of the endometrium with involvement of the cervix or metastatic disease from other sites (especially gastrointestinal sites). To differentiate between cervical and endometrial adenocarcinoma using HE staining alone can be difficult. p16 and E2 immunostaining would have been helpful but was not performed. Similarly, in a significant fraction (16 of 129) of non-HIV-associated squamous cervical cancers, no HPV type was detected and hence etiology remained unclear.

In conclusion, our data show that there is a contraction of HR-HPV diversity during carcinogenesis with HPV16, 18, and 45 causing the vast majority of CC regardless of HIV infection status, whereas a more diverse spectrum of HR-HPVs often causes cervical intraepithelial lesions. Diagnostic strategies in SSA settings would benefit from incorporating molecular diagnosis of individual HR-HPV types to identify and target women infected with the clinically most relevant HR-HPV+ infection instead of diagnoses of HR-HPV infections without further differentiation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Mbeya Medical Research and Ethics Review Committee (MRH/R.10/8/Vol. VI/107). The patients/

participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RM, TL, LM, MP, BM, MS, LH, MJ, and JF contributed to the clinical cohort work. LT and NS contributed to cytohistological diagnosis of cervical lesions and cancer. MG contributed to statistical data analyses and drafting of figures. NC and ES contributed to data management. WiM, WoM, AM, JM, and AB contributed to HPV genotyping analyses. RM contributed to analyses of other clinical data. MC coordinated laboratory workup of study samples. RL, LM, and MH provided senior advice and study-specific capacity building and contributed to manuscript writing. AK and CG conceived and coordinated the 2H study and secured funding. All authors contributed to manuscript writing and concur with the manuscript submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.763717/full#supplementary-material

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