



HPV induced cervical neoplasia and cancer

Characterization of local immune infiltration and mapping of T cell recognition towards HPV

Snejbjerg, Dorthe Blirup

Publication date:
2021

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
Snejbjerg, D. B. (2021). *HPV induced cervical neoplasia and cancer: Characterization of local immune infiltration and mapping of T cell recognition towards HPV*. DTU Health Technology.

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

HPV INDUCED
CERVICAL NEOPLASIA AND CANCER
CHARACTERIZATION OF LOCAL IMMUNE INFILTRATION AND
MAPPING OF T CELL RECOGNITION TOWARDS HPV

Dorthe Blirup Snebjerg

PhD Thesis
Kongens Lyngby
August 2021



DTU Health Tech
Department of Health Technology
Technical University of Denmark

Kemitorvet
Bygning 202
2800 Kgs. Lyngby, Denmark
healthtech-info@dtu.dk
www.healthtech.dtu.dk

PREFACE

The present thesis has been submitted to the Technical University of Denmark, Department of Health Technology, as part of the requirements for obtaining the degree as doctor of philosophy (PhD). The presented research was carried out at the Department of Micro- and Nanotechnology and then at the Department of Health Technology, under supervision of Professor Sine Reker Hadrup from August 2017 to August 2021.

The thesis consists of an overall introduction with relevant scientific, immunological and medical perspectives to understand the scope of the research. The two original manuscripts are presented, both being preliminary, and finally an epilogue discussing major findings and perspectives of the studies.



Dorthe Blirup Snebjerg
Kgs. Lyngby, August 2021

ABSTRACT

Human Papilloma Virus (HPV) is the primary cause of cervical cancer. It is evident that an impaired immune system plays an important role in the persistence of the viral infection, oncogenic transformation, and cancer development. Patients with advanced, recurrent, or metastatic cervical cancer still have poor prognosis and improved treatment strategies are needed. Recognition and elimination of cervical neoplasia and cancer is a multifaceted interplay of the immune system, which can both promote and reject tumor growth. Immune therapy has shown immense potentials and is now a major contributor in cancer treatment where immune checkpoint inhibitors (ICI) presents the most advanced therapy available. ICI treatments are capable of reinvigorating the functional capacity of exhausted T cells to kill the affected cells. With cancer immune therapy, the ambition is to achieve a long-lasting ability to detect and eliminate foreign tumor antigens. However, despite the promising developments within immune therapy, little is known in the tumor microenvironment about characteristics of immune infiltration which governs peptide-MHC T cell recognition and immune activation.

The overall goal of the research presented in this PhD thesis, was to characterize local and systemic immune infiltration, phenotype characteristics, state of activation, signs of T cell exhaustion in patients with high-grade intraepithelial neoplasia (CIN3) and cervical cancer compared to healthy individuals.

The main observation was detection of a late differentiated immune profile among CD8 and CD4 T cells in the cancer group. The frequency of terminally activated or even exhausted CD8 T cells was more abundant in CIN3 lesions and even further increased in the cancer patients, compared to the healthy individuals. Cells from biopsy and cytology were evaluated and strikingly, these specimens displayed identical signatures, hence suggesting cytology as a useful alternative to biopsies for evaluation of immune signature in cervical neoplasia and cancer. The analysis of blood demonstrated unique immune phenotypic characteristics associated with cancer, but different from those signatures found in cytology and biopsies.

For the investigated myeloid compartment we observed lower levels of classical antigen presenting cells, while myeloid populations in general expressed higher levels of PD-L1, compared to the same subsets of cells in the healthy individuals. All together, data suggest that immune recognition plays an active role in shaping the neoplastic development, and that immune inhibitory mechanisms emerge during cancer development.

Research presented in this thesis also included mapping of HPV-restricted T cell recognition. 685 potential distinct human leucocyte antigen (HLA)-binding peptides were evaluated covering E2, E6 and E7 genes of both HPV 16 and HPV 18. This was done to examine CD8 T cell recognition of Human Papilloma Virus. The cells were analyzed using DNA-barcoded peptide-MHC complex multimers, and we were thereby able to detect 127 immunogenic epitopes recognized by CD8 T cells. The majority of the predicted epitopes came from the E2 protein, and this was also where most epitopes were recognized. Conclusively, the E2 gene must be understood as a very immunogenic region of the HPV genome.

Our results were validated using tetramer staining assays on selected CD8 T cells and the recognized peptides were confirmed. Among the three study groups, a higher number of recognitions to HPV derived peptides were found in both the neoplasia and cancer group compared to the healthy individuals. The HLA-C05:01 allele turned out to be very dominant in the total number of identified epitopes but some skewing due to cross-reactivity is likely the case.

These results provide insight into the CD8 T cell recognition and the immunogenic hotspots of interest, and this can hopefully be of use in the future, when designing immune therapy and deciding the coveted targets of Human Papilloma Virus.

DANSK RESUMÉ

Human Papilloma Virus (HPV) er den primære årsag til livmoderhalskræft. Det er tydeligt, at et svækket immunsystemet spiller en vigtig rolle i forhold til persisterende virusinfektion, onkogen transformation og kræftudvikling. Patienter med fremskreden, tilbagevendende eller metastatisk livmoderhalskræft, har stadig dårlig prognose, og der er behov for forbedrede behandlingsstrategier. Genkendelse og eliminering af svære celleforandringer (CIN3) og livmoderhalskræft er et multifaktoriellet samspil af immunsystemet, som både kan fremme og forhindre tumorvækst.

Immunterapi har vist enormt potentiale og er nu en stor bidragsyder i kræftbehandling, hvor immun checkpoint-hæmmere (ICI) udgør den mest avancerede terapi på markedet. ICI-behandling muliggør genoplivning af den funktionelle kapacitet af svækkede T celler til at kunne bekæmpe de berørte celler. Med kræftimmunoterapi er ambitionen at opnå en langvarig evne til at opdage og eliminere fremmede tumorantigener. På trods af den lovende udvikling inden for immunoterapi, er der i tumor-mikromiljøet kun sparsom viden om egenskaber vedrørende den immuninfiltration der kontrollerer peptid-MHC T celle genkendelse og immunaktivering.

Det overordnede mål med forskningen præsenteret i denne PhD.-afhandling var at karakterisere lokal og systemisk immuninfiltration, fænotype-egenskaber, aktiveringstilstand, T celle svækkelse hos patienter med svær grad af celleforandringer (CIN3) og livmoderhalskræft, sammenlignet med raske individer.

Den væsentligste observation var påvisning af en sen differentieret immunprofil blandt CD8 og CD4 celler hos kræftpatienterne. Hyppigheden af de terminalt aktiverede og endda de helt svækkede CD8 T celler var øget hos CIN3 patienter og endda yderligere forøget hos livmoderhalskræftpatienterne sammenlignet med de raske individer. Celler fra biopsi og cytologi blev evalueret og viste overraskende en identisk signatur, hvilket tyder på, at cytologi er et brugbart alternativ til biopsier i forhold til at evaluere en immunsignatur hos patienter med svære celleforandringer og med livmoderhalskræft. Analysen af blod viste unikke immun-fænotypiske karakteristika forbundet med kræft, men adskilte sig fra de signaturer vi fandt i cytologi og biopsier.

Angående analysen af de myeloide celler, observerede vi lavere niveau af de klassiske antigenpræsenterende celler, mens de myeloide populationer generelt udtrykte højere niveauer af PD-L1 sammenlignet med de tilsvarende populationer hos de raske individer. Samlet set tyder data på, at immungenkendelse spiller en aktiv rolle i at forme den neoplastiske udvikling, og at immunhæmmende mekanismer allerede opstår under udviklingen af kræft.

Forskning præsenteret i denne afhandling omfattede også kortlægning af HPV-specifik T celle genkendelse. 685 potentielle distinkte humane leukocytantigen (HLA)-bindende peptider blev analyseret indbefattende E2-, E6- og E7-gener for både HPV16 og HPV18. Dette blev udført for at undersøge CD8 T cellegenkendelse af Human Papilloma Virus. Cellerne blev analyseret ved hjælp af DNA-barcoded peptid-MHC komplekse multimerer og vi var derved i stand til at påvise 127 immunogene epitoper genkendt af CD8 T celler. Størstedelen af de predikterede epitoper

stammer fra E2 proteinet og det var også her, de fleste epitoper blev genkendt. Konklusivt må E2 genet betragtes som en meget immunogen region af HPV-genomet.

Vores resultater blev valideret ved anvendelsen af tetramer farveanalyser på udvalgte CD8 T celler, og de genkendte peptider blev bekræftet.

Blandt de tre studerede grupper, blev der fundet et større antal genkendelser til HPV-afledte peptider i både neoplasie og kræftgruppen sammenlignet med de raske individer. HLA-C05:01 allelen viste sig at være meget dominerende i det samlede antal identificerede epitoper men en vis forskydning på grund af krydsreaktivitet er sandsynligvis tilfældet.

Disse resultater giver indsigt i CD8 T cellegenkendelse og de interessante immunogene hotspots, og dette kan forhåbentligt være nyttigt i fremtiden, når man designer immunterapi og udvælger de eftertragtede områder af Human Papilloma Virus.

ACKNOWLEDGEMENTS

After 4 years working as PhD student at DTU, I am now handing in my PhD thesis. The research presented here and all the work I have carried out, would not have been possible without the support I have been given.

In particular, my biggest thank you goes to Prof. Sine R. Hadrup for taking me in, trusting in me and allowing me to do a PhD in the research group even though I hadn't proven my worth. Furthermore, thank you for giving me the possibility to continue my career as a doctor while conducting the research. Your constant support and understanding has helped me to push forward and not give up. *To think that I actually made it is a very proud moment for me.* Thank you.

Thanks to Stine Kiær for teaching me the meaning of "RE-search", lab training and so many nice lunch breaks discussing all aspects of life. Thanks to Mo for my everyday guidance and co-supervising, for support in and outside the lab and for many pep-talks, constantly pushing and believing in me. For your caring personality, understanding, moral support and teaching me so much about doing research. I could not have done this without you - my greatest appreciation. Thanks to Marie for all the help when processing the samples, data analyses and making figures. Thanks both of you for hours spent in the FACS room, through ups and downs when a simple red marker made all the difference. Thanks to Bente and Annie for help when administering the samples and keeping track of them all. For everyone in the research group for great discussions, lunch, coffee breaks and group meetings. I have learnt a lot being a part of a very different but inspirational research environment.

Thanks to all participants and their willingness to donate samples despite some being in a difficult time of life - the results are in honor of all donors. Also thanks to my co-supervisors. Susanne Krüger Kjær for all her knowledge on HPV. Kirsten Marie Jochumsen, who made it possible to collect cancer samples. Benny Kirschner who collected patient biopsies from patients with neoplastic changes. All of you for helping designing the study, scientific discussions and feedback on the thesis. Jesper Bonde for kindly analyzing the HPV-tests and follow-up status.

Lastly, I would like to thank my family and friends. My dad for many scientific discussions, curiosity of my work, proofreading and relevant feedback. Mum and dad for your constant love and support and for convincing me to keep at it, even through tough times. All dear friends for positive spirits, always ready to listen, your joy and support in all aspects of life. To my husband Lasse for your love and support, especially in the final stages and for being my backup, helping me to finalize the work. I would not have made it through without your encouragement and confidence in me. *Thank you.*

I dedicate this thesis to our three children: Emilie, Frederik and August.

MANUSCRIPTS INCLUDED IN THE THESIS

The following research is included in this thesis:

Manuscript I

Dorthe Blirup Snebjerg, Mohammad Kadivar, Marie Viuff, Stine Kiær Larsen, Benny Kirschner, Kirsten Marie Jochumsen, Jesper Bonde, Susanne Krüger Kjær, Sine Reker Hadrup. "Characterization of immune infiltration In High-grade Cervical Intraepithelial Neoplasia and Cancer"

Manuscript II

Dorthe Blirup Snebjerg, Mohammad Kadivar, Marie Viuff, Stine Kiær Larsen, Benny Kirschner, Kirsten Marie Jochumsen, Jesper Bonde, Susanne Krüger Kjær, Sine Reker Hadrup. "Mapping of HPV-restricted T cell recognition in High-grade Cervical Intraepithelial Neoplasia and Cancer"

ABBREVIATIONS

ACT	Adoptive cell transfer
AIS	Adenocarcinoma in situ
APC	Antigen-presenting cell
CD	Cluster of differentiation
CDR	Complementarity-determining regions
CIN	Cervical intraepithelial neoplasia
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DNA	Deoxyribonucleic acid
E	Early region
EBV	Epstein-Barr virus
Eomes	Eomesdermin
FACS	Fluorescence activated cell sorting
FLU	Influenza virus
GZMB	Granzyme B
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HPV	Human papillomavirus
ICI	Immune checkpoint inhibition
IFN	Interferon
IL	Interleukin
KIR	Killer inhibitory receptor
L	Late region
LAG-3	Leucocyte-activation-gene 3
LBC	Liquid-based cytology
LCR	Long control region
LN	Lymph node
LR	Late region
MDSC	Myeloid derived suppressor cell
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MHC-I	MHC class-I molecule
NK	Natural killer
ORR	Objective response rate

Abbreviations

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate saline buffer
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
PD-L1	Programmed cell death-ligand 1
pDC	Plasmacytoid dendritic cell
pMHC	peptide-Major Histocompatibility Complex
PMN-MDSC	Polymorphonuclear myeloid derived suppressor cell
PT	Primary tumor
RR	Reverse primer region
RT	Room temperature
TCF-1	T cell factor 1
T _{CM}	Central memory T cell
TCR	T cell receptor
T _{EM}	Effector memory T cell
T _{EMRA}	Terminal differentiated effector memory T cell
T _{EX}	Exhausted T cell
TIL	Tumor infiltrating lymphocyte
TNF	Tumor necrosis factor
TOX	Thymocyte selection-associated high mobility group box protein
TMB	Tumor mutational burden
TME	Tumor microenvironment
Treg	regulatory T cell
T _{PEX}	Progenitor exhausted T cell
UMAP	Uniform manifold approximation and projection
V	Variable

CONTENTS

PREFACE	I
ABSTRACT	III
DANSK RESUMÉ	V
ACKNOWLEDGEMENTS	VII
MANUSCRIPTS INCLUDED IN THE THESIS	IX
ABBREVIATIONS	XI
SCOPE OF THIS THESIS	1
INTRODUCTION	3
HUMAN PAPILLOMA VIRUS	3
Burden of HPV	3
HPV facts	5
ANATOMY AND PATHOLOGY OF THE CERVIX	8
SCREENING IN DENMARK	9
VACCINATION AGAINST HPV	10
DISEASE AND TREATMENT	10
HPV pathogenesis	10
Cervical intraepithelial neoplasia (CIN)	11
Cervical cancer	12
CURRENT POSSIBILITIES OF TREATMENT OF CERVICAL CANCER	12
INTRODUCTION TO THE IMMUNE SYSTEM	13
T cell activation and maturation	14
CANCER IMMUNOLOGY	15
Tumor development and editing.....	15
Tumor microenvironment and immune suppression	17
CELL COMPONENTS OF INTEREST	19
T cells types of relevance in cancer	19
Myeloid cells types of relevance in cancer	24
MUCOSAL IMMUNITY OF THE CERVIX	26
Tumor antigens	27
Cancer immune therapy.....	28
COLLABORATORS AND FUNDING	30
SPECIMEN COLLECTION	31
Study population	32
The processing of specimens	32
MANUSCRIPT I	35
MANUSCRIPT II	77
EPILOGUE	109
BIBLIOGRAPHY	114

SCOPE OF THIS THESIS

Human Papilloma Virus (HPV) is a common sexually transmitted infection, which has the potential to develop into cervical intraepithelial neoplasia (CIN) and further into cancer. While screening programs have greatly reduced the risk of cervical cancer in developed countries, the traditional cervical screening based on cervical cytology, cannot discriminate between lesions that will become invasive and those that will not [1].

We therefore need a better understanding of the factors, which affect the balance between clearance of the virus, persistent infection and progression into cancer. Specifically, a deeper understanding of the immunological mechanisms of these processes would be very valuable - not only for more accurate diagnosis of precancerous lesions, but also for the development of novel immunotherapeutic approaches for inhibition of HPV-related cancers.

Aim of study:

1) To characterize both the local immune infiltration, state of activation and the microenvironment. To do this we will examine immune cell characteristics in:

- a) Patients with cervical high-grade intraepithelial neoplasia (CIN 3),
- b) Patients with cervical cancer, and
- c) healthy individuals (women without cervical neoplasia).

For all three groups, we will further evaluate systemic immune activation signatures to determine potential effects related to disease development.

This is done by multicolor flow cytometry which is used to analyze the infiltrating immune cells with respect to cell type, phenotype, function and activation status in biopsies, liquid based cytology (LBC) samples and blood. Immune characteristics in LBC could potentially improve diagnostics by easier and less invasive procedures for the patients.

2) To map T cell recognition towards oncogenic elements for the HPV in the same patient/control group.

We aim to map systemic CD8 T cell recognition of HPV 16/18 (protein E2 and oncoproteins E6 and E7). We will compare the T cell recognition profiles (width and intensity) of HPV16/18 positive controls with CIN3/cervical cancer patients positive for HPV16/18. Hereby we hope to obtain a deeper understanding of the characteristics between immune activation at the early stage and the late stage of disease, as well as the heterogeneity among patients.

HUMAN PAPILLOMA VIRUS

BURDEN OF HPV

Papilloma virus infect both humans and animals and thereby comprise a diverse group of viruses. Their origin appears to be linked to changes in the epithelium of their host, starting from reptiles moving on to birds, marsupials and mammals inclusive humans [2]. Between 1974 and 1976 researchers started to recognize and analyze a possible role of HPV in cervical carcinogenesis [3]. Professor Harald zur Hausen was the first to discover the link between HPV and cancer and was therefore awarded the Nobel prize in 2008 for his discovery [4].

HPV is the most common viral infection of the female reproductive tract [5]. More than 80% of all sexually active individuals will at some point in their lives be infected by the virus and some may even be infected repeatedly [6].

HPV is the cause of virtually all cases of cervical cancer and HPV is also associated with a significant number of oropharyngeal (15-25%), penile (40-50%), anal (88-90%), vaginal (99%) and vulva cancers (43%) [7][8].

Cervical cancer is the fourth most common malignancy diagnosed in women worldwide, with an estimated 604.127 cases (3.1% of all cancers) and 341.831 deaths (3.3% of all deaths caused by cancer) reported in 2020 [5][9][10]. This makes cervical cancer important and unfortunately still very relevant and the choice of disease to study further in this thesis. The overall incidence of cervical cancer in Europe is 9.9 per 100.000 (2020). In Denmark this number is: 12.0 (2019), 11.5 (2018) [10][11]. However, within Europe, the incidence of this disease differs significantly, being lower in Western Europe, where screening programs are more thoroughly implemented (Fig. 1). The incidence in Central and Eastern Europe is significantly higher, which is closely correlated with the rarity of organized screening programs. The mortality rate of HPV induced cervical cancers are 18 times higher in low- and middle-income countries compared with high-income countries [9][10]. A Danish study of more than 40.000 women has shown that the prevalence of HPV infection is generally high in the Danish population. The overall prevalence of high risk HPV was 20.6 % ranging from 46.0 % in women 20-23 years of age to 5.7 % in women \leq 65 years of age [12] (Fig. 2). Each year, 350-400 Danish women are diagnosed with cervical cancer and the disease accounts for the death of approximately 100 women every year [11][13].

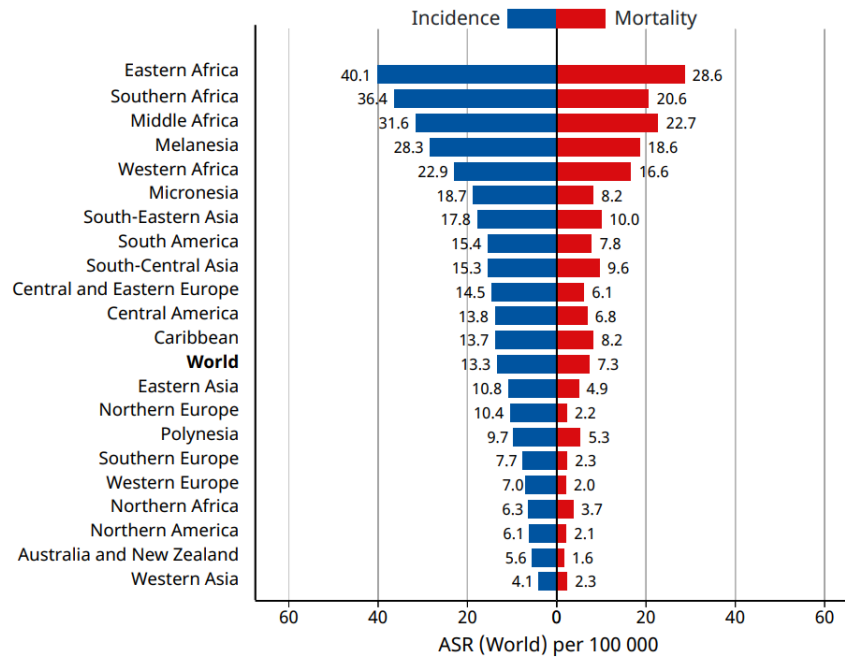


Fig. 1: Region-specific incidence and mortality rates

Age standardized (world) of cervical cancers in the cervix (Jan 2020). The bar chart shows incidence (blue) and mortality (red). Rates are shown in descending order, against age standardized in rate per. 100.000 women (W). Adapted from GLOBOCAN (2021) [10].

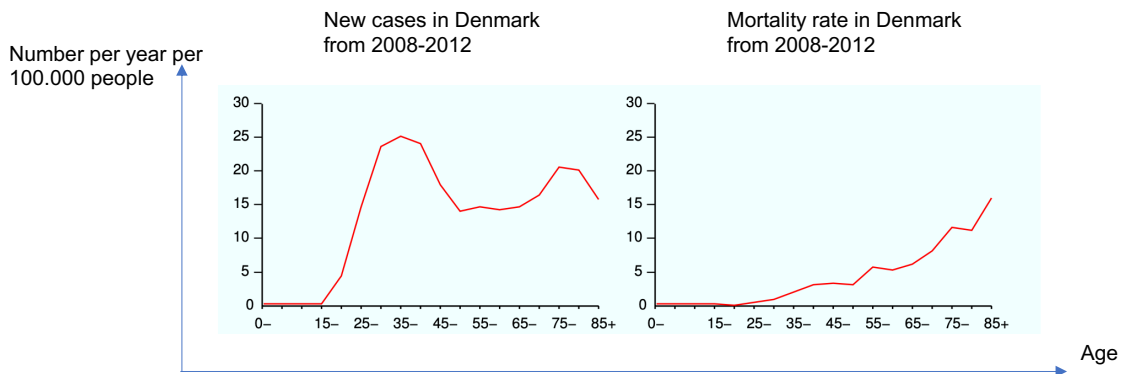


Fig. 2: Cervical cancer incidence and mortality in Denmark

These graphs show the number per year per 100.000 people in relation to age. New cases peak around the age of 35 and again very late around 80 years. The mortality rate is increasing gradually over the life span which is the persisting infection causing slow progression. Adapted from GLOBOCAN (2018) [14].

HPV FACTS

Viral genome

Human Papilloma Virus is a double-stranded (ds) DNA-virus. The viral particle consist of circular DNA (Fig. 3) which include eight open reading frames designated E6, E7, E1, E2, E4, E5 and L2 and L1 as well as a non-enveloped capsid [8].

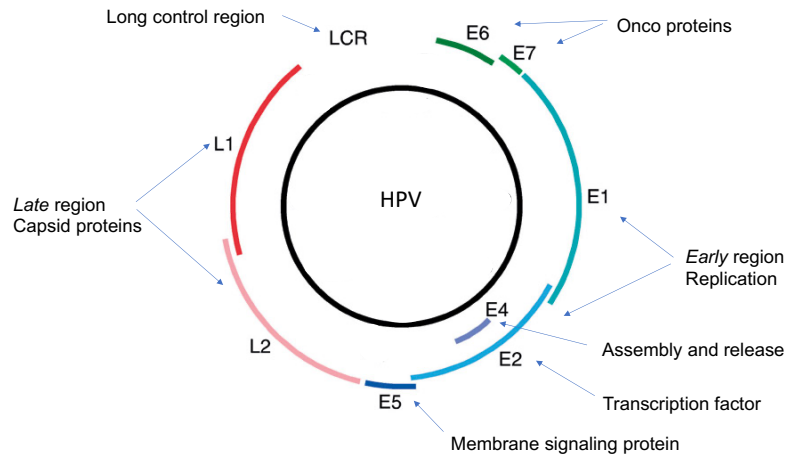


Fig. 3: HPV organization of the genome

Schematic representation of the HPV 16 circular genome showing the location of the *early* (E) and *late* genes (L1 and L2) and the long control region (LCR). The function of the eight proteins is indicated. Adapted from Tandläkartidningen, (2006), Graham (2006) [15].

HPV has three functional coding regions in their genome. The E region (*early*) encodes the regulatory function such as replication, transcription, cell cycle, cell signaling and apoptosis control, immune modulation and structural modification of the infected cell. The L (*late*) region is expressed late in the infection. Here the virus are often found in the upper part of the endothelium and L1 and L2 comprise the virus capsid required for virus transmission, spread and survival in the environment [16][17]. The *long control region* (LCR) is situated in between L1 and E6 and have shown to be the most variable region of the HPV genome and may play an important role in viral persistence and cancer development. It contains early promoter and various transcriptional regulatory sites for both viral and cellular proteins [18].

HPV is approximately 52-55 nm and composed of 72 pentameric capsomers [19]. The HPV genome contains between 6800 and 8000 base pairs [3]. There are more than 200 different papillomavirus types contained in 29 different genera with five human ones (Alpha, Beta, Gamma, Mu and Nu) and new types are continuously being found. HPV-induced pathologies are primarily related to the alpha type [20][21]. The alpha HPV types infect primarily anogenital and oropharyngeal mucosal areas. Based on their oncogenic potential, these HPV types are considered as either low risk or high risk also called oncogenic types. Altogether, 13 HPV types are classified as carcinogenic or probably carcinogenic by the International Agency for Research on Cancer (IARC): (group 1 and 2A) (HPV 16,18,31,33,35,39,45,51,52,56,58,59,68) [23][24].

Cervical HPV infection is most often an asymptomatic infection, but can also cause condyloma acuminata (HPV6 and 11), low- and high-grade intraepithelial neoplasia and cancer [24][25]. The most common serotypes of HPV in women leading to cervical cancer, in descending order of frequency, are 16, 18, 45, 31, 33, 52, 58, and 35. HPV 16 and 18 are reported to account for approximately >70% of cancer cases [26] however, essentially all cervical cancers contain DNA of an oncogenic HPV type [24][27]. In this thesis we chose to look further into both HPV 16 and 18.

A Danish study including more than 7000 women tested 2 years apart reported that 20% of the total group were still HPV positive (one or more types) after 2 years and this number increased to 32% if it was a high-risk HPV type. HPV16 was the most persistent type (95%) where HPV18 were less persistent (29%) [28].

Transmission

The peak age for acquiring HPV infection for both women and men is shortly after becoming sexually active. The impact of HPV infection is dependent on the different virus types as well as the anatomical site of infection [29]. Primary infection with HPV occurs in the cutaneous or mucosal surfaces of epithelial cells in the transformation zone (TZ) of the cervix through abrasion and microlesions, thereby allowing access of the virus to the basal membrane [30][31]. Infection with HPV requires the availability of epidermal or mucosal epithelial cells, which still are able to proliferate [3]. The TZ is especially susceptible to infections in particularly HPV [32]. The epithelial cells in the TZ are to some extent able to block, neutralize or kill microorganisms through physical (intercellular junctions, secretion of mucus) and immune defense (pathogen-recognition receptor-mediated pathways) which releases chemokines and or cytokines [33].

Life cycle and role of viral elements

Following microlesions in the epithelium, HPV virions attach to the basal epithelial cell receptors via the L1 capsid protein. This facilitates conformational changes of the capsid protein L2 causing cleavage [34]. L2 is conserved among all HPV subtypes [35]. Virions are then internalized by endocytosis and viral DNA is transported to the nucleus where it escapes intrinsic host defense mechanisms and are established in the genome as a stable extrachromosomal, autonomously replicating element [36][37]. The ring molecule is often opened within the E2 region. Once internalized, viral DNA replication starts and during the differentiation of daughter cells the viral genome is amplified concomitant with increasing levels of E1 and E2 proteins. E4 and E5 are frequently deleted during DNA integration [3]. The E2 protein plays an important role in the HPV life cycle. This protein contains a conserved C-terminal DNA-binding structure and a conserved N-terminal domain. These specific structural characteristics of the E2 protein allow them to be involved in viral transcription, replication and assembly into hexameric complexes (Fig. 4). They correspondingly interact with host proteins and by involvement of remodeling and modification of cellular chromatin [36] [38]. E2 acts as a transcriptional repressor of E6 and E7 and when the viral DNA becomes integrated the E2 sequence gets disrupted which leads to increased expression of E6 and E7. The overexpression of E6 and E7 oncoproteins promotes malignancy [39][40]. This is the reason, why the E2 gene was chosen for further examination and evaluation in this study and thesis.

E5 has been shown to form a complex with growth-factor receptors and has also been shown to prevent apoptosis following DNA damage [41][42]. Next step is entry into the supra basal layers where the transcription of the *late* genes is initiated. The circular DNA is then replicated, and proteins are formed. By now, the E5 protein is no longer obligatory in replication. Complete viral particles are assembled and released in the upper layers of the cervical epithelium. The E5 protein also mediates the immune evasion of the virus by downregulating major histocompatibility complex class I (MHC I), which reduces viral epitope recognition by CD8 T cells [43][44].

Expression of the *early* E5, E6 and E7 gene results in stimulated enhanced proliferation of the infected cells and they start to expand laterally [3]. E6 and E7 are referred to as the *onco proteins* and are the primary viral factors responsible for initiation and progression of cervical cancer [45]. They play a significant role in inducing DNA synthesis, telomerase activity, cell polarity and motility as well as regulation of the transcriptional co-activators and tumor suppressors [38]. These two onco genes are critical for the oncogenic transformation and continuous cancer cell growth. Consequently, they are valuable therapeutic targets. They are a part of the malignant transformation because their respective proteins are consistently expressed in malignant tissue and by inhibiting their expression, the malignant phenotype of cervical cancer cells is blocked. In tissue culture E6 and E7 are able to immortalize human cells and when expressed together, efficiency is increased and a malignant transformation has occurred [40][46][47]. The most important role of E6 is the degradation of p53, a tumor suppressor protein [40], preventing cell growth inhibition [45], and the major transforming characteristic of E7 is its inactivation of the retinoblastoma (Rb) also a tumor suppressor protein pRb. When the infected cells express E6/E7 proteins, it enhances genomic instability and induces epigenetic and further transcriptomic alterations that generate proteins that maintain a favorable micro environment for viral replication [8]. Elevated expression of E6 and E7 is directly related to the increasing severity of neoplasia [48] and enhancing cell proliferation [49]. Both E6 and E7 interfere with interferon signaling system [50] and promotes angiogenesis to (as a cancer hallmark) provide nutrients and oxygen to tumor cells [51].

The *late* genes L1 (major viral structural protein) and L2 (minor viral structural protein) are involved in encoding neutralizing epitopes and in assembling the capsomers and the capsid and facilitate virion assembly and are not expressed in neoplasia or malignant cells [3][8]. Therefore, we did not look further into these genes in this thesis.

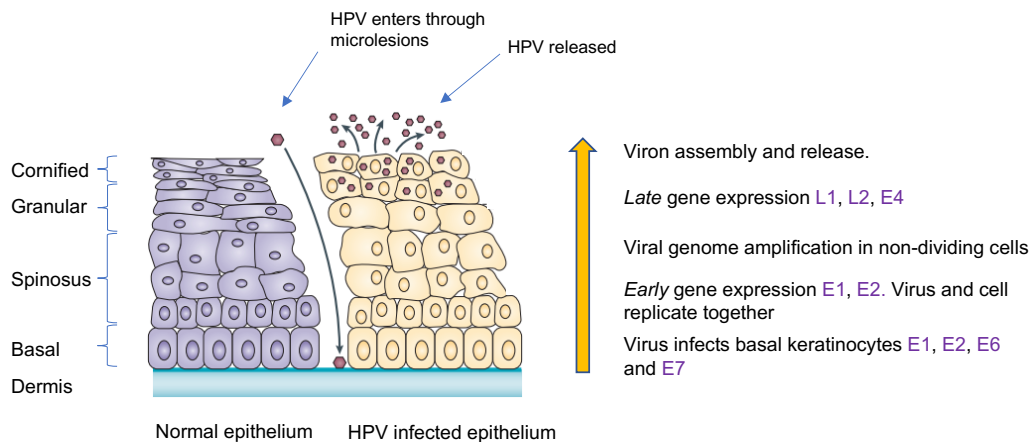


Fig. 4: Life cycle of HPV

The human papilloma virus infects the basal layer of the epithelium through microlesions. Healthy epithelium is shown to the left and HPV infected epithelium to the right. Once the cell gets infected the virus loses its capsid and the viral genome is established in the cell nucleus and *early* viral genes are expressed. The virus is dependent on the cells own replication and after cell division cells migrate upwards and undergo differentiation. E6 and E7 deregulates cell cycle control allowing viral genome amplification. The late phase occurs and L1 and L2 encapsulates newly synthesized virions and they are ready to be released (shown in red hexagons). Figure adapted from Moody (2017), Moody et al. (2010), Stanley (2012) [45][52][53].

Although HPV infection is very common, most infections - even with the most carcinogenic HPV types - clear spontaneously often within a few months [54] and about 90% of the lesions have cleared within 2 years [5]. It is believed to be the innate immune system as well as the adaptive CD8 T cells defense against early viral proteins, that clear the HPV infection [55]. If the infection is not cleared by the immune system it may cause mild cervical intraepithelial neoplasia (CIN 1) and this may eventually progress into moderate neoplasia (CIN 2), severe neoplasia (CIN 3) or even manifest cancer. HPV is a necessary cause (but not sufficient) cause of cervical cancer, and HPV DNA is found in 99.7% of all cases of cervical cancer [24][56]. Having intraepithelial lesions suggests underlying changes in the cells, which may predispose to cancer, however, also these lesions may regress spontaneously, whereas when malignant transformation has occurred, it is *irreversible*. It still remains unknown, why some women have cellular changes that progress and why others are able to clear the virus and yet other women can have CIN 2 for many years without progression. To this end, enhanced knowledge of immune reactivity and recognition of the virus may provide new leads to understand the observed differences.

ANATOMY AND PATHOLOGY OF THE CERVIX

The female lower genital tract consists of 4 regions (Fig. 5). 1. The skin covering the introitus resembles the rest of the skin and consists of keratinized stratified squamous epithelium. 2. The vagina is covered by a glandular, nonkeratinized stratified squamous epithelium 3. the ectocervix which resembles the vagina is covered by a mucosal layer. The vagina and the ectocervix presents a very resistant physical barrier to lymphocyte migration and 4. the endocervix has numerous mucus-secreting glands (pseudo glands) and consists of simple columnar epithelium. Components of the secretory (IgA antibody-mediated), humoral (IgG antibody-mediated) as well

as the cellular immune system are present in both the endo- and exocervix [57][58]. The transition from ectocervix to the endocervix is referred to as the transformation zone (TZ) or the squamocolumnar junction. It is covered by stratified epithelium constituting a physical and immunological barrier against pathogens [59].

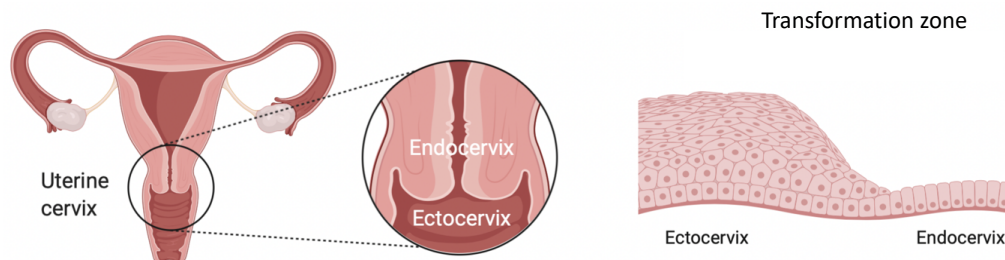


Fig. 5: Schematic overview of the uterus comprised of the cervix

The top of the vagina, the uterus with fallopian tubes and ovaries are shown to the left. Enlarged cervix is shown in the middle with the outer ectocervix and the inner endocervix. The different epithelium is shown to the right. Ectocervix contains stratified squamous epithelium and the endocervix consists of simple columnar epithelium. The zone in between is referred to as transformation zone (TZ).

The TZ changes during life. It is almost always located on the exposed portion of the cervix during youth and pregnancy. In older postmenopausal women it is often further inside the cervical canal [59][60]. This makes the second peak in incidence harder to diagnose because it is difficult to obtain sufficient biopsies and to visualize the neoplastic tissue. The penetrating vessels within the cervix, supply the epithelial cells and facilitates the rapid and efficient entry and exit for nutrition's, oxygen and migrating cells e.g. lymphocytes [59].

SCREENING IN DENMARK

Free of charge, screening programs against cervical cancer were initiated in Denmark in the 1960s', initially as opportunistic screening. In the following years, organized screening programs were implemented. Subsequently, incidence and mortality of HPV related disease have decreased. However, in the last 20 years the incidence has been stable or even increased for some age groups [61]. Women aged 23-49 years and 50-65 years are offered screening every 3 and 5 years, respectively. Despite the fact that cervical screening is free of charge in Denmark, screening coverage is only about 75%, and among the 25% not being screened, more than 50% of all cervical cancers are detected. It has been found, that women who did not have a cytology test within ten years or more, had a 12 times increased risk of developing cervical cancer [62].

Over the last two decades, the conventional cervical cytology (also known as Pap test/smear), has been replaced by a liquid-based method such as Liquid Based Cytology (LBC). It not only allows for a better cytological evaluation but also enables for HPV DNA testing directly from the same specimen. Cervical cancer screening can be performed using cytology screening and/or HPV testing, and HPV based primary screening is currently being implemented in several countries. The classification of cervical cytology using "The Bethesda System" was developed in 1991 and updated again in 2001. This classification is a uniform system of terminology which provides clear guidance for clinical management. ASCUS (Atypical squamous cells of

undetermined significance) is a term used if abnormal cells [63]. ASCUS may be a sign of infection with HPV or other types of infection, such as yeast infection. Epithelial cell abnormalities in cytology are classified as either LSIL (Low-grade squamous intraepithelial lesion) or HSIL (High-grade squamous intraepithelial lesion) and in biopsies encompassing moderate and severe neoplasia (CIN 2 and CIN 3) or carcinoma in situ.

VACCINATION AGAINST HPV

The concept behind prophylactic HPV vaccination is to achieve a high level of type-specific neutralizing antibodies directed against HPV in order to prevent cervical infection. Studies on developing a vaccine against HPV began in 1980's, but it was not until 2006, that two vaccines, containing L1 viral proteins, reached the market [8]. The critical discovery which led to the present vaccines is the fact that L1 could self-assemble into so-called virus-like particles without the genome. This were shown to be highly immunogenic with titers 10 to 100-fold higher than those induced by natural infection [64][65]. The vaccine gives rise to a humoral immune response. Immunization at younger age showed higher initial and remaining antibody titers, compared to immunization at older age (12 years compared to 17 years) [66][67][68].

Merck Sharp & Dohme introduced the fourvalent vaccine (Gardasil) in 2006 for women aged 19-26 years of age covering HPV type 6, 11, 16 and 18. The currently used vaccine is ninevalent and also covers also HPV 31, 33, 45, 52 and 58 [8]. In the clinical trials, the efficacy against HPV type 6, 11, 16 and 18 is almost 100%. In addition, long-term effectiveness up to 12 and 8 years respectively post-vaccination, has been documented for the fourvalent and ninevalent vaccine [69][70]. Several studies have shown that the current HPV vaccines on the market have a high efficacy against HPV 16/18 related cervical disease, when administered to HPV-naïve women [71]. There is no evidence that the vaccines have any therapeutic effects and vaccine efficacy has been shown to be lower when administered to an HPV *non*-naïve population [72]. Recently, a study of HPV vaccine effectiveness at population level, turned out to be high among girls vaccinated before the age of 20 [73]. The aim is therefore to vaccinate all girls and boys as soon as possible or preferably before they become sexually active.

DISEASE AND TREATMENT

HPV PATHOGENESIS

It is widely accepted that effective immune control is required to prevent persistent HPV infection [3][74][75]. HPV has the ability to avoid the immune system in otherwise healthy individuals and establish a persistent infection. Studies indicate that the immune system changes over time after HPV infection [75]. The virus causes a state of chronic inflammation and misleads the immune system because it has created a different microenvironment which plays a crucial role in the survival of the virus and the slowly progression of the disease [75].

It is believed to take around 15-20 years for cervical cancer to develop in women with normal immune systems and this indicates additional tumor promoting steps. For patients with immunosuppression, disease can develop in only 5-10 years [5][6][76]. These patients are at particular high risk of developing persistent HPV infection and HPV related diseases, further underlining the importance of the adaptive immune system for the control of HPV infection and

associated diseases [77]. However, in approximately 10% of patients, this transition into cancer can occur much faster [9].

Environmental factors have also been associated with development of cervical neoplasia. A high level of perceived stress is associated with impaired HPV-specific T cell response suggesting a potential mechanism by which stress may influence cervical disease progression [78][79]. The most consistently identified factors in HPV related carcinogenesis include: many sexual partners, high parity, long-term use of oral contraceptives, smoking concomitant infection with other sexually transmitted agents [80][81], the immune suppression, nutrition, endogenous and exogenous hormones as well as viral characteristics, such as HPV type, viral load and integration [82][83].

CERVICAL INTRAEPITHELIAL NEOPLASIA (CIN)

If the liquid based cervical cytology shows sign of abnormalities, histological sampling should be performed. If the histologic examination shows intraepithelial neoplasia, it will be classified into one of the following seven grades (Fig. 6):

- CIN 1 – up to 1/3 of the thickness of the lining covering the cervix has abnormal cells
- CIN 2 – Between 1/3-2/3 of the lining contains abnormal cells
- CIN 3 – The full thickness of the lining has abnormal cells
- CIN NOS (not otherwise specified)
- AIS – Adenocarcinoma in situ
- Squamous Cell Carcinoma
- Adenocarcinoma

Cytology has a false negative rate of ~9%, whilst this number is ~7.2% for colposcopy [84]. The high grade lesions (CIN2 or higher) detected during screening programs are often over treated since diagnostic tests are not able to discriminate between regressing and progressing precancerous lesions [1].

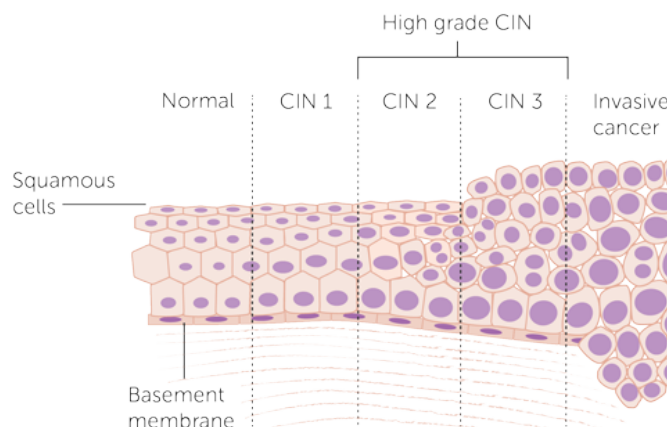


Fig. 6: The histology of the cervical cancer

This schematic step wise progression with healthy tissue to the left and then gradually changing from cervical intraepithelial neoplasia grade 1, 2 and 3 leading to invasive cancer where cells break through the basal layer/membrane. CIN 2 and 3 are also referred to as *high grade*. Figure from *cancer research UK webpage* [85].

CERVICAL CANCER

Tumors arising in the ectocervix are most often squamous cell carcinoma, which account for approximately 75-85% of invasive cervical lesions. In contrast, tumors arising in the endocervix are more likely to be adenocarcinomas and account for 5-10% of cervical cancers. Less common histological subtypes are: adenosquamous (3%), small cell or neuroendocrine, serous papillary, and clear cell carcinomas. Cervical cancer are staged according to the International Federation of Gynecology and Obstetrics (FIGO) just revised in 2020 [86].

Stage	Subdivision	5 year survival rate (%)
I	IA1, IA2, IB1, IB2	75-100
II	IIA1, IIA2, IIB	57-78
III	IIIA, IIIB	35-38
IV	IVA, IVB	5-15

Table adapted from [62][87].

Depending on the cancer stage, patients are offered either surgical treatment: conization, trachelectomy, simple or radical hysterectomy combined with pelvic lymphadenectomy (complete or sentinel nodes) and/or radiation and chemotherapy.

CURRENT POSSIBILITIES OF TREATMENT OF CERVICAL CANCER

In patients with early cervical cancers, surgery is recommended. The standard management of individuals with advanced cervical cancer includes external beam radiotherapy with concurrent cisplatin-based chemotherapy and brachy therapy. Brachy therapy is a curative-intent treatment of cervical cancer, and when compared with external beam radiotherapy alone, the results are clearly in favor of brachy therapy. For all stages combined, the 3-5-year survival rate from cervical

cancer for many developing countries is <50%. Surviving cervical cancer often implies significant suffering, including ureteral obstruction, pain, tightness of the vagina and fistulas [9].

Advanced cervical cancer patients do not substantially benefit from the conventional treatment options, i.e. surgery, chemotherapy and radiation. This has led to the development of a large number of clinical trials testing immune therapy both as monotherapy and as combination with chemotherapy. PD-L1-positive cervical cancer have received Food and Drug Association (FDA) approval for treatment with immune checkpoint inhibitors, 2nd line [88]. So far this strategy has not shown very promising results [89].

INTRODUCTION TO THE IMMUNE SYSTEM

The immune system is a highly evolved and complex system involved in many aspects of maintenance, defense, growth and death of cells in the human body. Despite the complex interplay of these mechanisms, the most important job of the immune system is quite simple: to detect and destroy invading microorganisms and malignant cells. It protects the body against different types of pathogens such as bacteria, virus, fungi and parasites. The immune system is divided into the rapid and unspecific innate immune response and the slow acting but antigen specific adaptive immune response.

The adaptive immune system is dependent on activation of T and B cells which upon activation will proliferate and then clonal expand into effector cells. All within 4-7 days and once the microorganism has been destroyed it leaves an immunological memory unlike the innate immune system [90].

T cells recognize peptide antigens presented on the surface of cells by Major Histocompatibility Complex (MHC), which in humans are named Human Leucocyte Antigen (HLA) [91]. It is a genetic system and the polygenic HLA consists of three different loci HLA-A, -B and -C. Every individual carries two gene copies per loci (inherited paternal and maternal), thereby up to six HLA molecules can be expressed in total. Most individuals are heterozygous and the frequency of HLA haplotypes changes between ethnical origin. Cytotoxic CD8 T cells mediate elimination of tumor and virus-infected cells by recognition of peptide antigens presented by the MHC class-I (MHC-I) through the T cell receptor (TCR) (Fig. 7). CD4 helper T cells mediate anti-tumor cytotoxicity through MHC class-II restricted peptide recognition.

The peptides presented in the binding groove of the MHC molecule have bound specifically to the MHC binding motif by its anchor residues stabilizing the MHC binding. The peptides are derived from cytosolic degraded proteins, defect ribosomal products and – in malignant cells – mutated or new protein products (neo antigens). This peptide-MHC (pMHC) is presented on all nucleated cells. Several studies suggest, that specific HLA alleles are associated with protection against neoplasia, while other alleles are associated with susceptibility to cancer [92][93][94].

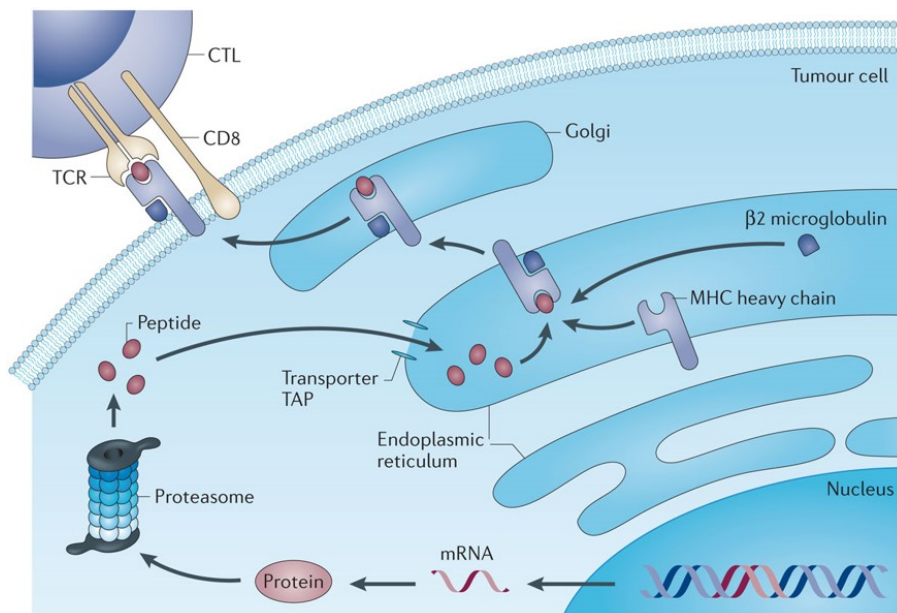


Fig. 7: Peptide antigen processing and presentation

Intracellular proteins are translated, processed by proteasomes in the cytosol, transported into the ER by TAP, loaded onto MHC-I, and translocated through the Golgi apparatus before the pMHC being presented to and recognized by the TCR of CD8 T cells. From Coulie et al. (2014) [95].

T CELL ACTIVATION AND MATURATION

Thymic maturation

T cells undergo development and maturation in the thymus by avidity selection. The cells of which TCR bind poorly to the peptide-MHC complex will be selected out and die. The rest will receive a survival signal and be positively selected and migrate from the thymic cortex to the medulla [96]. Since the TCR is made by random gene rearrangement, the possibility that T cells will bind too strongly to peptide-MHC complex - being autoreactive, exists. In that case they will be negatively selected for clonal deletion.

The T cells passing positive and negative selection will leave the thymus and enter circulation. Only around 5 % of the T cells will survive both positive and negative selection [97][98].

TCR re-arrangement and activation

TCR is the antigen receptor of T cells. The genes making up this T cell receptor are capable of rearranging, which makes them highly diverse. This somatic recombination of the genes V (variable), D (diversity) and J (joining) occurs for both the α (VJ) and the β (VDJ) chain which together constitutes the receptor. The most distal part of the receptor is made up by variable regions with three loops named complementarity-determining regions (CDR3) [99]. This area of the receptor determines the antigen binding site allowing limitless diversity in specificity. The human body has a confined number of unique TCRs. They hold the ability to cross-recognize and respond to several different MHC complexes presenting an even higher number of peptides. This gives the body the property of covering a broad antigenic repertoire of up to 10^6 different peptides.

After leaving the thymus T cells will express their specific TCR and co-receptor molecule. The co-receptors CD4 and CD8 stabilizes the binding between TCR and MHC-II and I, respectively [96]. When T cells encounter an antigen, they will proliferate and differentiates into functional effector types: Cytotoxic (CD8 T cells), helper (CD4 T cells) and regulatory T cells (T_{reg}). Some T cells will become memory cells and those are responsible for the long-lasting immunity.

The recognition of CD4/CD8 T cell and their TCR of the antigen (peptide) presented on the MHC molecule is called “*signal 1*”. Naïve T cells travel to T cell areas of secondary lymphoid tissues in search of antigen presenting cells (APC). Once activated they proliferate vigorously creating effector cells, which can migrate to B-cell areas or to inflamed tissue. The main focus in this thesis is on CD8 T cells, for the remaining referred to as just T cells. In order to fully engage a T cell, it also needs positive co-stimulation from the CD28 molecules, which then interact with B7 molecules on APC “*signal 2*”. These two signals will then drive a T cell response. The capability to efficiently mount a cytotoxic response and kill the tumor cell, depends on the state of activation, proliferation capacity and phenotype characteristics of the T cell.

CANCER IMMUNOLOGY

TUMOR DEVELOPMENT AND EDITING

Despite the heterogeneity of cancers in general, they share common characteristics in terms of driving the malignant transformation of cells. The hallmarks of cancer development are now defined as a conceptual framework for understanding cancer. They are now defined as 10 diverse principles of which one is avoiding immune destruction (Fig. 8). The immune system’s ability to control cancer growth is the constant surveillance of the body. Some cancer cells have the ability to survive immune recognition through additional mutations or increasing the immunosuppressive environment, resulting in low immunogenicity. This led to the recognition of the role the immune system represents, in controlling and shaping cancer through an adjusting process called “Cancer Immunoediting” theory [100][101].

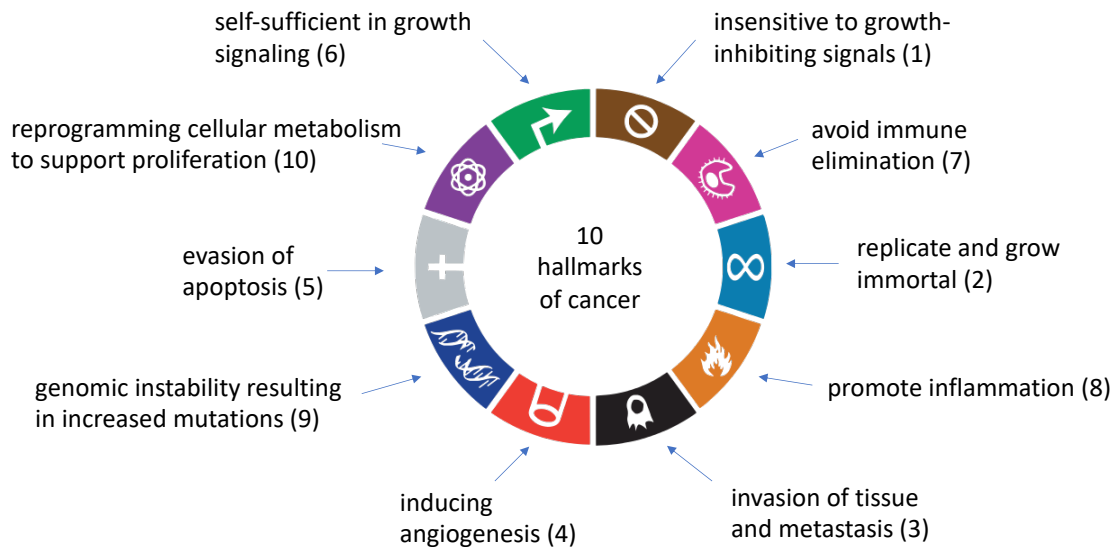


Fig. 8: The hallmarks of cancer

This framework comprises ten different acquired capabilities of cancer. The dependent principles for a cancer to survive and proliferate. Six of these hallmarks were first proposed in 2000 by Hanahan and Weinberg as crucial requirements for tumor mechanisms (1-6). A decade of intense research gave rise to further four key aspects (4-10). Adapted from [102][103].

This theory describes the interactions of a growing tumor with the immune system as three phases: elimination, equilibrium and escape (Fig. 9). In the first phase, the innate and adaptive immune cells detect and destroy the neoplastic cells before they become an apparent tumor. If incomplete, tumor cell variants will survive and enter the equilibrium phase. Here a balance occurs, where the adaptive immune system controls tumor growth, while shaping the immunogenicity of the tumor through constant selective pressure. In most cases, eventually immune-inhibitory mechanism develop and the tumor will regain uncontrolled growth. This is determined as the escape phase.

Not all tumors undergo all stages. Some will be destroyed during early elimination, where others will remain at equilibrium form for a very long time (e.g., cervical cancer). Some tumors will stay at this phase permanently, while other will progress rapidly and aggressively through the different phases. Many external factors such as stress, smoking, aging will affect the tumor microenvironment (TME) and affect the effectiveness of the immune system. These phases should be considered as dynamic and interconnected.

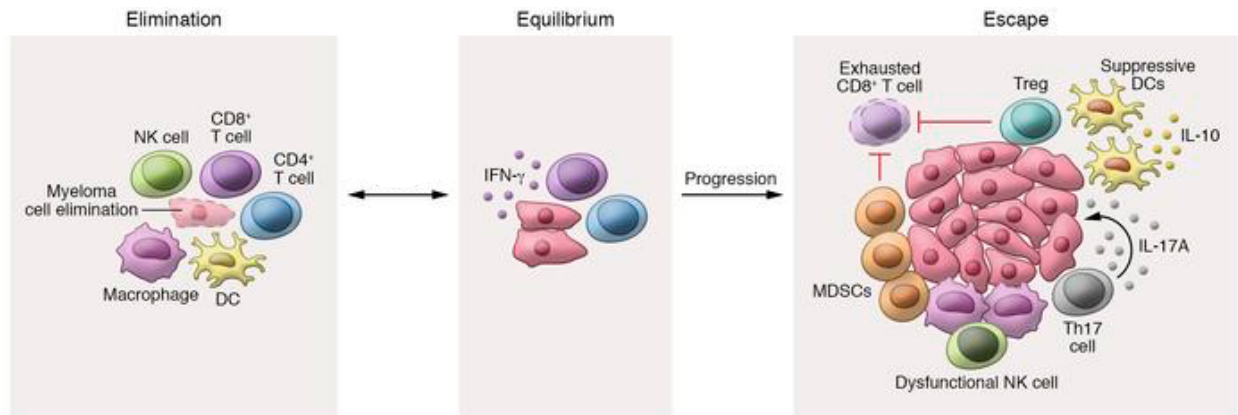


Fig. 9: The cancer immunoeediting theory

The tumor development can be divided into three phases: elimination, equilibrium and escape. The highly interplay between dysplastic cells and the immune system determines the cancer fate into either elimination or progression. Adapted from [100][104].

TUMOR MICROENVIRONMENT AND IMMUNE SUPPRESSION

Tumors are complex tissues in which the cancer cells evolve and communicate with their surrounding microenvironment. These stroma interactions are important determinants of tumor survival, growth and dissemination. By better understanding the tumor microenvironment (TME), we hopefully will be able to develop strategies and treatment options which neutralize its oncogenic influence and more effectively attack the tumor itself. Besides tumor cells, the TME also consists of fibroblasts, myofibroblasts, resident and transient immune cells, which are all nourished by blood vessels and drained by lymphatic vessels, all embedded in extracellular matrix. The TME will affect the tumor infiltrating T cells, and mediate T cell dysfunction through a range of different mechanisms and pathways. They are listed in the form of 6 different categories, but an interplay between them constantly occurs (Fig. 10). 1. Metabolic pathways, 2. Transcriptional regulation, 3. Inhibitory receptors, 4. Inhibitory cells, 5. Suppressive soluble mediators, 6. Epigenetic imprinting.

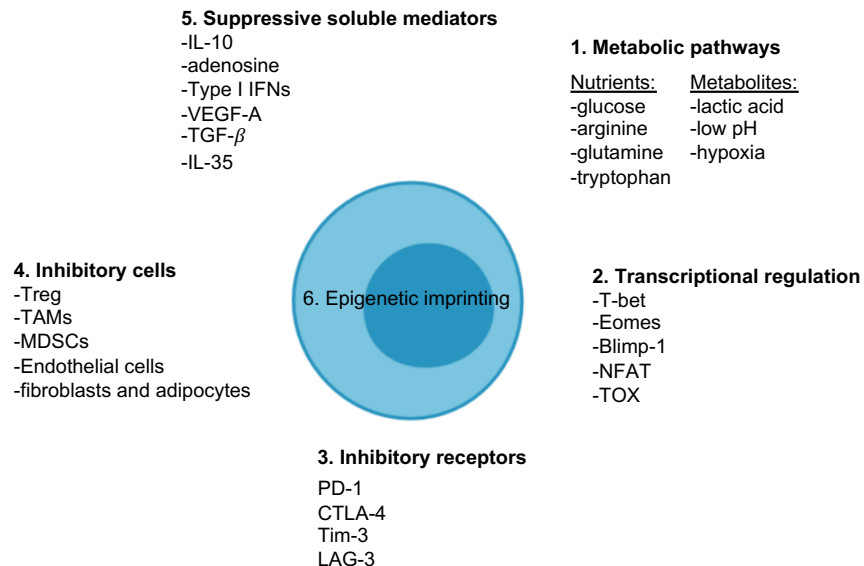


Fig. 10: Traits of character of dysfunctional T cells (overview version)

A grouped way of showing the many different aspects of the tumor microenvironment causing T cells to become dysfunctional. Not all mediators are shown here, and the interplay is complex, and researchers are still trying to solve it and get a better understanding. **1. Metabolic pathways.** T cells depend on these pathways for activation e.g., aerobic glycolysis, amino acid metabolism and fatty acid synthesis. Within the TME the cancer cells often compete with T cells to obtain sufficient nutrients. Also, the metabolites lactic acid, low pH and hypoxia are involved. **2. Transcriptional regulation** involves changes in the expression pattern and upregulation of transcription factors such as T-bet, Eomes, Blimp-1, NFAT and TOX. **3. Dysfunctional T cells** are characterized by the inhibitory receptors, their sustained expression and inhibiting effects, such as PD-1, CTLA-4, Tim-3, LAG-3. The higher the number of inhibitory receptors the more severe dysfunction of the T cells. **4. Inhibitory cells** also contribute to T cell dysfunction. These cells include regulatory T cells (Treg), tumor associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs), endothelial cells, cancer-associated fibroblasts and adipocytes. **5.** There are many suppressive soluble mediators some being IL-10, adenosine, Type I IFNs, VEGF-A, TGF- β and IL-35. **6. Epigenetic imprinting** covers persistent demethylation and changes in chromatin accessibility. Adapted from Xia et al. (2019) [105].

Tumors are often describes as either hot or cold meaning they can either contain low amount of infiltrating effector T cells and high levels of anti-inflammatory cytokines and metabolites are often referred to as immunologically “cold” tumors – non-immunogenic/non-inflamed. “Hot” tumors on the other hand are characterized by high levels of infiltrating effector CD8 T cells and are highly inflammatory. Patients with ‘hot’ tumors are more likely to to respond well to immunotherapy [106].

Cells are brought to the TME by blood vessels and studies often show abnormal angiogenesis, which causes hypoxia, low pH and elevated interstitial fluid pressure, which creates less exchange of oxygen. Poor profusion of the tumor has multiple consequences and causes a switch to anaerobic metabolism, fibrosis and thereby immune suppression.

Based on viral component being foreign to the body, the immune system is in theory more prone to induce tumor recognition than in other cancer diseases. However, tumor cells can learn to adapt to immune surveillance through these suppressive mechanisms and escape immune recognition from T cells by hiding their presentation of MHC-I antigen complexes [107][108].

Tumor associated immune cells infiltrating the TME, can be divided into two types: tumor-antagonizing and tumor promoting immune cells [109]. Effector T cells (CD8 and CD4), Natural Killer (NK), dendritic cells (DC) are mainly the antagonizing ones, where the tumor promoting immune cells consists of Regulatory T cells (Treg) and Myeloid-derived suppressor cells (MDSCs) among others.

When HPV reaches the state of persistent infection, a lower expression of E6 and E7 is observed and thereby a reduced activity of Langerhans cells, leading to immune-tolerant status and thereby potentially cancer development [29]. IL-10 being an immune-suppressive cytokine have demonstrated high expression levels in cervical cancer patients confirming an interesting link between cervical cancer and immune checkpoints [107].

The correlation of HPV mediated immune tolerance and tumor development is still not fully understood and the interplay of the TME is still to be further elucidated.

CELL COMPONENTS OF INTEREST

T CELLS TYPES OF RELEVANCE IN CANCER

Clusters of differentiation (CD) is a classification determinant used for cell *surface* molecules for identification and investigation of cells. Immunophenotyping is a test used to identify cells on the basis of the types of markers or antigens present on either the cells' *surface, nucleus or cytoplasm*. CD therefore provides surface targets for immunophenotyping of cells. Different fluochrome-conjugated antibodies are used as probes for staining target cells with high avidity and affinity, thereby rapidly detecting markers or antigens both by surface staining but also intracellularly after fixation and permeabilization. This allows for rapid phenotyping of each cell subset in a heterogeneous sample. Both their level of activation, effector function, migratory patterns, proliferative capacity among others. Some cells have very distinct markers and are easy to characterize, while others are far more challenging to identify and classify. This insight helps to describe the pathogenesis, the cellular changes in the affected tissue and the changes over time. Hopefully, this will give us insight in targets for immune therapy and better clinical outcome. This study interrogates both HPV infected cells in the microenvironment and in the blood. Since cervical cancer still have many unanswered questions, we need to look further into these cell signatures. Are there important differences in histological and cytological specimens? Can we detect signatures of immune activation in the blood, cytology and biopsies, and how does these different cellular compartments compare to each other. Are there any correlation between detected cells and state of disease?

By looking into the literature and previous research, selected markers of interest for cervical cancer were identified and these are listed below (Table 1, Fig. 11). Both well known but also more explorative markers have been chosen, to broadly describe the immune characteristics within the cervical tissue and blood.

Table 1: Scheme of both T- and myeloid cell markers

The marker that best characterizes the cell subtype, the expression and the function of the cell subtype.

Marker	Expression/cell type	Function
CD1a	Dendritic cells incl. Langerhans cells, CD4 and CD8 T cells	Presents lipopeptides to T cells independent of MHC class I and II
CD3	All stages of T-cell development	Identification of T cells and TCR signaling
CD11b	Myeloid cells especially macrophages, neutrophils and NK cells	Modulates immune cells in cell adhesion, migration and phagocytosis
CD11c	Myeloid cells including DCs, monocytes, and macrophages	Function in phagocytose, cell migration, cytokine production
CD14	Monocytes in blood and macrophages in tissue	Binds to lipopolysaccharide to detect bacterias. Used together with CD16 to distinguish between different subsets of monocytes
CD15	Neutrophils, eosinophils	Mediates neutrophil adhesion to DC for phagocytose and chemotaxis. Used to distinguish between PMN-MDSC (CD15+) and M-MDSC (CD15-)
CD16	Monocytes, granulocytes, tissue macrophages and a subset of monocytes, eosinophils and DC	Early activation of NK cells and in moderating a NK response. Defines the intermediate and especially the non-classical monocytes
CD19	B-cells	Biomarker for B cells and facilitates development and activation
CD27	Naive, T _{CM} , T _{EM}	Marker of early stages of activation
CD33	Myeloid lineage specific	Modulate immune cell functions, phagocytose, cytokine release and apoptosis
CD39	CD4 and CD8 T cells (especially exhausted, T reg), B cells	Key modulator with regulatory properties of activation and exhaustion by converting adenosine
CD45	All nucleated hematopoietic cells	Signalling gatekeeper in T cells, regulates cell growth, differentiation, mitotic cycle and oncogen transformation
CD45RA	T cells (naive, and Temra)	State of T cell differentiation/activation
CD56	NK and T cells	Constitutes cell-cell adhesion. Prototypic marker of NK cells
CD57	T cells and NK cells	Defines late T cell activation/exhaustion
CD64	Monocytes and macrophages	Binds IgG antibodies with high affinity with its Fc receptor. Distinguishes mDC from pDCs
CD103	T cells in the peripheral tissues and a subset of DC	Binds to E-cadherin (adhesion receptor) and important for T cell homing to the tissue and regulates mucosal immunity
CD123	Plasmacytoid DC (pDC), basophil granulocytes	Support proliferation and differentiation of hematopoietic cells
CD207	Langerhans cells (DCs)	Antigen presenting cells binds strongly to glycoproteins. Constitutes to binding of the CD1a antigen
CD274 (PD-L1)	Antigen presenting cells	Binds to the receptor PD1 and acts to block T cell activation and effector function. Induction and maintainance of immune tolerance to self
CCR7	T cells (naive, T _{CM}), B cells and mature DC	Responsible of directing the migration of DCs and lymphocytes to the lymph nodes
Eomes	T _{EM} and exhausted T cells, and NK cells	Transcription factor that regulates function and homeostasis of T _{EM} (resting and activated). High leves promote CD8+ T cell exhaustion
Granzyme B	T cells, NK cells, basophils, mast cells. High in T _{EMRA}	Cytotoxic agent that mediates apoptosis and induces inflammation. Distinguishes recently activated from resting memory CD8+ T cells
HLA-DR	Macrophages, DC and B-cells	Antigen presenting cell
Ki-67	Expressed in all phases of cell cycle except in resting cells	Marks the process of proliferation and growth fraction (prevents aggregation of mitotic chromosomes).
TCF-1	Naive and few exhausted T cells	Regulates T cell development, proliferation, survival and cytokine production
TOX	T _{EM} and detected in exhausted CD8+, CD4+ T cells and malignant cells	Drives T cell exhaustion, associated in tumor progression and essential in innate lymphoid development
PD-1	Activated T cells (T _{EM} , T _{EMRA} , T _{reg}), B cells and monocytes	Downregulates T cell effector functions and promotes apoptosis. Upregulates when activation but rapidly downregulates through chronic antigen stimulation

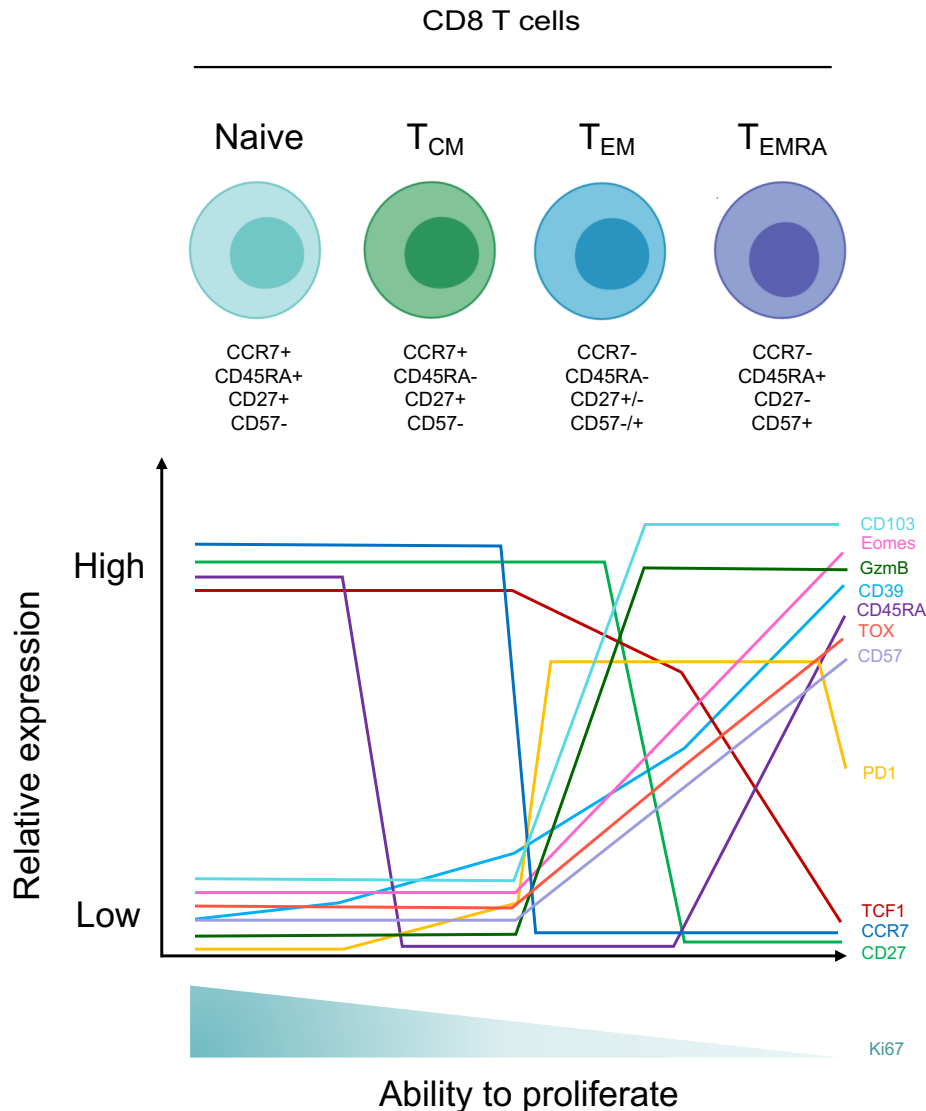


Fig. 11: CD8 T cell differentiation and phenotypic association (purposely simplified)

Four distinct subsets of circulation CD8 T cells are defined based on the expression of CCR7, CD45RA, CD27 and CD57. Their proliferative capacity deteriorates over time of activation. Expression of a variety of cell surface markers and intracellular molecules related to their state of activation, differentiation, regulation, homeostasis, homing potential and functional capacities are shown in a simplified schematic overview. Adapted from Appay et al. (2008) [110].

T cell exhaustion

When naïve T cells (T_N) becomes activated they turn into effector cells (T_{EF}). After eliminating the pathogen some T cells becomes functional memory cells (T_{Mem}) and retain the ability to reactivate upon new infection with the same antigen. If however the T cells do not succeed in destroying the infection and therefore are exposed to a persistently high antigen load and chronic T cell receptor (TCR) stimulation, they reach a more chronic state and can become “exhausted” over time (T_{Ex}) [111]. The term exhaustion and/or dysfunction was first defined in 1993 by Moskophidis and colleagues when they demonstrated impaired cytotoxic functions during viral persistence in murine models [112]. T_{Ex} is an adaptive state and these exhausted T cells are very heterogeneous

ranging from complete lack of effector function to altered functionality to prevent immunopathology [113].

T_{Ex} have been shown to have decreased (but not absent) cytokine production, increased chemokine expression, persistently high expression of multiple inhibitory receptors, reduced proliferative capacity when stimulated, an altered transcriptional program and a unique epigenetic landscape [105][113]. They also show cytotoxicity and poor survival ex vivo. Exhaustion probably exists as a spectrum because many factors contribute to this stage, leading to T_{Ex} with different profiles. The origin of T_{ex} is also currently being discussed. Do T_{ex}-cells arise from memory T cells, effector T cells or directly from naïve T cells upon antigen stimulation [113].

The first state of cytotoxic T cell exhaustion is mainly characterized by loss of Interleukin (IL) -2 production [114][115]. Subsequently the production of tumor necrosis factor (TNF- α) and other cytokines is dramatically reduced and in the most extreme stages of exhaustion production of Interferon (IFN)- γ is lost [116][117].

Even within the term exhaustion there are different characteristics. When the T cell becomes exhausted but potentially still have the capacity of self-renewing, they have recently been defined as progenitor exhausted T cells (T_{Pex}). They share similarities with memory T cells. Phenotype analyses of these T cells show TCF1⁺, PD-1⁺. As a continuum of this stage T cells become less proliferative, expressing more inhibitory receptors. A greater epigenetic enforcement in the end potentially causes the Terminally differentiated T cells to become exhausted (T_{Ex}) and probably not able to proliferate anymore. These subsets are often found in tumors or chronic infections. They have been shown to express TCF1⁻, PD-1⁺, GZmb⁺, Eomes⁺ [118][119].

So exhausted T cells are dysfunctional, but not all dysfunctional T cells are exhausted.

As seen in (Fig. 12) the dysfunctional T cells can also include anergic or senescent T cells. T_{ex} is a representative of T cell dysfunction. T_{dys} are not completely useless since they retain some level of residual function and this may limit the persistent pathogen and tumor progression. However T_{dys} fail to effectively eliminate infection and cancer [105].

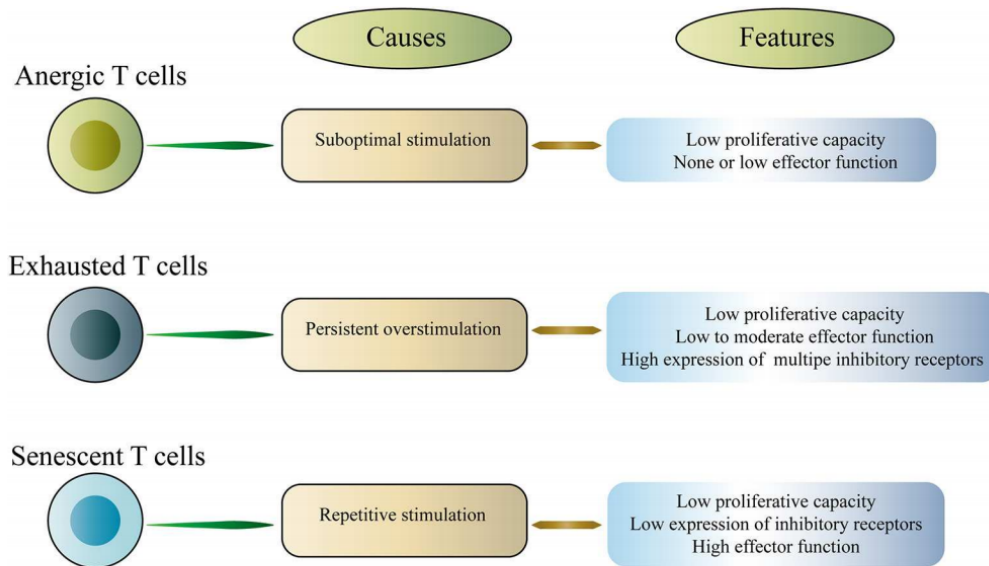


Figure 12: Dysfunctional T cells

These T cells are characterized by reduced proliferative capacity, decreased effector function and overexpression of multiple inhibitory receptors. This persistence of inhibitory signals in the complex TME causes different states of tumor-specific T cell dysfunction. During persistent overstimulation (e.g. chronic infection or cancer) these T cells are being constantly exposed to antigen exposure potentially making them *exhausted*. *Anergic* T cells are induced by suboptimal stimulation, whereas *senescent* T cells enter a terminally differentiated state due to repeated stimulation, which involves irreversible cell cycle arrest and telomere shortening [105].

Exhaustion has been mostly described for CD8 T cells responses although CD4 T cells have also been reported to be functionally unresponsive in several chronic infections [120][121].

The transcriptional factors involved in the altered profile are e.g., TCF-1, TOX, T-box transcription factor (T-bet) and eomesodermin (Eomes). Expression of TCF-1 promotes the effector function and self-renewal capacity of exhausted CD8 T cells.

Expression of TOX is driven by chronic TCR stimulation and nuclear factor of activated T cells (NFAT) which is associated with T cell exhaustion. By RNA sequencing (RNA-seq) the gene encoding the TOX was highly expressed in dysfunctional T cells. Cells expressing high levels of TOX correlates with inhibitory receptors and low expression of TCF-1. Moreover these cells fail to produce effector cytokines IFN- γ and TNF [122].

The gene transcription factor T-bet and Eomes control gene expression involved in the developmental processes and the regulation of the adaptive cell-mediated immunity through promoting infiltration of CD8 T cells to the tumor tissue. Both T-bet and Eomes enhances IFN- γ production and suppressing inflammatory IL-17 production and are required for the effector stage of T cell responses against tumor [123].

Granzyme B (Gzmb) is a protease cytokine secreted among other cells by CD8 T cells along with perforin to mediate apoptosis in target cells. Activated cells show upregulation of Gzmb, IFN- γ but exhausted CD8 T cells show impaired effector cytokine production including Gzmb, IFN- γ , IL-2, TNF- α [119] [124][125].

PD-1 is highly expressed in T_{ex} and the use of an inhibitor which blocks the interactions of PDL-1 with PD-1 receptor can prevent the cancer from escaping the immune system. The dominant role of PD-1 is regulating T_{Ex} in the form of an upregulation and blockade of the PD-1/PDL-1 pathway. Immunotherapy with these immune checkpoint inhibitors promotes T cell effector functions and significantly inhibits tumor growth in HPV positive cancers [126]. In HPV positive head and neck cancers strong infiltration of activated CD8 T cells have a favorable outcome and able to become reinvigorated upon PD-1 blockade [127]. High levels of PD1/PD-L1 are often expressed in cervical cancer patients and frequently expressed in dendritic cells CIN samples [128].

More detailed understanding of human T cells exhaustion and anti-viral immunity is still critical to develop novel immunotherapies to hopefully reverse the state of T_{Ex}.

MYELOID CELLS TYPES OF RELEVANCE IN CANCER

Both megakaryocyte, granulocyte and dendritic cells all originate from the myeloid progenitor cell. Monocytes (a subtype of granulocytes) migrate from the blood into the tissue where they develop into different types of macrophages or myeloid DC (mDC) [129]. Monocytes are classified into three subsets based on the expression of the surface markers CD14 and CD16. "Classical" CD14^{high}CD16⁺ (constitutes 85% of monocytes), "intermediate" CD14⁺CD16⁺ (only 5-10%) and "non-classical" CD14^{low}CD16^{high} (also only 5-10%) and they all play a key role in immune response [130]. CD14 is a marker for monocytes in blood and macrophages in tissue and is used to distinguish between macrophages and dendritic cells [131][132].

CD64 is a membrane glycoprotein also known as FC receptor and binds to monomeric IgG with high affinity. They are constitutively found on and a marker for macrophages and monocytes [133].

Dendritic cells

(DC) are the most important ones in initiating the adaptive immune response. They are a heterogeneous population of antigen presenting cells and abundant in the mucosal tissue where they were first discovered by Paul Langerhans in 1868 and they were described as having a striking dendritic or "tree-like" morphology [134] and named Langerhans cells [135]. Studies show that immature DC's in the mucosal tissue express CD1a [136] and langerin (CD207) and harbors Birbeck granules [137]. They are also found in the epithelial cells in the cervix and have been subject to controversial classification whether they should be classified as DC's or macrophages, but functionally they act as DC [135]. Presence of DC or Langerhans cells in the epithelial layer of the ectocervix is paramount in producing immune response [138][139]. Therefore langerin (CD207) was also included as a marker in this thesis.

Studies have shown that Langerhans cells in HPV lesions may be quantitatively reduced and functionally impaired, which may contribute to the persistence of infection [140].

When DC are immature, they specialize in phagocytose (receptor mediated) and pinocytosis (without receptor) but with low capacity to activate T cells. After cytokine maturation from e.g., TNF- α and IL-1, they will upon inflammation migrate to the secondary lymphoid organs. Here they will decrease their ability to phagocytose but in return they will be potent activator of T cells partly because they will upregulate their antigen presenting (MHC) and co-stimulatory molecules. Once

activated they will act as antigen presenting cells with a constitutive expression of the co-stimulatory molecules. They bridge the innate and the adaptive immune system and besides CD14+ myeloid cells the mature DC (CD83+) have also been detected at higher numbers in the stroma of neoplastic cervical tissues [136].

The diversity of DC include the plasmacytoid DC (pDC) found in blood defined as CD123+ and the conventional DC also known as myeloid DC (mDC) defined as CD11C+CD123- [135]. mDC are specialized at antigen presentation to naïve T cells and DC have been poorly investigated in cervical carcinogenesis so far despite the fact that their mechanisms are crucial for immune control/failure and progression.

Myeloid derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are immune cells from the myeloid lineage originated from the bone marrow. Both lymphocytes and myeloid cells are found in a band directly beneath the epithelium [57][58].

Myeloid cells are a heterogenous group and have emerged as major regulators of immune responses in cancer and other pathological conditions. MDSC is highly represented in tumor progression through immune suppression and are obstacle to many cancer immunotherapies.

However, a number of conditions associated with chronic inflammation, autoimmune diseases and cancer; may result in aberrant, sustained myelopoiesis characterized by the accumulation of immature myeloid cells which deviate from the standard path of differentiation. These cells are distinct from mature, terminally differentiated myeloid cells (macrophage, dendritic cells or neutrophils) and have an activation program (pathologic activation), which is different from that of mature myeloid cells.

In human peripheral blood mononuclear cell (PBMC), the PMN-MDSC are defined as CD11b+CD14-CD15+ and M-MDSC as CD11b+CD14+HLA-DR-/loCD15-. These gating criteria cannot discriminate monocytes from M-MDSCs (mononuclear) and neutrophils from PMN-MDSC (polymorphonuclear) since at present there are no combinations of markers unique to MDSC. A number of molecules produced by MDSCs have been implicated in suppression including arginases, NO, ROS, IDO, TGF β and PGE $_2$, among others. Although important for a thoroughly understanding of MDSC suppressive mechanism(s), evaluation of their expression cannot substitute for functional assays. In different settings MDSCs utilize different mechanisms of suppression and it is difficult to predict which will be more prevalent. It is also challenging to ascertain what level of production of any given effector molecule is sufficient for the MDSC suppressive activity. Pathological activation of MDSC is the result of persistent stimulation of the myeloid compartment with relatively low-strength signals coming from tumors or sites of chronic inflammation.

Early stages of cancer or initial stages of chronic inflammation may be associated with accumulation of cells with phenotypic and biochemical characteristics of MDSC but lacking potent suppressive activity [141]. MDSCs have been demonstrated to substantially impact the tumor reactivity negatively and also the patients response to immunotherapy [142].

MUCOSAL IMMUNITY OF THE CERVIX

Keratinocytes in the epithelium are the HPV host cells. They are armed with pathogen recognition receptors, host intrinsic restriction factors and an arsenal of inflammatory cytokines and chemokines orchestrating local immune responses.

HPV replication in the epithelium is non-cytolytic and involves only low gene expression in basal keratinocytes and lacks a viremic phase. HPV positive cells have shown to produce low levels of attracting chemokines and this interfere with inflammatory signaling in the infected epithelium [143][144]. As a consequence, HPV is allowed immune escape and persistence by avoiding eliciting immune responses in an active manner [29][53]. Not only does HPV avoid recognition, but it is also thought to actively counteract the immune response by suppressing epithelial inflammatory and interferon responses. It strongly impairs the recruitment of Langerhans cells to the lesioned mucosal or cutaneous epithelium [53][75][144]. The exact mechanism of the virus is still not fully understood, but studies propose that during progression the HPV infected cells initiate chronic stromal inflammation and immune deviation by autocrine growth factor Interleukin-6 (IL-6), which binds to the cytokine receptor on monocytes. This causes chemokine induction in mesenchymal, stromal and infiltrating immune cells [75].

In the stroma of cervical cancers, many mature DC lack the NF- κ B-regulated lymph node homing receptor CCR7 [144]. Being unable to migrate in response to lymph node homing chemokines [75] and as a consequence these immobile DC release the tumor promoting matrix-metalloproteinase MMP-9. Once released it is associated with poor prognosis for cervical cancer patients [144][145]. IL-6 has been shown to be a crucial regulator of both CCR7 suppression as well as MMP-9 induction in cervical cancer DC's [144]. This study also found an upregulation of CD1a in tumor-instructed cells and monocyte/macrophage marker CD14 was downregulated to levels comparable with the control cells.

The stroma of CIN 3 and cervical cancers have shown to be strongly infiltrated by CD14⁺ myeloid cells and were found to express high levels of matrix-metalloproteinase MMP-9 [145][146]. The endocrine state varies during menstrual cycle and during menopause. The CD3⁺ T cell population remains relatively constant which indicates that the hormonal imbalance does not alter either the presence or the function of the immune cells substantially [147]. The immune function have been shown to be affected by female sex hormones [148][149].

For post-menopausal women with inactive endometrium, CD3⁺ T lymphocytes have a higher cytolytic activity than pre-menopausal women. CD3⁺ T cells reside throughout both the vaginal and the ectocervical stroma. The CD3⁺ T cells with cytolytic activity are not presented as aggregates as seen in the uterus. In the cervix and the vagina, they are randomly distributed as individually CD4 or CD8 cells or in loose accumulations together with macrophages and DC. CD8 T cells were predominant compared to CD4 and T cells are more abundant in the cervix than B cells. The ectocervix mucosa is papillated and CD8 were present both in the epithelium and in these papillae. The abundance of CD4 lymphocytes occurred predominantly in the lamina propria [32][150].

The endometrium has been subject of several studies. Looking at activation markers showed no difference in the proliferative or luteal phase. Data showed an increased expression of CD69 and HLA-DR but not of CD25 or CD71 on endometrial T lymphocytes of nonpregnant women. These observations suggest a state of recent and persistent activation regardless of menstrual cycle [149]. The presence of cytotoxic T cells shows that the lower genital tract is fully capable of mounting cellular immunity. The appearance of DC has also been found scattered throughout the vagina and ectocervix mainly in the stratified squamous epithelium. They are shown to be HLA-DR+ dendritic cells and probably these are Langerhans cells and they might well be involved in HPV infection [151][152]. Data from the cervix and the vagina - all though not as well described as the endometrium, shows that IgG and IgA secreting plasma cells are scarce in the vagina and abundant in the lamina propria of the endocervix. This indicates that immunological microenvironments exist in the lower genital tract.

The ectocervix shows more CD45RO+ (memory) than CD45RA+ and more CD1a+ compared to the vaginal epithelium. No differences are seen for CD103+ between vagina and ectocervix. Women with cervicitis or chronic inflammation show higher concentration of CD8, CD4 and CD103+ in both vagina and cervix compared to the non-inflammation and with immature characteristics i.e., CD45RA. CD1a+ cells show only to be present in the ectocervix compared to the endocervix and in contract they were fewer in the inflamed vaginal and ectocervical tissue [32]. Other studies did find CD1a in the endocervix but cytobrush was used and it is therefore not possible to separate the ecto- from the endocervical sample [153]. Loss of CD1a+ cells have been suggested as a sign of migration to the lymph nodes and these changes in abundance have also been found in women infected with HPV [154][155]. Of all 4 regions in the lower female genital tract the transformation zone shows the highest concentration of CD4, CD8 lymphocytes (CD45RO+ CD103+), macrophages and more focal accumulations in the lamina propria of lymphocytes. Granulocytes are also numerous especially in the TZ. CD56+ and CD57+ NK cells are found in high numbers in both vaginal and ectocervical tissue. They have also been observed in the TZ of HPV infected cells [156]. CD57+ NK cells have been found in both vaginal and ectocervical tissue especially in the lamina propria [156].

TUMOR ANTIGENS

The number of mutations a tumor cell will facilitate is referred to as tumor mutational burden (TMB). Cervical cancer is defined as the eighth highest in TMB across 30 different cancer types (Fig. 10) [157]. If neoantigen specific CD8 T cells are found at tumor site, studies from different cancer types have reported, an association with improved prognosis [158][159]. Thereby stating that immune recognition has occurred. Neoantigen-derived peptides is bound to and presented by MHC class I molecules where neopeptides have furthermore been detected and recognized by T cells. Therefore, some neopeptides are more immunogenic than others and more prone to recognition.

HPV exploits the cells to be incorporated and these viral antigens are more dissimilar to "self"-antigens compared to neoantigens, so no tolerance exists. These are exclusively expressed by the onco virus infected tumor cells and shared between patients. This makes these markers possible to identify and potential candidates for use in cancer therapy and a key target to elucidate in manuscript two [160][161].

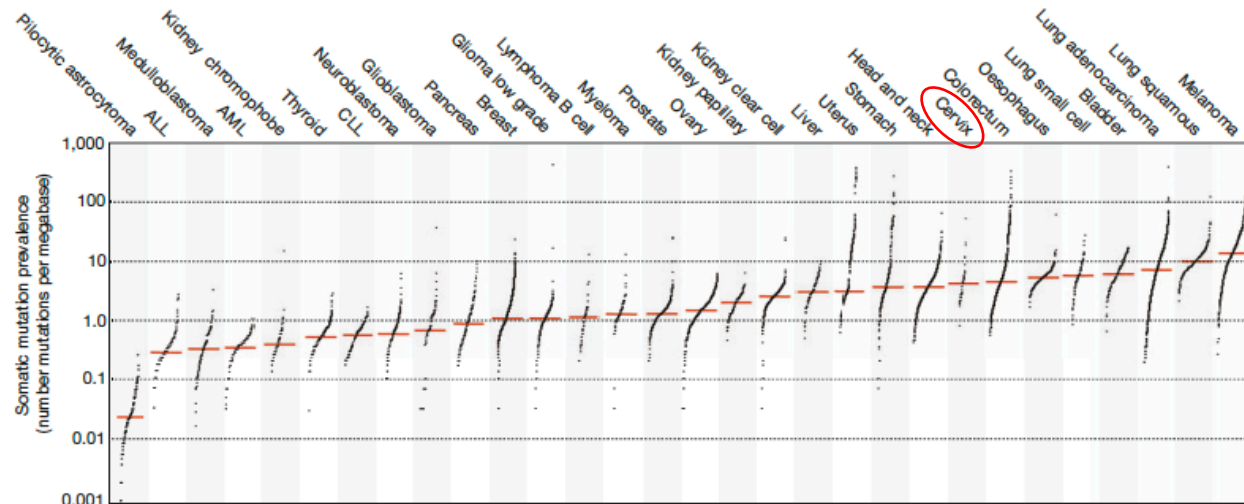


Figure 10: Tumor mutational burden across human cancer types

The prevalence of somatic mutation. Presented as a number of mutations per mega base with the red line representing median mutational load. Cervical cancer is the eighth highest. From Alexandrov et al. 2013 [157].

The landscape of immunogenic tumor antigens in HPV driven cancer still remains poorly understood. PD-1 blockage had been suggested as target for immunotherapy [160]. The viral HR oncoproteins (E6, E7) are potentially candidate tumor regression antigens, as they are foreign and constitutively expressed by cancer cells, but evidence supporting this is still limited [162][163]. Prophylactic vaccines target the major capsid protein L1 by neutralizing the virus before it enters the cell by inducing a humeral immune response [164]. Therapeutic vaccines induces T cell cytotoxicity to eliminate the virus infected cell. Due to the deletion of E1, E2, E4, E5, L1 and L2 encoded in the open reading frames of the malignant cell makes these antigens unsuitable as targets. E6 and E7 are highly conserved and much better candidates as tumor antigens.

In order to stimulate CD8 T cell response E6 and E7 have to be presented by class I MHC molecules after they are hydrolyzed to peptide fragments by the proteasome and only a fraction will engage to the MHC molecule with the affinity and interact with the TCR. Such peptide fragments or epitopes are valuable in determining T cell recognition. Failure to eliminate cancer cells might be because of alterations of the T cell phenotype and hampered function when trying to engage to the peptide-MHC and become activated [165].

These peptide of interest can be predicted using artificial intelligent algorithms such as NetMHCpan 4.0, and already known and tested epitopes can be found in the IEDB.org server or by web search. This topic will be reviewed further In manuscript II two.

CANCER IMMUNE THERAPY

The aim of immune therapy is to block or reverse the progression of these non-static and interconnected entities. The most promising therapy is immune checkpoint inhibition therapy (ICI) or adoptive cell transfer (ACT) which already have showed cases with partial and complete

response in clinical trials [166]. Checkpoint inhibition can lead to the killing of other subclones than intended and thereby unfortunately create selective advantage for the target clone and outgrowth killing by immune escape mechanisms. The immune system shows the ability to not only surveil, but also to influence tumor profile.

Another pathway of great interest, is the PD-1 and PDL-1. Treatment with anti-PD-1 against solid tumors shows remarkable results on tumors and metastasis. CD8 T cells show reinvigoration using anti-PD1 therapy [167]. Data also indicates that these PD-L1 treated cells are very similar to T_{Ex} and RNA-seq data indicates, that the treatment alters the gene expression without alterations of the chromatin. This leads to the fact that transcriptomic data (intracellular and extracellular signals and their response) is highly dynamic and is not as valuable as epigenomic stable information (what the cell is capable of doing, shaped by past and present experiences and its possibilities). Gaining this information is useful in understanding the fundamental identity of the T_{Ex} and if/how it can be changed by e.g., anti-PD1.

The high mutational burden found in cervical cancer patients and the fact that it almost always is virus that drives this process, makes it a theoretically perfect aim for immune therapy. KRAS, PIK3CA, TP53 and PTEN are all genes with genomic alterations found in cervical cancer patients [168]. Moreover, HPV integrates its genes in the host DNA and such 384 genes have been identified as being sites of T cell activation. This indicates, that HPV infection and immune surveillance are strongly correlated [169]. Several studies have assessed immune-checkpoint inhibitors. One study “KEYNOTE-028” phase Ib trial, where a group of 24 pretreated cervical cancer patients expressing PD-L1, received anti-PD-1 *pembrolizumab* biweekly, showed a overall response rate (ORR) of 17%. Four patients (17%) achieved a confirmed partial response and three patients (13%) had stable disease [170]. In the subsequent phase II “KEYNOTE-158” trial, 98 cervical cancer patients being positive for PD-L1, were given a higher dose every third week and showed ORR of 12%. Three patients showed complete response and nine patients showed partial response [171]. Based on these results, in June 2018 the FDA gave approval of *pembrolizumab* administration to patients with advanced PD-L1 positive cervical cancer, who experienced progression during or after chemotherapy [88]. Another anti-PD1 drug, *nivolumab* have also been tested and studies show divergent results from complete response in combination with radiotherapy [172] to poor results [173]. Several immune checkpoint inhibitors are currently being investigated in ongoing studies – often as combination therapy [174].

Given the fact that HPV vaccine has proven so effective against cervical cancer development, several studies focus on vaccination strategies. A *Listeria monocytogenes* (Lm) vaccine containing HPV-16 E7 oncoprotein “*Axalimogene filolisbac* (ADXS11-001)” was tested in a phase II study of 109 patients with advanced cervical cancer alone or in combination with chemotherapy. The two groups showed similar survival (17,1% and 14,7% respectively) [175]. Other studies with ADXS11-001 (50 patients) have shown severe treatment-related adverse events (TRAEs) in 43% of the cases and only 2% ORR [176]. A combination of *pembrolizumab* and ADXS11-001 have also been tried and showed ORR of 40% but TRAEs grade 3-4 in 36% of cases [177].

Despite many attempts, ICI has not shown the promising result as hoped and up to 55% of patients experience severe adverse events when ICIs are used [178]. Speculations on the

interplay of multiple different mechanisms in the tumor microenvironment have been suggested. Vaccine, screening programs and many ongoing ICI trials are all steps to gain new insight and potentially new strategies since cervical cancer still remains undefeated. Therefore this research can hopefully help elucidate the complex mechanisms and interplay of HPV and the immune system.

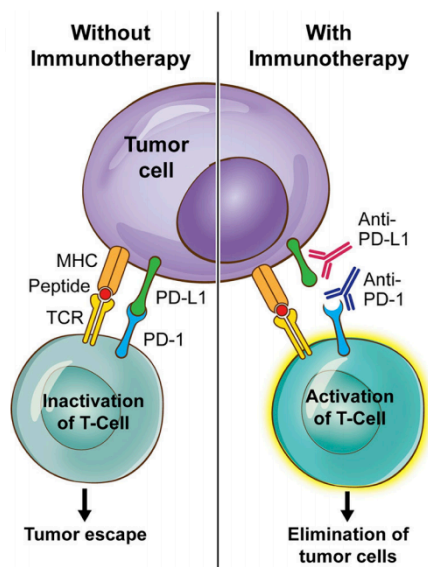


Fig 11: Role of programmed death-1 in immune regulation

PD-1 regulates T cell activation and by blocking its interactions with PD-L1 inhibition of T cell activation and their effector functions are blocked. PD-1 blockade by monoclonal antibodies (nivolumab and pembrolizumab) can provoke a peripheral antitumor immune reaction. Figure adapted from [179].

COLLABORATORS AND FUNDING

As co-supervisors we had the privilege to collaborate with Susanne Krüger Kjær, Professor, consultant at Rigshospitalet and the Danish Cancer Society Research Center, Copenhagen, Denmark. Her unique knowledge of the HPV has been of great importance also in terms of designing the study. As clinical collaborators we were honored to collaborate with Kirsten Marie Jochumsen, PhD., Associate Professor, senior consultant, Department of Gynecology and Obstetrics, Odense University Hospital, Odense, who made it possible to collect patient samples from the cancer patients. For all the cervical neoplastic patients we were fortunate to have Benny Kirschner, Clinical Associate Professor, senior consultant, Department of Gynecology and Obstetrics, Hvidovre Hospital, Hvidovre who collected both consent and patient material.

The Danish Cancer Society has awarded 1,000,000 DKK from the “Knæk Cancer” call, Danish Technical University has awarded 740,000 DKK. Aleris-Hamlet research foundation has awarded 105,000 DKK. No financial compensation was offered to the donors for participating in this study.

SPECIMEN COLLECTION

In this study we obtained liquid-based cytology (LBC) samples, cervical biopsies, and peripheral blood samples from three groups of Danish women; healthy individuals, patients diagnosed with high grade cervical intraepithelial neoplasia (CIN3) and patients diagnosed with cervical cancer at various disease stages.

From each individual in the study, we obtained:

- 2 LBC samples from the cervix
- 2 fresh cervical tissue biopsies (min.2 x 2 mm)
- 1 peripheral blood sample (50 mL)

The healthy individuals were recruited from Aleris-Hamlet private hospital, Søborg, Denmark. They were included in this project if they underwent hysterectomy for reasons unrelated to HPV. Healthy individuals with a history of cervical neoplasia were excluded from the study. The LBC and the biopsies were all performed during already planned surgery to minimize discomfort for the patient. If possible, the blood sample was taken prior to or during surgery.

Patients with CIN 3 were recruited from Department of Gynecology and Obstetrics, Hvidovre Hospital, Hvidovre. Biopsies and LBC samples were collected prior to having a cone biopsy, to avoid bleeding. The tissue collection procedure was made in agreement with the local pathologists and did not interfere with analyses of the cone biopsy. If possible, the blood samples were taken during that same consultation and were drawn from the cubital vein in the elbow joint.

Patients with cervical cancer were recruited from Department of Gynecology and Obstetrics, Odense University Hospital, Odense. Two LBC samples and two biopsies were collected prior to assessment of the cancer stage in full anesthesia.

I informed, drew blood, and obtained biopsies myself on all the healthy controls, since I also performed the hysterectomy. The neoplasia patients were all included at Hvidovre Hospital by senior consultant Benny Kirschner and samples were immediately after picked.

When a cancer patient was relevant for the study senior consultant Kirsten Jochumsen made sure to inform me and I then went to Odense on the day of examination. Here I informed the patient, drew blood, and went to the operation room and obtained all the biopsies myself.

All individuals were also given written information about the project and “Deltagerinformation” (participant information).

All specimens were marked with a study number and delivered to the laboratory at the Technical University of Denmark (DTU), Lyngby, Denmark. All specimens were handled by me, all within 6 hours prior to sampling.

The material will be kept anonymized for 8 years in a research bio bank, after which time it will be destroyed. If desired by the participant, the material can be destroyed at an earlier stage. All specimens were and will be used for this present study only.

The study was approved by The Danish Data Protection Agency. The study took almost four years and did not involve further visits or medical contact for the individuals included in this study.

We managed to include 57 participants. 24 healthy individuals, 16 patients with CIN 3, 17 patients with cervical cancer. 4 samples (two healthy, one CIN3 and one cancer) were used to test the experimental procedures. Three CIN3 patients were excluded because of missing one out of three patient specimens. In total we choose only to analyze specimens with cell count $>1 \times 10^4$. That ended up being 10 healthy, 10 neoplasia and 15 cervical cancer patients for analysis.

STUDY POPULATION

Inclusion criteria: Female participants, >18 years at inclusion and with informed consent.

Exclusion criteria: Patients on immunosuppressive treatment, e.g., larger doses of prednisolone ($>5\text{mg/ day}$), patients with previous cancer of any kind, controls with previous cervical neoplasia.

From all participants we also collected the following information on clinical parameters from the medical record: diagnosis, other diseases (especially immune mediated diseases), and medication. The information was used to select for the inclusion and exclusion criteria before asking the patient for signed consent. Additionally, following the signed consent, the participants was asked to fill in a questionnaire related to obtain information on -age, health status, history of smoking, and other environmental factors.

THE PROCESSING OF SPECIMENS

All samples both cytology and biopsy were collected from the transformation zone. The cytology and biopsies were only collected by medical doctors. Flow and sample collection is shown in (Fig. 1).

HPV genotyping

The first liquid-based cytology (LBC) samples were collected from each patient using the Cervix-Brush (Rovers) to collect cells from both endo- and ectocervix. The brushes were kept at room temperature (RT) in 10 mL SurePath™ Collection Vial (BD). Samples were sent to Department of Pathology, Hvidovre Hospital, Denmark, for HPV typing with Anyplex II HPV28 detection real-time PCR or BD Max DNA extraction, which can detect 19 high-risk and 9 low-risk HPV types.

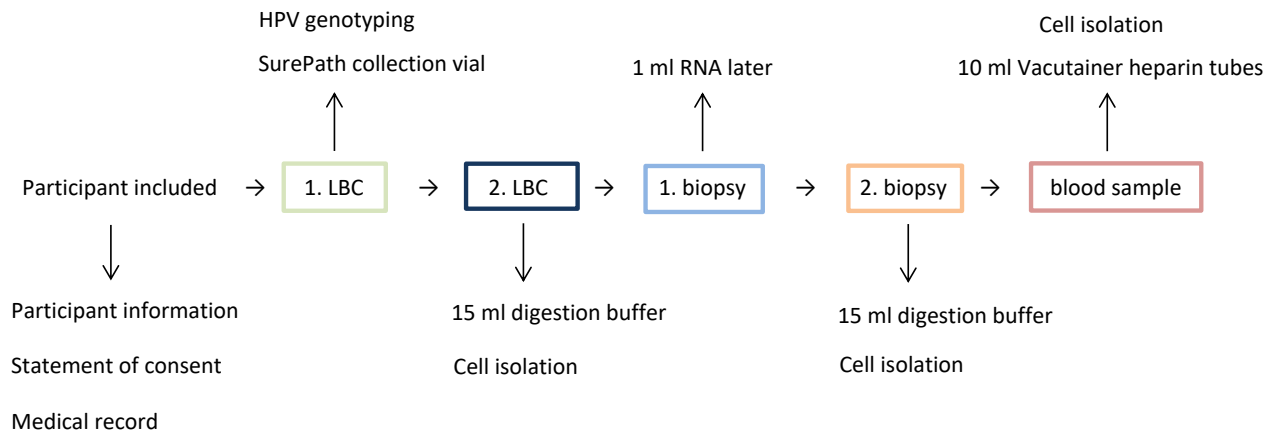


Fig. 1: Sample collection

Before sample collection oral and written information and consent was given. Liquid based cytology (LBC), biopsy and blood samples were all obtained on the same day.

Cell isolation

The second LBC was also obtained with Cervix-Brush (Rovers). These brushes were kept on ice in 15 mL digestion buffer (1:10 Hank's Balanced Saline Solution (HBSS) 50X + 15mM 1,5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) until cell isolation that was done the same day as sample collection. To isolate the cells, the brushes were rinsed, and the suspension filtered through a cell strainer. Cells were centrifuged 1500 rpm at 4°C for 5 min. Afterwards the cells were resuspended in PBS and counted on a NucleoCounter SCC-100TM (Chemometec), using the SCC-CassetteTM. The measurement detection range lies between 1×10^4 Cells/mL to 2×10^6 Cells/mL. After additional centrifugation of the remaining cell suspension, the cells were resuspended in 1 mL of freezing media 10% DMSO (Dimethyl Sulfoxide) Hybrid-Max (Sigma-Aldrich) a polar aprotic solvent for cryoprotectant vitrification agent to protect cells from ice crystal induced mechanical injury and 90% fetal calf serum (FCS) GibcoTM qualified, New Zealand. $5-10 \times 10^6$ cells/vial and distributed in cryotubes. The vials were slowly frozen by 1°C/min in freezing boxes placed at -80°C. Next day, the vials were transferred to a -180°C nitrogen tank for long-term storage until used for further analysis.

Two fresh cervical tissue biopsies were collected from each participant. Minimum 2 x 2 mm.

The first biopsy was harvested and directly submerged in 1,5 mL RNAlaterTM Stabilization Solution (ThermoFischer Scientific) to protect cellular RNA and stored at room temperature no longer than 6 hours and then frozen in -80-degree freezer in freezing box (-1 degree/min) and then after 12-24 hours the vials were transferred to -180 nitrogen tank and stored until further analyzes.

RNA isolation

The second biopsy was collected in the same digestion buffer as cytology, 7 mL and stored on ice. Cells were isolated within 6 hours from sampling. Using scalpel and tweezer, the biopsies were cut into smaller pieces which were transferred to gentleMACS C-tubes and centrifuged for homogenization for 1 min using gentleMAC Dissociator (Miltenyi Biotec). To avoid cell clumping

a enzymatic digest 2,5 mL (2U/ml) DNase I from bovine pancreas (Sigma-Aldrich, Merck) was added to the samples. Since this always is accompanied by rupture and lysis of some cells, DNA is released from these cells into the culture and dissociation medium respectively.

After 1-hour incubation at 37°C, the samples were further dissociated with gentleMACS for 1 min. The suspension was then filtered through a cell strainer, centrifuged 1500 rpm, in RT for 5 min, resuspended in PBS and counted. From this step the cells were treated as described for the cytology and blood samples.

Blood samples

Peripheral blood samples were collected in five 10 mL Vacutainer heparin tubes and kept at RT up to 6 hours before cell isolation. The blood samples were poured into 50 mL blood separation filter tubes (Falcon Leucosep) saturated with 15 mL Lymphoprep™ density gradient medium 1.077g/mL, (STEMCELL, cat. 07851), and phosphate saline buffer (PBS) was added for a final volume of 50 mL. After centrifuged at 1000 G at RT for 10min, low acceleration and deceleration to separate lymphocytes and peripheral mononuclear cells from erythrocytes by means of density gradient centrifugation, the buffy coat was carefully poured into a new tube centrifuged 1500 rpm at 4°C for 5 min and washed with PBS. Afterwards the cells were resuspended in 10 mL PBS, transferred to a new 15 mL tube and counted on a NucleoCounter (Chemometec).

To determine HLA type, $2 \cdot 10^6$ cells in suspension per donor/patient was transferred to an Eppendorf tube and centrifuged at 10.000 G for 5 min, 5 °C. The supernatant was discarded, and the sample was stored until all samples were collected and then sent off for HLA typing at IMG M Laboratories GmbH, Lochhamer Str. 29a, 82152 Martinsried, Germany. After additional centrifugation of the remaining cell suspension, the cells were resuspended and from this step the cells were treated as described for the cytology and biopsy samples.

Characterization of immune infiltration In High-grade Cervical
Intraepithelial Neoplasia and Cancer

Authors:

Dorthe Blirup Snejbjerg¹, Mohammad Kadivar¹, Marie Viuff¹, Stine Kiær Larsen², Benny Kirschner³, Kirsten Marie Jochumsen⁴, Jesper Bonde⁵, Susanne Krüger Kjær⁶, Sine Reker Hadrup^{1*}.

Affiliations:

¹ Department of Health Technology, Technical University of Denmark, Lyngby, Denmark

²The National Center for Cancer Immune Therapy, Herlev Hospital, Herlev, Denmark

³ Department of Gynecology and Obstetrics, Hvidovre Hospital, Hvidovre, Denmark

⁴ Department of Gynecology and Obstetrics, Odense University Hospital, Odense, Denmark

⁵ Department of Pathology, Hvidovre Hospital, Hvidovre, Denmark

⁶ Danish Cancer Society Research Center, Copenhagen, Denmark

*Correspondence

Corresponding Author Sine Reker Hadrup, sirha@dtu.dk

ABSTRACT

Human Papilloma Virus (HPV) is the primary cause of cervical cancer. It is evident that the immune system plays an important role for the persistence of the viral infection, oncogenic transformation, and cancer development. Patients with advanced, recurrent, or metastatic cervical cancer still have poor prognosis and improved treatment strategies are needed. The overall goal of this project was therefore to investigate the immune infiltration, the microenvironment, and alterations of the local and systemic immune system in patients with high-grade cervical intraepithelial neoplasia (CIN3) and cervical cancer compared to healthy individuals.

Liquid based cytology, biopsies and blood samples were collected from 10 healthy individuals, 10 patients with CIN3 and 10 cervical cancer patients of different stages. Flow cytometry was performed using phenotypic markers selected to characterize CD8, CD4 T cells, myeloid cells, and their individual subsets. The main observation was detection of a late differentiated immune profile among CD8 and CD4 T cells in the cancer group. The frequency of terminally activated or even exhausted CD8 T cells was more abundant in CIN3 lesions and even further increased in the cancer patients, compared to the healthy individuals.

This was found in both biopsy and cytology specimens, but not in peripheral blood. Cells from biopsy and cytology - hereafter defined as "tissue" were evaluated, and strikingly these specimens displayed identical signatures, hence suggesting cytology as a useful alternative to biopsies for evaluation of immune signature in cervical neoplasia cancer. Importantly, in this study we demonstrate increased frequency of activated and terminally differentiated T cells in both cytology and biopsies from cancer patients. This tendency was also observed in patients with CIN3, although not as pronounced. Further, in the myeloid compartment we observed lower levels of classical antigen presenting cells, while myeloid populations in general expressed higher levels of PD-L1, compared to the same cell subsets in the healthy individuals. Taken together these data suggest that immune recognition plays an active role in shaping the neoplastic development, and that immune inhibitory mechanisms emerge during cancer development.

INTRODUCTION

Human Papilloma Virus (HPV) is the most common viral infection of the female reproductive tract with preference for epithelial cells and it is furthermore the cause of virtually all cases of cervical cancer [1]. Cervical cancer is the fourth most common malignancy diagnosed in women worldwide, with an estimated 604,127 cases (3.1% of all cancers) and 341,831 deaths (3.3% of all deaths caused by cancer) reported in 2020 [2][3][4], hence HPV driven cervical cancer is still a major health challenge. The overall incidence of cervical cancer in Europe is 9.9 per 100,000 women despite HPV vaccination, screening programs and an advanced healthcare system [4][5]. While most infections with HPV are cleared by the immune system within 6-12 months, a minor percentage (10-12%) remains as a persistent infection [6]. The HPV infection can be associated with neoplastic changes of the epithelium together with an increased risk of T cell dysfunction and the development of carcinomas. This persistent infection starts to express viral oncogenes E6 and E7, leading to increased genomic instability, accumulation of somatic mutations, and in some cases integration of HPV into the host genome [7].

The overall five-year survival of cervical cancer remains around 66% (cancer.org) and treatment for recurring disease is still challenging [8]. As a result, novel therapeutic strategies against cervical cancer are strongly needed. The HPV vaccines target the major capsid protein L1 but once the virus is internalized via the endocytic route and trafficked to the endosome, low pH will trigger a disassembly of the capsid and vaccines will no longer have the intended effect [9].

The immune system plays a key role in the control of the infection and especially cytotoxic CD8 T cells are of vital importance in the clearance of viral infection as well as in killing of cancerous cells [15]. It has become evident that one of the reasons for the immune systems failure to eliminate cancers, may be due to an alteration of T cell phenotype and thereby hampered cell function [10]. The immunophenotype of exhausted T cell has been increasingly in focus in cancer patients since the immune system can be used to eradicate cancer by selective recognition of virus-associated tumor cells or by releasing the inhibition of the cytotoxic CD8 T cells allowing them to target neoplastic cells [11][12].

Solid tumor immunotherapies, such as immune checkpoint inhibition (ICI) using programmed cell death protein 1/programmed death ligand-1 (PD-1/PD-L1), are some of the most common molecules targeted [13], and has led to FDA approval of two new drugs. PD-1 is a immune suppressive molecule in the B7-CD28 family, which regulates T cell activation [14] and PD-L1 is a transmembrane protein, which can be expressed on myeloid or cancerous cells in the tumor microenvironment (TME) [10][15]. The clinical response to ICI is positively correlated with tumor neo-epitope load and since cervical cancer is the eighth highest in mutational load across cancer types, it enables the potential for successful use of ICIs as therapeutic target [16][17]. Furthermore, HPV-associated cancers express viral antigens and previous studies on HPV driven head and neck squamous cell carcinoma (HNSCC) show high titers of serum antibodies against HPV16 E2, E6 and E7, indicating immunogenicity and persistence of these antigens. Another study has reported that 8% of cervical cancers present genomic instability and therefore may also respond well to ICI [18].

These features all together (tumor inflammatory state, expression of PD-L1, high mutational load, immunogenicity, antigen persistence and genomic instability) all support the rationale for using ICIs [19]. Despite such features, ICI has still not shown the expected results in the treatment of cervical cancer with overall response rates (ORR) of only 12-26% and therefore many efforts are now employed to unveil this lack of response [8][11][20]. Expression of PD-L1 has shown to be strongly associated with HPV infection, both in the tumor but also in the surrounding inflammatory tissue [21]. It has been proposed to apply the expression of PD-L1 as a biomarker for the selection of patients for ICI treatment and as a threshold for timing of treatment [22].

Despite the HPV vaccines, screening programs and the many ongoing ICI trials, cervical cancer remains a serious problem and new insight and strategies are needed. Therefore, the aim of this study was to characterize the alteration of local and systemic immune infiltration and the microenvironment in patients with CIN3 and cervical cancer compared to healthy controls. Phenotypic markers characterizing both CD8 and CD4 T cell subsets and their state of activation, early/late differentiation, and exhaustion were analyzed in cervical tissue and blood.

Furthermore, we investigated the innate immune responses and the myeloid cell compartments in both cervical cancer and neoplasia, to determine a potential activation signature and phenotypic changes related to HPV infection/oncogenic transformation and cancer development.

Finally, we evaluated the potential of cytology, i.e., a minimally invasive cervical brush method, compared to conventional biopsies from the cervix to determine immune signatures. The 'cytology' method collects cells from both the endo- and ectocervix in contrast to more invasive biopsies which also include subepithelial connective tissue. Therefore, it was important to determine if the same immune infiltration could be detected using cytology vs. biopsies at different disease stages.

RESULTS

DETECTION OF LATE DIFFERENTIATED T CELL PHENOTYPE PROFILE

The goal was to analyse which T cells were infiltrating the tumor and/or circulating in the peripheral blood of patients with cervical cancer, CIN3 and healthy individuals. The phenotype panel was designed to examine T cell differentiation stages and local infiltration, as well as myeloid populations that may impact cancer immunogenicity.

Characterisation of CD8 T cells: When analysing the CD8 T cells, it was evident that the phenotypic characteristic in the cytology specimens resembled that of the specimens from biopsies. The analysis of blood demonstrated unique immune phenotypic characteristics associated with cancer being different from the tissue.

Looking at the manually gated CD8 T cell phenotypes in biopsy and cytology specimens (Fig. 1a), naïve cells were hardly present in the tissue compared to the circulatory system. On the other hand, most cells were classified as T_{EM}, and a smaller population of T_{EMRA} (Fig. S5). The CD8 T_{EM} is overall increasing from healthy to cancer cases for all three sample types, and the proportion of increase from healthy individuals to patients with neoplasia and cancer, seems to be the same. All groups had a majority of T_{EM}, and fewer naïve CD8 T cells, but since aging is associated with loss of naïve T cells this correlates with the age distribution among the three groups. The youngest women were found among the patients with CIN3 ($32.9 \pm 5,5$), and the eldest among the cancer patients ($60.0 \pm 13,6$) and the healthy individuals ($45.9 \pm 8,6$) (Fig. S2, Table S3).

The ratio between T_{EMRA} and T_{EM} for both CD8 and CD4 T cells is shown in (Fig. S3). The ratio of T_{EMRA}/T_{EM} in cancer patients for both CD4 and CD8 T cells were lower in all three compartments. Most evident is CD8 in cytology specimen where the decrease is significant.

Interestingly, the ratio between T_{EMRA} and T_{EM} cells in blood was decreasing with disease progression from healthy individuals to pre-cancerous CIN3 and cancer, which indicates less terminally differentiated effector cells (Fig. S3). When analysing each specific marker individually (Fig. 1b) the main noticeable differences between healthy individuals and patients with neoplasia and cancer, were the activation/exhaustion phenotype profile found in both biopsy and cytology specimens, and importantly, the observation that these two sources for tissue cells displayed the same characteristics.

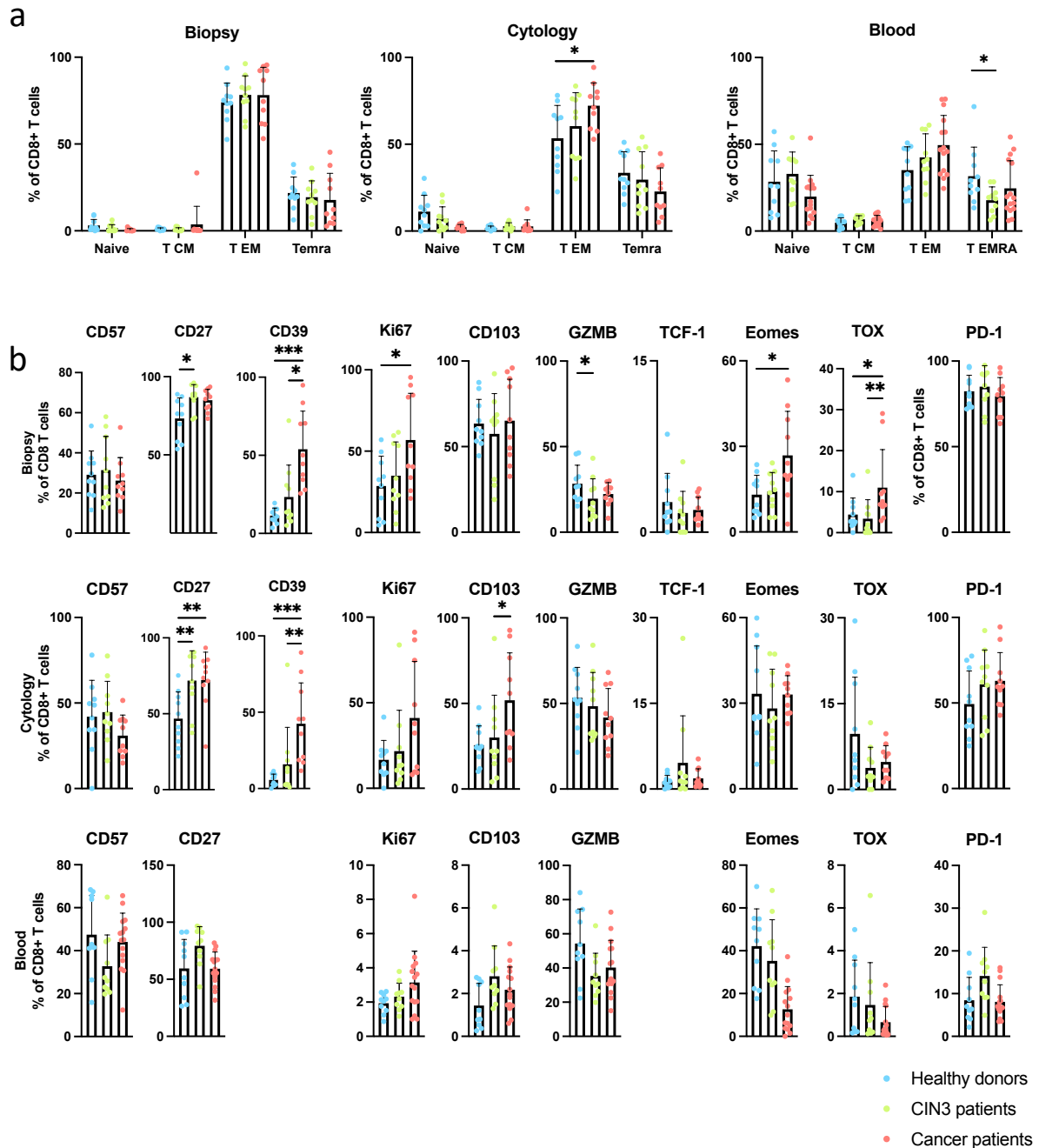


Fig 1: CD8 T cell phenotypes in biopsy, cytology, and blood

Healthy donors (n=10), CIN3 patients (n=10) and cancer patients (n=10, except blood n=15) were included. **a** Distribution of naïve, T_{CM}, T_{EM}, T_{EMRA} subsets in CD8 T cells for biopsy, cytology, and blood. **b** Frequencies of all CD8 T cells expressing various differentiation markers.

Plots show mean ± SD (Kruskal-Wallis uncorrected Dunn's test, *: p<0.05, **: p<0.01, ***: p< 0.001).

The similarity is also observed regarding the frequency of CD8 T cells that expressed both CD39 and CD27 in both cytology and biopsy specimens. They showed significantly increase in the cancer group.

CD27 being present on naïve, T_{CM} and at times on T_{EM} indicating early state of activation opposite expression of CD57 increases with antigen experience and an indication of the late differentiated T cell state - T_{EMRA}.

CD57⁺ CD8 T cells have the ability to be highly cytotoxic and not necessarily exhausted even though traditionally, CD57 has been reported to define T cells as terminally differentiated senescent cells [23][24].

Regarding Ki67 an increase was seen for all three compartments in the cancer patients, but this was only significant in cytology specimen. Increased numbers of CD103⁺ expression T cells are also observed in all three compartments, most clearly in biopsy and cytology specimens, indicating a role for tissue resident T cells.

Eomes, a T-box transcription factor that drives T cell differentiation and plays a role in initiation of T cell exhaustion programs [25] was more frequently observed in CD8 T cells from the cancer tissue.

The transcription factor TOX has been shown to be upregulated in tumor-specific T cells where it is a key regulator of other exhaustion markers [26][27]. In this study, TOX was also found to be expressed more frequently in the biopsies from cancer patients' CD8 T cells compared to the healthy individuals and the group with CIN3, indicating that more T cells entered T cell exhaustion programmes.

Looking at GZMB a decrease was observed from the healthy individuals to patients with neoplasia and thereafter cancer for all three compartments, but this was only significant, when comparing biopsies from healthy individuals with those in patients with neoplastic changes.

The overall significantly higher frequency of T cells expressing both CD39, CD27, Ki67, Eomes and TOX in biopsies and partly also in cytology specimens, indicates a signature of T cell activity and exhaustion. This is probably due to the CD8 T cells in the cancer patients have been exposed to chronic antigen stimulation throughout the preceding HPV infection. The signatures of T cell activity/exhaustion were observed both in cytology and biopsies specimens from cancer patients (Fig.1b).

One other surprising finding was the fact that TCF-1 was not upregulated in either of the three compartments and the frequency in cancer cases actually decreases. The transcription factor TCF-1 has been highlighted as a key indicator of a progenitor exhausted phenotypes with high proliferative capacity and ability to respond to immune checkpoint inhibition which contradicts our findings [28][29][30]. One explanation could be that the exhausted phenotypes are terminally differentiated, hence no longer expressing TCF-1.

One of the markers of interest was the immune checkpoint molecule PD-1. A higher frequency of PD-1 positive CD8 T cells was observed in cytology but not so evident in biopsy and the opposite

is seen in blood. This increase of PD-1 could indicate activated T cells and the decrease in cancer could indicate persistent TCR activation caused by the chronic infection and thereby less activation of T cells.

It seems like blood has a different profile regarding phenotype markers. This is seen for CD103, Eomes and TOX that does not resemble biopsy and cytology. The lower frequency of TOX⁺ CD8 T cells in the neoplasia group compared to cancer patients indicates activation from recent infection.

The analysis of the specimens regarding marker CD39 and TCF-1 in blood unfortunately failed and therefore they are not shown (Fig.1b).

Characterisation of CD4 T cells: When evaluating CD4 T cells we observe that CD4 T_{EM} is clearly enriched in the tissues, evident in both cytology and biopsy. In blood there is a more even distribution of Naïve, T_{CM} and T_{EM} but lower numbers of T_{EMRA}. There are no significant differences between healthy, CIN3 and cancer related to these subpopulations of CD4 T cells (Fig. 2a). We again find a significantly increase in CD39⁺, EOMES, and TCF-1 between healthy and cancer specimens for tissue, whereas a significantly decrease was observed for EOMES and TCF-1 in blood (Fig. 2b) resembling the phenotype signature of CD8 T cells. Again, cytology resembles biopsy for most of the T cell signatures observed.

CD103 frequency was significantly increased for CD8 T cells in cytology and for CD4 T cells we saw a tendency for increase in blood. CD57 frequency tend to decrease in tissue (biopsy and cytology) but is significantly increased in blood, which was also the case for CD8 T cells. Overall, the blood does not show the same activated phenotype signature as the tissue, suggesting that immune activation is primarily happening in the local tissue environment.

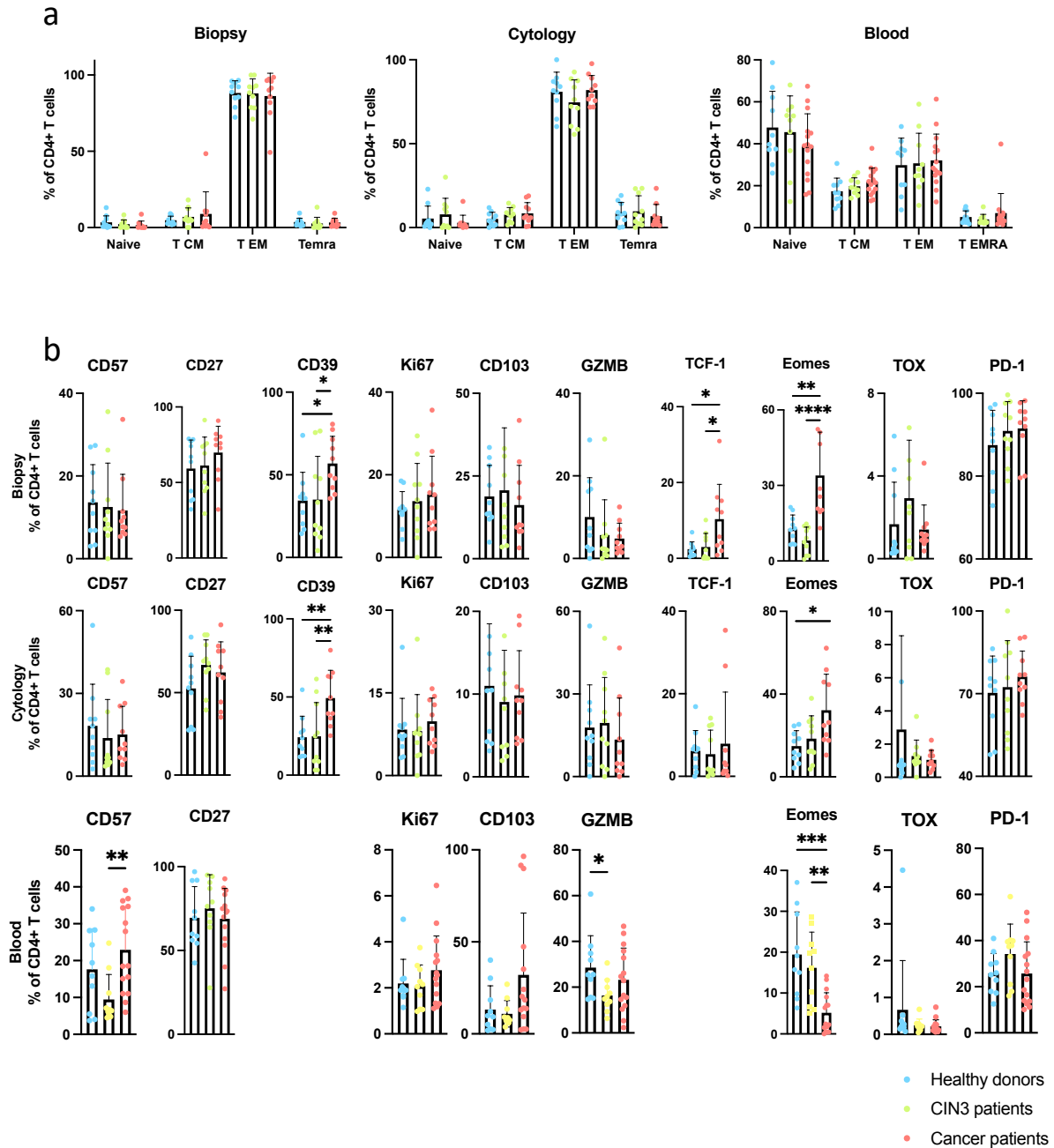


Fig. 2: CD4 T cell phenotypes in biopsy, cytology, and blood

Healthy donors (n=10), CIN3 patients (n=10) and cancer patients (n=10, except blood n=15) were included. **a**

Distribution of naïve, T_{CM}, T_{EM}, T_{EMRA} subsets in CD4 T cells for biopsy, cytology, and blood. **b** Frequencies of all CD4 T cells expressing various differentiation markers.

Plots show mean ± SD (Kruskal-Wallis uncorrected Dunn's test, *: p<0.05, **: p<0.01, ***: p< 0.001).

Multidimensional unsupervised clustering for the flow cytometry data: Using Uniform manifold approximation and projections (UMAP) the populations of CD8 and CD4 T cells in biopsy was visualized by clustering based on their relative co-expression of the parameters they were stained for (fig. 3a).

Interestingly, the UMAP plots revealed two populations of CD8 T cells which were mainly found in the cancer group. By using the unsupervised clustering algorithm FlowSom population 1 and 2 were defined (Fig. 3b). Pop 1 and pop 2 are both increased in cancer patients and very similar in phenotypic profile, classified by CD39⁺PD-1⁺CD103⁺CD27⁺ but pop 2 differs by also expressing CD57⁺. Pop 1 and pop 2 were named “early/late terminally differentiated T_{EM} cells respectively. Looking at the different cancer stages an interesting correlation between cancer stage and the frequency of CD8 T cells being more abundant in the tumor of the advanced cancer stage, were seen (Fig. 3c). The specific markers characterizing the different populations are shown in the heatmap (Fig. 3d). Population 1 and 2 are both highlighted with red boxes. The functional difference between the two exhausted populations remains unknown. The unsupervised clustering tool FlowSOM, illustrated by UMAP also find CD27 and CD39 as being significant but does not find Eomes or TOX but instead PD-1, CD103 and CD27.

Regarding CD4 T cells in biopsies (fig. 3e-h), the analyses revealed two clusters displaying a significantly different phenotypic profile. Pop 1 revealed a phenotypic profile defined as CD27⁺PD-1⁺CD57⁺ and was overall significantly decreased when comparing healthy individuals to cancer patients (Fig. 3f).

Pop2 was positive for CD27⁺CD39⁺PD-1⁺ and significantly increased, when comparing healthy individuals to patients with neoplasia and cancer (Fig. 3f). Noticeably, the markers defining these two clusters are identical to those seen regarding CD8 T cells, except CD103 which are not significantly upregulated in CD4 T cells. We did not observe any correlation of CD4 T cell subpopulation frequency and cancer stage.

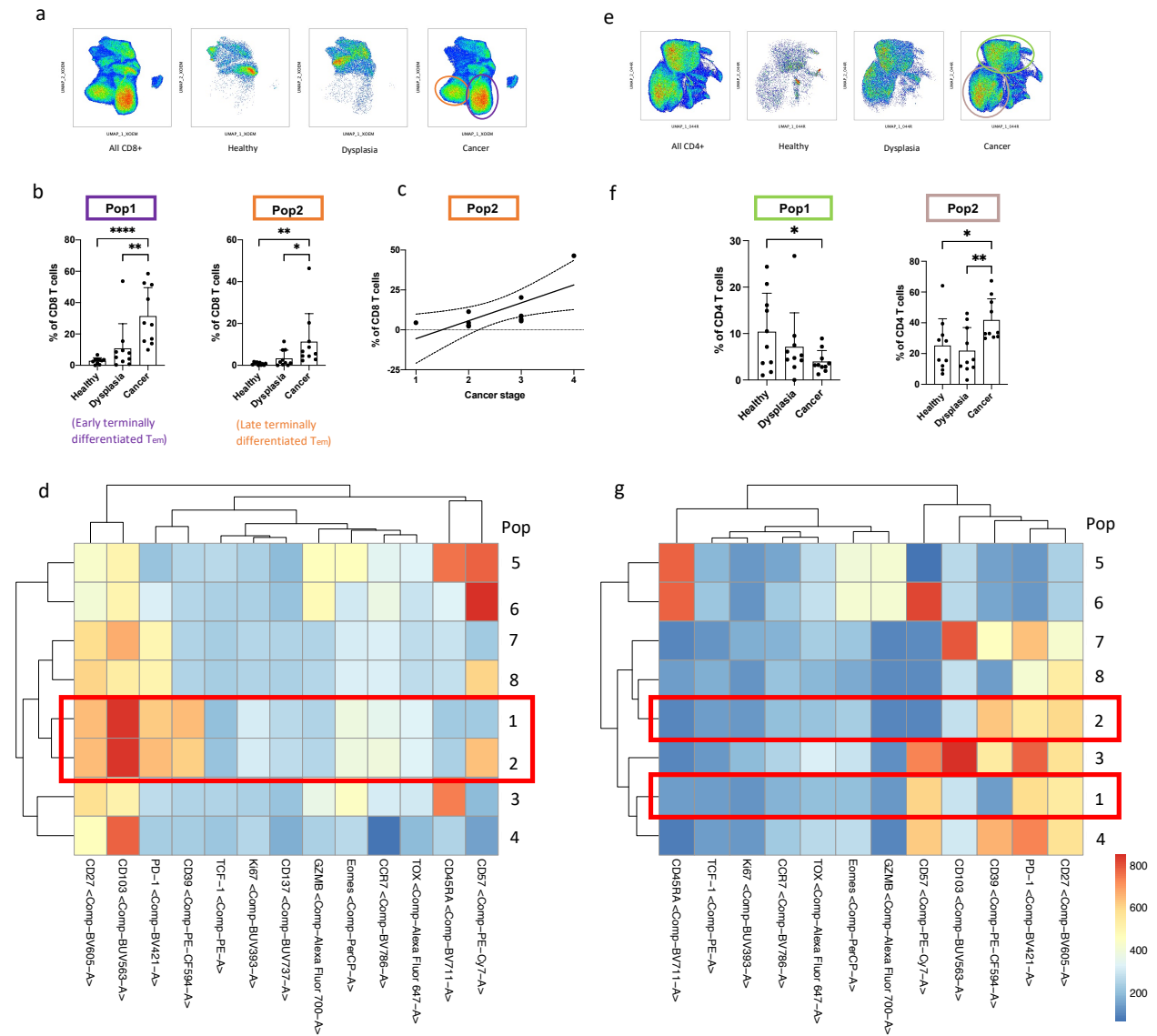


Fig. 3: T cells in biopsy a-d: CD8, e-g: CD4

a + e clusters generated using UMAP algorithm showing all CD8/CD4 T cells split into healthy, neoplasia and cancer. The two clusters that stand out are in the cancer group and therefore marked with red circles. **b + f** the same clusters using FlowSOM clustering self-organizing maps by colour. These two clusters are shown in purple (pop1) and orange (pop2) for CD8 T cells and green (pop1) and brown (pop2) for CD4 T cells. **c** shows the frequency of CD8 or CD4 T cells in each of the two significant populations split into healthy, neoplasia and cancer. **d + g** heatmap of all 8 populations against each marker. The relative expression level of all markers (Z-score) is coloured from blue (low) to red (high). The significant populations are marked with red boxes. For CD8 T cells: CD103 and CD27 are markers that characterizes all populations. Pop 1 increasing and positive for CD39, CD27, PD-1, CD103. Pop 2 increasing and positive for CD39, CD27, PD-1, CD103, CD57. Pop 1 and 2 are alike and differs only by CD57. For CD4 T cells: Pop1 decreasing and positive for CD27, PD-1, CD57. Pop2 increasing and positive for CD39, CD27, PD-1. Plots show mean \pm SD (Kruskal-Wallis uncorrected Dunn's test, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

SIGNATURES OF T CELL ACTIVITY/EXHAUSTION WAS OBSERVED IN CYTOLOGY AND BIOPSY SPECIMENS FROM CANCER PATIENTS

Analysing cytology specimens, the UMAP plot showed similar phenotypes characteristics as observed for biopsy specimens (Fig. 4a), particularly the clusters marked with red circles. By FlowSOM (Fig. 4b) they are marked as purple (pop1) and orange (pop2). Pop1 is expressing CD39⁺PD-1⁺CD103⁺CD27⁺ identical to the pop1 found in biopsy displaying an activated and early terminally differentiated phenotype. Pop2 in cytology is expressing CD39⁺PD-1⁺CD103⁺CD27⁺CD57⁺ which again correlates exactly with pop2 expressed in biopsy being late terminally differentiated and to some extent exhausted; however, any significant correlation between these subsets of CD8 and CD4 T cells and disease severity in the cancer group was not possible to determine (Fig. S6).

In the heatmap (Fig. 4d) all populations express CD103⁺, CD27⁺ as also seen in biopsy specimen. Interestingly the same markers defined the significant populations detected in specimens obtained by two different methods, cytology, and biopsy. The results indicate that when looking at CD8 and CD4 immune cells and their phenotypes we can obtain similar results from cytology as from biopsies specimen. This is important since cytology is a much less invasive method for the women and easier to perform for the clinician.

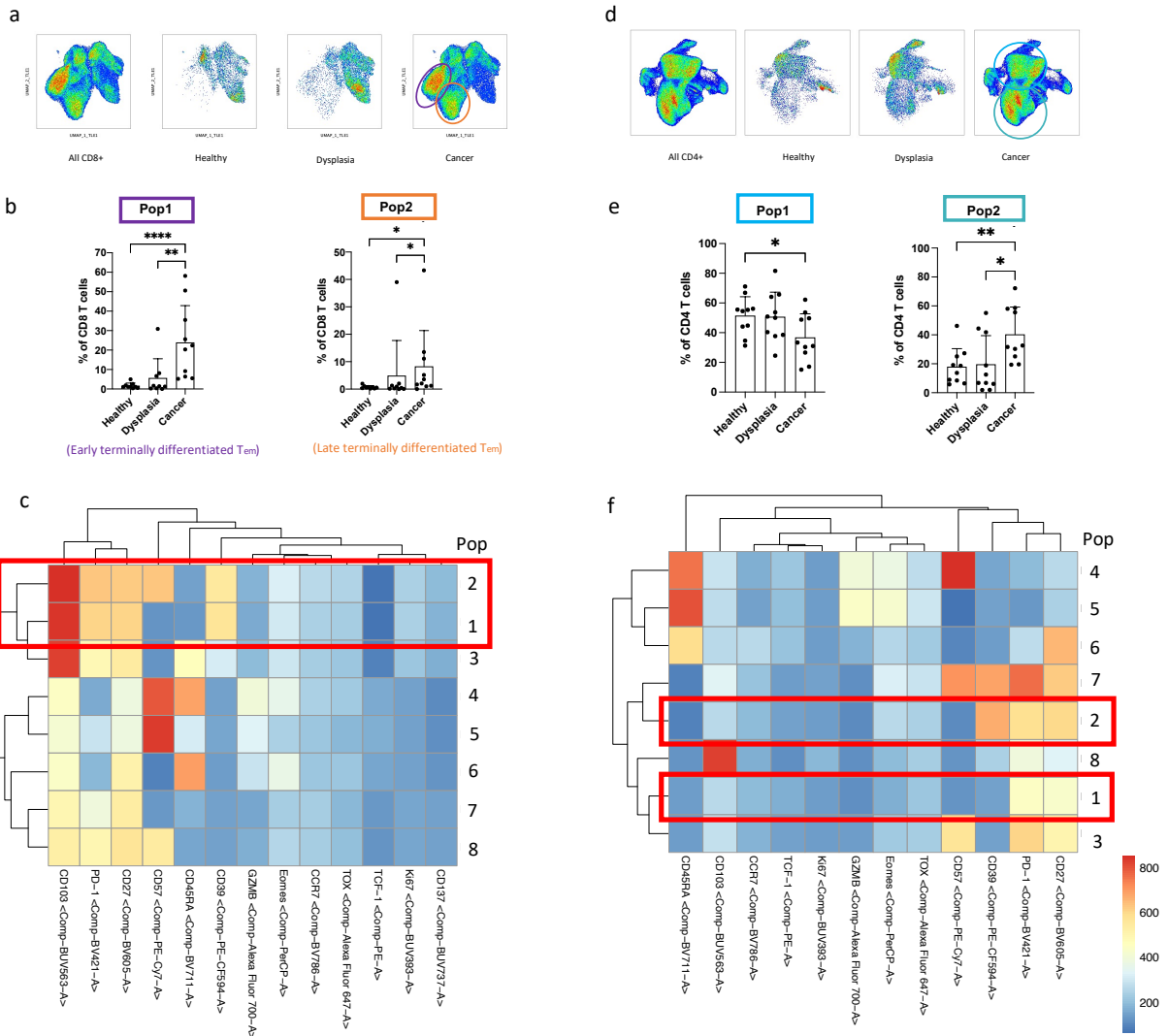


Fig. 4: T cells in cytology

a-c: CD8, d-f: CD4. **a + d** clusters generated using UMAP algorithm showing all CD8/CD4 T cells split into healthy, neoplasia and cancer. The two clusters that stand out are in the cancer group and they are marked with red circles. **b + e** the same clusters using FlowSOM clustering self-organizing maps by colour. These two clusters are shown in purple (pop1) and orange (pop4) for CD8 T cells and blue (pop1) and turquoise (pop2) for CD4 T cells. **c + f** heatmap of all 8 populations against each marker. The relative expression level of all markers (Z-score) is coloured from blue (low) to red (high). The significant populations are marked with red boxes. For CD8 T cells: CD103 the only marker that characterizes all populations. Pop 1 increasing and positive for CD39, CD27, PD-1, CD103. Pop 2 increasing and positive for CD39, CD27, PD-1, CD103, CD57. Pop 1 and 2 are alike being positive for PD-1⁺CD39 and differs only by CD57. For CD4 T cells: Pop1 decreasing and positive for CD27, PD-1. Pop2 increasing and positive for CD39, CD27, PD-1. Plots show mean ± SD (Kruskal-Wallis uncorrected Dunn's test, *: p<0.05, **: p<0.01, ***: p<0.001).

BLOOD DEMONSTRATES UNIQUE IMMUNE PHENOTYPIC CHARACTERISTICS ASSOCIATED WITH CANCER.

T cell differentiation in peripheral blood was also visualized using UMAP. Here again the two clusters of interest are marked with red circles. For CD8 T cells two populations of interest stand out; population 1 (red) is increased among cancer patient, whereas population 2 (orange) is decreased. Pop 1 being positive for CD103, CD27 and Pop 2 being positive for CD103, CD27 but also CCR7⁺ and CD45RA⁺. CCR7 and CD45RA could indicate that population 2 is naïve T cells and this correlates with the decreasing frequency (Fig. 5b). Overall, it is not the same picture when correlating blood with biopsy and cytology. Although not significant, it is worth mentioning that population 4 being PD-1⁺CD27⁺CD103⁺Ki67⁺ in blood resembles pop 1 for both biopsy and cytology and for pop 5 being PD-1⁺CD27⁺CD103⁺CD57⁺CD45RA correlates with pop 2 for both biopsy and cytology (Fig. 5c).

Analyzing CD4 T cells in peripheral blood, two small clusters stand out in their phenotypic profile (Fig. 5d). Pop 1 defined by expression of PD-1⁺ and CD103⁺ and significantly increased in frequency of CD4 T cells between healthy and cancer and between neoplasia and cancer. Pop 2 is defined by a cluster of CD4 T cells expressing PD-1⁺CD57⁺GZMB⁺, and when correlating the different groups, we see a significantly decrease between healthy individuals and patients with neoplasia; and between healthy and cancer patients (Fig. 2 e + f). Furthermore, when comparing these immune phenotypic profiles found in the blood to the clusters and T cell signatures from the tissue (both cytology and biopsy), the characteristic T cell populations are different in the two compartments, again supporting a strong infiltration or activation of the local immune environment.

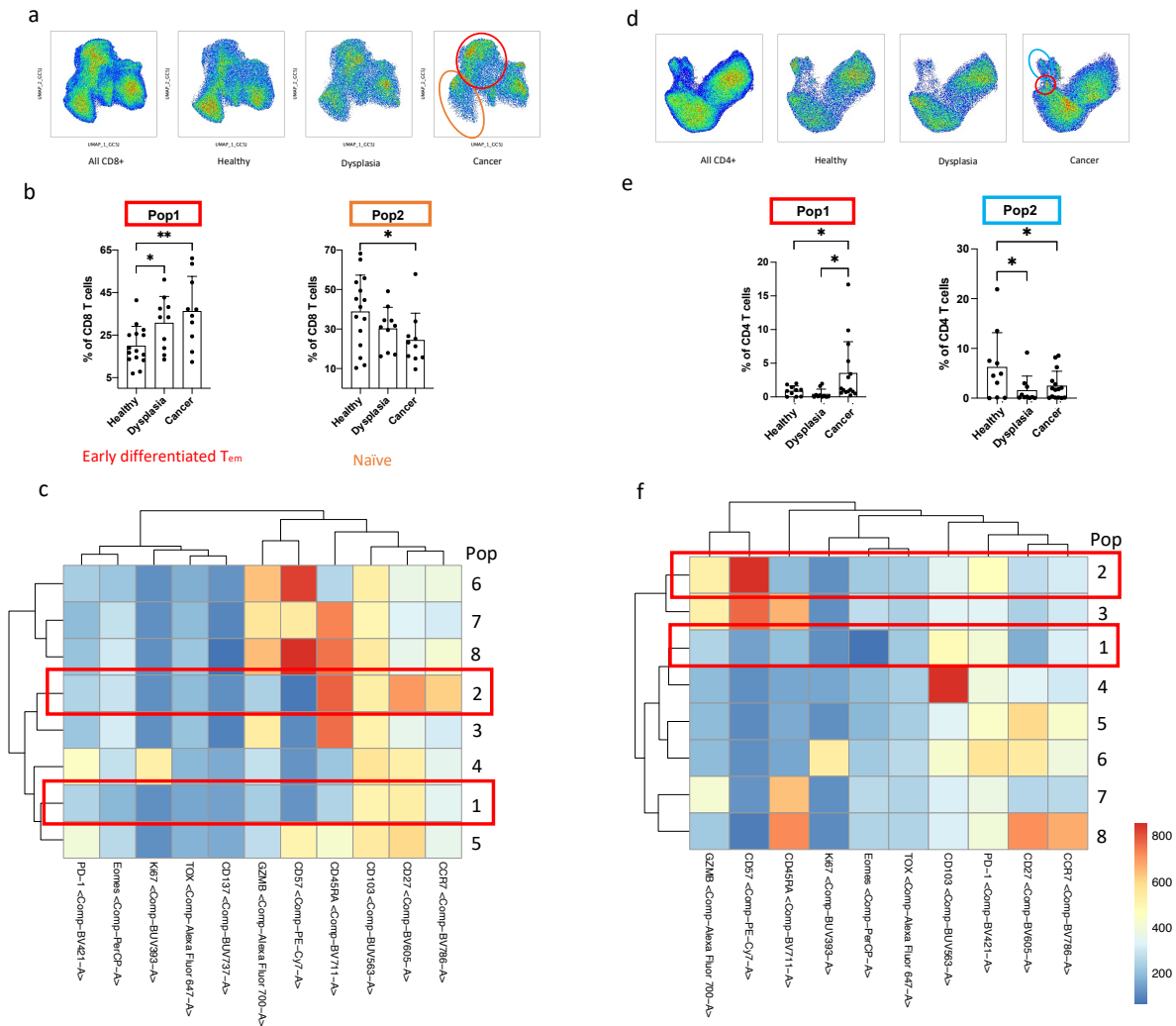


Fig. 5: T cells in blood a-c: CD8, d-f: CD4

a + d clusters generated using UMAP algorithm showing all CD8/CD4 T cells split into healthy, neoplasia and cancer. The two clusters that stand out are in the cancer group and they are marked with red circles. **b + e** the same clusters using FlowSOM clustering self-organizing maps by colour. These two clusters are shown in red (pop1) and orange (pop2) for CD8 T cells and red (pop1) and light blue (pop2) for CD4 T cells. **c + f** heatmap of all 8 populations against each marker. The relative expression level of all markers (Z-score) is coloured from blue (low) to red (high). The significant populations are marked with red boxes. For CD8 T cells: CD103 the only marker that characterizes all populations. Pop 1 increasing and positive for CD103, CD27 and Pop 2 decreasing and positive for CD103, CD27, CCR7, CD45RA. For CD4 T cells: Pop1 increasing and positive for CD103 and Pop2 decreasing and positive for CD57, GZMB, PD-1. Plots show mean \pm SD (Kruskal-Wallis uncorrected Dunn's test, *: p < 0.05, **: p < 0.01, ***: p < 0.001).

Table 1: Overview of which markers defining a specific population and the respective sample type – for both CD8 and CD4 T cells. All populations in CD8 T cells were positive for CD103. The markers found in CD8 T cells were the same for biopsy and cytology. The same was the case for CD4 T cells except CD57 being only positive in biopsy. All populations in CD4 T cells were positive for PD-1.

	Sample type	Significant population	Markers defining populations	Overall change from healthy to cancer
CD8 T cells				
	Biopsy	pop 1	PD-1, CD27, CD39, CD103	increase
		pop 2	PD-1, CD27, CD39, CD103, CD57	increase
	Cytology	pop 1	PD-1, CD27, CD39, CD103	increase
		pop 2	PD-1, CD27, CD39, CD103, CD57	increase
	Blood	pop 1	CD27, CD103	increase
		pop 2	CD27, CD103, CCR7, CD45RA	decrease
CD4 T cells				
	Biopsy	pop 1	PD-1, CD27, CD57	decrease
		pop 2	PD-1, CD27, CD39	increase
	Cytology	pop 1	PD-1, CD27	decrease
		pop 2	PD-1, CD27, CD39	increase
	Blood	pop 1	PD-1, CD103	increase
		pop 2	PD-1, CD57, GZMB	decrease

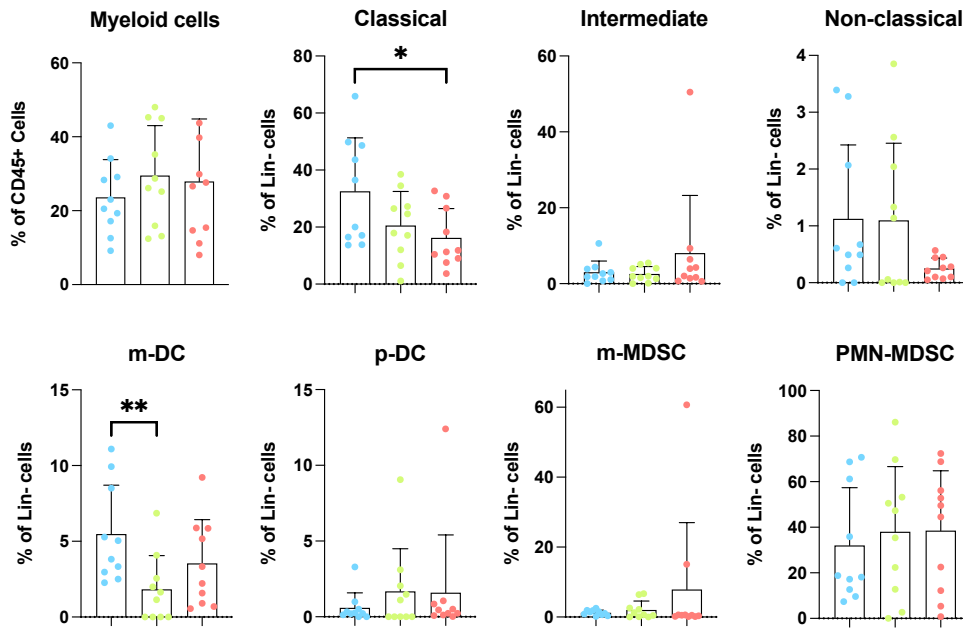
MYELOID CELLS SHOW SIGNS OF IMPAIRED FUNCTION AND SUPPRESSION

The immune profile of the investigated myeloid cells was somewhat different when looking at the cervical tissue, compared to the peripheral blood (PB). Tissue harbours both monocytes that have been recruited recently but also monocyte-derived macrophages which are often very heterogeneous and harder to define [31].

The same staining and gating strategy for biopsy, cytology, and blood samples (Fig. S7) was applied; hence, monocytes cannot fully be distinguished from macrophages or be certain of their pro/anti-inflammatory immune function. For the classical monocytes in biopsies (Fig. 6a) a significant reduction in the frequency of the CD14⁺ CD16⁻ monocytes/macrophages in the biopsies from the cervix was observed. A significant decrease in m-DC is seen between healthy and neoplasia. Moreover, non-classical CD14⁻CD16⁺ monocytes/macrophages were almost absent in the cervix of cancer patients.

In the cytology specimens, significantly fewer classical CD14⁺ CD16⁻ monocytes/macrophages was observed in the cancer group (Fig. 6b). That specific signature was also observed in the biopsy of the cancer specimens (Fig. 6a). The same tendency for non-classical monocytes/macrophages was also observed in the biopsy, although not significant, and no apparent difference was seen for the other subsets. An overall increase in PMN-MDSC was seen for both biopsy and cytology specimens which could indicate, a more immune inhibitory environment, especially in a subset of cancer patients.

A



B

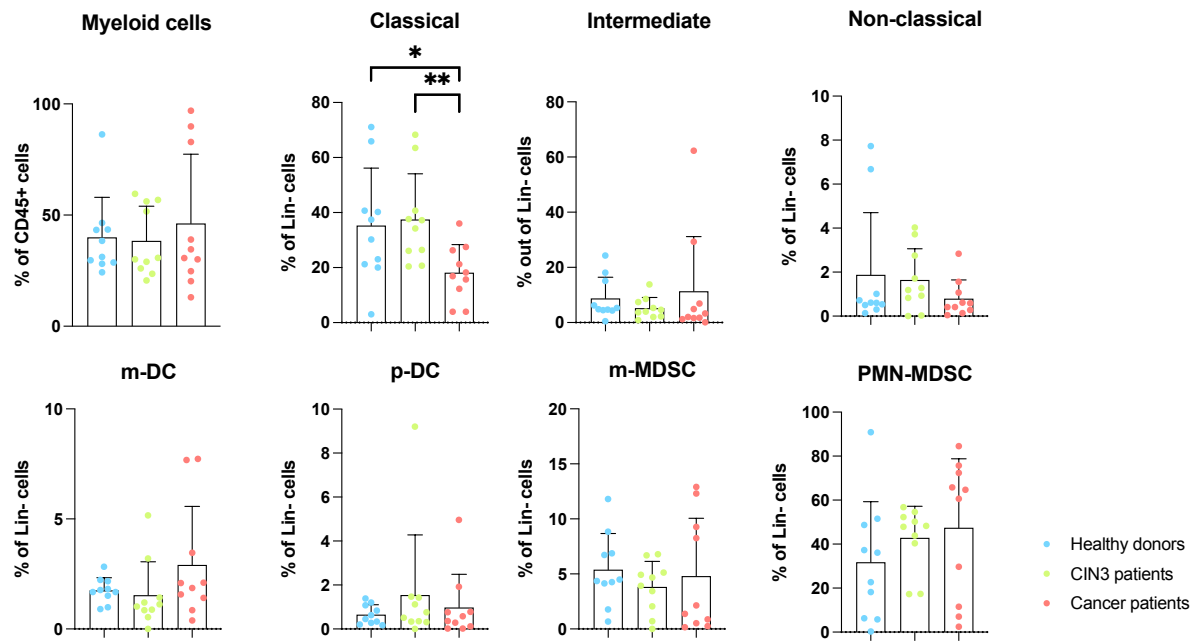


Fig. 6: Frequencies of myeloid cells and subsets out of all leucocytes in biopsies

(A) and in cytology specimens (B) of the cervix. Healthy donors (n=10), CIN3 patients (n=10) and cancer patients (n=10) were included. Plots show mean \pm SD (Kruskal-Wallis uncorrected Dunn's test, *: p<0.05, **: p<0.01, ***: p<0.001).

Looking specifically at the Langerhans cells (CD207⁺, CD1a⁺), no significant difference was observed in frequency of such cells present in biopsies from healthy, CIN3 and cancer patients, although numbers tended to decline in cancer (Fig. 7). This decrease correlates to previous studies, showing a reduction in Langerhans cells in HPV lesions. A reason for this observation, could be that they are functionally impaired, which may contribute to the persistence of infection [32]. We further investigated the level of expression of the immune checkpoint ligand PD-L1 on each of the myeloid subsets, based on the MFI of PD-L1 staining, comparing the different groups. We observed increased levels of PD-L1 expression on both classical, intermediate, non-classical monocytes, m-DC, p-DC and m-MDSCs in the cancer group, relative to either the healthy or the CIN3 population (Fig. 8). For the Langerhans population a trend for increase was likewise observed, whereas PMC-MDSC did not differ in their PD-L1 expression, which in general was low compared to the other myeloid subsets (Fig. 8). High levels of PD-1/PD-L1 have previously been shown to be expressed in cancer patients [15][19]. When comparing MFI (Fig. 8) with frequency of those same cells (Fig. S4), we also see an increase in the cervical cancer group for classical, intermediate, and non-classical monocytes but only significantly for the latter two. Indicating that such cell population are both increased in numbers and in their inhibitory capacity.

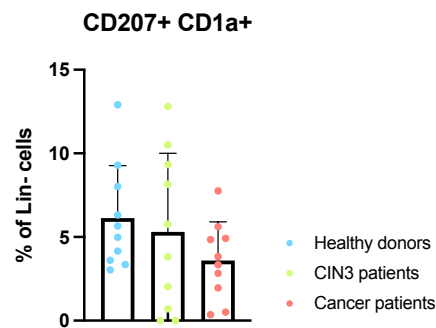


Fig. 7: Frequencies of myeloid CD207+CD1a+ subsets in biopsies.
Healthy donors (n=10), CIN3 patients (n=10) and cancer patients (n=10).

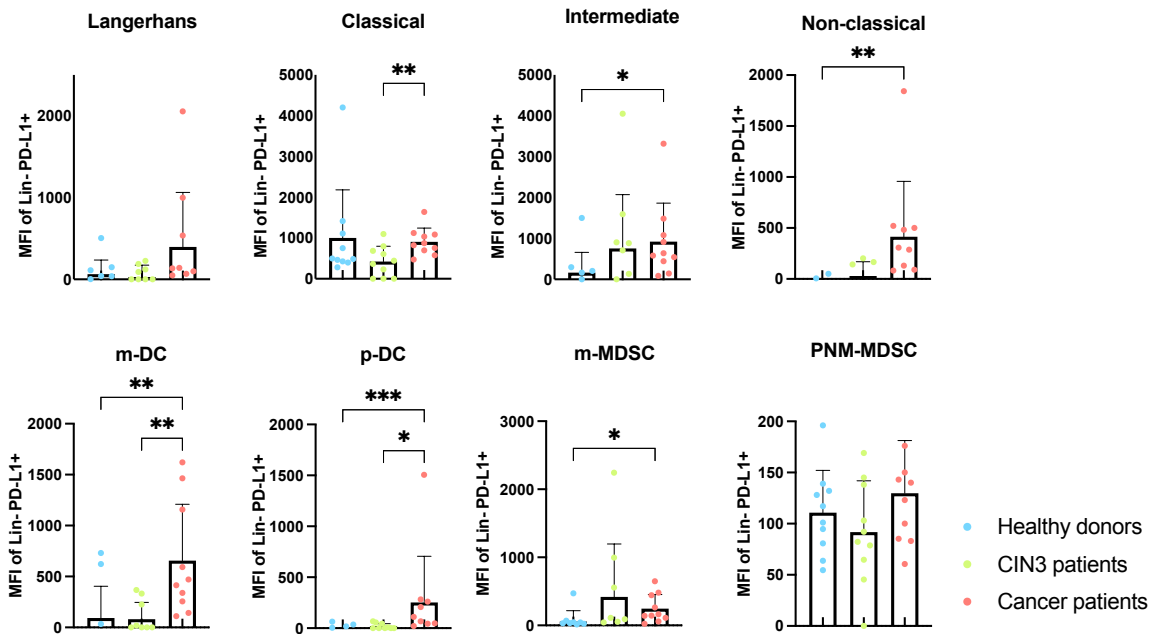


Fig. 8: Mean Fluorescence Intensity of myeloid cells and subsets out of all CD45⁺ Lineage⁻, PD-L1⁺ leucocytes in biopsies from the cervix. Healthy donors (n=10), CIN3 patients (n=10) and cancer patients (n=10) were included. Only samples with populations >20 cells were included. Plots show mean \pm SD (Kruskal-Wallis uncorrected Dunn's test, *: p<0.05, **: p<0.01, ***: p< 0.001).

MYELOID CELLS IN BLOOD

Out of all immune cells in the peripheral blood (CD45⁺), the cancer patients had statistically higher levels of myeloid cells (Fig. 9). Given their association with pro-inflammatory immune response, this suggests that the chronic inflammation has become systemic in the cancer patients.

However, when evaluating the individual subsets, only the mDCs and pDCs displayed a difference, with lower levels observed in cancer, while the numbers in CIN3 was either unaffected or increased compared to the group of healthy women (Fig. 9). For the other cell subsets, no difference was observed.

The role of pDCs is largely linked to anti-viral immunity, because they produce large amounts of type I-IFNs upon viral infection [33]. Thus, their presence in neoplasia patients indicates an ongoing anti-viral immune response, which is in consistence with the fact that these patients generally had current,

active HPV infection. The decrease seen for the cancer group could indicate less IFNs and thereby signs of a more chronic viral state or a pro-inflammatory state or perhaps even an exhausted profile.

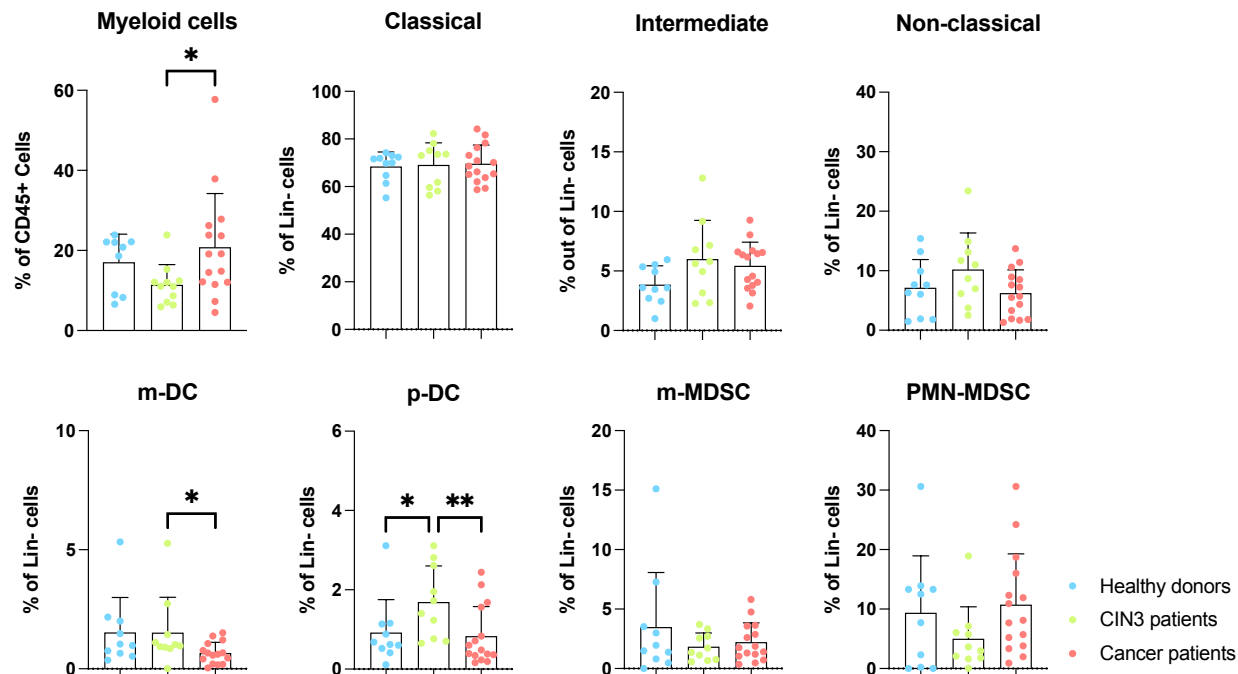


Fig. 9: Myeloid subsets in blood

Healthy donors (n=10), CIN3 patients (n=10) and cancer patients (n=15). Frequencies of myeloid cells and subsets out of all leucocytes. Plots show mean \pm SD (Kruskal-Wallis uncorrected Dunn's test, *: $p < 0.05$, **: $p < 0.01$).

DISCUSSION

Based on the presented results, it was shown that HPV-induced cervical cancers have a high degree of immune infiltration in the tumor, stemming from both innate and adaptive immune cell compartment.

Through immunophenotyping, we could demonstrate that late differentiated effector T cells (T_{EMRA}) were significantly increased in cancer patients, and it is possible that these dysfunctional T cells fail to promote tumor elimination.

These late differentiated effector T cells (T_{EMRA}) are also denoted “terminally differentiated”, “exhausted” or “dysfunctional” T cells. They are currently subject to heavy research and investigation to elucidate whether they can continue to elicit effective anti-tumor immunity, while maintaining proliferative and self-renewing capacities or if they are exhausted by upregulation of inhibitory markers or extrinsic chemokines/cytokines making them unable to execute immune effector functions and killing.

The patients diagnosed with CIN3 did not yet exhibit clear signs of chronic inflammation or T cell exhaustion, but some neoplasia displayed a phenotypic shift towards more activated/exhausted CD8 T cell phenotypes. This can be a sign of either the immune system of these patients mounting an immune response because the cells are activated, or a sign of T cell exhaustion and a less pronounced cytotoxic response towards the neoplastic changes - fighting but not necessarily defeating HPV. If the latter is the case, they might be candidates for immunotherapeutic interventions by reinvigoration of the T cells and thereby activating the patients' own immune defence. Potentially, they may clear the infection if capable of mounting the required response. Immune phenotype profiles from those CIN3 patients who did not show signs of an activation/exhaustion signature could be an indication of immune failure of recognizing the ongoing viral infection.

Since 30-40% of all CIN3 cases will progress into cancer if left untreated [34], the correlation of a potentially exhausted phenotype in those patients would be of valuable knowledge and perhaps in future studies potentially help to differentiate between follow-up and intervention.

In the present study, all CIN3 patients had a follow up 6 months after receiving a cone biopsy and eight out of ten were later tested HPV negative. One had adenocarcinoma in situ and had a hysterectomy and one was compound naevus. This shows a remarkably high efficacy after undergoing cone biopsy and it was not possible to distinguish between the neoplasia patients in our study in a prognostic manner.

The CIN3 patients have likely been infected with HPV for a prolonged period before the epithelial cells turned neoplastic, why the persisting HPV infection leading to the development of CIN is often defined as a chronic infection. However, our results show that the exhausted phenotypes have not yet been fully manifested in these patients, because no significant difference was observed between CIN3 patients and the healthy controls who were mainly HPV negative.

Looking at the phenotype distribution between Naïve, T_{CM}, T_{EM} and T_{EMRA} the decrease in naïve cells in cancer patients and the increase in CIN3 patients is probably due to the age difference with the cancer group being older. This is not surprising since CIN3 is discovered early among Danish women who enter the national screening program, whereas cervical cancer can take additional 10-20 years to develop [35] [36].

The compartmentalization of T cell differentiation based on surface markers is not linear but increasingly described as a back-and-forth development process. Further characterization of these chosen cell subsets is ongoing and new markers are constantly emerging. It probably is an interplay where T cells subsets are interconnected and can change and de-differentiate when necessary. But in this study, it was confirmed that these T cell subsets starting with naïve can differentiate into both T_{CM} and T_{EM} and upon constant antigen stimulation differentiate into T_{EMRA}.

By means of additional data in the future, T cell exhaustion could be evaluated as a marker to distinguish which CIN3 patients should be selected for earlier treatment and which patients (having already activated the right immune profile for cancer cell killing) should be selected for immunotherapy.

Surely a larger group of patients is required to validate these interesting findings, but it is an indication that the dysfunctional exhausted phenotypes, in particularly the CD57⁺ population (pop2), play a role in the reduced disease control.

One of the most prominent markers in this study was CD39 and the significantly increased frequency of CD39⁺ CD8 T cells in cancer patients. Tumor infiltrating immune cells have shown to express CD39, which is an enzyme involved in adenosine metabolism [37]. CD39 is upregulated as a response to recent antigen exposure and thereby signs of recognition and activation. CD39 also upregulates when exposed to tissue damage, hypoxia, chronic inflammation and is a key marker defining the terminally differentiated T_{EX} phenotype. This study confirms these previous observations and are shown to be significantly upregulated.

CD39 is also associated with an upregulation of inhibitory receptors such as PD-1 [37][38]. PD-1 expression indicates activation of the T cells. It is expressed when TCR is activated and decreases in the absence of signalling [39][40]. PD-1 is highly expressed in exhausted T cells and the use of ICIs which blocks the interactions of PDL-1 with PD-1 receptor might prevent the cancer from escaping the immune system. Furthermore, co-expression of tissue residence CD103 defines specific tumor-infiltrating exhausted CD8 T cells [41]. This again correlates with findings in this study.

Several trials in cervical cancer, both previously and on-going using ICI, show an ORR of 12-21% [8][11] and therefore not as promising as ICI have demonstrated for several other cancers e.g., malignant melanoma and lung cancer (~50%) [42][43]. One on-going study combines Pembrolizumab (anti-PD1) with chemoradiation and brachytherapy in order to assess if cell death by radiation can release tumor antigens and improve the immune response towards the tumor [11].

An additionally important observation from this study was the similarity between phenotypes in biopsy and cytology for all significant populations. This was shown both by manually gating and by unsupervised clustering with FlowSOM and illustrated with UMAP. The immune phenotype profile that characterises the different populations is the same. Demonstrating the identification of similar CD8 T cell population exhibiting exhaustion profiles in both biopsies and the cytology specimens, reveals the feasibility to use the less invasive cytology brush for collecting immune cells from the cervix in future analysis. This will be beneficial because cytology (liquid-based cytology) can be performed without local analgesia and without any precautions afterwards. Taking a cytology specimen, can easily be handled by the general practitioner and does therefore not require a referral to a specialist. This increases the likelihood of the patients having the liquid-based cytology sample performed.

Since the systemic T cell phenotype profile in blood is unique, when comparing with phenotypic profiles in tissue, it demonstrated the importance of cervical tissue analysis, to determine the relevant immune characteristics.

The level of activated infiltrating immune cells as well as the level of inflammation in the TME is likely to influence the clinical outcome positively [44][45][46]. In this study increased levels of infiltrating T cells was observed in cancer, but no major difference was observed for the myeloid subsets, although several of these showed increased expression of PD-L1 indicating immune inhibition, all though further analyses with additional phenotypic markers are required to fully elucidate the true functional implications. The ability to characterize the immune cell composition of a tumor is of favourable prognostic value to sensitize the TME to therapy. The myeloid cells are hard exactly to define, and sub categorize, but upregulation of the co-inhibitory molecule PD-L1 has been seen in immunogenic tumors which correlates with our findings.

The immunosuppressive cells m-MDSC and PNM-MDSC are upregulated although not statistically significant but indicating an impaired TME and increased inflammatory signatures. It is important to note that MDSCs are generally not present in healthy individuals. Still, we were able to detect MDSCs in all groups. This could be an indication that our gated population might not only comprise true MDSC but also other myeloid cells which are phenotypically closely related like neutrophils, eosinophils, and basophils. Due to limitations of markers in our panel the current markers did not make it possible to fully distinguish between these groups. In order to verify the nature of the gated MDSCs, further analysis is needed to elucidate their actual function and cytokine release profile [47].

Studies indicate that immature dendritic cells (DC's) in the mucosal tissue express CD1a [48] together with langerin (CD207) and harbor Birbeck granules [49]. These dendritic cells are also found in the epithelial cells in the cervix and have been subject to controversial classification weather they should be classified as DC's or macrophages, but functionally they act as DC's [50]. Presence of DC's or Langerhans cells in the epithelial layer of the ectocervix is paramount in producing immune response [51][52]. Studies have shown that Langerhans cells in HPV lesions may be quantitatively reduced and functionally impaired, which may contribute to the persistence of infection [32]. Therefore langerin (CD207) and CD1a were also included as markers in this phenotype panel.

The present study shows DC's (Langerhans (CD14⁻, CD16⁻), m-DC and p-DC) are significantly upregulated in tissue implying antigen presenting cells attempting to activate T cells.

To accurately distinguish macrophages from monocytes, additional surface markers are required. The myeloid panel was designed as more explorative and therefore not fully designed to distinguish between all subsets because of limitations of the numbers of markers.

The HPV subtypes of our three groups show a tendency of more HPV16 than HPV18 as also seen in head and neck HPV driven cancers. Only two cancer patients were HPV18 positive which is lower than expected.

Analyses of the data from this group, manual gating and unsupervised clustering was used. By using two different approaches when analysing the flow cytometry data, we gain greater insight in cell subpopulations, and they help support each other when trying to elucidate cells of interest.

To establish further evidence of the exhaustion phenotypes, additional characteristics could be done in the future. Co-expression of multiple inhibitory receptors such as PD-1, TIM3, LAG3 and CTLA4 is associated with more severe exhaustion, which is why these could be future targets of interest [53]. Moreover, it would be interesting to further investigate the difference between the CD57⁺ and CD57⁻ exhausted phenotypes in terms of functionality. To investigate this question further, it could be relevant to perform a cell cytotoxicity assay, intracellular cytokine staining or even single cell RNA sequencing. Those investigations would also be relevant when describing and categorizing the DC's and suppressor cells, their functionalities and effect on CD8 T cells and the TME.

Regarding the myeloid cells a deeper analysis using unsupervised clustering analysis could hopefully provide additional information regarding phenotype differences.

Overall, this study provides an important contribution to both CD4 and CD8 T cell phenotype signatures in both healthy, CIN3 and cervical cancer patients and the observations add to our knowledge of immune infiltration in cytology, biopsy, and blood samples.

MATERIALS AND METHODS

INCLUSION OF PARTICIPANTS

In this study liquid-based cytology (LBC) specimens, cervical biopsies (min. 2 x 2 mm), both from the transformation zone and 50 mL peripheral blood was obtained - a full set from each participant.

We included healthy donors, patients diagnosed with severe neoplasia (CIN3) and patients diagnosed with cervical cancer at various disease stages.

The healthy donors were recruited from Aleris-Hamlet private hospital, Søborg, Denmark, if they underwent hysterectomy for reasons unrelated to HPV. Participants with medical record of previous cervical neoplasia were excluded from the study but were not tested for HPV-DNA. Specimens were obtained during already planned surgery to minimize discomfort for the patient.

Patients with CIN 3 recruited from Department of Gynecology and Obstetrics, Hvidovre Hospital, Hvidovre. Biopsies and cytology specimens were collected prior to having a cone biopsy, to limit blood contamination into the specimen. If possible, the blood samples were taken during that same consultation.

Patients with cervical cancer were recruited from Department of Gynecology and Obstetrics, Odense University Hospital, Odense. Specimens were collected prior to assessment of the cancer stage in full anesthesia.

All specimens were obtained anonymously, marked with a study number, delivered to the laboratory at the Technical University of Denmark (DTU), Lyngby, Denmark and were all handled within 6 hours after sampling.

STUDY POPULATION

We included 57 participants, i.e., 24 healthy controls, 16 patients with severe neoplasia (CIN 3) and 17 patients with cervical cancer. We used 4 specimens (two healthy, one neoplasia and one cancer) to test the experimental procedures. Three patients with neoplasia had to be excluded because of lack of material, either blood, cytology, or biopsy. In total we choose only to analyze specimens where cell count was $>1 \times 10^4$. That ended up being 10 healthy, 10 neoplasia and 10 cervical cancer patients (invasive stage) for analysis and 15 blood samples from cancer patients. More cells from blood than from tissue was obtained. Patients were enrolled based on the following criteria: *Inclusion criteria*: Female participants, >18 years at inclusion and with informed consent. *Exclusion criteria*: Patients receiving immunosuppressive treatment, e.g., larger doses of prednisolone (>5mg/ day), previous cancer of any kind, healthy controls with previous cervical neoplasia.

Clinical parameters e.g., diagnosis, other diseases (especially immune mediated diseases), medication, age, health status, history of smoking, and other environmental factors was also registered.

In total, 11 different types of HPV (16, 18, 33, 39, 45, 51, 52, 53, 61, 70, 82) were detected among all participants (Table S3), 17% being positive for >1 type. Out of these subtypes, HPV 53, 61 and 82 are not classified as high-risk HPV types associated with cervical cancer.

70% of the healthy controls was tested negative for HPV, whereas the vast majority of CIN 3 (90%) and cancer patients (93%) were HPV positive (Table S3). The three HPV positive healthy patients were positive for only one HPV type each. One being low risk and two being high risk HPV type. Two healthy individuals were smoking, and they both had high risk HPV. One of the CIN 3 patients was negative for HPV but showed adenocarcinoma in situ in the cone biopsy and had a hysterectomy, without neoplasia and cancer. The nine HPV positive CIN 3 patients were all positive for more than one of high-risk types. The cancer patients had the highest prevalence of HPV infection, 60% being positive for HPV 16 and HPV 18 was only detected in few of the CIN 3 and cancer patients.

The cervical cancer patients were classified by disease progression according to the FIGO grading scale [54] and 87% had invasive stage II or higher (Table S3). 73,3% was squamous cell carcinomas, 20% was adenocarcinomas, one adeno-squamous and adenocarcinoma of mucinous type. BMI was non-significant; (healthy: 27 ± 4), (CIN 3: 25 ± 5) and (cancer: 29 ± 10).

SAMPLE COLLECTION AND PROCESSING

The liquid-based cytology specimens were collected using two Cervix-Brush (Rovers) technique. The first sample was kept on ice in 15 mL digestion buffer (1:10 Hank's Balanced Saline Solution (HBSS) 50X + 15mM 1,5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) until cell isolation. To isolate the cells, the brush was rinsed, and suspension filtered through a cell strainer. Cells were centrifuged and resuspended in phosphate saline buffer (PBS), counted on NucleoCounter SCC-100TM (Chemometec), using SCC-CassetteTM. Cells were again centrifuged and resuspended in 1 mL of freezing media 10% DMSO (Dimethyl Sulfoxide) Hybrid-Max (Sigma-Aldrich) and 90% fetal calf serum (FCS) GibcoTM qualified, New Zealand. $5-10 \times 10^6$ cells/vial and distributed in cryotubes. The vials were frozen by $1^\circ\text{C}/\text{min}$ in freezing boxes placed at -80°C and next day transferred to a -180°C nitrogen tank for long-term storage until used for further analysis.

The second specimen were kept at room temperature (RT) in 10 mL SurePathTM Collection Vial (BD). Specimens were sent to Department of Pathology, Hvidovre Hospital, Denmark, for HPV typing using Anyplex II HPV28 detection real-time PCR or BD Max DNA extraction.

The biopsy was collected in the same digestion buffer as cytology, 7 mL and stored on ice. Using scalpel and tweezer, the biopsies were cut into smaller pieces, centrifuged for homogenization for 1 min using gentleMAC Dissociator (Miltenyi Biotec). 2,5 mL (2U/ml) DNase bovine pancreas (Sigma-Aldrich, Merck) was added to the specimen. After 1-hour incubation (37°C), the specimen were further dissociated (1 min) using gentleMACS. The suspension was then filtered through a cell strainer, centrifuged, resuspended, and counted. From this step the cells were treated as described for the cytology and blood sample.

Peripheral blood samples were collected in five 10 mL Vacutainer heparin tubes and kept at RT until separated using filter tubes (Falcon Leucosep) saturated with 15 mL LymphoprepTM

1.077g/mL, (STEMCELL, cat. 07851), and PBS. After separation, lymphocytes were resuspended and counted on NucleoCounter (Chemometec) and were treated as described for the cytology and biopsy specimens.

IMMUNE PHENOTYPING – SURFACE AND INTRACELLULAR STAINING

Cell specimens were thawed in 10 ml RPMI (Gibco) + 10% FCS preheated to 37°C, centrifuged, washed twice in RPMI + 10% FCS and mixed with the surface antibodies diluted in equal amounts of FACS buffer and Brilliant Stain Buffer (BD) giving a total of 50 µl per sample. Incubation on ice (30 min), washed twice with FACS buffer and fixated with 100 µl fixation/permeabilization buffer (Invitrogen, cat. 00-5523-00) for 1 hour (RT) or ON (4°C). Following fixation, specimens were washed twice with washing buffer (10% permeabilization buffer (Invitrogen, cat. 00-5523-00) in MilliQ water). Intracellular antibodies were diluted in washing buffer to a total of 100 µl per sample and was incubated with the cells (30 min. on ice). After two additional washing steps with washing buffer, cells were resuspended in FACS buffer or PBS and filtered into FACS tubes just prior to acquisition.

FLOW CYTOMETRY

Compensation setup of the fluorescent conjugated antibodies was made with 1 drop of OneComp or UltraComp compensation beads with 0.5 µl of the respective antibody. PBS was added after 10 min incubation at RT. The compensation beads for the live/dead staining were made with 1 drop ArC amine reactive beads (Invitrogen, A10346) + 1 µl near-IR viability dye (Invitrogen, L10119). After 30 min incubation (on ice), beads were washed twice with PBS, 1 drop of ArC amine negative beads (Invitrogen, A10346) was added, and beads were suspended in PBS.

Flow cytometry experiments were performed on LSR-Fortessa (BD Biosciences). Data was analyzed in FACSDiva software (BD Biosciences) and FlowJo v10.7 (TreeStar, Inc.).

ANTIBODY TITRATION

Titration of antibodies was done prior to staining cell specimens, for optimal concentration and separation of positive and negative population while maintaining low unspecific binding (Table S2). Accordingly, $1-2 \cdot 10^6$ PBMCs or cells from healthy cervical tissue were stained with viability dye (Invitrogen, L10119) and different concentration of the antibodies, and the optimal concentration was determined by visual inspection of the separation and by calculation of the staining index and separation index for each concentration (Fig. S1).

STATISTICAL ANALYSES

Uniform manifold approximation and projections (UMAP) were made in FlowJo using the UMAP plugin a dimensionality reduction technique. DownSampleV3 was applied on the specimen before FlowSOM was used on concatenated files (clustering and visualization algorithm) to analyze and detect data subsets using self-organizing maps. All plugins for FlowJo from flowjo.com/exchange. Data was analyzed with a non-parametric Kruskal-Wallis test with Dunn's correlation for multiple comparisons. These statistical analyses were conducted using GraphPad Prism 9.0. Scripts to reproduce figures can be obtained from the corresponding author upon request.

T CELL PHENOTYPES

The specimens from all participants were grouped into healthy women, CIN3 and cervical cancer. The immunophenotypes and the frequencies of CD4 and CD8 T cell subsets in biopsy (n=10), liquid-based cytology (n=10), and blood (n=15) specimens were analyzed. The cells were stained with antibodies targeting surface and intracellular markers and analyzed using multicolor flow cytometry (Table S1), to possibly identify subsets of T cells, differentiating between groups and sample types.

After acquisition on the flow cytometer, the T cells were gated out as shown in (Fig. S5). Both CD4 and CD8 T cells were divided into naïve (CD45RA⁺ CCR7⁺), T_{CM} (CD45RA⁻ CCR7⁺), T_{EM} (CD45RA⁻ CCR7⁻) and T_{EMRA} (CD45RA⁺ CCR7⁻), and these subsets were afterwards analyzed for the expression of differentiation markers; PD-1, TOX, CD39, CD103, Ki67, CD27, CD57, Eomes, GZMB, TCF-1. This panel was designed mainly to characterize and evaluate each subset's expression of differentiation markers related to T cell activation and exhaustion status of CD8 T cells. However, the CD4 T cells were also analysed but the markers were not specifically designed for this purpose.

MYELOID PHENOTYPES

The same patient material was used for analyzing myeloid subsets. Cells were stained with antibodies targeting surface markers and analyzed using multicolor flow cytometry (panel of markers see Table S1) to identify subsets of monocytes (classical, intermediate, or non-classical), suppressor cells (PMN-MDSC, M-MDSC) and dendritic cells (p-DC, m-DC).

Myeloid subsets are harder to define, and several markers are of interest and the academic field is constantly changing. Having a limited numbers of markers available in our phenotype panel, markers were selected based on the ability to distinguish myeloid subsets defined as CD45⁺ and Lin⁻ (CD3⁻, CD19⁻, CD56⁻) to include leukocytes while excluding T cells, B cells and NK cells respectively. Monocytes were divided into classical (HLADR⁺, CD14⁺, CD16⁻, CD64⁺), intermediate (HLA-DR⁺, CD14⁺, CD16⁺) and non-classical monocytes (HLA-DR⁺, CD14⁻, CD16⁺). DCs were defined as mDC (HLA-DR⁺, CD14⁻, CD16⁻, CD33⁺, CD11b⁺) and pDC (HLA-DR⁺, CD14⁻, CD16⁻, CD33⁻, CD11b⁻, CD123⁺, CD11c⁻). The MDSCs (including granulocytes) were defined as (HLA-DR⁻, CD14^{+/-}, CD33⁺, CD11b⁺) cells and divided into PMN-MDSC (CD15⁺) and M-MDSC (CD15⁻). See (Fig. S7) for gating strategy.

ETHICS STATEMENT

Approval for the study design and sample collection was obtained from the Committee on Health Research Ethics in the Capital Region of Denmark. All included participants gave their informed written consent for inclusion. All specimens were kept anonymously. The remaining material will be kept anonymized for 8 years in a research bio bank, after which time it will be destroyed. If desired by the participant, the material can be destroyed at an earlier stage. All specimens were and will be used for this present study only. The study was approval by The Danish Data Protection Agency. The study took four years and did not involve further visits or medical contact for the participants.

FUNDING

Danish Cancer Society Research Center has awarded 1,000,000 DKK from “Knæk Cancer puljen”, Danish Technical University has awarded 740,000 DKK. Aleris-Hamlet research foundation has awarded 105,000 DKK.

ACKNOWLEDGEMENTS AND AUTHOR CONTRIBUTIONS

As first author I would like to thank all participants included in this study who contributed by donating specimens. No financial compensation was offered to the donors for participating in this study.

As co-supervisors we had the privilege to collaborate with Susanne Krüger Kjær, Professor, consultant at the Danish Cancer Society Research Center, Copenhagen, Denmark. Her unique knowledge of the HPV virus has been of great importance also in terms of designing the study and scientific discussions.

As clinical collaborators we were honored to collaborate with Kirsten Marie Jochumsen, PhD., Associate Professor, senior consultant, Department of Gynecology and Obstetrics, Odense University Hospital, Odense, who made it possible to collect patient specimens from cancer patients. For all the cervical neoplasia patients we were fortunate to have Benny Kirschner, Clinical Associate Professor, consultant, Department of Gynecology and Obstetrics, Hvidovre Hospital, Hvidovre who collected both consent and patient material. Aleris Hamlet private hospital for approval of inclusion of patients for healthy patient material done by the author. Jesper Bonde for analyzing HPV status and follow-up.

Marie Viuff for great lab assistance and data analyzes and Mohammad Kadivar for excellent supervision, lab assistance and close guidance. Lastly my main supervisor Sine Reker Hadrup for overall assistance, great support, and scientific discussions.

REFERENCES

- [1] "WHO." [https://www.who.int/news-room/fact-sheets/detail/human-papillomavirus-\(hpv\)-and-cervical-cancer](https://www.who.int/news-room/fact-sheets/detail/human-papillomavirus-(hpv)-and-cervical-cancer) (accessed Dec. 04, 2020).
- [2] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal, "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries," *CA. Cancer J. Clin.*, vol. 68, no. 6, pp. 394–424, 2018, doi: 10.3322/caac.21492.
- [3] W. Small et al., "Cervical cancer: A global health crisis," *Cancer*, vol. 123, no. 13, pp. 2404–2412, 2017, doi: 10.1002/cncr.30667.
- [4] GLOBOCAN, "Cervix uteri Source: Globocan 2020," *Int. Agent Res. Cervic Uteri*, vol. 419, pp. 1–10, 2020.
- [5] Sundhedsdatastyrelsen. Nye kræfttilfælde i Danmark., "Cancerregisteret," *Cancerregisteret*, pp. 30–31, 2019, [Online]. Available: https://sundhedsdatastyrelsen.dk/da/tal-og-analyser/analyser-og-rapporter/sygdomme/kraeft-_nyetilfaelde.
- [6] S. Shanmugasundaram and J. You, "Targeting persistent human papillomavirus infection," *Viruses*, vol. 9, no. 8, 2017, doi: 10.3390/v9080229.
- [7] C. A. Moody and L. A. Laimins, "Human papillomavirus oncoproteins: Pathways to transformation," *Nat. Rev. Cancer*, vol. 10, no. 8, pp. 550–560, 2010, doi: 10.1038/nrc2886.
- [8] C. Orbegoso, K. Murali, and S. Banerjee, "The current status of immunotherapy for cervical cancer," *Reports Pract. Oncol. Radiother.*, vol. 23, no. 6, pp. 580–588, 2018, doi: 10.1016/j.rpor.2018.05.001.
- [9] H. Papillomavirus and M. Capsid, "crossm Protein L1 Remains Associated with the Incoming Viral Genome throughout the," vol. 91, no. 16, pp. 1–17, 2017.
- [10] K. E. Pauken and E. J. Wherry, "Overcoming T cell exhaustion in infection and cancer," *Trends Immunol.*, vol. 36, no. 4, pp. 265–276, 2015, doi: 10.1016/j.it.2015.02.008.
- [11] E. Borcoman and C. Le Tourneau, "Pembrolizumab in cervical cancer: latest evidence and clinical usefulness," *Ther. Adv. Med. Oncol.*, vol. 9, no. 6, pp. 431–439, Jun. 2017, doi: 10.1177/1758834017708742.
- [12] H. Tashiro and M. K. Brenner, "Immunotherapy against cancer-related viruses," *Cell Res.*, vol. 27, no. 1, pp. 59–73, 2017, doi: 10.1038/cr.2016.153.
- [13] S. Duranti et al., "Role of immune checkpoint inhibitors in cervical cancer: From preclinical to clinical data," *Cancers (Basel)*, vol. 13, no. 9, pp. 1–12, 2021, doi: 10.3390/cancers13092089.
- [14] M. Sznol and L. Chen, "Antagonist Antibodies to PD-1 and B7-H1 (PD-L1) in the Treatment of Advanced Human Cancer-Response," *Clin. Cancer Res.*, vol. 19, no. 19, p. 5542, 2013, doi: 10.1158/1078-0432.CCR-13-2234.
- [15] P. Sharma and J. P. Allison, "The future of immune checkpoint therapy," *Science (80-.)*, vol. 348, no. 6230, pp. 56–61, 2015, doi: 10.1126/science.aaa8172.
- [16] T. N. Schumacher and R. D. Schreiber, "Neoantigens in cancer immunotherapy," *Science (80-.)*, vol. 348, no. 6230, pp. 69–74, 2015, doi: 10.1126/science.aaa4971.
- [17] L. B. Alexandrov et al., "Signatures of mutational processes in human cancer," *Nature*, vol. 500, no. 7463, pp. 415–421, 2013, doi: 10.1038/nature12477.
- [18] P. A. Lazo, "The molecular genetics of cervical carcinoma," *Br. J. Cancer*, vol. 80, no. 12, pp. 2008–2018, 1999, doi: 10.1038/sj.bjc.6690635.

- [19] O. L. Reddy, P. I. Shintaku, and N. A. Moatamed, "Programmed death-ligand 1 (PD-L1) is expressed in a significant number of the uterine cervical carcinomas," *Diagn. Pathol.*, vol. 12, no. 1, pp. 1–11, 2017, doi: 10.1186/s13000-017-0631-6.
- [20] R. K. Shrimali, Z. Yu, M. R. Theoret, D. Chinnasamy, N. P. Restifo, and S. A. Rosenberg, "Antiangiogenic agents can increase lymphocyte infiltration into tumor and enhance the effectiveness of adoptive immunotherapy of cancer," *Cancer Res.*, vol. 70, no. 15, pp. 6171–6180, 2010, doi: 10.1158/0008-5472.CAN-10-0153.
- [21] L. Mezache, B. Paniccia, A. Nyinawabera, and G. J. Nuovo, "Enhanced expression of PD L1 in cervical intraepithelial neoplasia and cervical cancers," *Mod. Pathol.*, vol. 28, no. 12, pp. 1594–1602, 2015, doi: 10.1038/modpathol.2015.108.
- [22] L. Festino et al., "Cancer Treatment with Anti-PD-1/PD-L1 Agents: Is PD-L1 Expression a Biomarker for Patient Selection?," *Drugs*, vol. 76, no. 9, pp. 925–945, 2016, doi: 10.1007/s40265-016-0588-x.
- [23] H. Kared, S. Martelli, T. P. Ng, S. L. F. Pender, and A. Larbi, "CD57 in human natural killer cells and T-lymphocytes," *Cancer Immunol. Immunother.*, vol. 65, no. 4, pp. 441–452, 2016, doi: 10.1007/s00262-016-1803-z.
- [24] F. Simonetta et al., "High Eomesodermin Expression among CD57+ CD8+ T Cells Identifies a CD8+ T Cell Subset Associated with Viral Control during Chronic Human Immunodeficiency Virus Infection," *J. Virol.*, vol. 88, no. 20, pp. 11861–11871, 2014, doi: 10.1128/jvi.02013-14.
- [25] J. Li, Y. He, J. Hao, L. Ni, and C. Dong, "High Levels of Eomes Promote Exhaustion of Anti-tumor CD8+ T Cells," *Front. Immunol.*, vol. 9, no. December, p. 2981, 2018, doi: 10.3389/fimmu.2018.02981.
- [26] A. C. Scott et al., "TOX is a critical regulator of tumour-specific T cell differentiation," *Nature*, vol. 571, no. 7764, pp. 270–274, 2019, doi: 10.1038/s41586-019-1324-y.
- [27] O. Khan et al., "TOX transcriptionally and epigenetically programs CD8+ T cell exhaustion," *Nature*, vol. 571, no. 7764, pp. 211–218, 2019, doi: 10.1038/s41586-019-1325-x.
- [28] C. U. Blank et al., "Defining 'T cell exhaustion,'" *Nat. Rev. Immunol.*, vol. 19, no. 11, pp. 665–674, 2019, doi: 10.1038/s41577-019-0221-9.
- [29] L. M. McLane, M. S. Abdel-Hakeem, and E. J. Wherry, "CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer," *Annu. Rev. Immunol.*, vol. 37, no. 1, pp. 457–495, Apr. 2019, doi: 10.1146/annurev-immunol-041015-055318.
- [30] Z. Chen et al., "TCF-1-Centered Transcriptional Network Drives an Effector versus Exhausted CD8 T Cell-Fate Decision," *Immunity*, vol. 51, no. 5, pp. 840-855.e5, 2019, doi: 10.1016/j.immuni.2019.09.013.
- [31] Q. Wang, A. Steger, S. Mahner, U. Jeschke, and H. Heidegger, "The formation and therapeutic update of tumor-associated macrophages in cervical cancer," *Int. J. Mol. Sci.*, vol. 20, no. 13, pp. 1–18, 2019, doi: 10.3390/ijms20133310.
- [32] F. Walker, H. Adle-Biassette, P. Madelenat, D. Hénin, and T. Lehy, "Increased apoptosis in cervical intraepithelial neoplasia associated with HIV infection: Implication of oncogenic human papillomavirus, caspases, and langerhans cells," *Clin. Cancer Res.*, vol. 11, no. 7, pp. 2451–2458, 2005, doi: 10.1158/1078-0432.CCR-04-1795.
- [33] F. Tang, Q. Du, and Y. J. Liu, "Plasmacytoid dendritic cells in antiviral immunity and autoimmunity," *Sci. China Life Sci.*, vol. 53, no. 2, pp. 172–182, 2010, doi: 10.1007/s11427-010-0045-0.
- [34] M. R. McCredie et al., "Natural history of cervical neoplasia and risk of invasive cancer in

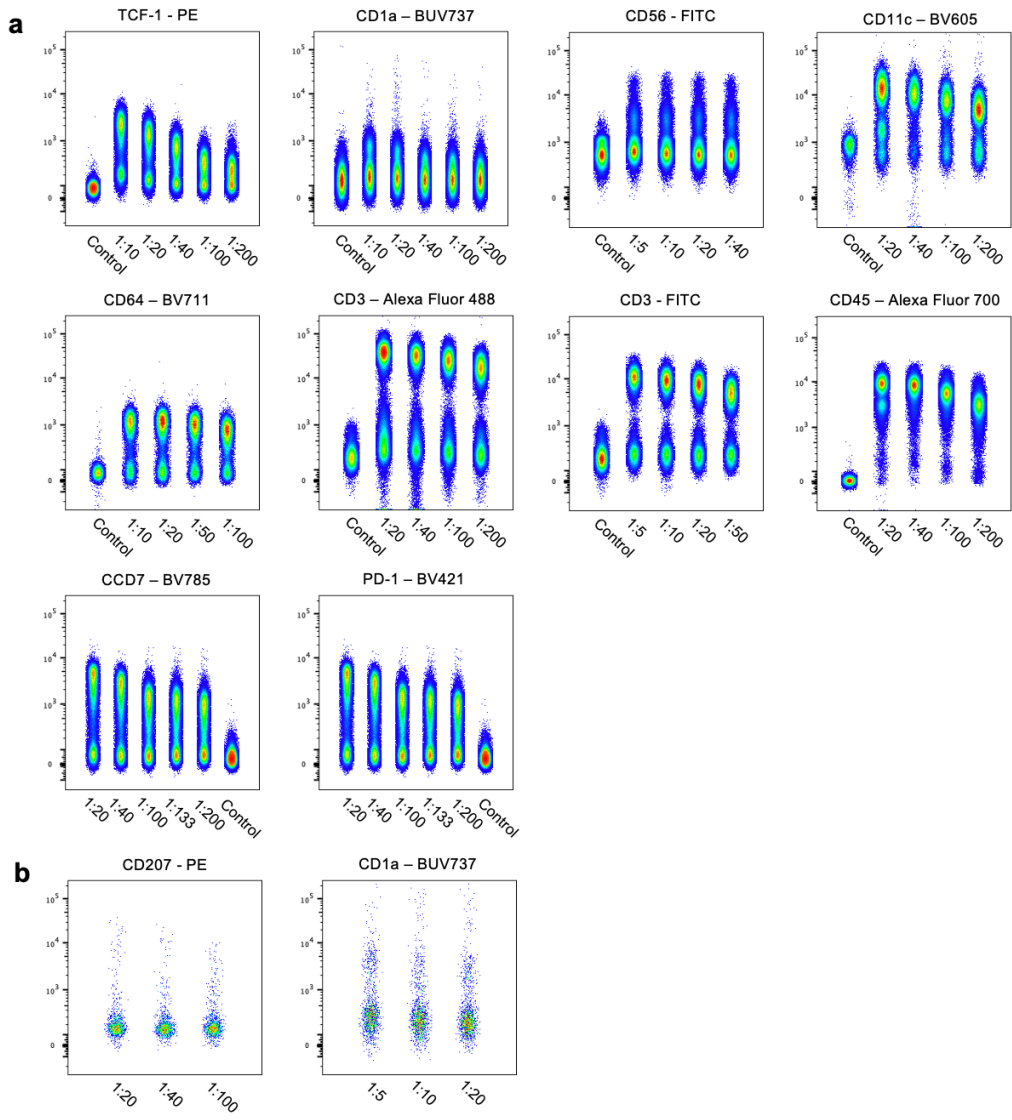
- women with cervical intraepithelial neoplasia 3: a retrospective cohort study,” *Lancet Oncol.*, vol. 9, no. 5, pp. 425–434, 2008, doi: 10.1016/S1470-2045(08)70103-7.
- [35] H. W. Chesson, E. F. Dunne, S. Hariri, and L. E. Markowitz, “The Estimated Lifetime Probability of Acquiring Human Papillomavirus in the United States,” *Sex. Transm. Dis.*, vol. 41, no. 11, pp. 660–664, Nov. 2014, doi: 10.1097/OLQ.000000000000193.
- [36] S. Stokley et al., “Human papillomavirus vaccination coverage among adolescents, 2007–2013, and postlicensure vaccine safety monitoring, 2006–2014--United States.,” *MMWR. Morb. Mortal. Wkly. Rep.*, vol. 63, no. 29, pp. 620–4, 2014, [Online]. Available: <http://www.ncbi.nlm.nih.gov/pubmed/25055185%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5779422>.
- [37] A. K. Moesta, X. Y. Li, and M. J. Smyth, “Targeting CD39 in cancer,” *Nat. Rev. Immunol.*, vol. 20, no. 12, pp. 739–755, 2020, doi: 10.1038/s41577-020-0376-4.
- [38] P. K. Gupta et al., “CD39 Expression Identifies Terminally Exhausted CD8+ T Cells,” *PLoS Pathog.*, vol. 11, no. 10, pp. 1–21, 2015, doi: 10.1371/journal.ppat.1005177.
- [39] Y.-Y. Yan et al., “PD-1/PD-L1 Inhibitors in Cervical Cancer,” 2019, doi: 10.3389/fphar.2019.00065.
- [40] S. Simon and N. Labarriere, “PD-1 expression on tumor-specific T cells: Friend or foe for immunotherapy?,” *Oncoimmunology*, vol. 7, no. 1, pp. 1–7, 2018, doi: 10.1080/2162402X.2017.1364828.
- [41] T. Duhon et al., “Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors,” no. 2018, doi: 10.1038/s41467-018-05072-0.
- [42] C. Robert et al., “Pembrolizumab versus Ipilimumab in Advanced Melanoma,” *N. Engl. J. Med.*, vol. 372, no. 26, pp. 2521–2532, 2015, doi: 10.1056/nejmoa1503093.
- [43] G. M. Blumenthal et al., “Overall response rate, progression-free survival, and overall survival with targeted and standard therapies in advanced non-small-cell lung cancer: US Food and Drug Administration trial-level and patient-level analyses,” *J. Clin. Oncol.*, vol. 33, no. 9, pp. 1008–1014, 2015, doi: 10.1200/JCO.2014.59.0489.
- [44] N. Nagarsheth, M. S. Wicha, and W. Zou, “Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy,” *Nat. Rev. Immunol.*, vol. 17, no. 9, pp. 559–572, 2017, doi: 10.1038/nri.2017.49.
- [45] T. F. Gajewski, “The Next Hurdle in Cancer Immunotherapy: Overcoming the Non-T-Cell-Inflamed Tumor Microenvironment,” *Semin. Oncol.*, vol. 42, no. 4, pp. 663–671, Aug. 2015, doi: 10.1053/j.seminoncol.2015.05.011.
- [46] C. Groth et al., “Immunosuppression mediated by myeloid-derived suppressor cells (MDSCs) during tumour progression,” *Br. J. Cancer*, vol. 120, no. 1, pp. 16–25, 2019, doi: 10.1038/s41416-018-0333-1.
- [47] V. Bronte et al., “Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards,” *Nat. Commun.*, vol. 7, 2016, doi: 10.1038/ncomms12150.
- [48] A. R. Hayati and M. Zulkarnaen, “An immunohistochemical study of CD1a and CD83-positive infiltrating dendritic cell density in cervical neoplasia,” *Int. J. Gynecol. Pathol.*, vol. 26, no. 1, pp. 83–88, 2007, doi: 10.1097.
- [49] V. Pena-Cruz et al., “HIV-1 replicates and persists in vaginal epithelial dendritic cells,” *J. Clin. Invest.*, vol. 128, no. 8, pp. 3439–3444, 2018, doi: 10.1172/JCI98943.
- [50] J. W. Rhodes, O. Tong, A. N. Harman, and S. G. Turville, “Human dendritic cell subsets, ontogeny, and impact on HIV infection,” *Front. Immunol.*, vol. 10, no. MAY, 2019, doi: 10.3389/fimmu.2019.01088.

- [51] M. Chopin and S. L. Nutt, "Establishing and maintaining the Langerhans cell network," *Semin. Cell Dev. Biol.*, vol. 41, pp. 23–29, 2015, doi: 10.1016/j.semcdb.2014.02.001.
- [52] Y. Belkaid and S. Tamoutounour, "The influence of skin microorganisms on cutaneous immunity," *Nat. Rev. Immunol.*, vol. 16, no. 6, pp. 353–366, 2016, doi: 10.1038/nri.2016.48.
- [53] E. J. Wherry and M. Kurachi, "Molecular and cellular insights into T cell exhaustion," *Nat. Rev. Immunol.*, vol. 15, no. 8, pp. 486–499, 2015, doi: 10.1038/nri3862.
- [54] G. Salvo, D. Odetto, R. Pareja, M. Frumovitz, and P. T. Ramirez, "Revised 2018 International Federation of Gynecology and Obstetrics (FIGO) cervical cancer staging: A review of gaps and questions that remain," *Int. J. Gynecol. Cancer*, vol. 30, no. 6, pp. 873–878, 2020, doi: 10.1136/ijgc-2020-001257.

SUPPLEMENTARY

Table S1: List of markers (targets), fluochrome, clone and supplier.

Myeloid panel			
Target	Fluochrome	Clone	Supplier
CD33	BUV395	WM53	BD
CD1a	BUV737	HI149	BD
HLA-DR	BV421	G46-6	BD
CD14	BV480	MφP9	BD
CD11c	BV605	B-ly6	BD
CD123	BV650	7G3	BD
CD64	BV711	10.01	BD
CD15	BV786	HI98/HIM1	BD
CD3	FITC	UCHT1	BD
CD56	FITC	NCAM16.2	BD
CD19	FITC	4G7	BD
CD11b	PECy7	ICRF44	BD
CD274 (PD-L1)	PE-CF594	MIH1	BD
CD207	PE	2G3	BD
Live/dead	Near-IR		Invitrogen
CD45	AlexaFluor 700	HI30	BD
CD16	APC	B73.1	BD
T-cell panel			
Target	Fluochrome	Clone	Supplier
Ki67	BUV395	B56	BD
CD103	BUV563	Ber-ACT8	BD
CD137 (4-1BB)	BUV737	4B4-1	BD
PD1	BV421	EH12.2H7	BioLegend
CD8	BV480	RPA-T8	BD
CD27	BV605	O323	BioLegend
CD4	BV650	SK3	BD
CD45RA	BV711	HI100	BD
CCR7	BV786	G043H7	BioLegend
CD3	Alexa Fluor 488	UCHT1	BD
Eomes	PerCP-eFluor 710	WD1928	Thermo
CD39	PE-CF594	Tu66	BD
CD57	PECy7	QA17A04	BioLegend
TCF1	PE	7F11A10	BioLegend
Live/dead	Near-IR		Invitrogen
GZMB	AlexaFluor 700	QA16A02	BioLegend
TOX	APC	REA473	Miltenyi



c

$$\text{Staining index} = \frac{\text{Median}_{\text{positive population}} - \text{Median}_{\text{Negative population}}}{2 \cdot \text{SD}_{\text{negative population}}}$$

$$\text{Separation index} = \frac{\text{Median}_{\text{positive population}} - \text{Median}_{\text{Negative population}}}{\frac{84\text{th percentile}_{\text{negative population}} - \text{Median}_{\text{negative population}}}{0.995}}$$

Fig. S1: Titration of antibodies

a Titration on PBMCs. Specimens were either unstained (control) or staining with different antibody concentrations. **b** Titration on cervical tissue biopsies from healthy donors. **c** Equation for calculation of staining index and separation index.

Table S2: Titration of antibodies

Antigen	Fluorophore	Sample	Dilution	Staining index	Separation index	Chosen concentration
TCF-1	PE	PBMC	1:10	9.28	16.8	1:40
			1:20	9.03	16.3	
			1:40	6.38	11.4	
			1:100	3.16	5.38	
			1:200	2.68	5.07	
CD1a	BUV737	PBMC	1:10	2.82	5.04	1:10
			1:20	2.37	4.19	
			1:40	1.92	3.21	
			1:100	2.34	3.93	
			1:200	2.63	4.66	
		Cervical tissue	1:5	10.2	18.3	
			1:10	6.6	12.4	
CD56	FITC	PBMC	1:5	5.42	9.89	1:50
			1:10	5.61	10.2	
			1:20	5.89	11.7	
			1:40	6.01	11.8	
CD11c	BV605	PBMC	1:20	7.72	13.9	1:66
			1:40	6.35	9.29	
			1:100	6.35	9.21	
			1:200	7.47	11.9	
CD64	BV711	PBMC	1:10	5.72	9.75	1:33
			1:20	5.76	9.5	
			1:50	5.34	9.54	
			1:100	4.69	7.55	
CD3	AlexaFlour 488	PBMC	1:20	1.44	97.8	1:133
			1:40	1.33	106	
			1:100	1.22	112	
			1:200	1.1	95.6	
CD3	FITC	PBMC	1:5	31.9	92.8	1:10
			1:10	33.7	86.3	
			1:20	26.1	65.7	
			1:50	19.8	46.8	
CD45	AlexaFlour 700	PBMC	1:20	3.24	7.24	1:200
			1:40	3.7	8.08	
			1:100	3.82	8.94	
			1:200	4.47	8.83	
CD207	PE	Cervical tissue	1:20	4.42	8.71	1:40
			1:40	3.68	7.17	
			1:100	6.03	13	
CCR7	BV785	PBMC	1:20	29.9	53.1	1:20
			1:40	18.5	33.4	
			1:100	11.2	19.1	
			1:133	9.73	16.7	
			1:200	8.46	14.5	
PD-1	BV421	PBMC	1:20	6.05	12.7	1:50
			1:40	5.52	11.6	
			1:100	4.86	10.1	
			1:133	5.06	10.4	
			1:200	5.12	10.3	

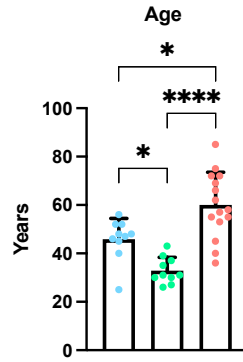


Fig. S2: Age distribution of the included groups at the time of **sample collection**. Plots show mean±SD (Kruskal-Wallis uncorrected Dunn’s test, *: p<0.05, **: p<0.01, ***: p< 0.001, ****: p< 0.0001).

Table S3: Study description

Cohort	Age	HPV status	Disease stage	Cancer type	Followup
Healthy control	40				
	25	51			2020 LBC normal and HPV-
	48				
	45				
	47				
	52				
	56				
	46	53			
	48	16			
52					
<i>Mean</i>	45,9				
<i>SD</i>	8,6				Followup
CIN3	43	16			2000 biopsy Compound naevus, nothing malignant
	27	33			2019 LBC normal and HPV-, normal and HPV-
	31				2019 AIS in cone biopsy, hysterectomy normal
	26	18			2000 LBC CIN I HPV-, 2000 LBC normal HPV-
	37	33			2021 LBC normal and HPV-
	30	39			2020 LBC normal and HPV-
	30	16, 33, 61			2020 LBC normal and HPV-
	39	16			2020 LBC normal and HPV-
	31	16			2020 LBC normal and HPV-
35	52			2020 LBC normal and HPV-	
<i>Mean</i>	32,9				Screening history
<i>SD</i>	5,4				
Cervical cancer	62	16	IV	Squamous	2000 last LBC, normal
	85	53	IIIB	Squamous	2017 last LBC not representative
	40	16, 45	IA1	Adenoc.	2013 normal LBC, 2019 HPV+, cone biopsy shows cancer
	36	16	IIIC1	Adenoc.	2012, 2018, 2019 normal LBC, Nov 2019 biopsy shows cancer
	45	16	IB2	Squamous	2005 dysplasia, no follow up
	72	16, 18	IIIB	Squamous	2002 last LBC, normal
	75	45	IIIB	Squamous	1997 last LBC, normal
	55	18	IIB	Adenosquamous	2004, 2014 last LBC, normal
	66	51, 70	IIB	Squamous	2019 last LBC, normal
	72	16	IIIB	Squamous	2008 last LBC and biopsies, normal
	53	16	IIIB	Squamous	2009 cervical polyp, never LBC
	58	33, 70	IB2	Squamous	2018 last LBC, normal
	57	16, 53, 70, 82	IIB	Squamous	2019 last LBC, inflammation
	69	neg	IIB	Adenoc. Mucinous	2015, 2018 last LBC HPV-
	55	16	IIB	Squamous	2004 last LBC, normal
<i>Mean</i>	60				
<i>SD</i>	13,6				

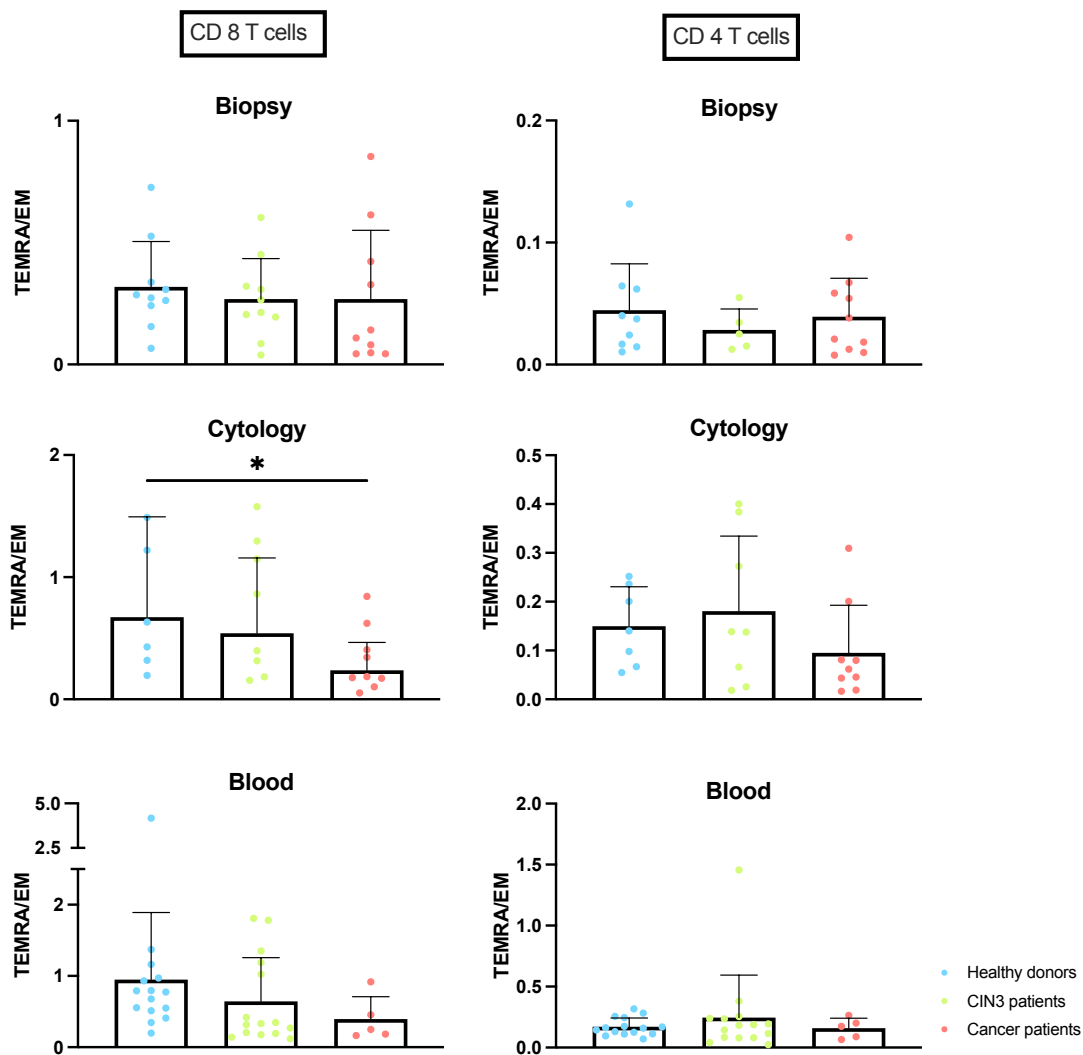


Fig. S3: The ratio of T_{EMRA} out of EM for CD8 and CD4 T cells for all three sample types

Healthy donors (n=10), CIN3 patients (n=10) and cancer patients (n=10 for tissue and n=15 for blood) were included. Plots show mean \pm SD (Kruskal-Wallis uncorrected Dunn's test, *: p<0.05, **: p<0.01, ***: p< 0.001).

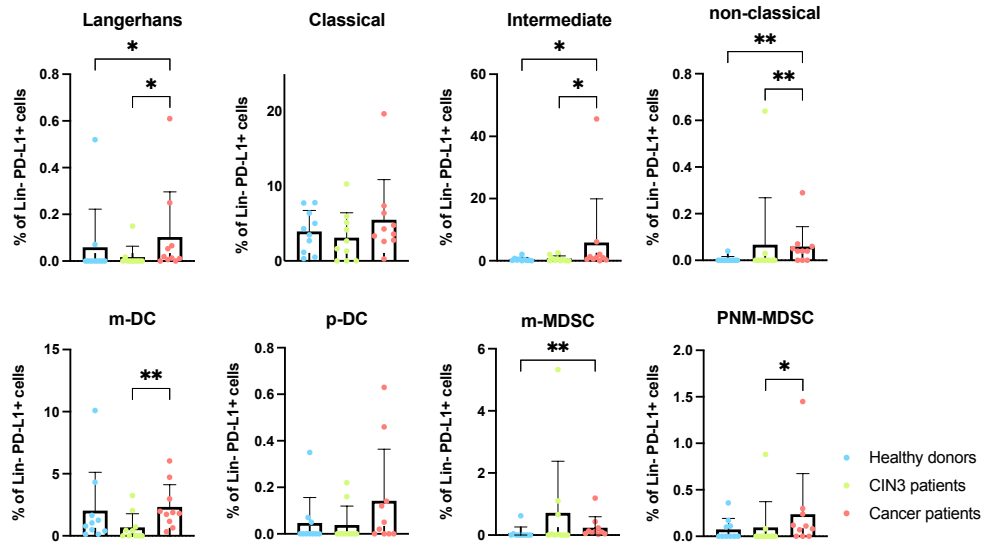


Fig. S4: Frequency of myeloid cells and subsets out of all CD45⁺ lineage PD-L1⁺ leucocytes in biopsies from the cervix. Langerhans cells are here defined as CD14⁻CD16⁻. Healthy donors (n=10), CIN3 patients (n=10) and cancer patients (n=15) were included. Plots show mean ± SD (Kruskal-Wallis uncorrected Dunn's test, *: p<0.05, **: p<0.01, ***: p< 0.001).

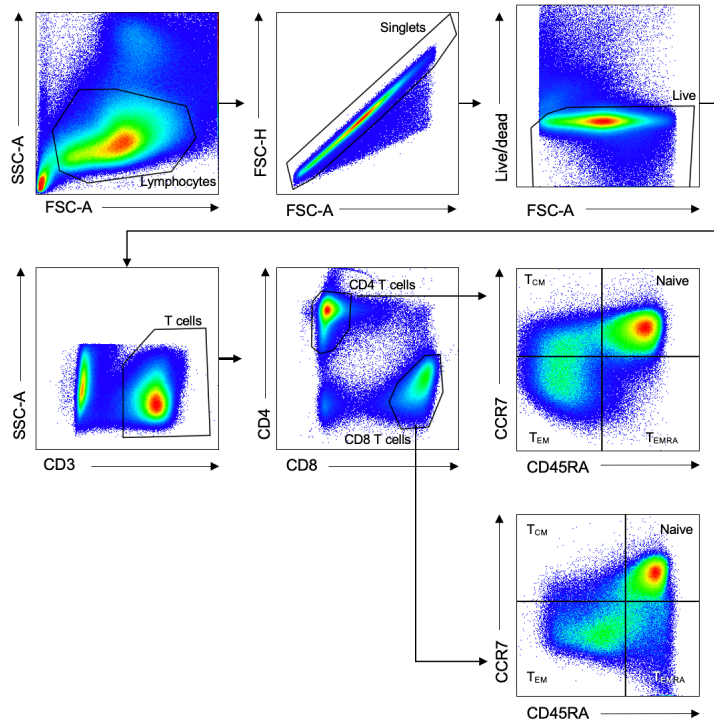


Fig. S5: General gating strategy for T cells used for blood, biopsy, and cytology specimens. All lymphocytes were defined by size. T cells were defined as CD3⁺ and the differentiation between CD8 and CD4 were gated and respectively split into two dot plots each defining Naive (CD45RA⁺, CCR7⁺), T_{CM} (CD45RA⁻, CCR7⁺), T_{EM} (CD45RA⁺, CCR7⁻) and T_{EMRA} (CD45RA⁺, CCR7⁻).

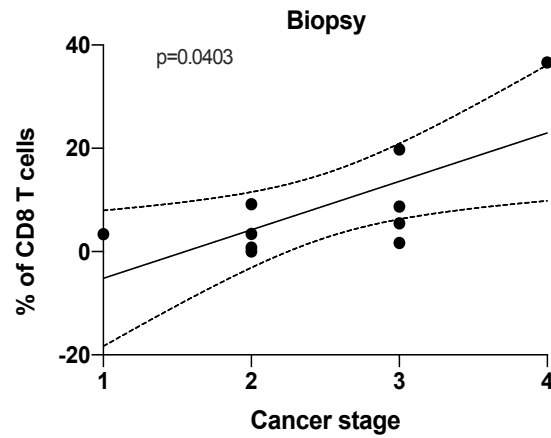


Fig. S6: Percentage of CD8 T cells according to cancer stage

Correlation between disease stage and proportion of CD8 T cells in cancer patients. Spearman's rank correlation with one-tailed p values were calculated. The bands show the 95% confidence intervals of the linear regression slopes.

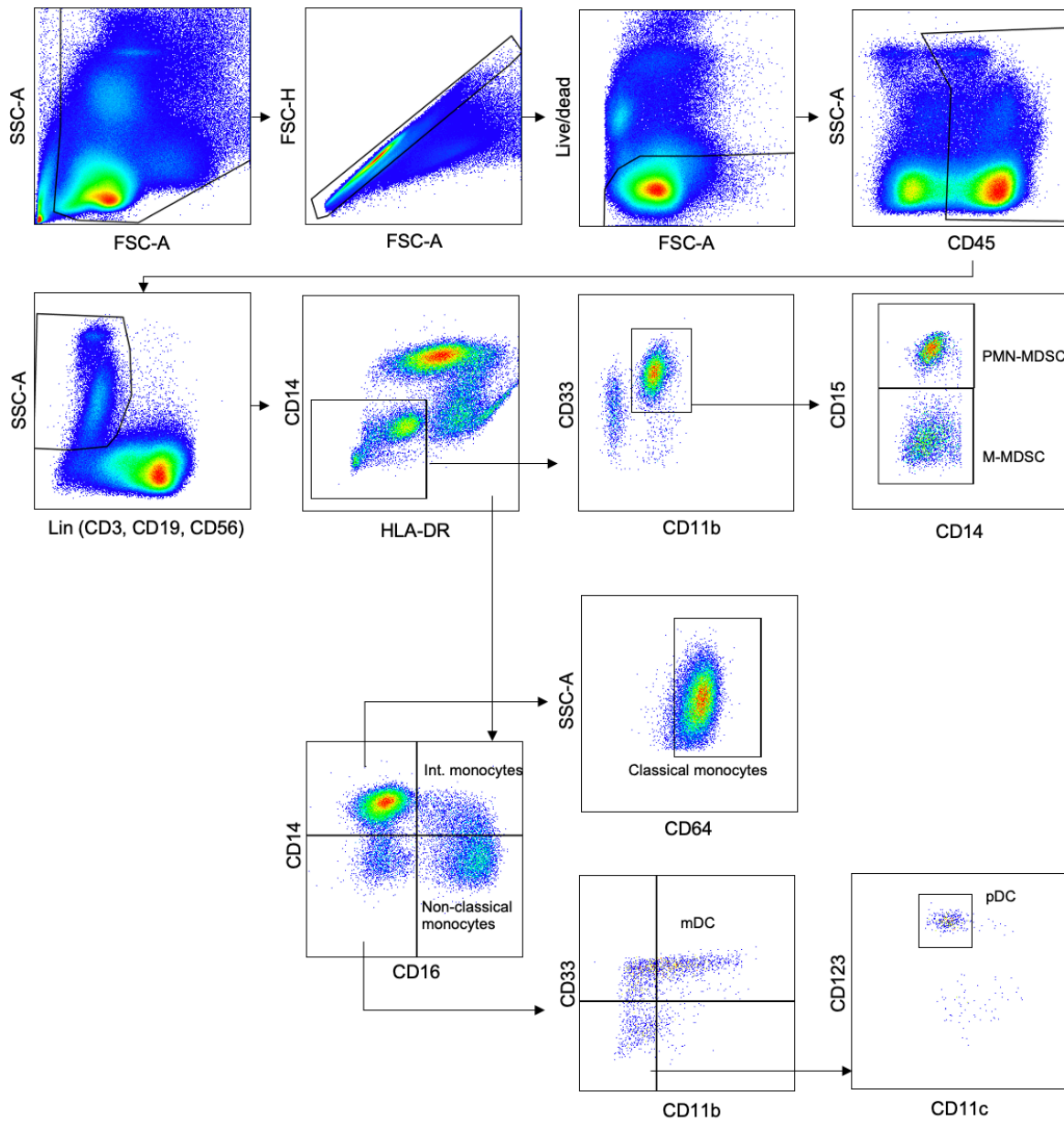


Fig. S7: General gating strategy that was employed in the tissue specimens and in the blood for myeloid cells. All myeloid subsets were defined as CD45⁺ and Lin⁻ (CD3, CD19, CD56) to include leukocytes while excluding T cells, B cells and NK cells respectively. Monocytes were divided into classical (HLADR⁺ CD14⁺ CD16⁻ CD64⁺), intermediate (HLADR⁺ CD14⁺ CD16⁺) and non-classical monocytes (HLADR⁺ CD14⁻ CD16⁺). DCs were defined as mDC (HLADR⁺ CD14⁻ CD16⁻ CD33⁺ CD11b⁺) and pDC (HLADR⁺ CD14⁻ CD16⁻ CD33⁻ CD11b⁻ CD123⁺, CD11c⁻). The MDSCs (including granulocytes) were defined as HLA-DR⁻ CD14^{+/-} CD33⁺ CD11b⁺ cells and divided into PMN-MDSC (CD15⁺) and M-MDSC (CD15⁻)

Mapping of HPV-restricted T cell recognition in Cervical
Intraepithelial Neoplasia and Cancer
Characterization of immune
infiltration In High-grade Cervical Intraepithelial Neoplasia and
Cancer

Authors:

Dorthe Blirup Snebjerg¹, Mohammad Kadivar¹, Marie Viuff¹, Stine Kiær Larsen², Benny Kirschner³, Kirsten Marie Jochumsen⁴, Jesper Bonde⁵, Susanne Krüger Kjær⁶, Sine Reker Hadrup^{1*}.

Affiliations:

¹ Department of Health Technology, Technical University of Denmark, Lyngby, Denmark

²The National Center for Cancer Immune Therapy, Herlev Hospital, Herlev, Denmark

³ Department of Gynecology and Obstetrics, Hvidovre Hospital, Hvidovre, Denmark

⁴ Department of Gynecology and Obstetrics, Odense University Hospital, Odense, Denmark

⁵ Department of Pathology, Hvidovre Hospital, Hvidovre, Denmark

⁶ Danish Cancer Society Research Center, Copenhagen, Denmark

*Correspondence

Corresponding Author Sine Reker Hadrup, sirha@dtu.dk

ABSTRACT

The immune system plays an active role in viral clearance and especially our T cells are prone to kill and eliminate virus-infected cells - when activated. 685 potential distinct human leucocyte antigen (HLA)-binding peptides were evaluated covering E2, E6 and E7 genes of both HPV 16 and HPV 18, to examine CD8 T cell recognition of Human Papilloma Virus.

The cells were analyzed using DNA-barcoded peptide-MHC complex multimers and was thereby able to detect 127 immunogenic epitopes recognized by CD8 T cells. The majority of the predicted epitopes came from the E2 protein, and this was also where most epitopes were recognized. This makes the E2 gene a very immunogenic region of the HPV genome.

To validate our results, the tetramer staining assay was used on selected CD8 T cell recognized peptides which were able to confirm our results.

Among the three groups (healthy individuals, neoplasia, and cancer patients) a higher number of recognitions to HPV derived peptides were found in both the neoplasia and cancer group compared to the healthy individuals. The HLA-C05:01 allele turned out to be very dominant in the total number of identified epitopes and some skewing due to cross reactivity is likely the case.

These results provide insight into the CD8 T cell recognition and the immunogenic hotspots of interest and can hopefully be of use in the future, when designing immune therapy and the coveted targets of HPV.

INTRODUCTION

Cervical cancer is the fourth most common malignancy diagnosed in women worldwide, with 604,127 cases (3.1% of new cancers cases all ages, both sexes registered in WHO 2020) and 341,831 deaths (3.3% all ages, both sexes registered in WHO 2020) [1][2][3]. During their lifespan exposure to Human Papilloma Virus (HPV) causes 80% of Danish women to be infected with the virus. The immune system is essential for the ability to control and clear the viral infection and in particular cytotoxic CD8 T cells are important. Activated CD8 T cells alongside with supporting CD4 T cells are in 78-80% able to clear the virus whereas 10-12% do not seem to be able to defeat the infection and a persistent infection occurs. This may result in dysplastic transformation of the epithelial cells and over time progression into cancer.

Cervical HPV infection is most often an asymptomatic infection and is divided into low- and high-grade serotypes [4][5][6][7]. The most common serotypes of HPV in women leading to cervical cancer, are in descending order of frequency 16, 18, 45, 31, 33, 52, 58, and 35 [8][9][10]. HPV 16 and 18 are reported to account for approximately >70% of cancer cases [11]. However, essentially all cervical cancers contain DNA of an oncogenic HPV type [4][12].

The HPV genome being a circular double-stranded DNA encodes six early genes (E1, E2, E4, E5, E6 and E7) and two late genes (L1 and L2), along with a non-coding region. The two viral genes E6 and E7 are of particular significance due to their role in inactivation of the host tumor-suppressor genes. They are continuously expressed in high-risk types and their expression is required to induce and maintain the neoplastic phenotype and oncogenic progression. They are therefore referred to as oncoproteins in the literature [13][14][15]. The E2 gene acts as a transcriptional repressor of E6 and E7 but when the viral DNA becomes integrated in the host cell the E2 sequence gets disrupted which leads to increased expression of E6 and E7. E2 therefore plays a critical role in oncogenic progression of HPV 15][16][17]. These three genes were therefore chosen for further investigations.

The biological role of how individuals respond when infected, their ability to clear this virus and the contribution of infiltrating immune cells remains to be fully determined. Furthermore, immune checkpoint inhibition has not shown the promising result as hoped and speculations on the interplay of multiple different mechanisms in the tumor microenvironment are still ongoing [18].

CD8 T cells are activated when their T-cell receptor (TCR) are interacting with the major histocompatibility complex class I (MHC-I) molecules and the peptide antigen (minimal peptide

epitope) they present on their surface of virus-infected cells. Regarding HPV, the spectrum of exact epitopes within the viral genome being presented and therefore becoming immunogenic is not fully described.

This study aims to map T cell recognition from the early gene region E2 and oncoprotein E6 and E7 genes of both HPV 16 and 18 and identify the exact epitopes recognized by HPV specific CD8 T cells and the immunodominance of these epitopes. The T cell recognition profiles (breadth and intensity) in healthy individuals were compared to patients diagnosed with severe neoplasia and with cervical cancer patients. Thereby obtaining a deeper understanding of the characteristics between immune activation at the early stage and at the late stage of the disease. Previously published large-scale T cell detection technology based on DNA-barcoded peptide-MHC (pMHC) multimers from this research group was applied [19].

A potential immune recognition was expected in healthy individuals due to prior HPV infection.

The study included 8 healthy, 9 with severe neoplasia and 10 with cervical cancer and their specific CD8 T cell recognition of predicted peptide epitopes was evaluated. Previous studies [20][21] determines that the oncoproteins E6 and E7 possibly incite the neoplastic changes. E2 encodes the transcription function, and all early proteins regulate immune modulation and structural modifications of the infected cell.

This study identified a relatively increased rate of T cell responses in CIN 3, and cancer patients compared to healthy. We identified 65 unique HPV-derived peptide-MHC complexes recognized by HPV specific CD8 T cells in 27 individuals and nine of these epitopes were immunodominant. This study also points out early region E2 (HPV16/18) as hotspot of interest for further analyzes harboring 68% of all peptides recognized by HPV specific CD8 T cells.

RESULTS

HPV-SPECIFIC CD8 T CELLS RECOGNIZED EPITOPES COMING FROM THE E2 GENE

To identify the minimal peptide epitopes recognized by CD8 T cells it was decided to investigate E2, E6 and E7 of both HPV16 and HPV18. The genome sequence from these six genes was analyzed and found no overlap in the predicted epitope sequences from the two genes. Regarding size, E2 is the largest, E6 approximately half the size and E7 the smallest in both HPV 16 and HPV 18 E2 (Fig. 1A, B and Fig. S1). Using NetMHCpan 4.0 algorithm, 685 potential distinct human leucocyte antigen (HLA)-binding peptides (9 to 11 amino acids) were selected in the library. 14 of the most common HLA-A, B and C alleles across European Caucasian populations Belgium (n=99), Germany (n=11407, n=8862), Sweden (n=966), Norway (n=576), The Netherlands (n=1305) was covered being: HLA-A (A01:01, A02:01, A03:01, A11:01, A24:02), HLA-B (B07:02, B08:01, B15:01, B35:01) and HLA-C (C03:04, C04:01, C05:01, C07:01, C07:02) loci (Fig. 1B and Fig. S1). They were predicted to bind one or more allele, and the E2 is by far the biggest gene region and hence contributed with the highest number of predicted peptide epitopes for both HPV 16 and HPV 18 (Fig. 1C). The highest number of predicted epitopes was found from HLA-A01:01, HLA-A11:01, HLA-C07:01 and HLA-C07:02.

The T cell reactivity of 27 individuals towards these predicted peptides were analyzed (8 healthy, 9 with cervical intraepithelial neoplasia (CIN) grade 3 and 10 with cervical cancer). Blood samples (50 mL) were collected prior to surgery or any kind of treatment. The healthy group had hysterectomy for reasons other than HPV. The severe neoplasia group had a cervical cone biopsy and the cervical cancer patients had gynecology examination in full anesthesia to plan further treatment. All cervical cancer patients had invasive stage and were classified by disease progression according to the FIGO grading scale [22]. Most cancers patients were classified as invasive (stage II or higher). 73% were squamous cell carcinomas, 20% were adenocarcinomas, remaining two patients had tumor of adeno-squamous and adenocarcinoma of mucinous type.

The mean HLA coverage that could be obtained using the 14 selected MHC-I molecules was 3.3 per patient and the average DNA-barcoded pMHC multimers used pr. patient was 276.

Briefly, pMHC complexes are attached to a PE (phycoerythrin)-labeled or APC (allophycocyanin)-labeled dextran backbone and labelled with a unique DNA barcode. To generate an HLA-matching patient-specific pMHC multimer panel, DNA-barcoded pMHC multimers were then pooled and incubated with patient-derived PBMCs (peripheral blood

mononuclear cells). Those multimers bound to CD8 T cells were then sorted and sequenced to identify the T cell recognition toward the probed pMHC complexes (Fig. 1D).

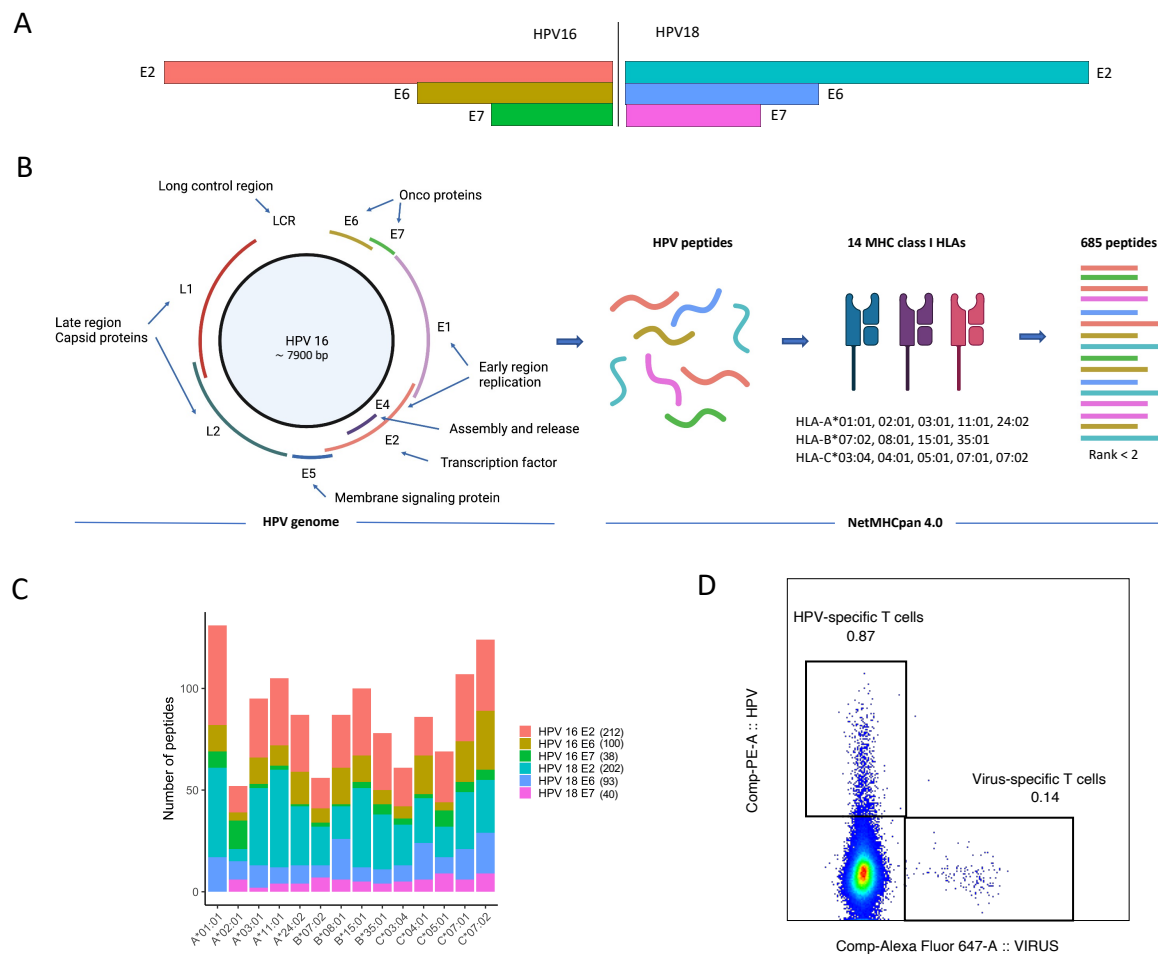


Fig. 1. Prediction of HPV CD8 T cell epitopes. (A) Schematic illustration of the early region E2, E6 and E7 genes of HPV 16/18 and their relative size, no overlap of the genes. (B) Schematic representation of the complete HPV 16 circular genome with all genes represented and used for identification of 685 peptides with predicted binding rank (NetMHCpan 4.0) of <2 for 14 prevalent HLA-A, HLA-B and HLA-C alleles. (C) Bar plot showing the distribution of HPV peptides related to their HLA-restriction (1238 peptide-HLA pairs) across the E2, E6 and E7 of both HPV 16 and HPV18. Total pMHC specificities analyzed for each protein are shown in parentheses next to the respective HPV protein. (D) Representative plot of flow cytometry pMHC multimer staining of CD8 T cells from a HPV positive patient stained with pMHC multimer panel showing HPV (PE) and CEF (APC) multimer⁺ T cells that were sorted for DNA barcode analysis to identify epitope recognition.

For comparative evaluation, 39 T cell epitopes from common viruses was also included in the panel, containing: cytomegalovirus (CMV), Epstein-Barr virus (EBV), and influenza (flu) virus (CEF-pool) (Fig. 2A).

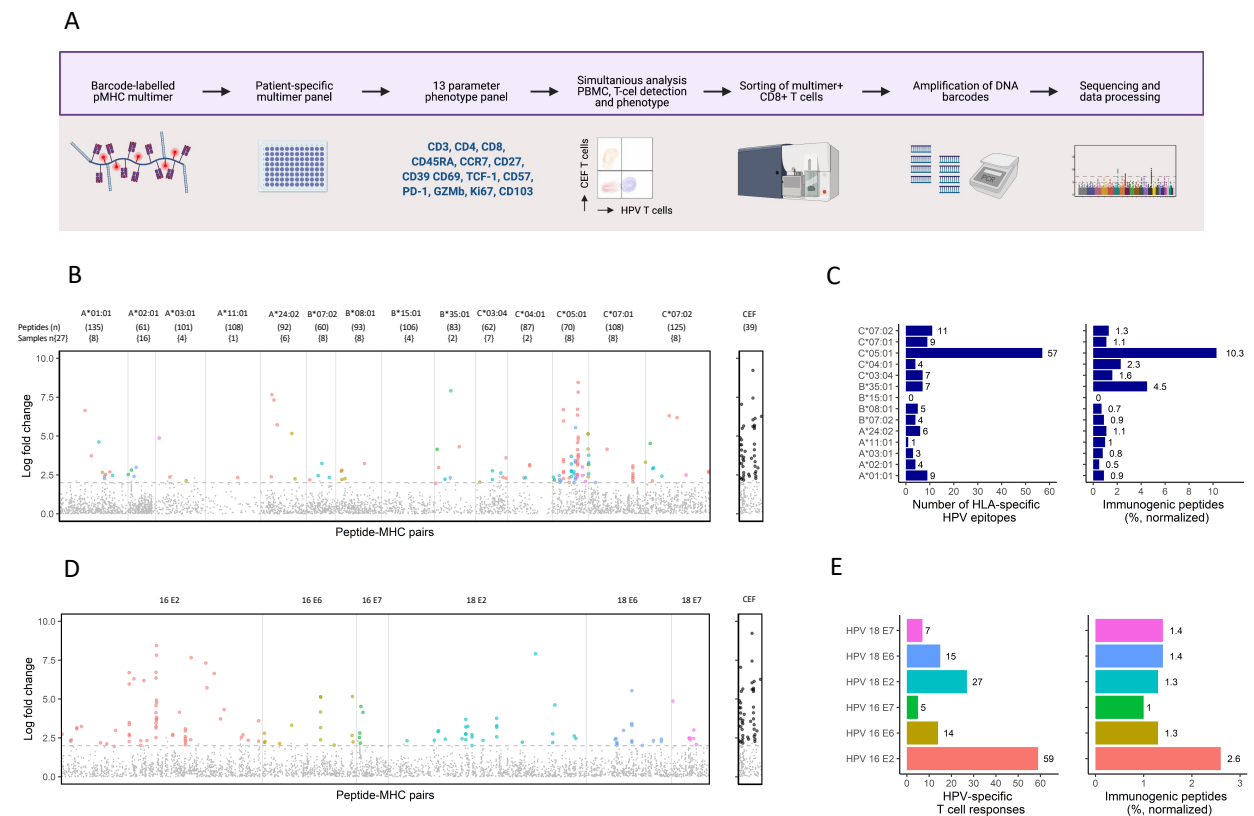
All specimens were on average stained with 276 different peptides and a total of 1238 distinct barcodes (peptide-MHC complexes) were in the library. 127 HLA specific HPV epitopes across

all individuals were identified from the 14 different HLAs analyzed (Table S3). They contained 65 unique peptide-MHC complexes and in total 64 specific peptide sequences (Fig. 2B). 44 T cell responses towards CEF-derived peptides across the 27 individuals was also identified, an average of 2.3 per patient. Regarding the HPV derived peptides, HLA-C05:01 turned out to be very dominant in the total number of identified epitopes even though 10 of the 14 HLAs had a higher predicted number of epitopes. HLA-A01:01, HLA-C07:01 and HLA-C07:02 are increased (Fig. 2 C). The immunogenic peptides (the number of T cell responses normalized to the number of probing pMHC multimers and the number of individuals analyzed) still show HLA-C05:01 to be very immunodominant but also HLA-B35:01 is increased. The high number of responses towards HLA-C05:01 might possibly be unspecific interaction, possibly driven by the killing inhibitory receptor (KIR) [23][24]. HLA-B15:01 showed no T cell reactivity and HLA-A01:01, HLA-02:01, HLA-A03:01, HLA-B07:02 and HLA-B08:01 had less than 1% immunogenic peptides (9, 4, 3, 4 and 5 epitopes each) despite being analyzed in 8, 22, 6, 10, 8 patients respectively (Fig. 2 C). Most of the immunogenic epitopes were mapped to the E2 gene especially HPV 16 but also HPV 18 E2 followed by HPV 16/18 E6 (Fig. 2D + E). Given the size difference of the viral proteins where E2 being over twice as long (Fig. 1A and 2E), their relative contribution to T cell recognition was evaluated by the immunogenicity score. We observed that peptides derived from HPV E2 displayed the highest relative immunogenicity (in terms of T cell recognition), especially from HPV 16. In summary we found HPV specific CD8 T cell immunity towards several epitopes and a substantial presence of HPV-specific T cells in both CIN3 and cancer patients. This HPV-specific T cells infiltration of the cervical tissue indicate activity of the immune system, which is advantageous, but does not fully elucidate if these CD8 T cells can defeat and clear the virus.

SIX OUT OF NINE HPV-DERIVED IMMUNODOMINANT EPITOPES ARE RESTRICTED TO HLA-C05:01

Out of the 65 unique peptide-MHC complexes we found 9 immunodominant epitopes from which we detected T cell recognition in >50% of the tested specimens according to the specific HLA. Although for some HLA alleles, the tested population size was small (Fig. 2F). HLA-C05:01 turned out to be extremely immunodominant accounting for six out of the nine most prevalent epitopes (SVDSAPIL, ICEEASVTV (and its variant YICEEASVTV), YRDGNPYAV, YVAWDSVYYM, FAFKDLFVV) and the rest being restricted to HLA-A11:01 (RLECAIYYK), HLA-C04:01 (HYTNWTHIY), HLA-C07:01 (YRFKKHCTL). Six of the nine immunodominant epitopes originate from the HPV16 E2 gene which correlates nicely to the distribution of the predicted peptides (Fig. 1 C) and HPV 16 E6, HPV 18 E2 and HPV 18 E6 accounts for the latter three (Fig. 2 G).

Exposure to HPV is highly likely and therefore preexisting T cell immunity is expected. After prediction and specific T cell recognition, the position of such T cell recognition was mapped to the six different proteins sequences. We observed clear “immunogenic hotspots” where T cell recognition is clustered to certain areas of the protein, while other areas are not recognized by T cells although epitopes were predicted and included for T cell screening (Fig. 2 H). As for HPV E2-protein these hotspots were spread out over the entire protein sequence with high epitope count, while for HPV 16/18 E7, hotspots clustered in three minor groups with few epitope-counts in each. This mapping helps elucidate the potential hotspots of interest for T cell recognition of the HPV 16/18 E2, E6 and E7 genes.



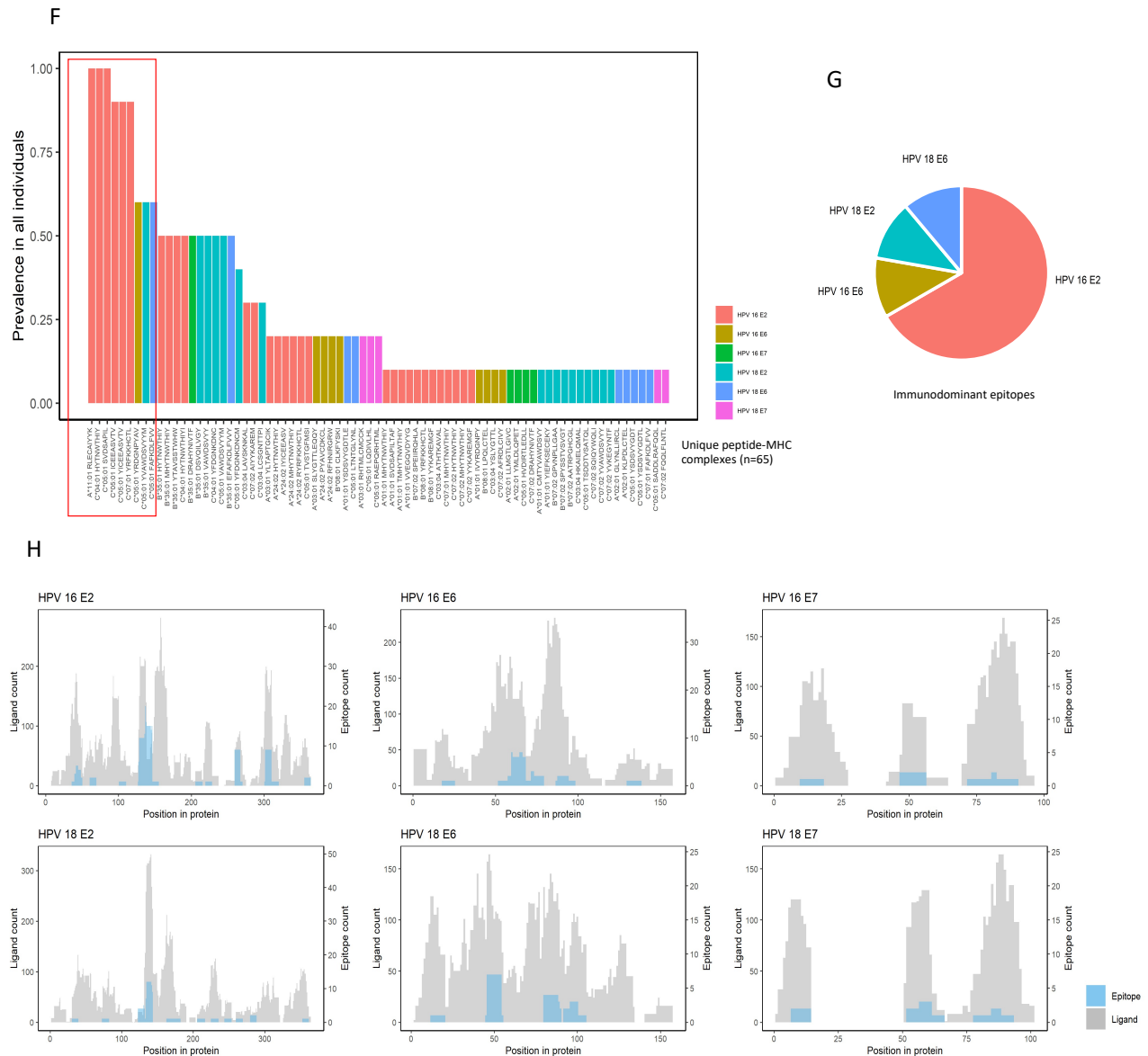


Fig. 2. Mapping of HPV CD8 T cell epitopes. (A) Experimental pipeline of T cell recognition analyzes towards HPV-derived HLA-binding peptides in PBMCs using peptide-MHC multimers. The specimens were stained with a 16-antibody panel and sorted on the basis of PE (HPV-specific) or APC (CEF specific) signal, amplified by PCR and sequenced in order to identify antigen-specific CD8 T cells. (B) Dot-plot showing summary of all T cell recognition to HPV derived peptides identified in the group (n=27) by HLA alleles. In parentheses, number of peptides tested for each HLA (top row) and the number of individuals analyzed for each HLA pool (bottom row). Each dot represents one peptide-HLA combination per patient and is colored according to their origin of protein, same colors as shown in (Fig. 1). The black dots show CD8 T cells reactive to the CEF peptides in all analyzed individuals. (C) Bar plots summarize the number of HLA-specific HPV epitopes identified and the HLA-restricted immunogenicity (% immunogenic peptides) in the analyzed patient group. Immunogenicity represents the fraction of T cell recognized peptides out of the total number of peptides analyzed for a given HLA restriction across the HLA-matching donors (% normalized). (D) Comparable to (C), a summary of HPV-specific responses separated based on the protein of origin. (E) Bar plots showing the number of epitopes derived from each of the HPV-proteins and their immunogenicity score (% immunogenic peptides). (F) Bar plot illustrating the prevalence of T cell recognition towards the individual peptide epitope detected in HPV+ patients. The red box indicates the immunodominant epitopes based on the presence of T cell recognition in >50% of the analyzed patients. Bars are colored according to their protein of origin, as shown in Fig. 1. (G) Pie chart of immunodominant epitopes distributed according to their protein of origin. (H) HPV T cell

immunogenicity map across the six different viral proteomes comparing the distribution of identified HPV-epitopes (patient group, blue line; n=27 patients) with the total peptides analyzed (grey line). The height of a peak indicates the number of ligands (left axis) analyzed in a particular region and the number of identified epitopes (right axis).

To validate the T cell recognition conventional fluorophore-labeled pMHC tetramer staining was performed on 2 healthy individuals, 2 CIN-3 patients and 4 cancer patients. From 15 immunodominant epitopes we could confirm a positive response in 69% of the CD8 T cell recognitions (Fig. 3). The range of the CD8 T cells were between 0.25-17.3% of CD8 T cells. We determined a confirmed response 4 times in cancer patients, once in CIN-3 patients and once in healthy individual. 15 of the responses were not easily defined because of less separation of the cell populations and therefore making them a borderline result (cancer n=2, CIN 3 n=7 all in the same patient, healthy n=6) (Fig. 3A upper left and lower right dot plot). pMHC-tetramers with no previous response were also included as negative control. We found that individually labeled pMHC tetramer results correlate to the responses detected by the DNA barcode-labeled MHC multimer assay.

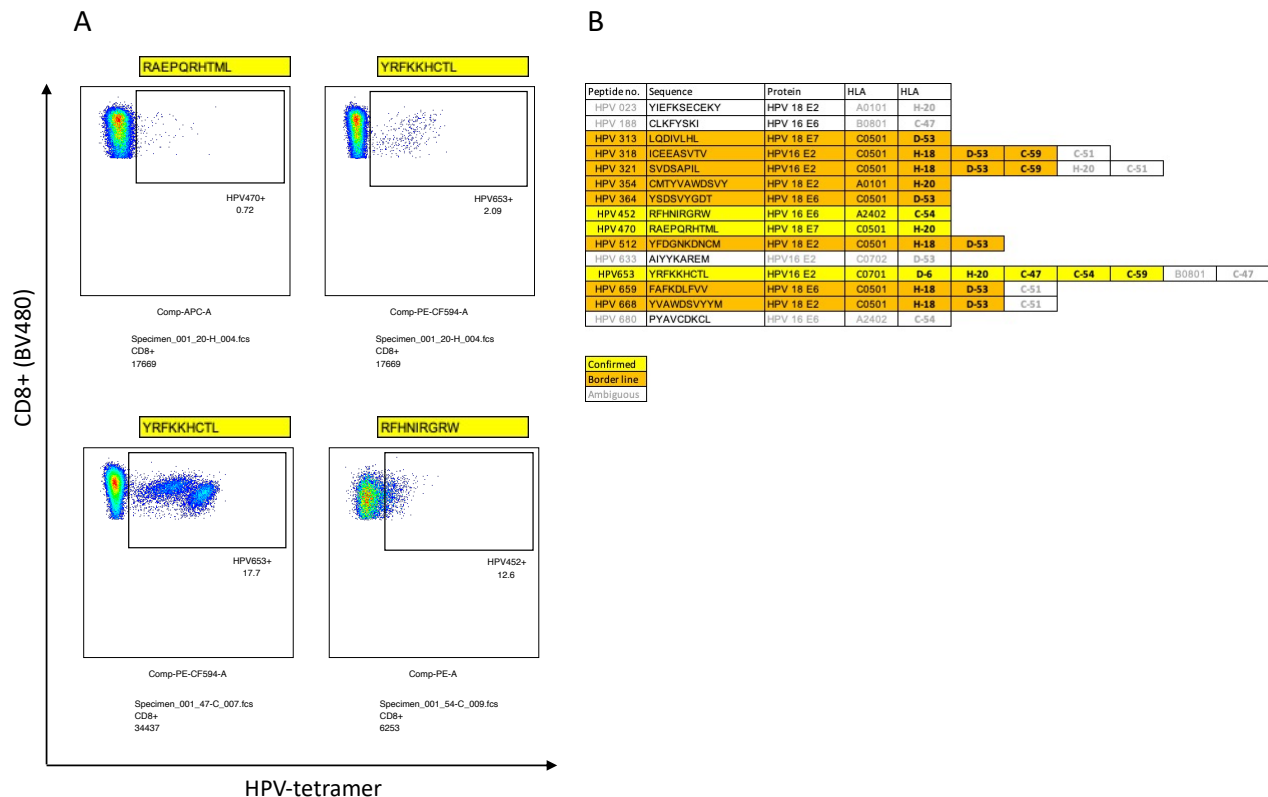


Fig. 3: Validation using tetramer stained analyzes. (A): Representative plots of tetramer-based analyses of CD8 T cells in PBMCs of eight individuals. Two healthy (HLA-01:01, HLA-C05:01, HLA-C07:01), two CIN-3 (HLA-C05:01, HLA-C07:01, HLA-C07:02) and five cancer patients (HLA-A24:02, HLA-B08:01, HLA-C05:01, HLA-C07:01). The gated populations show the percentage of T cells recognizing pMHC tetramers out of total CD8 T cells. The HPV

sequence of the validated peptides are marked with bright yellow (**B**) Scheme of the immunodominant predicted epitopes showing confirmed responses according to HLA and the corresponding individual patient sample. Bright yellows are confirmed by both DNA barcode-labeled MHC multimer technique and individually labeled pMHC tetramers with distinctive populations. Orange shows borderline separation of CD8 T cell populations and white indicates no separation and therefore no recognition of the CD8 T cells.

Furthermore, when dividing the group into cancer, CIN3 and healthy individuals and comparing number of T cell recognition to HPV derived peptides to all 14 HLA alleles it is striking the number of responses HLA-C*05:01 accounts for, especially in the healthy and CIN 3 group. Overall, there are a higher number of responses recognizing HPV derived peptides in CIN 3 and cancer group – as to be expected (Fig. 4A).

Looking at their number of T cell responses to HPV derived peptides pr. patient, the results were analyzed both with (Fig. 4 B, C and D) and without HLA-C alleles to account for any skewing because of the extremely high responses in HLA-C05:01 (Fig. 4 E, F and G).

The CIN 3 group shows a higher number of recognitions to HPV derived peptides compared to healthy and cancer individuals, but not significant and with no difference between cancer and healthy individuals (Fig. 4 B). When normalized to HLA coverage (Fig. 4 C) the results for CIN 3 are even more clear but probably HLA-C05:01 still accounts for some skewing. Regarding the T cell responses (normalized to total screen) HPV 16 E2 was found to be the most prevalent for all three groups, cancer (n=10), CIN 3 (n=9) and healthy (n=8).

When analyzing the same results except HLA-C, the results show a more pronounced change between the three groups. Still CIN 3 being high, but the difference between healthy and cancer groups is clearer (Fig. 4 E, F and G). Fig. 4 E shows few responses in the healthy group whereas the CIN 3 group is the high responder and the cancer group being in the middle. There are two outliers with extremely high T cell response which might indicate a strong immune level/activation (Fig. 4 E). When normalizing data to HLA coverage (Fig. 4 F) it is even more evident that the CIN 3 group have the highest T cell response of all three groups. This could be very valuable in selecting the CIN 3 patients according to their immune profile in terms of their immune response (T cell reactivity) and thereby differentiated need for follow-up. Lastly looking at the prevalence of T cell responses (normalized to total screen), a nice significant response was found in both the CIN 3 and cancer group, compared to healthy. HPV 16, E2, E6 and E7 seems to be very immunogenic in the CIN 3 and cancer groups, whereas the level of HPV 18 E2 have almost the same prevalence of T cell responses (Fig. 4 G).

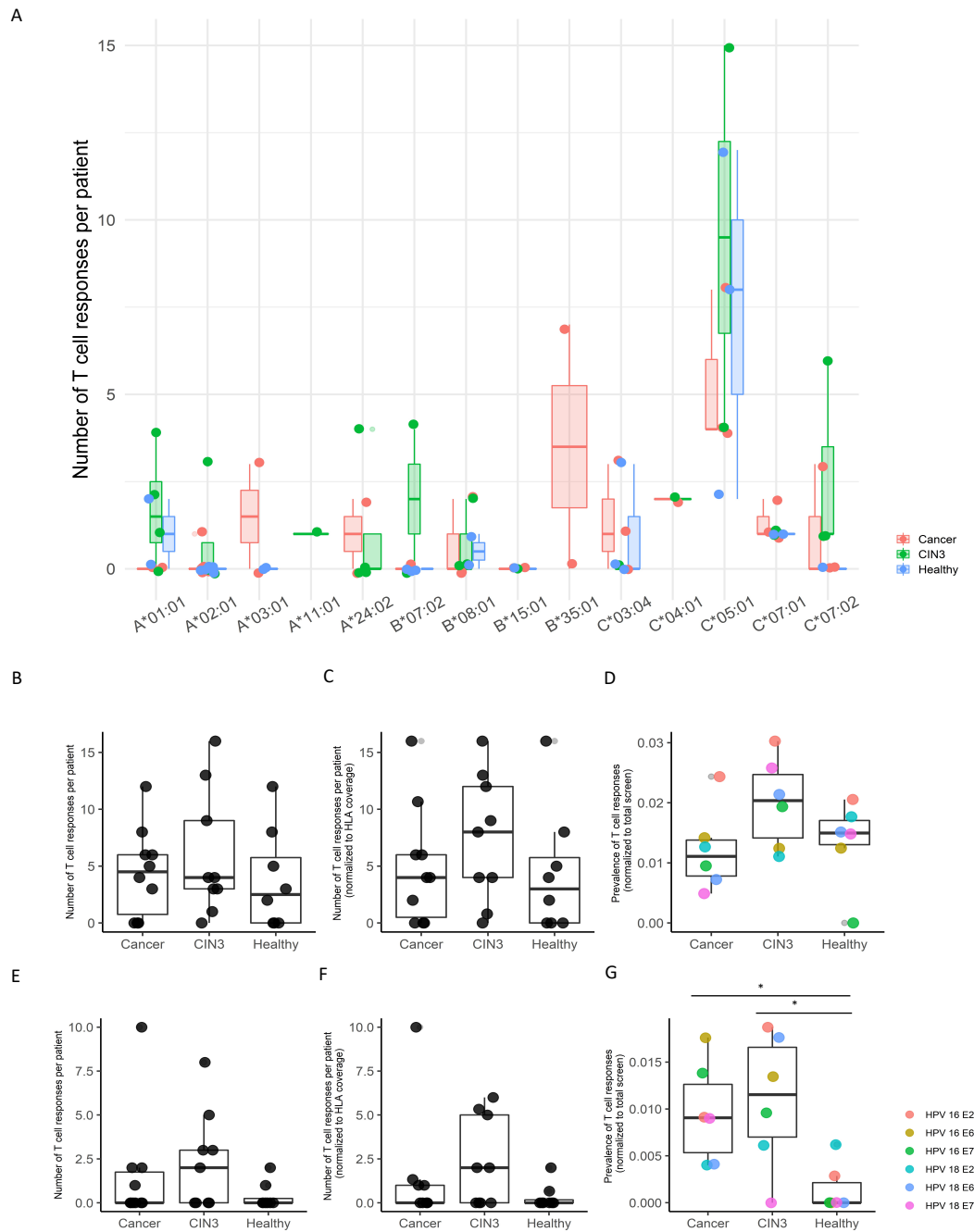


Fig. 4: (A) Box plot comparing number of T cell recognition to HPV derived peptides to all 14 HLA alleles. Dots and boxes are colored according to the three groups, cancer (n=10), CIN 3 (n=9) and healthy (n=8).

(B) Box plot comparing number of HPV epitopes recognized by T cells pr. patient in cancer (n=10), CIN 3 (n=9) and healthy (n=8). (C) Box plot comparing number of HPV epitopes recognized by T cells pr. patient normalized to HLA coverage for all three groups (the fraction of T cell-recognized peptides out of the total number of peptides analyzed for a given HLA restriction across the HLA-matching donors). (D) Box plot comparing prevalence of T cell responses, normalized to total screen recognized in cancer (n=10), CIN 3 (n=9) and healthy (n=8) (number of T cell-recognized peptides in a given HLA out of the total number of peptide-MHCs screened for this HLA (library size and number of patients screened for this HLA)). Dots are colored according to their origin of protein, same colors as shown in Fig. 1. Plots show mean \pm SD (Kruskal-Wallis uncorrected Dunn's test, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). (E), (F), (G) Box plots illustrating the same as J, K and L but without HLA-C. Plots show mean \pm SD (Kruskal-Wallis uncorrected Dunn's test, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

Over all the data shows a correlation between the predicted epitopes and the T cell recognition found in the patient group. The early region E2 especially from HPV 16 turned out to be the most immunogenic gene and is therefore as interesting to look further into as the more well-known oncoproteins E6 and E7. An enrichment of T cell recognition to the predicted peptide library was also found, although HLA-C 05:01 seems to be very dominant and one might speculate if it is an outlier or cross recognition. The attempt to validate some of our T cell recognition with tetramer staining showed confirmation of the DNA-barcoded peptide-MHC multimers positive responses in 69% of the CD8 T cell responses.

DISCUSSION

HPV is a well-known virus and is well described in the literature. This study gives novel insight into the characterization of T cell recognition of predicted minimal epitopes by large-scale T cell detection technology based on DNA-barcoded peptide-MHC (pMHC) multimers. The immunogenicity from the early gene region E2 and oncoprotein E6 and E7 genes of both HPV 16 and 18 was screened for T cell recognition based on 685 potential distinct peptide predicted sequences in the library from the protein sequences and selected based on their HLA-binding capacity. We identified 65 unique HPV-derived peptide-MHC complexes recognized by HPV specific CD8 T cells in 27 individuals and nine of these epitopes were immunodominant. Among the evaluated epitopes 669 were predicted using NetMHC-Pan 4.0 and further 15 epitopes were added to the list using IEDB.org [25] and web search. Out of the 15 added not already on the list only one epitope (LLMGTLGIVC, HPV16 E7, HLA-A02:01) was recognized by CD8 T cells in our group. This list was generated in March 2018 and new epitopes are updated continuously to the IEDB database.

The positive responses seen in the healthy group are not surprising since the lifetime risk of being infected with HPV is high and it is still not clear how long time after infection it is possible to trace T cell recognition and the correlation to current methods of viral HPV DNA detection. For healthy individuals it is highly likely that despite being HPV negative on PCR test and thereby clearance of the virus, there might still be HPV specific CD8T cells remaining, however the reasons remain unknown.

The lower number of T cell responses pr. patient in the cancer group might indicate either the immune system has not yet fully detected the virus and mounted a T cell response, or it could also be a sign of T cell exhaustion and immune defeat [15][18].

More research would be helpful to determine whether immune therapy would be helpful and thereby still be able to reinvigorate the immune cells or if it is too late. The CIN 3 group might be more interesting to investigate to distinguish those who need an immediate immune boost/therapy or those who just need active surveillance because their immune system already has detected the virus and ongoing killing is taking place.

The current vaccine against HPV has the virus capsid as target and does therefore not have the intended effect on already infected or possible re-infected persons. This study might help to point out hotspots of interest for further analyzes with focus on T cell immunity. This study suggest early region E2 might be highly relevant and requires further analyzes. A more longitudinal study of the dysplastic changes from the same group would also be highly interesting together with the development of T cell recognition of immunodominant epitopes over time from date of infection. Also, the TCR and the interaction with the peptide-MHC complexes have shown great cross reactivity and the technology to understand this is constantly improving. Over time this may allow us to gain insight and obtain more knowledge in these highly complex recognitions and interactions. Analyzing the rest of the HPV genes and predicting epitopes still needs further investigations.

In this study we identified a relatively increased rate of T cell responses in CIN 3, and cancer patients compared to healthy, and a substantial T cell recognition was validated by tetramer staining. All data verified CD8 T cell recognition of HPV epitopes after infection with HPV virus and we suggest immunodominant hotspots of interest for further functional analyzes to be able to offer vaccines, immune checkpoint inhibition or cell therapies.

MATERIAL AND METHODS

STUDY DESIGN

The aim of this study was to identify CD8 T cell recognition from the early gene region E2 and oncoprotein E6 and E7 genes of both HPV 16 and 18 and identify the exact epitopes recognized by HPV specific CD8 T cells and the immunodominance of these epitopes. We compared 685 peptides to the T cell recognition (breadth and intensity) in three groups (healthy, severe neoplasia and cervical cancer patients). Thereby obtain a deeper understanding of the characteristics between immune activation at the early stage and the late stage of disease. DNA barcoded MHC multimer T cell detection technology was applied in combination with a phenotypic flow cytometry panel of 16-parameters for T cell identification. This was done with PBMCs from a group of 27 individuals (healthy n=8, severe neoplasia n=9 and cervical cancer n=10).

ETHICAL STATEMENT

Approval for the study design and sample collection was obtained from the Committee on Health Research Ethics in the Capital Region of Denmark. All included individuals gave their informed written consent for inclusion. Liquid-based cytology (LBC) specimens from the transformation zone and 50 mL peripheral blood was obtained - a full set from each participant.

All specimens were obtained anonymously, marked with a study number and the remaining material will be kept anonymized for 8 years in a research bio bank, after which time it will be destroyed. If desired by the participant, the material can be destroyed at an earlier stage. All specimens were and will be used for this present study only. The study was approval by The Danish Data Protection Agency. The study took four years and did not involve further visits or medical contact for the individuals.

COLLECTION OF CLINICAL SPECIMENS

All specimens were delivered to the laboratory at the Technical University of Denmark (DTU), Lyngby, Denmark and were all handled within 6 hours prior to sampling to increase the number of live cells.

Full medical record regarding age, previous neoplasia from HPV, medical history, comorbidity, BMI, medications, and smoking was also collected.

Peripheral blood samples were collected in five 10 mL Vacutainer heparin tubes and kept at room temperature until separated using filter tubes (Falcon Leucosep) saturated with 15 mL Lymphoprep™ 1.077g/mL, (STEMCELL, cat. 07851), and PBS. After separation, lymphocytes were resuspended and counted on NucleoCounter (Chemometec), centrifuged, and resuspended in 1 mL of 10% DMSO (Dimethyl Sulfoxide) Hybrid-Max (Sigma-Aldrich) and 90% fetal calf serum (FCS) Gibco™ qualified, New Zealand. 2×10^6 cells from all individuals were used for genomic DNA isolation and subsequently high-resolution typing of HLA genotype (IMGH Laboratories GmbH, Martinsried, Germany) (Table S1).

The specimens were then split into $5-10 \times 10^6$ cells/vial and distributed in cryotubes and frozen by $1^\circ\text{C}/\text{min}$ in freezing boxes placed at -80°C for 24 hr. and thereafter kept in -180°C nitrogen tank for long-term storage until used for further analysis.

The healthy donors were recruited from Aleris-Hamlet private hospital, Søborg, Denmark, if they underwent hysterectomy for reasons unrelated to HPV. Individuals with medical record of

previous cervical neoplasia were excluded from the study but were not tested for HPV-DNA. Specimens were obtained during already planned surgery to minimize discomfort for the patient.

Patients with CIN 3 recruited from Department of Gynecology and Obstetrics, Hvidovre Hospital, Hvidovre and specimens were collected prior to having a cone biopsy. The procedure for tissue collection was made in agreement with the local pathologists in order not to interfere with analyses of the cone biopsy.

Patients with cervical cancer were recruited from Department of Gynecology and Obstetrics, Odense University Hospital, Odense. Specimens were collected prior to assessment of the cancer stage in full anesthesia.

Patients were included soon after diagnosis and prior to any kind of treatment; however, it was not possible to determine the exact time of infection. Specimens were collected from May 2019 to June 2020. A total of 57 individuals (healthy n=24, CIN 3 n=16 and cervical cancer n=17). 4 specimens were used (two healthy, one neoplasia and one cancer) to test the experimental procedures. Three patients with neoplasia had to be excluded because of lack of material, either blood, LBC, or biopsy. In total we choose only to analyze specimens with cell count $>1 \times 10^4$. This ended up being 8 healthy, 9 neoplasia and 10 cervical cancer patients (invasive stage). More cells from blood than from cytology were obtained.

SELECTION OF HPV PEPTIDES

The protein sequence of HPV 16 E2 (ID P03120), E6 (P03126) and E7 (P03129) and HPV 18 E2 (P06790), E6 (P06463) and E7 (P06788) was found at www.uniprot.org. (Fig. S1C) The result showed several sequences with minor differences and hence tested in netMHCpan 4.0 algorithm with the same result. Relevant HLA-A, HLA-B and HLA-C alleles were selected using allelefrequencies.net where only studies covering European Caucasian populations were chosen, being: Belgian (n=99), German (n=11407), Swedish (n=966), Norwegian (n=576) and Dutch (n=1305). The 14 most common were chosen, HLA-A (A01:01, A02:01, A03:01, A11:01, A24:02), HLA-B (B07:02, B08:01, B15:01, B35:01) and HLA-C (C03:04, C04:01, C05:01, C07:01, C07:02). The search criteria in the artificial neural network netMHCpan 4.0 [26] were: 8-11 peptide in length, percentile rank binding threshold of 2%. In total 1161 potential distinct human leucocyte antigen (HLA)-binding peptides, binding to one or more, were selected. Furthermore IEDB.org was used and settings were ≥ 1 references and ≥ 2 assays and maximum length of 14. This generated further 23 predicted peptides but 16 of those were already on the list therefore only 7 epitopes were added. A PubMed search for known CD8 T cell epitopes was also performed and

resulted furthermore 31 peptides, showing 28 duplicates and only 3 more to add to the prediction list. All predicted peptides were custom synthesized with an estimated purity of 70-92% by Pepscan Presto BV, Lelystad, The Netherlands. This generated in total 1238 peptide-HLA pairs for experimental evaluation.

PRODUCTION OF MHC-MONOMERS

The 14 different MHC-I monomers were produced using plasmids in *Escherichia coli* expressing the heavy chain and the human β_2 -microglobulin and their soluble denatured proteins were collected. UV-labile HLA-specific peptide ligands were used when folding of the MHC-I molecules. Some folded empty and some biotinylated using BirA biotin-protein ligase standard reaction kit (Avidity LLC, Aurora, CO). All MHC-I monomers were purified and stored at -80°C until further use [27].

PREPARATION OF DNA-BARCODED MULTIMERS

The DNA-barcode technique was applied to our results and the technique developed in our research group [28]. To prepare a multimer library, unique barcodes using a combination of single-stranded A and B oligos was used, as the 5' biotinylated DNA sequence. These barcodes were then attached to a conjugated fluorochrome PE (HPV epitope library) or APC (CEF multimer binding) and conjugated to the dextran backbone by a biotin-streptavidin interaction (Fina Biosolutions, Rockville, MD, USA). DNA barcodes (final concentration 17.8 mM) were mixed with dextran backbone (final concentration of 35 mM) and were incubated at 4°C , 30 min to generate a DNA barcode dextran library of the unique barcode specificities. The HPV library was generated by incubating 200 μM of each peptide with 100 $\mu\text{g/ml}$ of the specific MHC molecules for 1 hour and UV-mediated peptide exchange occurred, or direct binding, if the MHC-I molecule was empty (HLA 02:01 and A24:02). Incubation of the pMHC to their corresponding DNA barcode-labeled dextran at 4°C for 30 min generated the HLA-specific DNA-barcode multimer library, to select the respective T cell population. To select HLA-A, HLA-B and HLA-C, APC- and streptavidin-conjugated dextran attached with unique barcodes were used.

STAINING OF T CELLS WITH DNA-BARCODED PMHC MULTIMERS AND PHENOTYPE PANEL

One liquid-based cytology specimens from each participant were collected using two Cervix-Brush (Rovers) technique and collected in 10 ml SurePathTM Collection Vial (BD) and were HPV genotyped for HLA-A, HLA-B and HLA-C loci (Pathology department Hvidovre hospital, DK) using Anyplex II HPV28 detection real-time PCR or BD Max DNA extraction (Table S1). PBMCs from healthy, CIN 3 and cancer patients were thawed, washed twice in RPMI and 10% calf serum (FCS) and washed once in barcode cytometry buffer for 15 min at 37°C at a final volume of 50 μl

per sample and pooled according to their matching HLA. Cells were then stained with the phenotype panel of surface and intracellular marker antibodies (Table S2) and live/dead marker (Fixable Near-IR; Invitrogen, L101199) with final dilution of 1/1000 and incubated at 4°C for 30 min. The cells were washed twice with barcode cytometry buffer and fixed with 100 µl permeabilization buffer (Invitrogen, cat. 00-5523-00). Following fixation, intracellular antibodies were incubated with cells 30 min on ice. After two additional washing steps with washing buffer cells were resuspended in FACS buffer or PBS and filtered into FACS tubes just prior to acquisition.

FLOW CYTOMETRY ANALYSIS

After compensation with fluorescently conjugated antibodies, T cells were sorted on a FACSAria flow cytometry instrument (AriaFusion, Becton Dickinson) and gated using the FACSDiva acquisition program (Becton Dickinson) and FlowJo software v10.7 (TreeStar, Inc.). All PE-positive (HPV multimer binding) and APC-positive (CEF multimer binding) cells selected in the CD8 gate and sorted into pre-saturated tubes (2% bovine serum albumin and 100 µl of barcode cytometry buffer). Cells were centrifuged for 10 min at 5000g, and supernatant discarded. The barcodes associated to the corresponding sorted cells were then amplified by polymerase chain reaction (PCR), using a common reverse primer (Rx) and a sample-specific forward primer (Fx) A-key, mixed with Taq PCR Master Mix Kit (Qiagen, 201443). Nuclease free water was added to the individual PCR tubes containing 3 µl of distinct forward primer with a given sample ID to reach a total volume of 50 µl. Besides the sorted cells, three baseline specimens and two non-template controls (NTCs) were included in the amplification and sequencing. Gel electrophoresis was applied to the PCR product to verify the PCR amplification of the DNA barcodes. All specimens were then pooled with an estimated equal amount of DNA assessed based on the respective bands in the gel. The QIAquick PCR Purification kit (Qiagen, 28104) was used to purify the PCR-amplified DNA barcodes and then measured with Nanodrop 1000 spectrophotometer, and 50ng of barcode DNA was sequenced at PrimBio (USA) using an Ion Torrent PGM chip (Life Technologies).

ANALYSIS OF DNA BARCODE SEQUENCE AND IDENTIFICATION OF PMHC SPECIFICITIES

The specific software server “Barracoda 1.8” designed for processing of sequenced data and identifying the barcode sequences was used [29]. For each sequence read, the software identifies the sequence of the forward primer, annealing region and reverse primer and filters out any reads which does not contain at least two of these sequences. The quality of each DNA barcode

sequence is counted and assigned to each sample identified by the sample ID. To avoid amplification bias, clonal reduction was performed, details are given in [28].

DETECTION OF PEPTIDE-MHC SPECIFIC T CELLS BY FLUORESCENTLY LABELLED TETRAMERS

For selected immunodominant peptides, pMHC tetramers were generated for staining of neoepitope-specific T cells. Relevant peptides were selected based on the observed CD8 T cell responses from the DNA barcode-labelled multimer screening. Single-fluorochrome pMHC specificity tetramers were generated, using a library of streptavidin (SA)-conjugated fluochromes consisting of PE-SA, APC-SA, BV421-SA, PE-Cy7-SA, BV605-SA, PE-CF594-SA, BV650-SA, BUV395-SA. Up to eight patient-specific pMHC tetramers per sample were investigated. PBMC specimens were stained with respective library of pMHC tetramers and with an antibody mix, dump channel antibodies and a dead cell marker. Tetramer-specific T cells analyzed as lymphocytes, single, live, CD8, FITC- and tetramer+ cells. Due to staining strategy, tetramer+ cells were gated by being CD8.

DATA PROCESSING AND STATISTICS

The statistical analysis of the barcoded multimers was performed as previously described and details are given in [28]. Kruskal-Wallis uncorrected Dunn's test was applied for additional statistical analyses unless otherwise stated.

ACKNOWLEDGEMENTS AND AUTHOR CONTRIBUTIONS

As first author I would like to thank all individuals included in this study who contributed by donating specimens. No financial compensation was offered to the donors for participating in this study.

As co-supervisors we had the privilege to collaborate with Susanne Krüger Kjær, Professor, consultant at the Danish Cancer Society Research Center, Copenhagen, Denmark. Her unique knowledge of the HPV virus has been of great importance also in terms of designing the study and scientific discussions.

As clinical collaborators we were honored to collaborate with Kirsten Marie Jochumsen, PhD., Associate Professor, senior consultant, Department of Gynecology and Obstetrics, Odense University Hospital, Odense, who made it possible to collect patient specimens from cancer patients. For all the cervical neoplasia patients we were fortunate to have Benny Kirschner, Clinical Associate Professor, consultant, Department of Gynecology and Obstetrics, Hvidovre Hospital, Hvidovre who collected both consent and patient material. Aleris Hamlet private hospital for

approval of inclusion of patients for healthy patient material done by the author. Jesper Bonde for analyzing HPV status and follow-up. Stine Kiær Larsen for the initial help designing the protocols, predicting peptides and as a great lab teacher.

Marie Viuff for great lab assistance and data analyzes and especially to Mohammad Kadivar for excellent supervision, lab assistance and close guidance. Lastly my main supervisor Sine Reker Hadrup for overall assistance, great support, and scientific discussions.

REFERENCES

- [1] A. Kothari, "Human papilloma virus (HPV) and cervical cancer," *Indep. Nurse*, vol. 2020, no. 2, pp. 20–22, Feb. 2020, doi: 10.12968/indn.2020.2.20.
- [2] W. Small et al., "Cervical cancer: A global health crisis," *Cancer*, vol. 123, no. 13, pp. 2404–2412, 2017, doi: 10.1002/cncr.30667.
- [3] GLOBOCAN, "Cervix uteri Source: Globocan 2020," *Int. Agent Res. Cervic Uteri*, vol. 419, pp. 1–10, 2020.
- [4] J. M. M. Walboomers et al., "Human papillomavirus is a necessary cause of invasive cervical cancer worldwide," *J. Pathol.*, vol. 189, no. 1, pp. 12–19, 1999, doi: 10.1002/(SICI)1096-9896(199909)189:1<12::AID-PATH431>3.0.CO;2-F.
- [5] S. Duensing and K. Münger, "Mechanisms of genomic instability in human cancer: Insights from studies with human papillomavirus oncoproteins," *Int. J. Cancer*, vol. 109, no. 2, pp. 157–162, 2004, doi: 10.1002/ijc.11691.
- [6] M. K. Jang, D. E. Anderson, K. van Doorslaer, and A. A. McBride, "A proteomic approach to discover and compare interacting partners of papillomavirus E2 proteins from diverse phylogenetic groups," *Proteomics*, vol. 15, no. 12, pp. 2038–2050, 2015, doi: 10.1002/pmic.201400613.
- [7] A. Varsani, A.-L. Williamson, D. de Villiers, I. Becker, N. D. Christensen, and E. P. Rybicki, "Chimeric Human Papillomavirus Type 16 (HPV-16) L1 Particles Presenting the Common Neutralizing Epitope for the L2 Minor Capsid Protein of HPV-6 and HPV-16," *J. Virol.*, vol. 77, no. 15, pp. 8386–8393, 2003, doi: 10.1128/jvi.77.15.8386-8393.2003.
- [8] "No Title," p. <https://www.iarc.who.int/>, [Online]. Available: <https://www.iarc.who.int/>.
- [9] M. Stanley, "Pathology and epidemiology of HPV infection in females," *Gynecol. Oncol.*, vol. 117, no. 2 SUPPL., pp. S5–S10, 2010, doi: 10.1016/j.ygyno.2010.01.024.
- [10] V. Bouvard et al., "A review of human carcinogens--Part B: biological agents.," *Lancet Oncol.*, vol. 10, no. 4, pp. 321–322, 2009, doi: 10.1016/s1470-2045(09)70096-8.
- [11] G. M. Clifford, J. S. Smith, M. Plummer, N. Muñoz, and S. Franceschi, "Human papillomavirus types in invasive cervical cancer worldwide: A meta-analysis," *Br. J. Cancer*, vol. 88, no. 1, pp. 63–69, 2003, doi: 10.1038/sj.bjc.6600688.
- [12] Bosch F., Lorincz A., Muñoz N., Meijer C. J. L. M., and Shah K. V., "The causal relation between human papillomavirus and cervical cancer," *J. Clin. Pathol.*, vol. 55, no. 4, pp. 244–265, 2002, [Online]. Available: <http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L34293391>.
- [13] M. Knebel Doeberitz, C. Rittmuller, F. Aengeneyndt, P. Jansen-durr, and D. Spitkovsky, "Reversible Repression," *J. Virol.*, vol. 68, no. 5, pp. 2811–2821, 1994.
- [14] P. Melsheimer, S. Vinokurova, N. Wentzensen, G. Bastert, and M. Von Knebel Doeberitz, "DNA Aneuploidy and Integration of Human Papillomavirus Type 16 E6/E7 Oncogenes in Intraepithelial Neoplasia and Invasive Squamous Cell Carcinoma of the Cervix Uteri," *Clin. Cancer Res.*, vol. 10, no. 9, pp. 3059–3063, 2004, doi: 10.1158/1078-0432.CCR-03-0565.
- [15] S. Shanmugasundaram and J. You, "Targeting persistent human papillomavirus infection," *Viruses*, vol. 9, no. 8, 2017, doi: 10.3390/v9080229.
- [16] J. Doorbar, "Model systems of human papillomavirus-associated disease," *J. Pathol.*, vol. 238, no. 2, pp. 166–179, 2016, doi: 10.1002/path.4656.
- [17] M. Ghanaat et al., "Virus against virus: strategies for using adenovirus vectors in the

- treatment of HPV-induced cervical cancer,” *Acta Pharmacol. Sin.*, no. January, pp. 1–10, 2021, doi: 10.1038/s41401-021-00616-5.
- [18] S. Duranti et al., “Role of immune checkpoint inhibitors in cervical cancer: From preclinical to clinical data,” *Cancers (Basel)*, vol. 13, no. 9, pp. 1–12, 2021, doi: 10.3390/cancers13092089.
- [19] A. K. Bentzen et al., “Large-scale detection of antigen-specific T cells using peptide-MHC-I multimers labeled with DNA barcodes,” *Nat. Biotechnol.*, vol. 34, no. 10, pp. 1037–1045, 2016, doi: 10.1038/nbt.3662.
- [20] M. E. McLaughlin-Drubin and K. Münger, “Oncogenic activities of human papillomaviruses,” *Virus Res.*, vol. 143, no. 2, pp. 195–208, 2009, doi: 10.1016/j.virusres.2009.06.008.
- [21] C. A. Moody and L. A. Laimins, “Human papillomavirus oncoproteins: Pathways to transformation,” *Nat. Rev. Cancer*, vol. 10, no. 8, pp. 550–560, 2010, doi: 10.1038/nrc2886.
- [22] G. Salvo, D. Odetto, R. Pareja, M. Frumovitz, and P. T. Ramirez, “Revised 2018 International Federation of Gynecology and Obstetrics (FIGO) cervical cancer staging: A review of gaps and questions that remain,” *Int. J. Gynecol. Cancer*, vol. 30, no. 6, pp. 873–878, 2020, doi: 10.1136/ijgc-2020-001257.
- [23] B. Huard and L. Karlsson, “KIR expression on self-reactive CD8 + T cells is controlled by T-cell receptor engagement,” vol. 403, no. January, pp. 325–328, 2000.
- [24] A. Gati et al., “Tumor cells regulate the lytic activity of tumor-specific cytotoxic T lymphocytes by modulating the inhibitory natural killer receptor function,” *Cancer Res.*, vol. 61, no. 8, pp. 3240–3244, 2001.
- [25] “Immune Epitope Database (IEDB).” <https://www.iedb.org/>.
- [26] V. Jurtz, S. Paul, M. Andreatta, P. Marcatili, B. Peters, and M. Nielsen, “NetMHCpan-4.0: Improved Peptide–MHC Class I Interaction Predictions Integrating Eluted Ligand and Peptide Binding Affinity Data,” *J. Immunol.*, vol. 199, no. 9, pp. 3360–3368, 2017, doi: 10.4049/jimmunol.1700893.
- [27] S. K. Saini, E. T. Abualrous, A. S. Tigan, K. Covella, U. Wellbrock, and S. Springer, “Not all empty MHC class I molecules are molten globules: Tryptophan fluorescence reveals a two-step mechanism of thermal denaturation,” *Mol. Immunol.*, vol. 54, no. 3–4, pp. 386–396, 2013, doi: 10.1016/j.molimm.2013.01.004.
- [28] A. K. Bentzen et al., “Large-scale detection of antigen-specific T cells using peptide-MHC-I multimers labeled with DNA barcodes,” *Nat. Biotechnol.*, vol. 34, no. 10, pp. 1037–1045, 2016, doi: 10.1038/nbt.3662.
- [29] “Barracoda.” <https://services.healthtech.dtu.dk/service.php?Barracoda-1.8>).

SUPPLEMENTARY

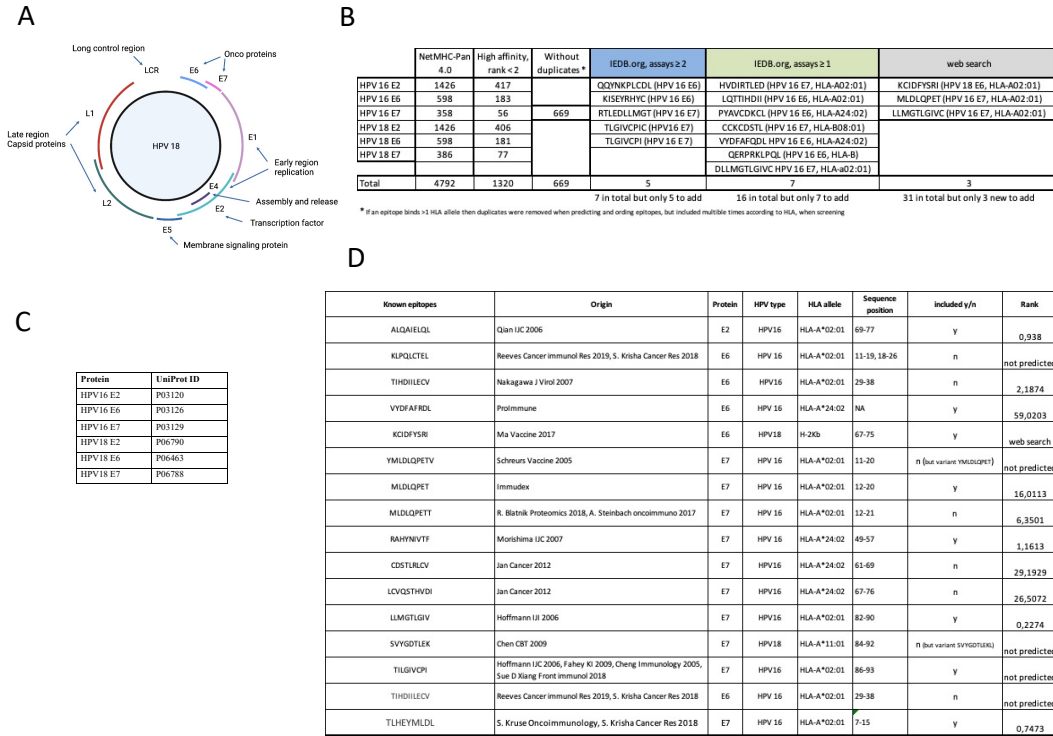


Fig. S1: Prediction of HPV epitopes. (A): Schematic representation of the complete HPV 18 circular genome with all genes represented and used for identification of 685 peptides with predicted binding rank (NetMHCpan 4.0) of <2 for 14 prevalent HLA-A, HLA-B and HLA-C molecules. (B): Table of the HPV epitopes prediction and the added epitopes by different algorithms and servers each providing prediction or previously found epitopes. In total 685 epitopes all used in patient specific HLA matching panels. (C): Amino acid sequences of HPV 16 and HPV 18 genes of interest and their ID, found at uniprot.org. (D): Schematic view of known epitopes, the reference and protein of origin, their corresponding HLA, sequence position and whether they were included for testing or not. Only strong binders (rank score <2 NetMHC Pan 4.0), or if previously described (IEDB.org) were included.

Table S1: Group overview. 8 healthy individuals, 9 CIN-3 and 10 cancer patients were included in this study. Age and BMI is listed and the average value. Also, HPV status when enrolled and their disease stage. Number of peptides screened according to their respective HLA alleles and the number of responses detected are listed too.

Cohort	Patient/donor ID	Age	BMI	HPV status	Disease stage	No of peptides screened	Responses	HLA-A	HLA-B	HLA-C
Benign	9	25	24	51		229	3	02:01		
	18	45	26			294	8	02:01	03:01	03:04
	20	47	29			332	5	01:01	08:01	05:01
	21	52	29			61	0	02:01		07:01
	24	56	31			191	12	02:01	07:02	05:01
	26	46	20	53		447	2	01:01	07:02	08:01
	31	48	34	16		123	0	02:01		03:04
	32	52	24		286	0	03:01	07:02	07:02	
Average		46	27			245	4			
CIN3	5	43	21	16	CIN3	61	3	02:01		
	6	27	26	33	CIN3	416	1	01:01	24:02	03:04
	38	26	23	18	CIN3	262	3	01:01	08:01	07:01
	40	37	29	33	CIN3	354	4	01:01	24:02	07:01
	42	30	21	39	CIN3	381	9	01:01	02:01	07:02
	45	30	25	16, 33, 61	CIN3	131	4	02:01		05:01
	52	39	29	16	CIN3	198	0	24:02	15:01	
	53	31	20	16	CIN3	316	17	02:01	07:02	05:01
	57	35	38	52	CIN3	439	13	11:01	24:02	04:01
Average		33	26			284	6			
Cervical cancer	13	62	29	16	IV	799	6	02:01	15:01	03:04
	35	40	26	16, 45	IA1	352	0	02:01	07:02	15:01
	43	45	36	16	IB2	208	6	02:01	07:02	03:04
	47	72	19	16, 18	IIB	397	4	01:01	02:01	08:01
	49	55	52	18	IIB	92	0	24:02		
	51	72	47	16	IIB	214	8	02:01	35:01	05:01
	54	53	27	16	IIB	579	2	03:01	24:02	07:02
	55	58	N/A	33, 70	IB2	123	0	02:01		03:04
	56	57	22	16, 53, 70, 82	IIB	271	12	03:01		04:01
	59	55	30	16	IIB	467	5	01:01	02:01	08:01
Average		57	32			310	4			

Table S2: Phenotype panel of surface and intracellular marker antibodies

Antigen	Fluorophore	Clone	Supplier	Cat no.	µl/stain
PD1	BV421	B56	BD	329920	2
CD8	BV480	Ber-ACT8	BD	566121	2
CD27	BV605	4B4-1	Biologend	302830	2,5
CD4	BV650	EH12.1 (EH12.2H7)	BD	563875	2,5
CD45RA	BV711	RPA-T8	BD	563733	2,5
CCR7	BV786	G043H7	BioLegend	353229	5
CD3	Alexa Flour 488	SK3	BD	557694	0,75
TCF-1	BB 700	S33-966	BD		2,5
CD39	PE-CF594	G043H7 (2-L1-A)	BD	563678	2,5
CD57	PECy7	UCHT1	Biologend	393310	2,5
HPV-antigen multimers	PE				
Viability	Near-IR	Tu66	Invitrogen	L34976	0,1
GZMb	AlexaFlour 700	QA17A04	Biologend	372222	2,5
Viral antigen multimers	APC				
Ki67	BUV395	B56	BD	564071	2,5
CD103	BUV563	QA16A02	BD	748503	5

Table S3: Table of predicted epitopes. All 685 predicted epitopes and their sequence, protein (colored according to their origin of protein, same colors as shown in (Fig. 1)), position, length, and corresponding 14 different HLA allene are listed. The enriched responses are marked in red together with the patient number and group (H: healthy, D: CIN-3 and C: cancer). In total 127 immunogenic epitopes all recognized by CD8 T cells was found.

Protein	Peptide	Position	Sekvens	Length	Disease	Patient No	HLA-type
HPV16 E2	HPV109	57	WPTLAVSKNK	11			HLA-A11:01
HPV16 E2	HPV110	246	TGNPCHTTK	9			HLA-A11:01
HPV16 E2	HPV111	281	NSNTTPVHLK	11			HLA-A11:01
HPV16 E2	HPV112	284	TTPIVHLK	8			HLA-A11:01
HPV16 E2	HPV113	295	NTLKLRYR	9			HLA-A11:01
HPV16 E2	HPV114	295	NTLKLRYRFK	11			HLA-A11:01
HPV16 E2	HPV115	333	TLTYDSEWQR	10			HLA-A11:01
HPV16 E2	HPV116	334	LTYSDEWQR	9			HLA-A11:01
HPV16 E2	HPV136	40	AIYKAREMGF	11			HLA-A24:02
HPV16 E2	HPV137	41	IYKAREMGFK	11			HLA-A24:02
HPV16 E2	HPV138	81	IYNSQYSNEKW	11			HLA-A24:02
HPV16 E2	HPV139	84	SQYSNEKWTL	10			HLA-A24:02
HPV16 E2	HPV140	100	WYLTAPTGI	10			HLA-A24:02
HPV16 E2	HPV141	129	HYTNWTHI	8			HLA-A24:02
HPV16 E2	HPV142	136	IYICEASVT	11			HLA-A24:02
HPV16 E2	HPV143	136	IYICEASV	9	D	57	HLA-A24:02
HPV16 E2	HPV144	153	YYGLYYHEGI	11			HLA-A24:02
HPV16 E2	HPV145	176	KYSKNKWVEV	10			HLA-A24:02
HPV16 E2	HPV146	308	CTLYTAVSSTW	11			HLA-A24:02
HPV16 E2	HPV147	309	TLTYTAVSSTW	10			HLA-A24:02
HPV16 E2	HPV148	310	LYTAVSSTWHW	11			HLA-A24:02
HPV16 E2	HPV149	310	LYTAVSSTWH	10			HLA-A24:02
HPV16 E2	HPV150	317	TWHWTGHNV	9			HLA-A24:02
HPV16 E2	HPV177	205	SSPEIRQHL	10			HLA-B07:02
HPV16 E2	HPV178	206	SPEIRQHL	9			HLA-B07:02
HPV16 E2	HPV179	216	NHPAATHKAV	11			HLA-B07:02
HPV16 E2	HPV180	217	HPAATHKAVA	11			HLA-B07:02
HPV16 E2	HPV181	248	NPCHTTKLL	9			HLA-B07:02
HPV16 E2	HPV182	264	APLTAFTNS	10			HLA-B07:02
HPV16 E2	HPV183	350	KIPKTTVST	10			HLA-B07:02
HPV16 E2	HPV184	351	IPKTTVST	9			HLA-B07:02
HPV16 E2	HPV220	32	WHRMRECAV	10			HLA-B08:01
HPV16 E2	HPV221	32	WHRMRECA	9			HLA-B08:01
HPV16 E2	HPV222	34	HMRLECA	8			HLA-B08:01
HPV16 E2	HPV223	60	TLAVSKNKGL	10			HLA-B08:01
HPV16 E2	HPV224	108	QKHGTYT	9			HLA-B08:01
HPV16 E2	HPV225	109	KKHGTYT	8			HLA-B08:01
HPV16 E2	HPV226	221	THTKAVL	8			HLA-B08:01
HPV16 E2	HPV227	295	NTLKLRYRF	10			HLA-B08:01
HPV16 E2	HPV228	323	HNNKHSAL	9			HLA-B08:01
HPV16 E2	HPV229	323	HNNKHSALV	10			HLA-B08:01
HPV16 E2	HPV230	324	NWRKHSAL	8			HLA-B08:01
HPV16 E2	HPV231	324	NWRKHSALV	9			HLA-B08:01
HPV16 E2	HPV232	324	NWRKHSALVTL	11			HLA-B08:01
HPV16 E2	HPV249	32	WHRMRECAIV	11			HLA-B15:01
HPV16 E2	HPV250	33	KHRMRECAIV	10			HLA-B15:01
HPV16 E2	HPV251	118	VQFDGDCNTM	11			HLA-B15:01
HPV16 E2	HPV252	149	GGVDYGYLY	9			HLA-B15:01
HPV16 E2	HPV253	162	GIRTYFVQF	9			HLA-B15:01
HPV16 E2	HPV254	168	VQFDGDAEKY	10			HLA-B15:01
HPV16 E2	HPV255	189	GGVLCPTSVF	11			HLA-B15:01
HPV16 E2	HPV256	190	QVLCPTSVF	10			HLA-B15:01
HPV16 E2	HPV257	192	ILCPTSVF	8			HLA-B15:01
HPV16 E2	HPV258	261	VDSAPILTAF	10			HLA-B15:01
HPV16 E2	HPV259	353	KTTVSTGFDM	10			HLA-B15:01
HPV16 E2	HPV265	145	TWEGQVDY	9			HLA-B35:01
HPV16 E2	HPV266	194	CPTSVFSSN	9			HLA-B35:01
HPV16 E2	HPV267	214	LANHPPAATH	9			HLA-B35:01
HPV16 E2	HPV277	190	QVLCPTSV	9			HLA-C03:04
HPV16 E2	HPV278	196	TSVFSNEV	9			HLA-C03:04
HPV16 E2	HPV297	17	HYENDSTDL	9			HLA-C04:01
HPV16 E2	HPV298	90	KWTLQDVSL	9			HLA-C04:01
HPV16 E2	HPV299	119	QFDGDCNTM	10			HLA-C04:01
HPV16 E2	HPV317	93	LQDVSLEV	8			HLA-C05:01
HPV16 E2	HPV318	138	IYICEASVT	9	B,B,D,C,C,C,C	13,18,24,45,51,53,59	HLA-C05:01
HPV16 E2	HPV319	228	LGTEETQTTI	10			HLA-C05:01
HPV16 E2	HPV320	229	GTEETQTTI	9			HLA-C05:01
HPV16 E2	HPV321	260	SVDSAPIL	8	B,B,B,D,D,C,C,C	13,18,20,24,45,51,53,59	HLA-C05:01
HPV16 E2	HPV322	340	WQRDQFLSQV	10			HLA-C05:01
HPV16 E2	HPV326	110	KKHGYTVEV	9			HLA-C07:01
HPV16 E2	HPV327	128	MHTNWTYHI	11			HLA-C07:01
HPV16 E2	HPV328	257	HRDSVDSAPIL	11			HLA-C07:01
HPV16 E2	HPV329	302	YRFKHKCTLYT	11			HLA-C07:01
HPV16 E2	HPV339	41	IYKAREM	8			HLA-C07:02
HPV16 E2	HPV34	8	NVCCDKILTHY	11			HLA-A01:01
HPV16 E2	HPV340	156	LYVHEGIRTY	11			HLA-C07:02
HPV16 E2	HPV341	163	IRTYFVQF	8			HLA-C07:02
HPV16 E2	HPV35	9	VQDKILTHY	10			HLA-A01:01
HPV16 E2	HPV36	10	QDKILTHY	9			HLA-A01:01
HPV16 E2	HPV37	21	DSTDLRDHIDY	11			HLA-A01:01
HPV16 E2	HPV38	22	STDLRDHIDY	10			HLA-A01:01
HPV16 E2	HPV39	22	STDLRDHIDYW	11			HLA-A01:01
HPV16 E2	HPV40	36	RLECAIV	8			HLA-A01:01
HPV16 E2	HPV41	76	LTLETYNSQY	11			HLA-A01:01
HPV16 E2	HPV42	91	WTLQDVSLEVY	11			HLA-A01:01
HPV16 E2	HPV43	120	FDGDCNTMRY	11			HLA-A01:01
HPV16 E2	HPV44	221	DGDCNTMRYH	10			HLA-A01:01
HPV16 E2	HPV45	244	VTVREGQVDY	11			HLA-A01:01
HPV16 E2	HPV46	246	WVSDQVRYV	10	D	42	HLA-A01:01
HPV16 E2	HPV47	148	EGQDQYGLYY	11			HLA-A01:01
HPV16 E2	HPV48	150	QVDYGLY	8			HLA-A01:01
HPV16 E2	HPV49	150	QVDYGLYYH	11			HLA-A01:01
HPV16 E2	HPV50	151	VDYGLY	8			HLA-A01:01
HPV16 E2	HPV51	167	VQFDQDAEKY	11			HLA-A01:01
HPV16 E2	HPV52	260	SVDSAPILTA	10			HLA-A01:01
HPV16 E2	HPV53	294	ANTLKLRY	9			HLA-A01:01
HPV16 E2	HPV54	311	YTAIVSSTWH	9			HLA-A01:01
HPV16 E2	HPV55	326	KHSALVTLTY	11			HLA-A01:01

Protein	Peptide	Position	Sekvens	Length	Disease	Patient No	HLA-type
HPV16E2	HPV56	336	YDSEWQRDOF	10			HLA-A01:01
HPV16E2	HPV73	68	ALQAIQLQLTL	11			HLA-A02:01
HPV16E2	HPV74	90	KWTLQDVSLEW	11			HLA-A02:01
HPV16E2	HPV75	92	TLQDVSLEWIL	11			HLA-A02:01
HPV16E2	HPV76	154	YGLYYWHEGI	10			HLA-A02:01
HPV16E2	HPV77	155	GLYYWHEGI	9			HLA-A02:01
HPV16E2	HPV78	158	YVHEGIRTYFV	11			HLA-A02:01
HPV16E2	HPV89	36	RLECAIYYKA	10			HLA-A03:01
HPV16E2	HPV90	101	YLTAPTGCICK	11			HLA-A03:01
HPV16E2	HPV91	155	GLYYWHEGIR	10			HLA-A03:01
HPV16E2	HPV92	289	HLKGDANTLK	10			HLA-A03:01
HPV16E2	HPV472	206	SPEIRQHILA	10			HLA-B07:02
HPV16E2	HPV493	147	VEGQVDYGLY	11			HLA-B15:01
HPV16E2	HPV501	312	TAVSSTWHW	9			HLA-B35:01
HPV16E2	HPV383	68	ALQAIQLQL	9			HLA-A02:01
HPV16E2	HPV384	137	VICEASVTV	10	B,B,D,D,C,C,C	13,18,24,45,51,53,59	HLA-A02:01
HPV16E2	HPV427	34	HMRLECAIYYK	11			HLA-A03:01
HPV16E2	HPV428	35	MRLECAIYYK	10			HLA-A03:01
HPV16E2	HPV429	36	RLECAIYYK	9	D	57	HLA-A03:01
HPV16E2	HPV430	56	QWPTLAVSK	10			HLA-A03:01
HPV16E2	HPV431	57	WPTLAVSK	9			HLA-A03:01
HPV16E2	HPV432	58	WPTLAVSKNK	10			HLA-A03:01
HPV16E2	HPV433	80	TIYNSQVSNK	11			HLA-A03:01
HPV16E2	HPV434	101	YLTAPTGCICK	10			HLA-A03:01
HPV16E2	HPV435	102	LTAPTGCICK	10			HLA-A03:01
HPV16E2	HPV436	102	LTAPTGCICK	9			HLA-A03:01
HPV16E2	HPV437	162	GIRTYVQFK	10			HLA-A03:01
HPV16E2	HPV438	164	RTYVQFK	8			HLA-A03:01
HPV16E2	HPV439	266	LTAFNSSHK	10	C	56	HLA-A03:01
HPV16E2	HPV440	267	LTAFNSSHK	9			HLA-A03:01
HPV16E2	HPV441	282	SNTTPIVHLK	10			HLA-A03:01
HPV16E2	HPV442	283	NTTPIVHLK	9			HLA-A03:01
HPV16E2	HPV443	294	ANTLKLRYR	10			HLA-A03:01
HPV16E2	HPV444	296	TLKCLRYPFK	10			HLA-A03:01
HPV16E2	HPV445	296	TLKCLRYPFK	11			HLA-A03:01
HPV16E2	HPV446	316	STWHVGTGHNVK	11			HLA-A03:01
HPV16E2	HPV447	344	QFLSQKIPK	10			HLA-A03:01
HPV16E2	HPV448	345	FLSQKIPK	9	C	56	HLA-A03:01
HPV16E2	HPV463	310	LYTAVSSTW	9			HLA-A24:02
HPV16E2	HPV464	335	TYDSEWQRDOF	11			HLA-A24:02
HPV16E2	HPV473	217	HPAATHTKAW	10			HLA-B07:02
HPV16E2	HPV474	217	HPAATHTKA	9			HLA-B07:02
HPV16E2	HPV475	351	IKTKITVSTGF	11			HLA-B07:02
HPV16E2	HPV482	86	YSNEKWTI	8			HLA-B08:01
HPV16E2	HPV483	303	RFKKHCTL	8			HLA-B08:01
HPV16E2	HPV489	34	HMRLECAIY	9			HLA-B15:01
HPV16E2	HPV490	55	HQVPTLAV	9			HLA-B15:01
HPV16E2	HPV491	69	LQAIQLQLTL	10			HLA-B15:01
HPV16E2	HPV492	74	LQLTLETIY	9			HLA-B15:01
HPV16E2	HPV498	70	QAIQLQLTL	9			HLA-B35:01
HPV16E2	HPV499	112	HGYTVEVGF	9	C	56	HLA-B35:01
HPV16E2	HPV500	262	DSAPILTAF	9			HLA-B35:01
HPV16E2	HPV510	356	TVSTGFMSI	9	B,D	24,53	HLA-C03:04
HPV16E2	HPV514	93	LQDVSLEWYL	10			HLA-C04:01
HPV16E2	HPV538	35	MRLECAIYY	9			HLA-C07:01
HPV16E2	HPV539	50	FKHINHQVW	9			HLA-C07:01
HPV16E2	HPV540	53	INHQVWPTL	9			HLA-C07:01
HPV16E2	HPV541	185	WVAGSQVLL	9			HLA-C07:01
HPV16E2	HPV542	301	RYRFKHKCTLY	11			HLA-C07:01
HPV16E2	HPV543	303	RFKKHCTLY	9			HLA-C07:01
HPV16E2	HPV544	326	KHKSAVTL	9			HLA-C07:01
HPV16E2	HPV545	341	QRDQFLSQV	9			HLA-C07:01
HPV16E2	HPV546	349	WKPKITIV	9			HLA-C07:01
HPV16E2	HPV375	149	GQVDYGLYYV	11			HLA-A01:01
HPV16E2	HPV365	73	ELQLTLETIY	10			HLA-A01:01
HPV16E2	HPV366	77	TLETIYNSQY	10			HLA-A01:01
HPV16E2	HPV367	92	TLQDVSLEW	10			HLA-A01:01
HPV16E2	HPV368	93	LQDVSLEWY	9			HLA-A01:01
HPV16E2	HPV369	127	TMNYTNWTHIY	11	D	38	HLA-A01:01
HPV16E2	HPV370	130	YTNWTHIY	8			HLA-A01:01
HPV16E2	HPV371	144	YTWEGQVDY	10			HLA-A01:01
HPV16E2	HPV372	145	TWEGQVDY	10			HLA-A01:01
HPV16E2	HPV373	146	WEGQVDY	9			HLA-A01:01
HPV16E2	HPV374	148	EGQVDYGLY	10			HLA-A01:01
HPV16E2	HPV376	260	SDSAPILT	9			HLA-A01:01
HPV16E2	HPV377	293	DANTLKCLRY	10			HLA-A01:01
HPV16E2	HPV378	311	YTAVSSTWHW	10	C	56	HLA-A01:01
HPV16E2	HPV561	149	GQVDYGLYY	10			HLA-A01:01
HPV16E2	HPV563	260	SDSAPILTAF	11	D	42	HLA-A01:01
HPV16E2	HPV564	327	HKSAVTLTY	10			HLA-A01:01
HPV16E2	HPV565	329	SAIVLTY	8			HLA-A01:01
HPV16E2	HPV560	91	WTLQDVSLEW	10			HLA-A01:01
HPV16E2	HPV562	150	QVDYGLYYV	10			HLA-A01:01
HPV16E2	HPV567	92	TLQDVSLEW	9			HLA-A02:01
HPV16E2	HPV576	41	YYKAREMGF	10			HLA-A24:02
HPV16E2	HPV577	85	QVSNKWTI	9			HLA-A24:02
HPV16E2	HPV578	128	MNYTNWTHI	9			HLA-A24:02
HPV16E2	HPV579	157	YVWEGIRTYF	11			HLA-A24:02
HPV16E2	HPV580	296	TLKCLRYPFK	9			HLA-A24:02
HPV16E2	HPV586	219	HATHTKAWL	9			HLA-B07:02
HPV16E2	HPV590	300	LYRFKHKCTLY	11			HLA-B08:01
HPV16E2	HPV591	302	YRFKHKCTLY	10			HLA-B08:01
HPV16E2	HPV594	353	KTIYVSTGF	9			HLA-B15:01
HPV16E2	HPV600	101	YLTAPTGCIC	9			HLA-C03:04
HPV16E2	HPV606	159	VHEGIRTYF	9			HLA-C04:01
HPV16E2	HPV614	28	HIDYWRKMR	10			HLA-A01:01
HPV16E2	HPV627	191	MLCPTSVF	9			HLA-A24:02
HPV16E2	HPV628	301	RYRFKHKCTLY	10	D	57	HLA-A24:02

Protein	Peptide	Position	Sekvens	Length	Disease	Patient No	HLA-type
HPV16 E2	HPV629	61	LAIVKNIKAL	9	B,C	9,43	HLA-B07:02
HPV16 E2	HPV633	40	AIYKAREM	9	D,D	42,53	HLA-B08:01
HPV16 E2	HPV635	157	YYVHEGIRTY	10			HLA-B15:01
HPV16 E2	HPV639	34	HMRLKCAIYY	10			HLA-A01:01
HPV16 E2	HPV640	128	MHYTNWTHIY	10	D,D,2XD,C	38,40,56,57,57	HLA-A01:01
HPV16 E2	HPV641	150	QVDYVGLYY	9			HLA-A01:01
HPV16 E2	HPV647	42	YKAREMEGF	9	B,C	26,43	HLA-A24:02
HPV16 E2	HPV648	129	HYTNWTHIY	10	C	56	HLA-A24:02
HPV16 E2	HPV649	129	HYTNWTHIY	9	3XD,2XC	56,56,57,57,57	HLA-A24:02
HPV16 E2	HPV650	220	ATHKAWAL	9		43	HLA-B07:02
HPV16 E2	HPV652	177	YSKNKWEV	9			HLA-B08:01
HPV16 E2	HPV653	302	YRFKHKCTL	9	B,B,D,D,2XC,CC	6,20,26,38,47,47,54,59	HLA-B08:01
HPV16 E2	HPV654	158	YVHEGIRTY	9			HLA-B15:01
HPV16 E2	HPV657	328	KSAILTYLT	9			HLA-A01:01
HPV16 E2	HPV665	158	YVHEGIRTYF	10			HLA-B15:01
HPV16 E2	HPV662	130	YTNWTHIYI	9	D	53	HLA-A01:01
HPV16 E6	HPV1	22	CTELQTHH	9			HLA-A01:01
HPV16 E6	HPV117	65	PVAVCDKCLXF	11			HLA-A24:02
HPV16 E6	HPV118	79	ISEYHYCYSL	11			HLA-A24:02
HPV16 E6	HPV119	84	HYCYSLYGTTL	11			HLA-A24:02
HPV16 E6	HPV120	97	QYNKPLCDLLI	11			HLA-A24:02
HPV16 E6	HPV151	14	RPRKLPQLC	9			HLA-B07:02
HPV16 E6	HPV152	14	RPRKLPQLCT	10			HLA-B07:02
HPV16 E6	HPV153	117	CREEKQRHL	9			HLA-B07:02
HPV16 E6	HPV154	135	RGRWTRGRCM	9			HLA-B07:02
HPV16 E6	HPV185	0	MHOKRTAM	8			HLA-B08:01
HPV16 E6	HPV186	36	CYKQKQL	8			HLA-B08:01
HPV16 E6	HPV187	41	QQLLRREV	8			HLA-B08:01
HPV16 E6	HPV188	72	GLKYSKI	8	D,C	40,47	HLA-B08:01
HPV16 E6	HPV189	124	HLDKKQRFHNI	11			HLA-B08:01
HPV16 E6	HPV190	125	LDKKQRFHNI	10			HLA-B08:01
HPV16 E6	HPV191	126	DKKQRFHNI	9			HLA-B08:01
HPV16 E6	HPV192	148	SSRTTRRETQL	10			HLA-B08:01
HPV16 E6	HPV2	68	VCDKCLKIFY	9			HLA-A01:01
HPV16 E6	HPV233	40	KQQLLRREY	10			HLA-B15:01
HPV16 E6	HPV234	41	QQLLRREV	9			HLA-B15:01
HPV16 E6	HPV235	56	LGVYRDGNPY	11			HLA-B15:01
HPV16 E6	HPV236	58	IVYRDGNPYA	10			HLA-B15:01
HPV16 E6	HPV260	66	YAVCDKCLKF	10			HLA-B35:01
HPV16 E6	HPV261	90	YGTTLQEQY	9			HLA-B35:01
HPV16 E6	HPV268	51	FAFRDLCT	8			HLA-C03:04
HPV16 E6	HPV269	87	YSLVGTTL	8	B	9	HLA-C03:04
HPV16 E6	HPV279	17	KLPQLCTEL	9			HLA-C04:01
HPV16 E6	HPV280	29	IHDILLECV	9			HLA-C04:01
HPV16 E6	HPV281	46	REWDFAFRDL	11			HLA-C04:01
HPV16 E6	HPV282	47	EYDFAFRDL	10			HLA-C04:01
HPV16 E6	HPV283	48	YDFAFRDL	10			HLA-C04:01
HPV16 E6	HPV284	49	YDFAFRDL	10			HLA-C04:01
HPV16 E6	HPV285	49	YDFAFRDL	8			HLA-C04:01
HPV16 E6	HPV3	77	SKISEYRHYCY	11			HLA-A01:01
HPV16 E6	HPV300	8	FQDPGERPRKL	11			HLA-C05:01
HPV16 E6	HPV301	58	IVYRDGNPYAV	11			HLA-C05:01
HPV16 E6	HPV330	52	AFRDLQVY	9	D	57	HLA-C07:02
HPV16 E6	HPV331	75	FYSKISEY	8			HLA-C07:02
HPV16 E6	HPV332	75	FYSKISEYR	8			HLA-C07:02
HPV16 E6	HPV333	83	RHYCYSLY	8			HLA-C07:02
HPV16 E6	HPV334	123	RHLDKKQRF	9			HLA-C07:02
HPV16 E6	HPV4	79	ISEYRHYCY	9			HLA-A01:01
HPV16 E6	HPV5	79	ISEYRHYCY	10			HLA-A01:01
HPV16 E6	HPV79	74	KFYSKISEYR	10			HLA-A03:01
HPV16 E6	HPV80	141	RCMSSCRSSR	10			HLA-A03:01
HPV16 E6	HPV81	142	CMSSCRSSR	9			HLA-A03:01
HPV16 E6	HPV93	90	YGTTLQEQYNK	11			HLA-A11:01
HPV16 E6	HPV671	96	QQYNKPLCDL	10			HLA-A02:01
HPV16 E6	HPV673	78	KISEYRHYC	9			HLA-A02:01
HPV16 E6	HPV679	25	LQTHDII	9			HLA-A02:01
HPV16 E6	HPV680	65	PVAVCDKCL	9			HLA-A24:02
HPV16 E6	HPV682	48	VYDFAFCDL	9			HLA-A24:02
HPV16 E6	HPV683	12	QERPRKLPQL	10			HLA-B07:02
HPV16 E6	HPV344	76	YSKISEYRHY	10			HLA-A01:01
HPV16 E6	HPV342	66	YAVCDKCLKIFY	11			HLA-B35:01
HPV16 E6	HPV343	67	AVCDKCLKIFY	10			HLA-A01:01
HPV16 E6	HPV345	78	KISEYRHYCY	10			HLA-A01:01
HPV16 E6	HPV385	32	IILECVCK	9			HLA-A03:01
HPV16 E6	HPV386	36	CYKQKQLR	10			HLA-A03:01
HPV16 E6	HPV387	91	GTTLQEQYNK	10			HLA-A03:01
HPV16 E6	HPV388	92	TTLQEQYNK	9			HLA-A03:01
HPV16 E6	HPV389	105	LIRICNCOQ	10			HLA-A03:01
HPV16 E6	HPV390	106	LIRICNCOQ	9			HLA-A03:01
HPV16 E6	HPV450	48	WDFAFRDLCT	11			HLA-A24:02
HPV16 E6	HPV451	97	QYNKPLCDLL	10			HLA-A24:02
HPV16 E6	HPV452	130	RPHNRGRW	9	C	54	HLA-A24:02
HPV16 E6	HPV465	14	RPRKLPQL	8			HLA-B07:02
HPV16 E6	HPV466	18	LPQLCTEL	8	D	40	HLA-B07:02
HPV16 E6	HPV476	1	HQKRTAMF	8			HLA-B08:01
HPV16 E6	HPV477	43	LLREVDVDF	9			HLA-B15:01
HPV16 E6	HPV478	43	LLREVDVDF	11			HLA-B15:01
HPV16 E6	HPV484	73	LKFYSKISEY	10			HLA-B15:01
HPV16 E6	HPV511	97	QYNKPLCDL	9			HLA-C04:01
HPV16 E6	HPV516	13	ERPRKLPQL	9			HLA-C07:01
HPV16 E6	HPV517	44	LRREVDVDF	10			HLA-C07:01
HPV16 E6	HPV518	53	FRDLCTVY	8			HLA-C07:01
HPV16 E6	HPV519	81	EYRHYCYSLY	10			HLA-C07:01
HPV16 E6	HPV520	82	YRHYCYSLY	9			HLA-C07:01
HPV16 E6	HPV521	82	YRHYCYSLY	10			HLA-C07:01
HPV16 E6	HPV522	134	IRGRWTRGRCM	10			HLA-C07:01
HPV16 E6	HPV523	149	SRTTRRETQL	9			HLA-C07:01
HPV16 E6	HPV569	81	EYRHYCYSL	9			HLA-A24:02

Protein	Peptide	Position	Sekvens	Length	Disease	Patient No	HLA-type									
HPV16E6	HPV587	36	CYWCKQQLL	9				HLA-B08:01	HLA-C07:01	HLA-C07:02						
HPV16E6	HPV588	82	YRYHCVSL	8				HLA-B08:01	HLA-C07:01	HLA-C07:02						
HPV16E6	HPV601	45	RREWDFAF	9				HLA-C04:01	HLA-C07:01	HLA-C07:02						
HPV16E6	HPV602	59	YVRDGNPYA	9				HLA-C04:01	HLA-C07:01	HLA-C07:02						
HPV16E6	HPV621	80	SEYRHCVSYL	10				HLA-A24:02	HLA-B08:01	HLA-C07:02						
HPV16E6	HPV547	57	CIYVRDGNPY	10				HLA-A01:01	HLA-B15:01	HLA-B35:01						
HPV16E6	HPV607	51	FAFRDLCLVY	10				HLA-A01:01	HLA-B15:01	HLA-B35:01	HLA-C03:04					
HPV16E6	HPV608	88	SLVGTLLQOYV	11				HLA-A01:01	HLA-B15:01	HLA-A03:01	HLA-A11:01					
HPV16E6	HPV617	74	KEYSKISEY	9				HLA-A03:01	HLA-C04:01	HLA-C07:01	HLA-C07:02					
HPV16E6	HPV619	48	YVDFAFRDL	9				HLA-A24:02	HLA-C04:01	HLA-C07:01	HLA-C07:02					
HPV16E6	HPV620	59	YVRDGNPYAV	10				HLA-A24:02	HLA-C04:01	HLA-C07:01	HLA-C07:02					
HPV16E6	HPV622	86	CYSLYGTTL	9				HLA-A24:02	HLA-C04:01	HLA-C07:01	HLA-C07:02					
HPV16E6	HPV637	60	YVRDGNPYAV	9	B.B.D.C.C	18,24,51,53,59		HLA-C04:01	HLA-C05:01	HLA-C04:01	HLA-C07:01					
HPV16E6	HPV642	51	FAFRDLCLV	9				HLA-A02:01	HLA-C03:04	HLA-C05:01	HLA-C07:01	HLA-B35:01				
HPV16E6	HPV643	0	MHQKRTAMF	9				HLA-A24:02	HLA-B08:01	HLA-C04:01	HLA-C07:01	HLA-C07:02				
HPV16E6	HPV644	85	YCVSYLGTTL	10				HLA-A24:02	HLA-C03:04	HLA-C04:01	HLA-C07:01	HLA-C07:02				
HPV16E6	HPV655	58	IYVRDGNPY	9	D	42		HLA-A01:01	HLA-B15:01	HLA-B35:01	HLA-C03:04	HLA-A03:01	HLA-A11:01			
HPV16E7	HPV237	42	GQAEPDRAHY	10				HLA-B15:01								
HPV16E7	HPV286	78	LEDLLMGTL	9				HLA-C04:01								
HPV16E7	HPV302	11	MLDLQPETTDL	11				HLA-C05:01								
HPV16E7	HPV303	11	MLDLQPETT	9				HLA-C05:01								
HPV16E7	HPV304	72	HVDIRTLLEDL	10				HLA-C05:01								
HPV16E7	HPV305	76	RTLEDLLMGTL	11				HLA-C05:01								
HPV16E7	HPV306	77	TLEDLLMGTL	10				HLA-C05:01								
HPV16E7	HPV494	15	QPETDLVYCY	10				HLA-B35:01								
HPV16E7	HPV57	10	YMLDLQPET	9	D	5		HLA-A02:01								
HPV16E7	HPV58	10	YMLDLQPETT	10				HLA-A02:01								
HPV16E7	HPV59	80	DLLMGTLGIV	10				HLA-A02:01								
HPV16E7	HPV6	1	HGDTPTLHEY	10				HLA-A01:01								
HPV16E7	HPV60	81	LLMGTLGIV	9				HLA-A02:01								
HPV16E7	HPV61	82	LMGTLGIVCFI	11				HLA-A02:01								
HPV16E7	HPV7	13	DLQPETTDLY	10				HLA-A01:01								
HPV16E7	HPV8	17	ETDLVYCY	10				HLA-A01:01								
HPV16E7	HPV9	18	TTDLVYCYEQ	9				HLA-A01:01								
HPV16E7	HPV670	76	RTLEDLLMGTL	10				HLA-A02:01								
HPV16E7	HPV672	85	TLGIVCPIC	9				HLA-A02:01								
HPV16E7	HPV674	84	TLGIVCPI	8				HLA-A02:01								
HPV16E7	HPV676	11	MLDLQPET	8				HLA-A02:01								
HPV16E7	HPV677	81	LLMGTLGIVC	10	C	13		HLA-A02:01								
HPV16E7	HPV678	72	HVDIRTLLED	9				HLA-A02:01								
HPV16E7	HPV681	57	CKCKDSTL	8				HLA-B08:01								
HPV16E7	HPV684	80	DLLMGTLGIVC	11				HLA-A02:01								
HPV16E7	HPV346	14	LQPETTDLY	9				HLA-A01:01	HLA-B15:01							
HPV16E7	HPV347	18	ITDLVYCYEQ	10				HLA-A01:01	HLA-C05:01							
HPV16E7	HPV348	43	GQAEPDRAHY	9				HLA-A01:01	HLA-B35:01							
HPV16E7	HPV349	72	HVDIRTLLEDLL	11	D	53		HLA-A01:01	HLA-C05:01							
HPV16E7	HPV379	84	GTLGIVCPI	9				HLA-A02:01	HLA-C03:04							
HPV16E7	HPV391	87	GIVCPICSQK	10				HLA-A03:01	HLA-A11:01							
HPV16E7	HPV392	88	IVCPICSQK	9				HLA-A03:01	HLA-A11:01							
HPV16E7	HPV467	4	TPTLHEYML	9				HLA-B07:02	HLA-B35:01							
HPV16E7	HPV524	75	IRTELDLLM	9				HLA-C07:01	HLA-C07:02							
HPV16E7	HPV566	6	TLHEYMLDI	9				HLA-A02:01	HLA-C07:01	HLA-C07:02						
HPV16E7	HPV595	47	DRAHYNIYVF	10	D	57		HLA-B35:01	HLA-C07:01	HLA-C07:02						
HPV16E7	HPV599	70	STHVDIRTL	9				HLA-C03:04	HLA-C07:01	HLA-C07:02						
HPV16E7	HPV669	48	RAHYNIYVF	9				HLA-A24:02	HLA-B15:01	HLA-C05:01	HLA-B35:01	HLA-C03:04	HLA-C07:01	HLA-C07:02	HLA-C04:01	HLA-B07:02
HPV18E2	HPV10	12	SCVQKIIDHY	11				HLA-A01:01								
HPV18E2	HPV100	169	NTFYIEFK	8				HLA-A11:01								
HPV18E2	HPV101	227	YSSVSVGTAK	11				HLA-A11:01								
HPV18E2	HPV102	229	STVSVGTAKT	10				HLA-A11:01								
HPV18E2	HPV103	268	AATPTGNKK	9				HLA-A11:01								
HPV18E2	HPV104	282	SGNTPIPHLK	11				HLA-A11:01								
HPV18E2	HPV105	285	TIPIHLK	8				HLA-A11:01								
HPV18E2	HPV106	333	TVTVHSETQR	10				HLA-A11:01								
HPV18E2	HPV11	13	CVQDKIIDHY	10				HLA-A01:01								
HPV18E2	HPV12	14	VQDKIIDHY	9				HLA-A01:01								
HPV18E2	HPV121	33	IYVQQLIRW	9				HLA-A24:02								
HPV18E2	HPV122	34	IYVQQLIRW	8				HLA-A24:02								
HPV18E2	HPV123	35	IYVQQLIRWENA	11				HLA-A24:02								
HPV18E2	HPV124	45	IFFAAREHGI	10				HLA-A24:02								
HPV18E2	HPV125	88	SAYKTDWTL	10				HLA-A24:02								
HPV18E2	HPV126	139	SVYMTDAGTW	11				HLA-A24:02								
HPV18E2	HPV127	140	VYMTDAGTW	10				HLA-A24:02								
HPV18E2	HPV128	141	YVMTDAGTWD	10				HLA-A24:02								
HPV18E2	HPV129	166	EGYNTFYIEF	10				HLA-A24:02								
HPV18E2	HPV13	28	DIDSQIQY	8				HLA-A01:01								
HPV18E2	HPV130	168	YNTFYIEF	8				HLA-A24:02								
HPV18E2	HPV131	181	KYGNITGTWEV	10				HLA-A24:02								
HPV18E2	HPV132	181	KYGNITGTW	8				HLA-A24:02								
HPV18E2	HPV133	335	TYHSETQRTKF	11				HLA-A24:02								
HPV18E2	HPV14	55	QTLNHQVWPAY	11				HLA-A01:01								
HPV18E2	HPV15	95	WTLQDTCCEELW	11				HLA-A01:01								
HPV18E2	HPV155	2	TPKELSERL	10				HLA-B07:02								
HPV18E2	HPV156	48	AREHEGIQT	9				HLA-B07:02								
HPV18E2	HPV157	222	HTPSYSSTV	10				HLA-B07:02								
HPV18E2	HPV158	223	TPSYSSTVSV	11				HLA-B07:02								
HPV18E2	HPV159	224	PSYSSTVSV	10				HLA-B07:02								
HPV18E2	HPV16	141	YVMTDAGTWOK	11				HLA-A01:01								
HPV18E2	HPV160	225	SPYSSTVSVG	10				HLA-B07:02								
HPV18E2	HPV161	225	SPYSSTVS	8				HLA-B07:02								
HPV18E2	HPV162	225	SPYSSTVSVGT	11	D	42		HLA-B07:02								
HPV18E2	HPV163	244	AATRPGHCGI	10	D	42		HLA-B07:02								
HPV18E2	HPV164	247	RPGHCGLA	9				HLA-B07:02								
HPV18E2	HPV165	260	GPVNPVLLGAA	10	D	42		HLA-B07:02								
HPV18E2	HPV166	260	GPVNPVLLGA	9				HLA-B07:02								
HPV18E2	HPV167	270	TPTGNKKRKL	11				HLA-B07:02								
HPV18E2	HPV17	143	MTDAGTWDKTA	11				HLA-A01:01								
HPV18E2	HPV18	143	MTDAGTWDKT	10				HLA-A01:01								
HPV18E2	HPV19	143	MTDAGTWD	8				HLA-A01:01								

Protein	Peptide	Position	Sekvens	Length	Disease	Patient No	HLA-type
HPV18 E2	HPV193	36	WQLIRWENAI	10			HLA-B08:01
HPV18 E2	HPV194	67	ISKAKHAKAI	10			HLA-B08:01
HPV18 E2	HPV195	272	TGNNKRRKL	9			HLA-B08:01
HPV18 E2	HPV196	275	NKRRKLCSG	9			HLA-B08:01
HPV18 E2	HPV197	288	IHLKGDNRSL	11			HLA-B08:01
HPV18 E2	HPV198	296	NSLKLRYRL	10			HLA-B08:01
HPV18 E2	HPV199	297	SLKCLRYRL	9			HLA-B08:01
HPV18 E2	HPV20	152	TATCVSHRGLY	11			HLA-A01:01
HPV18 E2	HPV200	297	SLKCLRYRLR	10			HLA-B08:01
HPV18 E2	HPV201	298	LKCLRYRL	8			HLA-B08:01
HPV18 E2	HPV202	340	TQRTKFLNTV	10			HLA-B08:01
HPV18 E2	HPV203	340	TQRTKFLNT	9			HLA-B08:01
HPV18 E2	HPV21	156	VSHRGLYY	8			HLA-A01:01
HPV18 E2	HPV22	163	YVKEGYNTFYI	11			HLA-A01:01
HPV18 E2	HPV23	172	YIEFKSECEKY	11	B	20	HLA-A01:01
HPV18 E2	HPV238	36	WQLIRWENAI F	11			HLA-B15:01
HPV18 E2	HPV239	37	QLIRWENAI F	10			HLA-B15:01
HPV18 E2	HPV24	295	RNSLKCLRY	9			HLA-A01:01
HPV18 E2	HPV240	78	LQMALQGLA	9			HLA-B15:01
HPV18 E2	HPV241	217	VKQLQHTPSPY	11			HLA-B15:01
HPV18 E2	HPV242	220	LQHTPSPY	8			HLA-B15:01
HPV18 E2	HPV243	256	KQHCQPNPL	10			HLA-B15:01
HPV18 E2	HPV244	302	RYRLRKHSDDHY	11			HLA-B15:01
HPV18 E2	HPV245	355	VQILVGYMTM	10			HLA-B15:01
HPV18 E2	HPV25	327	EKTGILT VTY	10			HLA-A01:01
HPV18 E2	HPV262	135	VAVDSVYY	8	C	56	HLA-B35:01
HPV18 E2	HPV270	212	SATQLVKQL	9			HLA-C03:04
HPV18 E2	HPV271	237	KTYGQTSAA	9			HLA-C03:04
HPV18 E2	HPV272	280	LCSGNTTPI	9	B,C	9,43	HLA-C03:04
HPV18 E2	HPV273	349	VAI PDSVQIL	10			HLA-C03:04
HPV18 E2	HPV274	356	QILVGYMTM	9			HLA-C03:04
HPV18 E2	HPV287	40	RWENAIFFA	9			HLA-C04:01
HPV18 E2	HPV288	122	YFVDGNKDNCM	11			HLA-C04:01
HPV18 E2	HPV289	123	YFVDGNKDC	9	D	57	HLA-C04:01
HPV18 E2	HPV290	136	AWDSVYYM	8			HLA-C04:01
HPV18 E2	HPV291	136	AWDSVYYMT	9			HLA-C04:01
HPV18 E2	HPV292	148	TWDKATCV	9			HLA-C04:01
HPV18 E2	HPV307	96	TLQDTCCEL	9			HLA-C05:01
HPV18 E2	HPV308	206	TSDDTVSATQL	11	C	51	HLA-C05:01
HPV18 E2	HPV309	206	TSDDTVSAT	9			HLA-C05:01
HPV18 E2	HPV310	349	VAI PDSVQILV	11			HLA-C05:01
HPV18 E2	HPV311	350	AI PDSVQILV	10			HLA-C05:01
HPV18 E2	HPV323	70	SKAHKAIEL	9			HLA-C07:01
HPV18 E2	HPV324	156	VSHRGLYYV	9			HLA-C07:01
HPV18 E2	HPV325	312	YRDISSTW	8			HLA-C07:01
HPV18 E2	HPV335	171	FYIEFKSEC	9			HLA-C07:02
HPV18 E2	HPV359	326	NEKTGILT VTY	11			HLA-A01:01
HPV18 E2	HPV458	165	KEGYNTFYIEF	11			HLA-A24:02
HPV18 E2	HPV459	309	SDHYRDISSTW	11			HLA-A24:02
HPV18 E2	HPV460	311	HYRDISSTWHW	11			HLA-A24:02
HPV18 E2	HPV502	312	YRDISSTWHW	10			HLA-B44:02
HPV18 E2	HPV62	56	TLNHQVWPA	9			HLA-A02:01
HPV18 E2	HPV63	345	FLNTVAIPDSV	11			HLA-A02:01
HPV18 E2	HPV82	62	VPAYNISKSK	10			HLA-A03:01
HPV18 E2	HPV83	65	YNISKSKAHK	10			HLA-A03:01
HPV18 E2	HPV84	84	GLAQSAVK	8			HLA-A03:01
HPV18 E2	HPV85	290	HLKGDNRSLK	10	C	56	HLA-A03:01
HPV18 E2	HPV94	17	KIDHYENDSK	11			HLA-A11:01
HPV18 E2	HPV95	31	SQIQYWQLIR	10			HLA-A11:01
HPV18 E2	HPV96	83	QGLAQSAVK	9			HLA-A11:01
HPV18 E2	HPV97	106	NTEPTFCFK	10			HLA-A11:01
HPV18 E2	HPV98	118	QTVQWFDGNK	11			HLA-A11:01
HPV18 E2	HPV99	168	YNTFYIEFK	9			HLA-A11:01
HPV18 E2	HPV350	56	TLNHQVWPA	10			HLA-A01:01 HLA-B15:01
HPV18 E2	HPV351	81	ALQGLAQSAVK	10			HLA-A01:01 HLA-B15:01
HPV18 E2	HPV352	105	WNTPTFCFK	10			HLA-A01:01 HLA-A11:01
HPV18 E2	HPV353	106	NTEPTFCFK	9			HLA-A01:01 HLA-A11:01
HPV18 E2	HPV354	131	CMTYVAVDWSVY	11	B	20	HLA-A01:01 HLA-B15:01
HPV18 E2	HPV355	143	MTDAGTWDK	9			HLA-A01:01 HLA-A11:01
HPV18 E2	HPV356	153	ATCVSHRGLYY	11			HLA-A01:01 HLA-A11:01
HPV18 E2	HPV357	154	TCVSHRGLYY	10			HLA-A01:01 HLA-A03:01
HPV18 E2	HPV358	229	STVSVGTAKTY	11			HLA-A01:01 HLA-B15:01
HPV18 E2	HPV360	351	IPDSVQILVGY	11			HLA-A01:01 HLA-B35:01
HPV18 E2	HPV361	353	DSVQILVGY	9	C	56	HLA-A01:01 HLA-B35:01
HPV18 E2	HPV380	6	TLSERLSCV	9			HLA-A02:01 HLA-B08:01
HPV18 E2	HPV393	60	QVWPAYNISK	10			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV394	61	WPAYNISKSK	11			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV395	61	WPAYNISK	9			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV396	81	ALQGLAQSAVK	11			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV397	119	TVQVYFDGNK	10			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV398	151	KTATCVSHR	9			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV399	155	CVSHRGLYYK	11			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV400	156	VSHRGLYYK	10			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV401	159	RGLYYKEGY	10			HLA-A03:01 HLA-B15:01
HPV18 E2	HPV402	160	GLYYKEGY	9			HLA-A03:01 HLA-B15:01
HPV18 E2	HPV403	172	YIEFKSECEK	10			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV404	209	DTVSATQLVK	10			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV405	210	TVSATQLVK	9			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV406	218	KQLQHTPSPY	10			HLA-A03:01 HLA-B15:01
HPV18 E2	HPV407	228	SSTVSVGTAK	10			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV408	229	STVSVGTAK	9			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV409	237	KTYGQTSATR	11			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV410	267	GAATPTGNANK	10			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV411	283	GNTTPIIHLK	10			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV412	284	NTPPIIHLK	9			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV413	295	RNSLKCLRYR	10			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV414	297	SLKCLRYRLRK	11			HLA-A03:01 HLA-A11:01
HPV18 E2	HPV415	304	RLRKHSDDHYR	10			HLA-A03:01 HLA-B15:01

Protein	Peptide	Position	Sekevns	Length	Disease	Patient No	HLA-type
HPV18E6	HPV337	51	LFVWRDSI	9			HLA-C07:02
HPV18E6	HPV338	54	VYRDSIPHA	9			HLA-C07:02
HPV18E6	HPV363	38	LELTVFEFAF	11			HLA-A01:01
HPV18E6	HPV382	38	LELTVFEFA	10			HLA-A02:01
HPV18E6	HPV480	121	NEKRRFHNI	9			HLA-B08:01
HPV18E6	HPV503	2	RFEDPTRRPY	10			HLA-B44:02
HPV18E6	HPV504	44	FEFAKDLF	9			HLA-B44:02
HPV18E6	HPV505	44	FEFAKDLFV	10			HLA-B44:02
HPV18E6	HPV64	23	SLQDIETCV	10			HLA-A02:01
HPV18E6	HPV65	35	KTVLETEV	9			HLA-A02:01
HPV18E6	HPV66	91	KLNTGLYLN	10			HLA-A02:01
HPV18E6	HPV67	96	GLYNLLIRCL	10	D	5	HLA-A02:01
HPV18E6	HPV86	96	GLYNLLIRCLR	11			HLA-A03:01
HPV18E6	HPV87	101	LIRLRCQK	9			HLA-A03:01
HPV18E6	HPV88	116	KLRLHNEK	8			HLA-A03:01
HPV18E6	HPV675	66	KGDIFYSR	9			HLA-A02:01
HPV18E6	HPV362	23	SLQDIETCV	11			HLA-A01:01
HPV18E6	HPV364	80	YSDSVYGDGT	9	D	53	HLA-A01:01
HPV18E6	HPV381	11	YKLPDLCTEL	10			HLA-A02:01
HPV18E6	HPV419	53	VWRDIPH	9			HLA-A03:01
HPV18E6	HPV420	58	SIPHAACKH	9			HLA-A03:01
HPV18E6	HPV421	81	SDSVYGDTEK	11			HLA-A03:01
HPV18E6	HPV422	82	DSVYGDTEK	10			HLA-A03:01
HPV18E6	HPV423	83	SVYGDTEK	9			HLA-A03:01
HPV18E6	HPV424	100	LIRLRCQK	10			HLA-A03:01
HPV18E6	HPV461	31	CVYCKTLEL	10			HLA-A24:02
HPV18E6	HPV469	112	NPAEKLRLH	9			HLA-B07:02
HPV18E6	HPV479	3	FEDPTRRPYKL	11			HLA-B08:01
HPV18E6	HPV508	92	LTNTGLYLN	9	B,D	24,53	HLA-C03:04
HPV18E6	HPV529	8	RRPVKPD	9			HLA-C07:01
HPV18E6	HPV530	68	IDFYSRIREL	10			HLA-C07:01
HPV18E6	HPV531	72	SRIRELRYH	9			HLA-C07:01
HPV18E6	HPV532	77	LRHYSVDSVY	9			HLA-C07:01
HPV18E6	HPV533	124	RRFHNIAGHY	10			HLA-C07:01
HPV18E6	HPV534	125	RHNIAGHY	9			HLA-C07:01
HPV18E6	HPV535	126	FHNIAGHYR	9			HLA-C07:01
HPV18E6	HPV572	43	VFEFAKDLF	10			HLA-A24:02
HPV18E6	HPV598	3	FEDPTRRPY	9			HLA-B35:01
HPV18E6	HPV556	24	LQDIETCVY	10			HLA-A01:01
HPV18E6	HPV557	40	LTVFEFAF	9			HLA-A01:01
HPV18E6	HPV558	40	LTVFEFAFK	10			HLA-A01:01
HPV18E6	HPV559	71	YSRIRELRYH	10			HLA-A01:01
HPV18E6	HPV573	79	HYSDSVYGDTL	11			HLA-A24:02
HPV18E6	HPV574	84	YVYDTELEK	9			HLA-A24:02
HPV18E6	HPV589	7	TRRPVKLPDL	10			HLA-B08:01
HPV18E6	HPV604	55	YRDSIPHAA	9			HLA-C04:01
HPV18E6	HPV612	46	FAKDLFVY	10			HLA-A01:01
HPV18E6	HPV613	80	YSDSVYGDTL	10	B	24	HLA-A01:01
HPV18E6	HPV615	12	KLPDLCTEL	9	D	5	HLA-A02:01
HPV18E6	HPV618	83	SVYGDTELEK	10			HLA-A03:01
HPV18E6	HPV624	32	WYCKTLEL	9			HLA-A24:02
HPV18E6	HPV625	36	TVELTEVF	9			HLA-A24:02
HPV18E6	HPV626	97	LYNLLIRCL	9			HLA-A24:02
HPV18E6	HPV631	69	DFYSRIREL	9			HLA-B08:01
HPV18E6	HPV632	70	FYSRIREL	8			HLA-B08:01
HPV18E6	HPV659	46	FAKDLFVY	9	B,B,D,C,C	18,24,45,47,51,53	HLA-A02:01
HPV18E7	HPV107	56	QRHTMLCMCKC	11			HLA-A11:01
HPV18E7	HPV108	58	HTMLCMCKC	10			HLA-A11:01
HPV18E7	HPV135	88	LFLNTLSF	8			HLA-A24:02
HPV18E7	HPV173	1	HGPKATLQDI	10			HLA-B07:02
HPV18E7	HPV174	2	GPKATLQDI	9			HLA-B07:02
HPV18E7	HPV175	2	GPKATLQDIVL	11			HLA-B07:02
HPV18E7	HPV176	2	GPKATLQDIV	10			HLA-B07:02
HPV18E7	HPV217	54	EPQRHTML	8			HLA-B08:01
HPV18E7	HPV218	68	EARIKLW	8			HLA-B08:01
HPV18E7	HPV219	81	DLRAFQQL	8			HLA-B08:01
HPV18E7	HPV248	85	FQQLFLNTLSF	11			HLA-B15:01
HPV18E7	HPV264	76	ESSADLRAF	10			HLA-B35:01
HPV18E7	HPV296	88	LFLNTLSFV	9			HLA-C04:01
HPV18E7	HPV313	7	LQDVLHL	8	B,D	53,24	HLA-C05:01
HPV18E7	HPV314	74	WESSADDL	9			HLA-C05:01
HPV18E7	HPV315	78	SADDLRAFQQL	11	D	53	HLA-C05:01
HPV18E7	HPV316	78	SADDLRAF	8			HLA-C05:01
HPV18E7	HPV497	91	NTLSFVCPVW	9			HLA-B35:01
HPV18E7	HPV68	4	KATLQDIVLHL	11			HLA-A02:01
HPV18E7	HPV69	5	ATLQDIVLHL	10			HLA-A02:01
HPV18E7	HPV70	87	QLFLNTLSFV	10			HLA-A02:01
HPV18E7	HPV71	89	FLNTLSFV	8			HLA-A02:01
HPV18E7	HPV72	92	TLSFVCPWCA	10			HLA-A02:01
HPV18E7	HPV425	57	RHTMLCMCKC	10			HLA-A03:01
HPV18E7	HPV426	58	HTMLCMCKC	9			HLA-A03:01
HPV18E7	HPV462	84	AFQQLFLNTL	10			HLA-A24:02
HPV18E7	HPV470	52	RAEQRHTM	10	B,D	20,53	HLA-C05:01
HPV18E7	HPV471	54	EPQRHTMLCM	10			HLA-B07:02
HPV18E7	HPV481	81	DLRAFQQLFL	10			HLA-B08:01
HPV18E7	HPV509	4	KATLQDIVL	9			HLA-C03:04
HPV18E7	HPV536	51	RRAEQRHTM	11			HLA-C07:01
HPV18E7	HPV537	82	LRAFQQLF	8			HLA-C07:01
HPV18E7	HPV575	86	QLFLNTLSF	10			HLA-A24:02
HPV18E7	HPV585	51	RRAEQRHTM	10	D	42	HLA-B07:02
HPV18E7	HPV605	82	LRAFQQLFL	9			HLA-C04:01
HPV18E7	HPV616	6	TLQDIVLHL	9			HLA-A02:01
HPV18E7	HPV634	77	SSADDLRAF	9			HLA-B15:01
HPV18E7	HPV646	87	QLFLNTLSF	9			HLA-A24:02
HPV18E7	HPV651	85	FQQLFLNTL	9	D	57	HLA-B08:01
HPV18E7	HPV660	52	RAEQRHTM	9			HLA-B07:02

EPILOGUE

The research presented in this PhD thesis elucidates immune cells in cervical tissue (cytology and biopsies) and blood samples from healthy individuals, patients with severe cervical intraepithelial neoplasia (CIN3) and cervical cancer. Specimens were analyzed for immune infiltration, phenotypic characteristics, state of activation, signs of exhaustion and CD8 T cell recognition of HPV derived peptides. All with the purpose of gaining insight in immune infiltrating cells and their dynamics in cervical neoplasia and cancer. The immune system plays a key role in the control of HPV infection and is the cause of cervical cancer. Somehow the virus seems to be able to escape immune recognition, persist, and may eventually lead to neoplastic transformation and cancer. The changes in the tumor microenvironment (TME) and the interplay with the virally infected keratinocytes determines the course of the disease. HPV-driven cancers have a relatively high tumor mutational burden (TMB), (Fig. 10) which indicates increased likelihood of neoepitopes being potential targets for T cell recognition. As a consequence of both the high TMB and the viral components from HPV, these cancers would be expected to be relatively immunogenic, and consequently prone to T cell recognition and immune therapy [220]. To date, treatment with immune checkpoint inhibition (ICI) is the most widely used immune therapy available across several solid cancers e.g., malign melanoma, kidney, and lung cancer. However, for HPV-driven cancers, ICI has not shown the same promising result when applied to cervical cancer patients with overall response rate (ORR) of only 12-26% [182][184][191], which is in stark contrast to other viral-driven cancers, such as Merkel cell carcinoma, where ORR for treatment with ICI is as high as ~70%. Despite having three approved HPV vaccines on the market (Gardasil, Gardasil 9 and Cervarix), all three of which target the conserved L2 protein, they are only prophylactic vaccines and therefore insufficient in cases of existing infection [221]. Patients with advanced, recurrent, or metastatic cervical cancer still have poor prognosis and the median overall survival for advanced cervical cancer is only 16.8 months [222]. The overall 5-year survival for all stages of cervical cancer is 68% [182]. For these reasons, improved treatment strategies are greatly needed.

In **Manuscript I**, we studied the immune infiltration, the microenvironment, and the alterations of the local and systemic immune system in patients with high-grade cervical intraepithelial neoplasia (CIN3) and cervical cancer compared to healthy individuals. We characterized both immune phenotypes of CD8 and CD4 T cells and myeloid cells in cytology, biopsies, and peripheral blood samples. Our results show a high prevalence of immune infiltration in cancer

patients. Comparing immune cells between the 3 groups, our main observation was an immune signature characterized by late differentiated/exhausted CD8 and CD4 T cells in the cancer group. Looking into signs of exhaustion we found CD8 T cells were more abundant in CIN3 lesions and in cancer patients, compared to healthy individuals. This indicates that there are immune cells present on site, but their function seems to be impaired. The interplay of inhibitory mechanisms has not been fully elucidated, but if the T cells are dysfunctional due to persistent overstimulation and therefore in a state of fully exhaustion, they will not be able to be invigorated. Regardless of ICI and thereby blocking of inhibitory receptors such as (PD-1/PD-L1), T cells might still be terminally exhausted and thereby unable to mount a significant immune response. These inhibitory receptors, cytokine production, inflammation in the TME and level of infiltrating immune cells, all influence the clinical outcome. One might speculate that the reason for low ORR when treated with ICI is the abundance of late differentiated/exhausted immune signatures as demonstrated in this study.

To investigate this question further, it would be relevant to perform either a cell cytotoxicity assay, intracellular cytokine staining or even single cell RNA sequencing. If T cells are irreversibly exhausted, therapeutic initiatives generating novel T cell responses to HPV or other tumor-associated antigens such as vaccination or adoptive T cell therapies could potentially be helpful, especially in the combination with ICI. Further studies investigating this matter would be highly valuable.

This study also provides novel insight to the striking identical immune signatures we found in cells from biopsies compared to cytology. We hereby suggest cytology as a fully useful alternative to biopsies when obtaining cells for immune evaluation. This would also greatly increase compliance in the clinical setting, as there would be no need for more invasive biopsies that demand administration of local analgesia and entails complications as for example the risk of post-operative bleeding and infection.

This study showed increased level of infiltrating T cells in cervical cancer patients. However, no major differences were observed for the myeloid subsets, except increased expression of PD-L1, indicating immune inhibition and supporting the finding of an immune suppressive TME of complex interplay of multiple factors. Previous studies have found an increased frequency of MDCs in cervical cancer biopsies and blood samples [223][224], but our data did not support these earlier findings. To our present knowledge, these discrepancies cannot be explained, and further studies are therefore needed.

Manuscript II: This study provides novel insight into the CD8 T cell recognition of peptides of the early region genes E2, E6 and E7 of Human Papilloma Virus genotype 16 and 18.

As cervical cancer is driven by HPV infection, and the HPV-encoded oncogenes will be integrated in the cancer cell genome, proteins encoded by such oncogenes could be potential valuable targets for T cell recognition of cervical cancer and other HPV-driven cancers. These viral-derived antigens are foreign to the immune systems T cells, and hence should not be affected by T cell tolerance. Furthermore, such antigens will be shared among patients with HPV-driven cancers, and therefore provides a potentially ideal set of antigens for T cell targeting.

To date, our knowledge is still limited in terms of the CD8 T cell recognition toward HPV-derived oncoproteins, i.e., which epitopes are presented and recognized by CD8 T cells and to what extent will patients with HPV driven cancers or neoplasia mount T cell recognition towards such antigens. Although several HPV-derived T cell epitopes has been identified [225], a comprehensive screening of T cell recognition across multiple HLA haplotypes has not previous been conducted.

Here, we conducted such a T cell screening based on the current most high-throughput technology for investigating a large pool of potential epitopes, utilizes DNA barcodes to label each pMHC specifically. This makes it possible to include more than 1000 different potent T cell epitopes in one simultaneous analysis [218]. Based T cell epitopes predicted from HPV 16 & 18 E2, 6 & 7 gene sequences and previous identified T cell epitopes, 685 potentially distinct human leucocyte antigen binding peptides were predicted and evaluated. We included 14 different HLAs covering the most common HLA-A, B and C alleles across European Caucasian populations. 27 individuals were included in the study: eight healthy individuals, nine patients with CIN3 and ten patients with cervical cancer. We examined both the width and intensity of T cell recognition and compared healthy individuals to patients diagnosed with severe cervical neoplasia or cancer. We were able to detect T cell recognition against 127 HPV-derived peptides. We found high prevalence of T cell recognition towards nine of these HPV-derived peptides, of which six originated from the HPV16 E2 gene. The HPV E2 protein is substantially larger than E6 and E7 and consequently more epitopes were predicted from this protein (60% of predicted peptides), however, when T cell recognition was evaluated and normalized to the size of such proteins, E2 seems to mount a broader T cell recognition than E6 and E7. This underlines the importance of further investigations of the E2 gene, as a target for T cell recognition of HPV-driven cancers.

Although this study extended our knowledge of T cell epitopes in HPV oncogenes, the level in T cell reactivity in terms of the frequency of CD8 T cells recognizing such epitopes were relatively low across all evaluated groups. This could be explained by the exhausted T cell profile and immune suppressive environment as elucidated in manuscript I, but also be a consequence of additional antigens being more immunogenic than the HPV-derived sequences. It has been observed that T cell recognition towards HPV-driven cancers, following adoptive T cell therapy with tumor infiltrating lymphocytes, is driven by other antigens than those of HPV origin [225]. Herein, neoantigens and cancer-germline antigens were recognized over those of HPV origin. It has been observed that E6 and E7 expression is downregulated in late-stage cancer [226], which could potentially be an effect of immunoediting and early selection of less immunogenic cancer cells carrying lower levels of these viral elements. But numerous other mechanisms may also play a role in the effect.

An additional interesting observation from the T cell screening, was the apparent highly prevalent T cell recognition of a given epitope presented in HLA-C05:01. To our knowledge, no other comparative studies have investigated this broad HLA coverage in cervical neoplasia and cancer patients, and hence T cell recognition of HPV in the context from HLA-C has not previously been explored. However, it is important to note, that our findings may be impacted by novel knowledge demonstrating a potential interaction between certain peptide-HLA-C complexes and KIR receptors expressed on the surface of CD8 T cell [213][214] (and ongoing studies by colleagues, Saini et al.). This KIR driven interaction can result in a false positive interpretation of TCR directed recognition of the pHLA-C complexes. For future analysis a KIR receptor blocker would be recommendable to block this interaction and hence mitigate the risk of false positive identification.

CD8 T cell recognition of HPV derived epitopes is the first step in understanding the interactions between virus infected keratinocyte and the immune response. Having detected this recognition, we still don't know for sure whether the specific epitopes are truly immunogenic and capable of activating the T cells to a functional level of sufficient killing and clearance of the virus.

Given the knowledge of T cell epitopes in HPV this can be exploited for immunotherapeutic strategies such as vaccination, or adoptive T cells therapy. Both with the purpose to increase the level of functional HPV-targeting CD8 T cells. HPV-specific T cells can be isolated to determine the T cell receptor sequence for use in TCR gene therapy, where the TCR gene could be inserted in T cells of irrelevant specificities. Ongoing studies also examine targeted therapeutic vaccines

as potential options for treatment. Multiple investigations are being conducted with the goal of finding the optimal way to target the cancer through immunotherapy.

A combination of therapies targeting several mechanisms, probably holds the key to future treatments. The research presented in this thesis, provides novel insight into which epitopes to select for further investigations and into characterization of an exhausted immune profile in cancer patients. Hopefully, this may help to utilize or burst the immune system to fully mount a response and thereby combat the cancer.

BIBLIOGRAPHY

- [1] M. Schiffman *et al.*, “Carcinogenic human papillomavirus infection,” *Nat. Rev. Dis. Prim.*, vol. 2, 2016, doi: 10.1038/nrdp.2016.86.
- [2] J. Doorbar, “Papillomavirus life cycle organization and biomarker selection,” *Disease Markers*, vol. 23, no. 4. IOS Press, pp. 297–313, 2007, doi: 10.1155/2007/613150.
- [3] H. Zur Hausen, “Papillomaviruses and cancer: From basic studies to clinical application,” *Nat. Rev. Cancer*, vol. 2, no. 5, pp. 342–350, 2002, doi: 10.1038/nrc798.
- [4] H. Zur Hausen, “The search for infectious causes of human cancers: Where and why (Nobel Lecture),” *Angew. Chemie - Int. Ed.*, vol. 48, no. 32, pp. 5798–5808, 2009, doi: 10.1002/anie.200901917.
- [5] A. Kothari, “Human papilloma virus (HPV) and cervical cancer,” *Indep. Nurse*, vol. 2020, no. 2, pp. 20–22, Feb. 2020, doi: 10.12968/indn.2020.2.20.
- [6] H. W. Chesson, E. F. Dunne, S. Hariri, and L. E. Markowitz, “The Estimated Lifetime Probability of Acquiring Human Papillomavirus in the United States,” *Sex. Transm. Dis.*, vol. 41, no. 11, pp. 660–664, Nov. 2014, doi: 10.1097/OLQ.000000000000193.
- [7] M. Stanley, “Pathology and epidemiology of HPV infection in females,” *Gynecol. Oncol.*, vol. 117, no. 2 SUPPL., pp. S5–S10, 2010, doi: 10.1016/j.ygyno.2010.01.024.
- [8] D. Boda *et al.*, “Human papilloma virus: Apprehending the link with carcinogenesis and unveiling new research avenues (Review),” *Int. J. Oncol.*, vol. 52, no. 3, pp. 637–655, 2018, doi: 10.3892/ijo.2018.4256.
- [9] W. Small *et al.*, “Cervical cancer: A global health crisis,” *Cancer*, vol. 123, no. 13, pp. 2404–2412, 2017, doi: 10.1002/cncr.30667.
- [10] GLOBOCAN, “Cervix uteri Source: Globocan 2020,” *Int. Agent Res. Cervic Uteri*, vol. 419, pp. 1–10, 2020.
- [11] Sundhedsdatastyrelsen. Nye kræfttilfælde i Danmark., “Cancerregisteret,” *Cancerregisteret*, pp. 30–31, 2019, [Online]. Available: https://sundhedsdatastyrelsen.dk/da/tal-og-analyser/analyser-og-rapporter/sygdomme/kraeft-_nyetilfaelde.
- [12] S. K. Kjær, C. Munk, J. Junge, and T. Iftner, “Carcinogenic HPV prevalence and age-specific type distribution in 40,382 women with normal cervical cytology, ASCUS/LSIL, HSIL, or cervical cancer: What is the potential for prevention?,” *Cancer Causes Control*, vol. 25, no. 2, pp. 179–189, 2014, doi: 10.1007/s10552-013-0320-z.
- [13] <http://www-dep.iarc.fr/NORDCAN/DK/frame.asp>, “Kræftstatistik : Nøgletal og figurer (Danmark - Livmoderhals),” 2016, [Online]. Available: <http://www-dep.iarc.fr/NORDCAN/DK/StatsFact.asp?cancer=150&country=208>.
- [14] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal, “Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries,” *CA. Cancer J. Clin.*, vol. 68, no. 6, pp. 394–424, 2018, doi: 10.3322/caac.21492.
- [15] S. V. Graham, “Late events in the life cycle of Human papillomaviruses,” *Papillomavirus Research: from natural history to vaccines and beyond*. p. 195, 2006.
- [16] J. Doorbar *et al.*, “Characterization of events during the late stages of HPV16 infection in vivo using high-affinity synthetic Fabs to E4,” *Virology*, vol. 238, no. 1, pp. 40–52, 1997, doi: 10.1006/viro.1997.8768.
- [17] S. V. Graham, “Human papillomavirus: Gene expression, regulation and prospects for

- novel diagnostic methods and antiviral therapies," *Future Microbiol.*, vol. 5, no. 10, pp. 1493–1506, 2010, doi: 10.2217/fmb.10.107.
- [18] J. Xi *et al.*, "Genetic variability and functional implication of the long control region in HPV-16 variants in Southwest China," *PLoS One*, vol. 12, no. 8, pp. 1–11, 2017, doi: 10.1371/journal.pone.0182388.
- [19] T. Ramqvist, N. Grün, and T. Dalianis, "Human papillomavirus and tonsillar and base of tongue cancer," *Viruses*, vol. 7, no. 3, pp. 1332–1343, 2015, doi: 10.3390/v7031332.
- [20] D. Bzhalava, C. Eklund, and J. Dillner, "International standardization and classification of human papillomavirus types," *Virology*, vol. 476, pp. 341–344, 2015, doi: 10.1016/j.virol.2014.12.028.
- [21] H.-U. Bernard, R. D. Burk, Z. Chen, D. van Koenraad, and E. Al, "Classification of Papillomaviruses (PVs) Based on 189 PV Types and Proposal of Taxonomic Amendments," *Virology*, vol. 401, no. 1, pp. 70–79, 2012, doi: 10.1016/j.virol.2010.02.002.Classification.
- [22] "No Title," p. <https://www.iarc.who.int/>, [Online]. Available: <https://www.iarc.who.int/>.
- [23] V. Bouvard *et al.*, "A review of human carcinogens--Part B: biological agents.," *Lancet Oncol.*, vol. 10, no. 4, pp. 321–322, 2009, doi: 10.1016/s1470-2045(09)70096-8.
- [24] J. M. M. Walboomers *et al.*, "Human papillomavirus is a necessary cause of invasive cervical cancer worldwide," *J. Pathol.*, vol. 189, no. 1, pp. 12–19, 1999, doi: 10.1002/(SICI)1096-9896(199909)189:1<12::AID-PATH431>3.0.CO;2-F.
- [25] S. Duensing and K. Münger, "Mechanisms of genomic instability in human cancer: Insights from studies with human papillomavirus oncoproteins," *Int. J. Cancer*, vol. 109, no. 2, pp. 157–162, 2004, doi: 10.1002/ijc.11691.
- [26] G. M. Clifford, J. S. Smith, M. Plummer, N. Muñoz, and S. Franceschi, "Human papillomavirus types in invasive cervical cancer worldwide: A meta-analysis," *Br. J. Cancer*, vol. 88, no. 1, pp. 63–69, 2003, doi: 10.1038/sj.bjc.6600688.
- [27] Bosch F., Lorincz A., Muñoz N., Meijer C. J. L. M., and Shah K. V., "The causal relation between human papillomavirus and cervical cancer," *J. Clin. Pathol.*, vol. 55, no. 4, pp. 244–265, 2002, [Online]. Available: <http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L34293391>.
- [28] A. Nielsen, S. K. Kjaer, C. Munk, M. Osler, and T. Iftner, "Persistence of high-risk human papillomavirus infection in a population-based cohort of Danish women," *J. Med. Virol.*, vol. 82, no. 4, pp. 616–623, Apr. 2010, doi: 10.1002/jmv.21750.
- [29] M. H. Einstein *et al.*, "Clinician's guide to human papillomavirus immunology: knowns and unknowns," *Lancet Infect. Dis.*, vol. 9, no. 6, pp. 347–356, 2009, doi: 10.1016/S1473-3099(09)70108-2.
- [30] I. N. Mamas, G. Sourvinos, and D. A. Spandidos, "The paediatric story of human papillomavirus," *Oncol. Lett.*, vol. 8, no. 2, pp. 502–506, 2014, doi: 10.3892/ol.2014.2226.
- [31] J. T. Schiller, P. M. Day, and R. C. Kines, "Current understanding of the mechanism of HPV infection," *Gynecol. Oncol.*, vol. 118, no. 1 SUPPL. 1, pp. S12–S17, 2010, doi: 10.1016/j.ygyno.2010.04.004.
- [32] J. Pudney, A. J. Quayle, and D. J. Anderson, "Immunological microenvironments in the human vagina and cervix: Mediators of cellular immunity are concentrated in the cervical transformation zone," *Biol. Reprod.*, vol. 73, no. 6, pp. 1253–1263, 2005, doi: 10.1095/biolreprod.105.043133.
- [33] J. B. De Tomasi, M. M. Opata, and C. N. Mowa, "Immunity in the cervix: Interphase

- between immune and cervical epithelial cells," *J. Immunol. Res.*, vol. 2019, 2019, doi: 10.1155/2019/7693183.
- [34] C. A. Horvath, G. A. Boulet, V. M. Renoux, P. O. Delvenne, and J. P. J. Bogers, "Mechanisms of cell entry by human papillomaviruses: An overview," *Viol. J.*, vol. 7, pp. 1–7, 2010, doi: 10.1186/1743-422X-7-11.
- [35] R. M. Richards, D. R. Lowy, J. T. Schiller, and P. M. Day, "Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 5, pp. 1522–1527, 2006, doi: 10.1073/pnas.0508815103.
- [36] M. K. Jang, D. E. Anderson, K. van Doorslaer, and A. A. McBride, "A proteomic approach to discover and compare interacting partners of papillomavirus E2 proteins from diverse phylogenetic groups," *Proteomics*, vol. 15, no. 12, pp. 2038–2050, 2015, doi: 10.1002/pmic.201400613.
- [37] P. M. Day, D. R. Lowy, and J. T. Schiller, "Heparan Sulfate-Independent Cell Binding and Infection with Furin-Precleaved Papillomavirus Capsids," *J. Virol.*, vol. 82, no. 24, pp. 12565–12568, 2008, doi: 10.1128/jvi.01631-08.
- [38] I. D. Lyronis, S. Baritaki, I. Bizakis, E. Krambovitis, and D. A. Spandidos, "K-ras mutation, HPV infection and smoking or alcohol abuse positively correlate with esophageal squamous carcinoma," *Pathol. Oncol. Res.*, vol. 14, no. 3, pp. 267–273, 2008, doi: 10.1007/s12253-008-9032-1.
- [39] J. Doorbar, "Model systems of human papillomavirus-associated disease," *J. Pathol.*, vol. 238, no. 2, pp. 166–179, 2016, doi: 10.1002/path.4656.
- [40] M. Ghanaat *et al.*, "Virus against virus: strategies for using adenovirus vectors in the treatment of HPV-induced cervical cancer," *Acta Pharmacol. Sin.*, no. January, pp. 1–10, 2021, doi: 10.1038/s41401-021-00616-5.
- [41] E. S. Hwang, T. Nottoli, and D. Dimaio, "The HPV16 E5 protein: Expression, detection, and stable complex formation with transmembrane proteins in COS cells," *Virology*, vol. 211, no. 1, pp. 227–233, 1995, doi: 10.1006/viro.1995.1395.
- [42] B. Zhang, D. F. Spandau, and A. Roman, "E5 Protein of Human Papillomavirus Type 16 Protects Human Foreskin Keratinocytes from UV B-Irradiation-Induced Apoptosis," *J. Virol.*, vol. 76, no. 1, pp. 220–231, 2002, doi: 10.1128/jvi.76.1.220-231.2002.
- [43] G. H. Ashrafi, M. R. Haghshenas, B. Marchetti, P. M. O'Brien, and M. S. Campo, "E5 protein of human papillomavirus type 16 selectively downregulates surface HLA class I," *Int. J. Cancer*, vol. 113, no. 2, pp. 276–283, 2005, doi: 10.1002/ijc.20558.
- [44] M. S. Campo *et al.*, "HPV-16 E5 down-regulates expression of surface HLA class I and reduces recognition by CD8 T cells," *Virology*, vol. 407, no. 1, pp. 137–142, 2010, doi: 10.1016/j.virol.2010.07.044.
- [45] C. A. Moody and L. A. Laimins, "Human papillomavirus oncoproteins: Pathways to transformation," *Nat. Rev. Cancer*, vol. 10, no. 8, pp. 550–560, 2010, doi: 10.1038/nrc2886.
- [46] K. Münger, W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel, "The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes," *J. Virol.*, vol. 63, no. 10, pp. 4417–4421, 1989, doi: 10.1128/jvi.63.10.4417-4421.1989.
- [47] W. C. Hahn, "Immortalization and transformation of human cells," *Mol. Cells*, vol. 13, no. 3, pp. 351–361, 2002.
- [48] J. Doorbar *et al.*, "The biology and life-cycle of human papillomaviruses," *Vaccine*, vol.

- 30, no. SUPPL.5, pp. F55–F70, 2012, doi: 10.1016/j.vaccine.2012.06.083.
- [49] S. G. Hwang, D. Lee, J. Kim, T. Seo, and J. Choe, “Human papillomavirus type 16 E7 binds to E2F1 and activates E2F1-driven transcription in a retinoblastoma protein-independent manner,” *J. Biol. Chem.*, vol. 277, no. 4, pp. 2923–2930, 2002, doi: 10.1074/jbc.M109113200.
- [50] W. K. Songock, S. man Kim, and J. M. Bodily, “The human papillomavirus E7 oncoprotein as a regulator of transcription,” *Virus Res.*, vol. 231, pp. 56–75, 2017, doi: 10.1016/j.virusres.2016.10.017.
- [51] E. Toussaint-Smith, D. B. Donner, and A. Roman, “Expression of human papillomavirus type 16 E6 and E7 oncoproteins in primary foreskin keratinocytes is sufficient to alter the expression of angiogenic factors,” *Oncogene*, vol. 23, no. 17, pp. 2988–2995, 2004, doi: 10.1038/sj.onc.1207442.
- [52] C. A. Moody, “Mechanisms by which HPV induces a replication competent environment in differentiating keratinocytes,” *Viruses*, vol. 9, no. 9, pp. 1–21, 2017, doi: 10.3390/v9090261.
- [53] M. A. Stanley, “Epithelial cell responses to infection with human papillomavirus,” *Clin. Microbiol. Rev.*, vol. 25, no. 2, pp. 215–222, 2012, doi: 10.1128/CMR.05028-11.
- [54] A. C. Rodríguez *et al.*, “Rapid clearance of human papillomavirus and implications for clinical focus on persistent infections,” *J. Natl. Cancer Inst.*, vol. 100, no. 7, pp. 513–517, 2008, doi: 10.1093/jnci/djn044.
- [55] Y. L. Woo *et al.*, “A prospective study on the natural course of low-grade squamous intraepithelial lesions and the presence of HPV16 E2-, E6- And E7-specific T-cell responses,” *Int. J. Cancer*, vol. 126, no. 1, pp. 133–141, 2010, doi: 10.1002/ijc.24804.
- [56] L. Alemany *et al.*, “Time trends of human papillomavirus types in invasive cervical cancer, from 1940 to 2007,” *Int. J. Cancer*, vol. 135, no. 1, pp. 88–95, 2014, doi: 10.1002/ijc.28636.
- [57] A. Manickam, M. Sivanandham, and I. L. Tourkova, “Immunological role of dendritic cells in cervical cancer,” *Adv. Exp. Med. Biol.*, vol. 601, no. Tindle 2002, pp. 155–162, 2007, doi: 10.1007/978-0-387-72005-0_16.
- [58] E. L. Johansson, A. Rudin, L. Wassén, and J. Holmgren, “Distribution of lymphocytes and adhesion molecules in human cervix and vagina,” *Immunology*, vol. 96, no. 2, pp. 272–277, 1999, doi: 10.1046/j.1365-2567.1999.00675.x.
- [59] T. C. Wright and B. M. Ronnett, *Blaustein’s Pathology of the Female Genital Tract*. 2019.
- [60] P. Autier, M. Coibion, F. Huet, and A. R. Grivegnee, “Transformation zone location and intraepithelial neoplasia of the cervix uteri,” *Br. J. Cancer*, vol. 74, no. 3, pp. 488–490, 1996, doi: 10.1038/bjc.1996.388.
- [61] B. Baldur-Felskov *et al.*, “Trends in the incidence of cervical cancer and severe precancerous lesions in Denmark, 1997–2012,” *Cancer Causes Control*, vol. 26, no. 8, pp. 1105–1116, 2015, doi: 10.1007/s10552-015-0603-7.
- [62] “No Title,” p. <http://www.gyncph.dk/procedur/gyn/cacerv.htm>.
- [63] D. Solomon *et al.*, “The 2001 Bethesda System: Terminology for reporting results of cervical cytology,” *J. Am. Med. Assoc.*, vol. 287, no. 16, pp. 2114–2119, 2002, doi: 10.1001/jama.287.16.2114.
- [64] M. Safaeian *et al.*, “Durable antibody responses following one dose of the bivalent human papillomavirus L1 virus-like particle vaccine in the Costa Rica vaccine trial,” *Cancer Prev. Res.*, vol. 6, no. 11, pp. 1242–1250, 2013, doi: 10.1158/1940-6207.CAPR-13-0203.
- [65] L. L. Villa *et al.*, “High sustained efficacy of a prophylactic quadrivalent human

- papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up,” *Br. J. Cancer*, vol. 95, no. 11, pp. 1459–1466, 2006, doi: 10.1038/sj.bjc.6603469.
- [66] A. R. Giuliano *et al.*, “Impact of baseline covariates on the immunogenicity of a quadrivalent (Types 6, 11, 16, and 18) human papillomavirus virus-like-particle vaccine,” *J. Infect. Dis.*, vol. 196, no. 8, pp. 1153–1162, 2007, doi: 10.1086/521679.
- [67] C. Pedersen *et al.*, “Immunization of Early Adolescent Females with Human Papillomavirus Type 16 and 18 L1 Virus-Like Particle Vaccine Containing AS04 Adjuvant,” *J. Adolesc. Heal.*, vol. 40, no. 6, pp. 564–571, 2007, doi: 10.1016/j.jadohealth.2007.02.015.
- [68] S. L. Block *et al.*, “Comparison of the immunogenicity and reactogenicity of a prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in male and female adolescents and young adult women,” *Pediatrics*, vol. 118, no. 5, pp. 2135–2145, 2006, doi: 10.1542/peds.2006-0461.
- [69] S. K. Kjaer *et al.*, “Long-term effectiveness of the nine-valent human papillomavirus vaccine in Scandinavian women: interim analysis after 8 years of follow-up,” *Hum. Vaccines Immunother.*, vol. 17, no. 4, pp. 943–949, 2020, doi: 10.1080/21645515.2020.1839292.
- [70] D. G. Ferris *et al.*, “4-valent human papillomavirus (4vHPV) vaccine in preadolescents and adolescents after 10 years,” *Pediatrics*, vol. 140, no. 6, pp. 1–11, 2017, doi: 10.1542/peds.2016-3947.
- [71] J. T. Schiller, X. Castellsagué, and S. M. Garland, “A review of clinical trials of human papillomavirus prophylactic vaccines,” *Vaccine*, vol. 30, no. SUPPL.5, pp. F123–F138, 2012, doi: 10.1016/j.vaccine.2012.04.108.
- [72] J. T. Bryan, B. Buckland, J. Hammond, and K. U. Jansen, “Prevention of cervical cancer: Journey to develop the first human papillomavirus virus-like particle vaccine and the next generation vaccine,” *Curr. Opin. Chem. Biol.*, vol. 32, pp. 34–47, 2016, doi: 10.1016/j.cbpa.2016.03.001.
- [73] S. K. Kjaer, C. Dehlendorff, F. Belmonte, and L. Baandrup, “Real-world Effectiveness of Human Papillomavirus Vaccination Against Cervical Cancer,” *JNCI J. Natl. Cancer Inst.*, vol. 00, no. January, pp. 1–7, 2021, doi: 10.1093/jnci/djab080.
- [74] K. U. Petry *et al.*, “Cellular immunodeficiency enhances the progression of human papillomavirus-associated cervical lesions,” *Int. J. Cancer*, vol. 57, no. 6, pp. 836–840, 1994, doi: 10.1002/ijc.2910570612.
- [75] S. Smola, “Immunopathogenesis of HPV-associated cancers and prospects for immunotherapy,” *Viruses*, vol. 9, no. 9, 2017, doi: 10.3390/v9090254.
- [76] S. Stokley *et al.*, “Human papillomavirus vaccination coverage among adolescents, 2007–2013, and postlicensure vaccine safety monitoring, 2006–2014--United States.,” *MMWR. Morb. Mortal. Wkly. Rep.*, vol. 63, no. 29, pp. 620–4, 2014, [Online]. Available: <http://www.ncbi.nlm.nih.gov/pubmed/25055185><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5779422>.
- [77] T. V. Ellerbrock *et al.*, “Incidence of cervical squamous intraepithelial lesions in HIV-infected women,” *J. Am. Med. Assoc.*, vol. 283, no. 8, pp. 1031–1037, 2000, doi: 10.1001/jama.283.8.1031.
- [78] C. Y. Fang *et al.*, “Perceived stress is associated with impaired t-cell response to HPV16 in women with cervical dysplasia,” *Ann. Behav. Med.*, vol. 35, no. 1, pp. 87–96, 2008, doi: 10.1007/s12160-007-9007-6.
- [79] A. L. Coker, S. Bond, M. M. Madeleine, K. Luchok, and L. Pirisi, “Psychosocial stress and

- cervical neoplasia risk," *Psychosom. Med.*, vol. 65, no. 4, pp. 644–651, 2003, doi: 10.1097/01.PSY.0000041471.57895.08.
- [80] X. Castellsagué, F. X. Bosch, and N. Muñoz, "Environmental co-factors in HPV carcinogenesis," *Virus Res.*, vol. 89, no. 2, pp. 191–199, 2002, doi: 10.1016/S0168-1702(02)00188-0.
- [81] "No Title." <https://www.nhs.uk/conditions/cervical-cancer/causes/>.
- [82] F. X. Bosch and N. Muñoz, "The viral etiology of cervical cancer," *Virus Res.*, vol. 89, no. 2, pp. 183–190, 2002, doi: 10.1016/S0168-1702(02)00187-9.
- [83] M. Steben and E. Duarte-Franco, "Human papillomavirus infection: Epidemiology and pathophysiology," *Gynecol. Oncol.*, vol. 107, no. 2 SUPPL., p. S2, 2007, doi: 10.1016/j.ygyno.2007.07.067.
- [84] J. Tan, M. A. Quinn, J. M. Pyman, P. M. Delaney, and W. J. McLaren, "Detection of cervical intraepithelial neoplasia in vivo using confocal endomicroscopy," *BJOG An Int. J. Obstet. Gynaecol.*, vol. 116, no. 12, pp. 1663–1670, 2009, doi: 10.1111/j.1471-0528.2009.02261.x.
- [85] "What are abnormal cervical cells? | Cervical cancer | Cancer Research UK." <https://www.cancerresearchuk.org/about-cancer/cervical-cancer/treatment-for-abnormal-cervical-cells/what-are-abnormal-cervical-cells> (accessed Dec. 30, 2020).
- [86] G. Salvo, D. Odetto, R. Pareja, M. Frumovitz, and P. T. Ramirez, "Revised 2018 International Federation of Gynecology and Obstetrics (FIGO) cervical cancer staging: A review of gaps and questions that remain," *Int. J. Gynecol. Cancer*, vol. 30, no. 6, pp. 873–878, 2020, doi: 10.1136/ijgc-2020-001257.
- [87] G. Engholm *et al.*, "NORDCAN - A Nordic tool for cancer information, planning, quality control and research," *Acta Oncol. (Madr.)*, vol. 49, no. 5, pp. 725–736, 2010, doi: 10.3109/02841861003782017.
- [88] G. Schepisi *et al.*, "Immunotherapy and its development for gynecological (Ovarian, endometrial and cervical) tumors: From immune checkpoint inhibitors to chimeric antigen receptor (car)-T cell therapy," *Cancers (Basel)*, vol. 13, no. 4, pp. 1–22, 2021, doi: 10.3390/cancers13040840.
- [89] S. Duranti *et al.*, "Role of immune checkpoint inhibitors in cervical cancer: From preclinical to clinical data," *Cancers (Basel)*, vol. 13, no. 9, pp. 1–12, 2021, doi: 10.3390/cancers13092089.
- [90] "No Title."
- [91] A. J. Yates, "Theories and quantification of thymic selection," *Front. Immunol.*, vol. 5, no. FEB, pp. 1–15, 2014, doi: 10.3389/fimmu.2014.00013.
- [92] M. Zoodsma *et al.*, "Analysis of the entire HLA region in susceptibility for cervical cancer: a comprehensive study.," *J. Med. Genet.*, vol. 42, no. 8, pp. 1–6, 2005, doi: 10.1136/jmg.2005.031351.
- [93] M. Engelmark, A. Beskow, J. Magnusson, H. Erlich, and U. Gyllensten, "Affected sib-pair analysis of the contribution of HLA class I and class II loci to development of cervical cancer," *Hum. Mol. Genet.*, vol. 13, no. 17, pp. 1951–1958, 2004, doi: 10.1093/hmg/ddh201.
- [94] M. Allen *et al.*, "HLA DQ-DR haplotype and susceptibility to cervical carcinoma: Indications of increased risk for development of cervical carcinoma in individuals infected with HPV 18," *Tissue Antigens*, vol. 48, no. 1, pp. 32–37, 1996, doi: 10.1111/j.1399-0039.1996.tb02602.x.
- [95] P. G. Coulie, B. J. Van Den Eynde, P. Van Der Bruggen, and T. Boon, "Tumour antigens

- recognized by T lymphocytes: At the core of cancer immunotherapy,” *Nat. Rev. Cancer*, vol. 14, no. 2, pp. 135–146, 2014, doi: 10.1038/nrc3670.
- [96] J. Santamaria, J. Darrigues, J. P. M. van Meerwijk, and P. Romagnoli, “Antigen-presenting cells and T-lymphocytes homing to the thymus shape T cell development,” *Immunol. Lett.*, vol. 204, no. July, pp. 9–15, 2018, doi: 10.1016/j.imlet.2018.10.003.
- [97] M. Egerton, R. Scollay, and K. E. N. Shortman, “Kinetics of Mature T-Cell Development in the Thymus Author (s): Mark Egerton , Roland Scollay and Ken Shortman Source : Proceedings of the National Academy of Sciences of the United States of America , Published by : National Academy of Sciences Stable , ” vol. 87, no. 7, pp. 2579–2582, 2021.
- [98] H. von Boehmer, “Positive selection of lymphocytes,” *Cell*, vol. 76, no. 2, pp. 219–228, 1994, doi: 10.1016/0092-8674(94)90330-1.
- [99] N. L. La Gruta, S. Gras, S. R. Daley, P. G. Thomas, and J. Rossjohn, “Understanding the drivers of MHC restriction of T cell receptors,” *Nat. Rev. Immunol.*, vol. 18, no. 7, pp. 467–478, 2018, doi: 10.1038/s41577-018-0007-5.
- [100] R. D. Schreiber, L. J. Old, and M. J. Smyth, “Cancer immunoediting: Integrating immunity’s roles in cancer suppression and promotion,” *Science (80-.)*, vol. 331, no. 6024, pp. 1565–1570, 2011, doi: 10.1126/science.1203486.
- [101] M. W. L. Teng, M. H. Kershaw, and M. J. Smyth, “Cancer Immunoediting: From Surveillance to Escape,” *Cancer Immunother. Immune Suppr. Tumor Growth Second Ed.*, vol. 3, no. 11, pp. 85–99, 2013, doi: 10.1016/B978-0-12-394296-8.00007-5.
- [102] D. Hanahan and R. A. Weinberg, “The Hallmarks of Cancer,” *Cell*, vol. 100, no. 1, pp. 57–70, Jan. 2000, doi: 10.1016/S0092-8674(00)81683-9.
- [103] D. Hanahan and R. A. Weinberg, “Hallmarks of cancer: The next generation,” *Cell*, vol. 144, no. 5, pp. 646–674, 2011, doi: 10.1016/j.cell.2011.02.013.
- [104] S. A. Minnie and G. R. Hill, “Immunotherapy of multiple myeloma,” *J. Clin. Invest.*, vol. 130, no. 4, pp. 1565–1575, 2020, doi: 10.1172/JCI129205.
- [105] A. Xia, Y. Zhang, J. Xu, T. Yin, and X. J. Lu, “T Cell Dysfunction in Cancer Immunity and Immunotherapy,” *Front. Immunol.*, vol. 10, no. July, p. 1719, 2019, doi: 10.3389/fimmu.2019.01719.
- [106] T. F. Gajewski, “The Next Hurdle in Cancer Immunotherapy: Overcoming the Non–T-Cell–Inflamed Tumor Microenvironment,” *Semin. Oncol.*, vol. 42, no. 4, pp. 663–671, Aug. 2015, doi: 10.1053/j.seminoncol.2015.05.011.
- [107] W. Yang, Y. Song, Y. L. Lu, J. Z. Sun, and H. W. Wang, “Increased expression of programmed death (PD)-1 and its ligand PD-L1 correlates with impaired cell-mediated immunity in high-risk human papillomavirus-related cervical intraepithelial neoplasia,” *Immunology*, vol. 139, no. 4, pp. 513–522, 2013, doi: 10.1111/imm.12101.
- [108] S. Lyford-Pike *et al.*, “Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma.,” *Cancer Res.*, vol. 73, no. 6, pp. 1733–41, Mar. 2013, doi: 10.1158/0008-5472.CAN-12-2384.
- [109] X. Lei *et al.*, “Immune cells within the tumor microenvironment: Biological functions and roles in cancer immunotherapy,” *Cancer Lett.*, vol. 470, no. July 2019, pp. 126–133, 2020, doi: 10.1016/j.canlet.2019.11.009.
- [110] V. Appay, R. A. W. Van Lier, F. Sallusto, and M. Roederer, “Phenotype and function of human T lymphocyte subsets: Consensus and issues,” *Cytom. Part A*, vol. 73, no. 11, pp. 975–983, 2008, doi: 10.1002/cyto.a.20643.
- [111] L. M. McLane, M. S. Abdel-Hakeem, and E. J. Wherry, “CD8 T Cell Exhaustion During

- Chronic Viral Infection and Cancer,” *Annu. Rev. Immunol.*, vol. 37, no. 1, pp. 457–495, Apr. 2019, doi: 10.1146/annurev-immunol-041015-055318.
- [112] P. M. Freedman, J. R. Autry, S. Tokuda, R. J. J. Williams, and M. Campbell, “Cancer fnst,” *Ullrich. A. SchleSSinger. J Cell*, vol. 56, no. 23, pp. 459–476, 1972.
- [113] C. U. Blank *et al.*, “Defining ‘T cell exhaustion,’” *Nat. Rev. Immunol.*, vol. 19, no. 11, pp. 665–674, 2019, doi: 10.1038/s41577-019-0221-9.
- [114] M. J. Fuller, A. Khanolkar, A. E. Tebo, and A. J. Zajac, “Maintenance, Loss, and Resurgence of T Cell Responses During Acute, Protracted, and Chronic Viral Infections,” *J. Immunol.*, vol. 172, no. 7, pp. 4204–4214, 2004, doi: 10.4049/jimmunol.172.7.4204.
- [115] E. J. Wherry, J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed, “Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment.,” *J. Virol.*, vol. 77, no. 8, pp. 4911–27, Apr. 2003, doi: 10.1128/jvi.77.8.4911-4927.2003.
- [116] K. J. Mackerness, M. A. Cox, L. M. Lilly, C. T. Weaver, L. E. Harrington, and A. J. Zajac, “Pronounced Virus-Dependent Activation Drives Exhaustion but Sustains IFN- γ Transcript Levels,” *J. Immunol.*, vol. 185, no. 6, pp. 3643–3651, Sep. 2010, doi: 10.4049/jimmunol.1000841.
- [117] S. D. Blackburn *et al.*, “Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection,” *Nat. Immunol.*, vol. 10, no. 1, pp. 29–37, 2009, doi: 10.1038/ni.1679.
- [118] J. Li, Y. He, J. Hao, L. Ni, and C. Dong, “High Levels of Eomes Promote Exhaustion of Anti-tumor CD8+ T Cells,” *Front. Immunol.*, vol. 9, no. December, p. 2981, 2018, doi: 10.3389/fimmu.2018.02981.
- [119] C. Y. Hu *et al.*, “Interleukin-2 reverses CD8+ T cell exhaustion in clinical malignant pleural effusion of lung cancer,” *Clin. Exp. Immunol.*, vol. 186, no. 1, pp. 106–114, 2016, doi: 10.1111/cei.12845.
- [120] J. S. Yi, M. A. Cox, and A. J. Zajac, “T-cell exhaustion: Characteristics, causes and conversion,” *Immunology*, vol. 129, no. 4, pp. 474–481, 2010, doi: 10.1111/j.1365-2567.2010.03255.x.
- [121] S. Han, A. Asoyan, H. Rabenstein, N. Nakano, and R. Obst, “Role of antigen persistence and dose for CD4+ T-cell exhaustion and recovery,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 47, pp. 20453–20458, 2010, doi: 10.1073/pnas.1008437107.
- [122] A. C. Scott *et al.*, “TOX is a critical regulator of tumour-specific T cell differentiation,” *Nature*, vol. 571, no. 7764, pp. 270–274, 2019, doi: 10.1038/s41586-019-1324-y.
- [123] Y. Zhu *et al.*, “T-bet and Eomesodermin Are Required for T Cell-Mediated Antitumor Immune Responses,” *J. Immunol.*, vol. 185, no. 6, pp. 3174–3183, 2010, doi: 10.4049/jimmunol.1000749.
- [124] Y. Jiang, Y. Li, and B. Zhu, “T-cell exhaustion in the tumor microenvironment,” *Cell Death Dis.*, vol. 6, no. 6, pp. 1–9, 2015, doi: 10.1038/cddis.2015.162.
- [125] L. M. Myers *et al.*, “A functional subset of CD8 + T cells during chronic exhaustion is defined by SIRP α expression,” *Nat. Commun.*, vol. 10, no. 1, 2019, doi: 10.1038/s41467-019-08637-9.
- [126] S. Simon and N. Labarriere, “PD-1 expression on tumor-specific T cells: Friend or foe for immunotherapy?,” *Oncoimmunology*, vol. 7, no. 1, pp. 1–7, 2018, doi: 10.1080/2162402X.2017.1364828.
- [127] C. Badoual *et al.*, “PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-Associated head and neck cancer,” *Cancer Res.*, vol. 73, no. 1, pp.

- 128–138, 2013, doi: 10.1158/0008-5472.CAN-12-2606.
- [128] L. Mezache, B. Paniccia, A. Nyinawabera, and G. J. Nuovo, “Enhanced expression of PD L1 in cervical intraepithelial neoplasia and cervical cancers,” *Mod. Pathol.*, vol. 28, no. 12, pp. 1594–1602, 2015, doi: 10.1038/modpathol.2015.108.
- [129] A. Coillard and E. Segura, “In vivo differentiation of human monocytes,” *Front. Immunol.*, vol. 10, no. AUG, pp. 1–7, 2019, doi: 10.3389/fimmu.2019.01907.
- [130] L. Ziegler-Heitbrock *et al.*, “Nomenclature of monocytes and dendritic cells in blood,” *Blood*, vol. 116, no. 16, pp. 5–7, 2010, doi: 10.1182/blood-2010-02-258558.
- [131] U. O’Doherty *et al.*, “Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature.,” *Immunology*, vol. 82, no. 3, pp. 487–93, 1994, [Online]. Available: <http://www.ncbi.nlm.nih.gov/pubmed/7525461> <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1414873>.
- [132] N. McGovern *et al.*, “Human dermal CD14+ cells are a transient population of monocyte-derived macrophages,” *Immunity*, vol. 41, no. 3, pp. 465–477, 2014, doi: 10.1016/j.immuni.2014.08.006.
- [133] D. D. Danikas, M. Karakantza, G. L. Theodorou, G. C. Sakellaropoulos, and C. A. Gogos, “Prognostic value of phagocytic activity of neutrophils and monocytes in sepsis. Correlation to CD64 and CD14 antigen expression,” *Clin. Exp. Immunol.*, vol. 154, no. 1, pp. 87–97, 2008, doi: 10.1111/j.1365-2249.2008.03737.x.
- [134] P. Langerhans, “Ueber die Nerven der menschlichen Haut,” *Arch. für Pathol. Anat. und Physiol. und für Klin. Med.*, vol. 44, no. 2–3, pp. 325–337, 1868, doi: 10.1007/BF01959006.
- [135] J. W. Rhodes, O. Tong, A. N. Harman, and S. G. Turville, “Human dendritic cell subsets, ontogeny, and impact on HIV infection,” *Front. Immunol.*, vol. 10, no. MAY, 2019, doi: 10.3389/fimmu.2019.01088.
- [136] A. R. Hayati and M. Zulkarnaen, “An immunohistochemical study of CD1a and CD83-positive infiltrating dendritic cell density in cervical neoplasia,” *Int. J. Gynecol. Pathol.*, vol. 26, no. 1, pp. 83–88, 2007, doi: 10.1097.
- [137] V. Pena-Cruz *et al.*, “HIV-1 replicates and persists in vaginal epithelial dendritic cells,” *J. Clin. Invest.*, vol. 128, no. 8, pp. 3439–3444, 2018, doi: 10.1172/JCI98943.
- [138] M. Chopin and S. L. Nutt, “Establishing and maintaining the Langerhans cell network,” *Semin. Cell Dev. Biol.*, vol. 41, pp. 23–29, 2015, doi: 10.1016/j.semcdb.2014.02.001.
- [139] Y. Belkaid and S. Tamoutounour, “The influence of skin microorganisms on cutaneous immunity,” *Nat. Rev. Immunol.*, vol. 16, no. 6, pp. 353–366, 2016, doi: 10.1038/nri.2016.48.
- [140] F. Walker, H. Adle-Biassette, P. Madelenat, D. Hénin, and T. Lehy, “Increased apoptosis in cervical intraepithelial neoplasia associated with HIV infection: Implication of oncogenic human papillomavirus, caspases, and langerhans cells,” *Clin. Cancer Res.*, vol. 11, no. 7, pp. 2451–2458, 2005, doi: 10.1158/1078-0432.CCR-04-1795.
- [141] V. Bronte *et al.*, “Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards,” *Nat. Commun.*, vol. 7, 2016, doi: 10.1038/ncomms12150.
- [142] C. Groth *et al.*, “Immunosuppression mediated by myeloid-derived suppressor cells (MDSCs) during tumour progression,” *Br. J. Cancer*, vol. 120, no. 1, pp. 16–25, 2019, doi: 10.1038/s41416-018-0333-1.
- [143] B. Walch-Rückheim *et al.*, “Cervical cancer-instructed stromal fibroblasts enhance IL23

- expression in dendritic cells to support expansion of Th17 cells," *Cancer Res.*, vol. 79, no. 7, pp. 1573–1586, 2019, doi: 10.1158/0008-5472.CAN-18-1913.
- [144] J. Pahne-Zeppenfeld *et al.*, "Cervical cancer cell-derived interleukin-6 impairs CCR7-dependent migration of MMP-9-expressing dendritic cells," *Int. J. Cancer*, vol. 134, no. 9, pp. 2061–2073, 2014, doi: 10.1002/ijc.28549.
- [145] N. Schröer, J. Pahne, B. Walch, C. Wickenhauser, and S. Smola, "Molecular pathobiology of human cervical high-grade lesions: Paracrine STAT3 activation in tumor-instructed myeloid cells drives local MMP-9 expression," *Cancer Res.*, vol. 71, no. 1, pp. 87–97, 2011, doi: 10.1158/0008-5472.CAN-10-2193.
- [146] M. Heusinkveld *et al.*, "M2 Macrophages Induced by Prostaglandin E 2 and IL-6 from Cervical Carcinoma Are Switched to Activated M1 Macrophages by CD4 + Th1 Cells," *J. Immunol.*, vol. 187, no. 3, pp. 1157–1165, 2011, doi: 10.4049/jimmunol.1100889.
- [147] R. T. Trifonova, J. Lieberman, and D. van Baarle, "Distribution of immune cells in the human cervix and implications for HIV transmission," *Am. J. Reprod. Immunol.*, vol. 71, no. 3, pp. 252–264, 2014, doi: 10.1111/aji.12198.
- [148] M. Cutolo *et al.*, "Sex hormones influence on the immune system: Basic and clinical aspects in autoimmunity," in *Lupus*, 2004, vol. 13, no. 9, pp. 635–638, doi: 10.1191/0961203304lu1094oa.
- [149] C. K. Chen, S. C. Huang, C. L. Chen, M. R. Yen, H. C. Hsu, and H. N. Ho, "Increased expressions of CD69 and HLA-DR but not of CD25 or CD71 on endometrial T lymphocytes of nonpregnant women," *Hum. Immunol.*, vol. 42, no. 3, pp. 227–232, 1995, doi: 10.1016/0198-8859(94)00105-Y.
- [150] H. D. White, G. R. Yeaman, A. L. Givan, and C. R. Wira, "Mucosal immunity in the human female reproductive tract: Cytotoxic T lymphocyte function in the cervix and vagina of premenopausal and postmenopausal women," *Am. J. Reprod. Immunol.*, vol. 37, no. 1, pp. 30–38, 1997, doi: 10.1111/j.1600-0897.1997.tb00190.x.
- [151] S. Bjercke, H. Scott, L. R. Braathen, and E. Thorsby, "AND CERVICAL EPITHELIUM," vol. 100, no. 8, pp. 585–589, 1983.
- [152] L. a Hussain, C. G. Kelly, R. Fellowes, E. Hecht, J. Wilsont, and M. Chapmant, "Expression and gene transcript of Fc receptors for IgG , HLA class II antigens and Langerhans cells in human cervico-vaginal epithelium virusyndromisthe (AiDS) gil ofacquired NofacquirthAmericanoWesiernc Europe andBrita HAIVS) has3 . Int," *Clin. Exp. Immunol.*, no. 90, pp. 530–538, 1992.
- [153] M. Prakash, M. S. Kapembwa, F. Gotch, and S. Patterson, "Chemokine receptor expression on mucosal dendritic cells from the endocervix of healthy women," *J. Infect. Dis.*, vol. 190, no. 2, pp. 246–250, 2004, doi: 10.1086/422034.
- [154] H. H. B. MORRIS, K. C. GATTER, G. SYKES, V. CASEMORE, and D. Y. MASON, "Langerhans' cells in human cervical epithelium: effects of wart virus infection and intraepithelial neoplasia," *BJOG An Int. J. Obstet. Gynaecol.*, vol. 90, no. 5, pp. 412–420, 1983, doi: 10.1111/j.1471-0528.1983.tb08936.x.
- [155] J. P. McArdle and H. K. Muller, "Quantitative assessment of Langerhans' cells in human cervical intraepithelial neoplasia and wart virus infection," *Am. J. Obstet. Gynecol.*, vol. 154, no. 3, pp. 509–515, 1986, doi: 10.1016/0002-9378(86)90592-2.
- [156] J. McKenzie, A. King, J. Hare, T. Fulford, B. Wilson, and M. Stanley, "Immunocytochemical characterization of large granular lymphocytes in normal cervix and HPV associated disease," *J. Pathol.*, vol. 165, no. 1, pp. 75–80, 1991, doi: 10.1002/path.1711650112.

- [157] L. B. Alexandrov *et al.*, “Signatures of mutational processes in human cancer,” *Nature*, vol. 500, no. 7463, pp. 415–421, 2013, doi: 10.1038/nature12477.
- [158] S. D. Brown *et al.*, “Neo-antigens predicted by tumor genome meta-analysis correlate with increased patient survival,” *Genome Res.*, vol. 24, no. 5, pp. 743–750, 2014, doi: 10.1101/gr.165985.113.
- [159] C. J. Cohen *et al.*, “Isolation of neoantigen-specific T cells from tumor and peripheral lymphocytes,” *J. Clin. Invest.*, vol. 125, no. 10, pp. 3981–3991, 2015, doi: 10.1172/JCI82416.
- [160] S. Stevanović *et al.*, “Landscape of immunogenic tumor antigens in successful immunotherapy of virally induced epithelial cancer,” *Science (80-.)*, vol. 356, no. 6334, pp. 200–205, 2017, doi: 10.1126/science.aak9510.
- [161] H. E. Wang *et al.*, “Prediction and identification of human leukocyte antigen-a2-restricted cytotoxic t lymphocyte epitope peptides from the human papillomavirus 58 e7 protein,” *Oncol. Lett.*, vol. 16, no. 2, pp. 2003–2008, 2018, doi: 10.3892/ol.2018.8875.
- [162] S. H. van der Burg and C. J. M. Melief, “Therapeutic vaccination against human papilloma virus induced malignancies,” *Curr. Opin. Immunol.*, vol. 23, no. 2, pp. 252–257, 2011, doi: 10.1016/j.coi.2010.12.010.
- [163] B. Ma *et al.*, “Emerging human papillomavirus vaccines,” *Expert Opin. Emerg. Drugs*, vol. 17, no. 4, pp. 469–492, 2012, doi: 10.1517/14728214.2012.744393.
- [164] N. Kash, M. Lee, R. Kollipara, C. Downing, J. Guidry, and S. Tyring, “Safety and Efficacy Data on Vaccines and Immunization to Human Papillomavirus,” *J. Clin. Med.*, vol. 4, no. 4, pp. 614–633, 2015, doi: 10.3390/jcm4040614.
- [165] K. E. Pauken and E. J. Wherry, “Overcoming T cell exhaustion in infection and cancer,” *Trends Immunol.*, vol. 36, no. 4, pp. 265–276, 2015, doi: 10.1016/j.it.2015.02.008.
- [166] S. A. Rosenberg and N. P. Restifo, “Adoptive cell transfer as personalized immunotherapy for human cancer,” *Science (80-.)*, vol. 348, no. 6230, pp. 62–68, 2015, doi: 10.1126/science.aaa4967.
- [167] A. C. Huang *et al.*, “T-cell invigoration to tumour burden ratio associated with anti-PD-1 response,” *Nature*, vol. 545, no. 7652, pp. 60–65, 2017, doi: 10.1038/nature22079.
- [168] R. D. Burk *et al.*, “Integrated genomic and molecular characterization of cervical cancer,” *Nature*, vol. 543, no. 7645, pp. 378–384, 2017, doi: 10.1038/nature21386.
- [169] X. Bin Pan, Y. Lu, J. L. Huang, Y. Long, and D. S. Yao, “Prognostic genes in the tumor microenvironment in cervical squamous cell carcinoma,” *Aging (Albany. NY)*, vol. 11, no. 22, pp. 10154–10166, 2019, doi: 10.18632/aging.102429.
- [170] J. S. Frenel *et al.*, “Safety and efficacy of pembrolizumab in advanced, programmed death ligand 1-positive cervical cancer: Results from the phase IB KEYNOTE-028 trial,” *J. Clin. Oncol.*, vol. 35, no. 36, pp. 4035–4041, 2017, doi: 10.1200/JCO.2017.74.5471.
- [171] H. C. Chung *et al.*, “Efficacy and safety of pembrolizumab in previously treated advanced cervical cancer: Results from the phase II KEYNOTE-158 study,” *J. Clin. Oncol.*, vol. 37, no. 17, pp. 1470–1478, 2019, doi: 10.1200/JCO.18.01265.
- [172] A. Sharabi *et al.*, “Exceptional Response to Nivolumab and Stereotactic Body Radiation Therapy (SBRT) in Neuroendocrine Cervical Carcinoma with High Tumor Mutational Burden: Management Considerations from the Center For Personalized Cancer Therapy at UC San Diego Moores Cance,” *Oncologist*, vol. 22, no. 6, pp. 631–637, 2017, doi: 10.1634/theoncologist.2016-0517.
- [173] A. D. Santin *et al.*, “Phase II evaluation of nivolumab in the treatment of persistent or recurrent cervical cancer (NCT02257528/NRG-GY002),” *Gynecol. Oncol.*, vol. 157, no. 1,

- pp. 161–166, 2020, doi: 10.1016/j.ygyno.2019.12.034.
- [174] L. Chitsike and P. Duerksen-Hughes, “The Potential of Immune Checkpoint Blockade in Cervical Cancer: Can Combinatorial Regimens Maximize Response? A Review of the Literature,” *Curr. Treat. Options Oncol.*, vol. 21, no. 12, 2020, doi: 10.1007/s11864-020-00790-4.
- [175] P. Basu *et al.*, “A Randomized Phase 2 Study of ADXS11-001 Listeria monocytogenes-Listeriolysin O Immunotherapy With or Without Cisplatin in Treatment of Advanced Cervical Cancer,” *Int. J. Gynecol. Cancer*, vol. 28, no. 4, pp. 764–772, 2018, doi: 10.1097/IGC.0000000000001235.
- [176] W. Huh *et al.*, “A prospective phase II trial of the listeria-based human papillomavirus immunotherapy axalimogene filolisbac in second- and third-line metastatic cervical cancer: A NRG oncology group trial,” *Gynecol. Oncol.*, vol. 145, no. 2017, p. 220, 2017, doi: 10.1016/j.ygyno.2017.03.506.
- [177] E. E. Cohen *et al.*, “Phase I/II study of ADXS11-001 or MEDI4736 immunotherapies alone and in combination, in patients with recurrent/metastatic cervical or human papillomavirus (HPV)-positive head and neck cancer,” *J. Immunother. Cancer*, vol. 3, no. S2, pp. 2–3, 2015, doi: 10.1186/2051-1426-3-s2-p147.
- [178] J. B. A. G. Haanen *et al.*, “Management of toxicities from immunotherapy: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up,” *Ann. Oncol.*, vol. 28, no. Supplement 4, pp. iv119–iv142, 2017, doi: 10.1093/annonc/mdx225.
- [179] E. Soularue *et al.*, “Enterocolitis due to immune checkpoint inhibitors: A systematic review,” *Gut*, vol. 67, no. 11, pp. 2056–2067, 2018, doi: 10.1136/gutjnl-2018-316948.
- [180] “WHO.” [https://www.who.int/news-room/fact-sheets/detail/human-papillomavirus-\(hpv\)-and-cervical-cancer](https://www.who.int/news-room/fact-sheets/detail/human-papillomavirus-(hpv)-and-cervical-cancer) (accessed Dec. 04, 2020).
- [181] S. Shanmugasundaram and J. You, “Targeting persistent human papillomavirus infection,” *Viruses*, vol. 9, no. 8, 2017, doi: 10.3390/v9080229.
- [182] C. Orbegoso, K. Murali, and S. Banerjee, “The current status of immunotherapy for cervical cancer,” *Reports Pract. Oncol. Radiother.*, vol. 23, no. 6, pp. 580–588, 2018, doi: 10.1016/j.rpor.2018.05.001.
- [183] H. Papillomavirus and M. Capsid, “crossm Protein L1 Remains Associated with the Incoming Viral Genome throughout the,” vol. 91, no. 16, pp. 1–17, 2017.
- [184] E. Borcoman and C. Le Tourneau, “Pembrolizumab in cervical cancer: latest evidence and clinical usefulness,” *Ther. Adv. Med. Oncol.*, vol. 9, no. 6, pp. 431–439, Jun. 2017, doi: 10.1177/1758834017708742.
- [185] H. Tashiro and M. K. Brenner, “Immunotherapy against cancer-related viruses,” *Cell Res.*, vol. 27, no. 1, pp. 59–73, 2017, doi: 10.1038/cr.2016.153.
- [186] M. Sznol and L. Chen, “Antagonist Antibodies to PD-1 and B7-H1 (PD-L1) in the Treatment of Advanced Human Cancer-Response,” *Clin. Cancer Res.*, vol. 19, no. 19, p. 5542, 2013, doi: 10.1158/1078-0432.CCR-13-2234.
- [187] P. Sharma and J. P. Allison, “The future of immune checkpoint therapy,” *Science (80-.)*, vol. 348, no. 6230, pp. 56–61, 2015, doi: 10.1126/science.aaa8172.
- [188] T. N. Schumacher and R. D. Schreiber, “Neoantigens in cancer immunotherapy,” *Science (80-.)*, vol. 348, no. 6230, pp. 69–74, 2015, doi: 10.1126/science.aaa4971.
- [189] P. A. Lazo, “The molecular genetics of cervical carcinoma,” *Br. J. Cancer*, vol. 80, no. 12, pp. 2008–2018, 1999, doi: 10.1038/sj.bjc.6690635.
- [190] O. L. Reddy, P. I. Shintaku, and N. A. Moatamed, “Programmed death-ligand 1 (PD-L1) is expressed in a significant number of the uterine cervical carcinomas,” *Diagn. Pathol.*,

- vol. 12, no. 1, pp. 1–11, 2017, doi: 10.1186/s13000-017-0631-6.
- [191] R. K. Shrimali, Z. Yu, M. R. Theoret, D. Chinnasamy, N. P. Restifo, and S. A. Rosenberg, “Antiangiogenic agents can increase lymphocyte infiltration into tumor and enhance the effectiveness of adoptive immunotherapy of cancer,” *Cancer Res.*, vol. 70, no. 15, pp. 6171–6180, 2010, doi: 10.1158/0008-5472.CAN-10-0153.
- [192] L. Festino *et al.*, “Cancer Treatment with Anti-PD-1/PD-L1 Agents: Is PD-L1 Expression a Biomarker for Patient Selection?,” *Drugs*, vol. 76, no. 9, pp. 925–945, 2016, doi: 10.1007/s40265-016-0588-x.
- [193] H. Kared, S. Martelli, T. P. Ng, S. L. F. Pender, and A. Larbi, “CD57 in human natural killer cells and T-lymphocytes,” *Cancer Immunol. Immunother.*, vol. 65, no. 4, pp. 441–452, 2016, doi: 10.1007/s00262-016-1803-z.
- [194] F. Simonetta *et al.*, “High Eomesodermin Expression among CD57+ CD8+ T Cells Identifies a CD8+ T Cell Subset Associated with Viral Control during Chronic Human Immunodeficiency Virus Infection,” *J. Virol.*, vol. 88, no. 20, pp. 11861–11871, 2014, doi: 10.1128/jvi.02013-14.
- [195] O. Khan *et al.*, “TOX transcriptionally and epigenetically programs CD8+ T cell exhaustion,” *Nature*, vol. 571, no. 7764, pp. 211–218, 2019, doi: 10.1038/s41586-019-1325-x.
- [196] Z. Chen *et al.*, “TCF-1-Centered Transcriptional Network Drives an Effector versus Exhausted CD8 T Cell-Fate Decision,” *Immunity*, vol. 51, no. 5, pp. 840-855.e5, 2019, doi: 10.1016/j.immuni.2019.09.013.
- [197] Q. Wang, A. Steger, S. Mahner, U. Jeschke, and H. Heidegger, “The formation and therapeutic update of tumor-associated macrophages in cervical cancer,” *Int. J. Mol. Sci.*, vol. 20, no. 13, pp. 1–18, 2019, doi: 10.3390/ijms20133310.
- [198] F. Tang, Q. Du, and Y. J. Liu, “Plasmacytoid dendritic cells in antiviral immunity and autoimmunity,” *Sci. China Life Sci.*, vol. 53, no. 2, pp. 172–182, 2010, doi: 10.1007/s11427-010-0045-0.
- [199] M. R. McCredie *et al.*, “Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study,” *Lancet Oncol.*, vol. 9, no. 5, pp. 425–434, 2008, doi: 10.1016/S1470-2045(08)70103-7.
- [200] A. K. Moesta, X. Y. Li, and M. J. Smyth, “Targeting CD39 in cancer,” *Nat. Rev. Immunol.*, vol. 20, no. 12, pp. 739–755, 2020, doi: 10.1038/s41577-020-0376-4.
- [201] P. K. Gupta *et al.*, “CD39 Expression Identifies Terminally Exhausted CD8+ T Cells,” *PLoS Pathog.*, vol. 11, no. 10, pp. 1–21, 2015, doi: 10.1371/journal.ppat.1005177.
- [202] Y.-Y. Yan *et al.*, “PD-1/PD-L1 Inhibitors in Cervical Cancer,” 2019, doi: 10.3389/fphar.2019.00065.
- [203] T. Duhon *et al.*, “Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors,” no. 2018, doi: 10.1038/s41467-018-05072-0.
- [204] C. Robert *et al.*, “Pembrolizumab versus Ipilimumab in Advanced Melanoma,” *N. Engl. J. Med.*, vol. 372, no. 26, pp. 2521–2532, 2015, doi: 10.1056/nejmoa1503093.
- [205] G. M. Blumenthal *et al.*, “Overall response rate, progression-free survival, and overall survival with targeted and standard therapies in advanced non-small-cell lung cancer: US Food and Drug Administration trial-level and patient-level analyses,” *J. Clin. Oncol.*, vol. 33, no. 9, pp. 1008–1014, 2015, doi: 10.1200/JCO.2014.59.0489.
- [206] N. Nagarsheth, M. S. Wicha, and W. Zou, “Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy,” *Nat. Rev. Immunol.*, vol. 17, no. 9, pp. 559–572, 2017, doi: 10.1038/nri.2017.49.

- [207] E. J. Wherry and M. Kurachi, "Molecular and cellular insights into T cell exhaustion," *Nat. Rev. Immunol.*, vol. 15, no. 8, pp. 486–499, 2015, doi: 10.1038/nri3862.
- [208] A. Varsani, A.-L. Williamson, D. de Villiers, I. Becker, N. D. Christensen, and E. P. Rybicki, "Chimeric Human Papillomavirus Type 16 (HPV-16) L1 Particles Presenting the Common Neutralizing Epitope for the L2 Minor Capsid Protein of HPV-6 and HPV-16," *J. Virol.*, vol. 77, no. 15, pp. 8386–8393, 2003, doi: 10.1128/jvi.77.15.8386-8393.2003.
- [209] M. Knebel Doeberitz, C. Rittmuller, F. Aengeneyndt, P. Jansen-durr, and D. Spitkovsky, "Reversible Repression," *J. Virol.*, vol. 68, no. 5, pp. 2811–2821, 1994.
- [210] P. Melsheimer, S. Vinokurova, N. Wentzensen, G. Bastert, and M. Von Knebel Doeberitz, "DNA Aneuploidy and Integration of Human Papillomavirus Type 16 E6/E7 Oncogenes in Intraepithelial Neoplasia and Invasive Squamous Cell Carcinoma of the Cervix Uteri," *Clin. Cancer Res.*, vol. 10, no. 9, pp. 3059–3063, 2004, doi: 10.1158/1078-0432.CCR-03-0565.
- [211] A. K. Bentzen *et al.*, "Large-scale detection of antigen-specific T cells using peptide-MHC-I multimers labeled with DNA barcodes," *Nat. Biotechnol.*, vol. 34, no. 10, pp. 1037–1045, 2016, doi: 10.1038/nbt.3662.
- [212] M. E. McLaughlin-Drubin and K. Münger, "Oncogenic activities of human papillomaviruses," *Virus Res.*, vol. 143, no. 2, pp. 195–208, 2009, doi: 10.1016/j.virusres.2009.06.008.
- [213] B. Huard and L. Karlsson, "KIR expression on self-reactive CD8 + T cells is controlled by T-cell receptor engagement," vol. 403, no. January, pp. 325–328, 2000.
- [214] A. Gati *et al.*, "Tumor cells regulate the lytic activity of tumor-specific cytotoxic T lymphocytes by modulating the inhibitory natural killer receptor function," *Cancer Res.*, vol. 61, no. 8, pp. 3240–3244, 2001.
- [215] "Immune Epitope Database (IEDB)." <https://www.iedb.org/>.
- [216] V. Jurtz, S. Paul, M. Andreatta, P. Marcatili, B. Peters, and M. Nielsen, "NetMHCpan-4.0: Improved Peptide–MHC Class I Interaction Predictions Integrating Eluted Ligand and Peptide Binding Affinity Data," *J. Immunol.*, vol. 199, no. 9, pp. 3360–3368, 2017, doi: 10.4049/jimmunol.1700893.
- [217] S. K. Saini, E. T. Abualrous, A. S. Tigan, K. Covella, U. Wellbrock, and S. Springer, "Not all empty MHC class I molecules are molten globules: Tryptophan fluorescence reveals a two-step mechanism of thermal denaturation," *Mol. Immunol.*, vol. 54, no. 3–4, pp. 386–396, 2013, doi: 10.1016/j.molimm.2013.01.004.
- [218] A. K. Bentzen *et al.*, "Large-scale detection of antigen-specific T cells using peptide-MHC-I multimers labeled with DNA barcodes," *Nat. Biotechnol.*, vol. 34, no. 10, pp. 1037–1045, 2016, doi: 10.1038/nbt.3662.
- [219] "Barracoda." <https://services.healthtech.dtu.dk/service.php?Barracoda-1.8>).
- [220] H. Kanaan, H. R. Kourie, and A. H. Awada, "Are virus-induced cancers more sensitive to checkpoint inhibitors?," *Futur. Oncol.*, vol. 12, no. 23, pp. 2665–2668, Dec. 2016, doi: 10.2217/fo-2016-0283.
- [221] G. Gupta, R. Glueck, and P. R. Patel, "HPV vaccines: Global perspectives," *Hum. Vaccines Immunother.*, vol. 13, no. 6, pp. 1421–1424, 2017, doi: 10.1080/21645515.2017.1289301.
- [222] M. Kagabu *et al.*, "Immunotherapy for uterine cervical cancer using checkpoint inhibitors: Future directions," *Int. J. Mol. Sci.*, vol. 21, no. 7, 2020, doi: 10.3390/ijms21072335.
- [223] L. Wu *et al.*, "Circulating and tumor-infiltrating myeloid-derived suppressor cells in cervical carcinoma patients," *Oncol. Lett.*, vol. 15, no. 6, pp. 9507–9515, 2018, doi:

- 10.3892/ol.2018.8532.
- [224] S. Mabuchi and T. Sasano, "Myeloid-Derived Suppressor Cells as Therapeutic Targets in Uterine Cervical and Endometrial Cancers," *Cells*, vol. 10, no. 5, pp. 1–14, 2021, doi: 10.3390/cells10051073.
- [225] S. Stevanović *et al.*, "Landscape of immunogenic tumor antigens in successful immunotherapy of virally induced epithelial cancer," *Science (80-.)*, vol. 356, no. 6334, pp. 200–205, 2017, doi: 10.1126/science.aak9510.
- [226] K. Hoppe-Seyler, F. Bossler, J. A. Braun, A. L. Herrmann, and F. Hoppe-Seyler, "The HPV E6/E7 Oncogenes: Key Factors for Viral Carcinogenesis and Therapeutic Targets," *Trends Microbiol.*, vol. 26, no. 2, pp. 158–168, 2018, doi: 10.1016/j.tim.2017.07.007.