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High-dimensional imaging of the HPV-induced tumor microenvironment to improve clinical outcomes

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Citation

Abdulrahman, Z. (2026, September 3). *High-dimensional imaging of the HPV-induced tumor microenvironment to improve clinical outcomes*. Retrieved from <https://hdl.handle.net/1887/4309067>

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High-dimensional imaging of the HPV-induced tumor microenvironment to improve clinical outcomes

Ziena Abdulrahman

High-dimensional imaging of the HPV-induced tumor microenvironment
to improve clinical outcomes

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ISBN 978-94-6537-728-5

Layout and cover design by Arul Raja
Printed by Ridderprint

The printing of this thesis was financially supported by Leiden University,
Nederlandse Vereniging voor Vulva Pathologie, Viatrix and Quanterix.

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High-dimensional imaging of the HPV-induced tumor microenvironment to improve clinical outcomes

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr. S. de Rijcke,
volgens besluit van het college voor promoties
te verdedigen op donderdag 3 september 2026
klokke 13:00 uur

door

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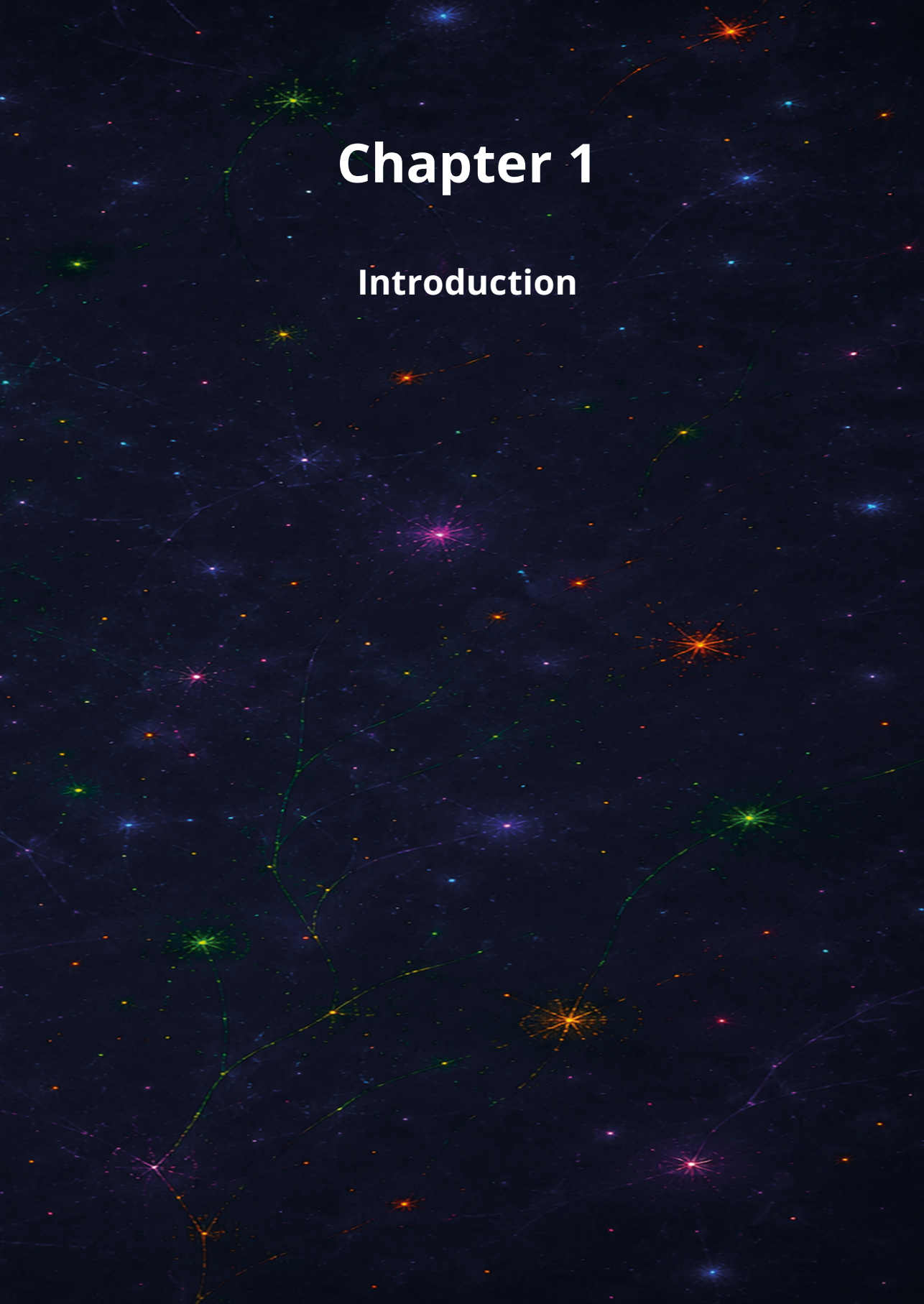
'Read'

To my family

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The background of the entire page is a deep space image showing a complex network of filaments and voids, known as the cosmic web. These structures are highlighted in various colors including green, orange, and blue against a dark, star-filled sky. Numerous stars of different colors (blue, white, orange, red) are scattered throughout the scene, some appearing as bright points and others as smaller, dimmer specks. The overall effect is one of vastness and cosmic scale.

Chapter 1

Introduction

Cancer represents one of the most intricate diseases in medicine, driven not only by the genetic alterations within tumor cells but by a dynamic interplay with the surrounding microenvironment. The tumor microenvironment (TME) is increasingly recognized as a decisive driver of antitumor immunity, determining whether immune cells mediate tumor eradication or contribute to immune escape. Advances in immuno-oncology have revealed that successful therapy depends as much on reprogramming this ecosystem as on targeting the tumor itself. Yet, the precise mechanisms by which spatial organization and cellular composition within the TME shapes therapeutic response remains incompletely understood. This thesis aims to deepen our understanding of these interactions in HPV-induced tumors, leveraging cutting-edge spatial profiling technologies to uncover how the immune landscape governs response to immunotherapy and how it impacts patient survival, ultimately striving to inform the rational design of more effective, personalized treatment strategies.

The tumor microenvironment

Composition of the tumor microenvironment

The TME is a complex ecosystem of cellular and molecular constituents that collectively shape tumor behavior and influence therapeutic outcome. In addition to malignant cells, it comprises diverse immune cell populations, including lymphocytes (e.g. T cells, B cells) and myeloid cells (e.g. macrophages, dendritic cells, neutrophils, mast cells), which together regulate immune surveillance and evasion. Stromal elements, such as fibroblasts and endothelial cells, provide structural integrity while promoting angiogenesis, invasion, and immune modulation. The extracellular matrix (ECM), composed of proteins such as collagen, laminin and fibronectin, acts as a dynamic scaffold that modulates cellular behavior. Soluble factors, including cytokines, chemokines and growth factors, orchestrate communication between tumor and stromal compartments (**Figure 1**).

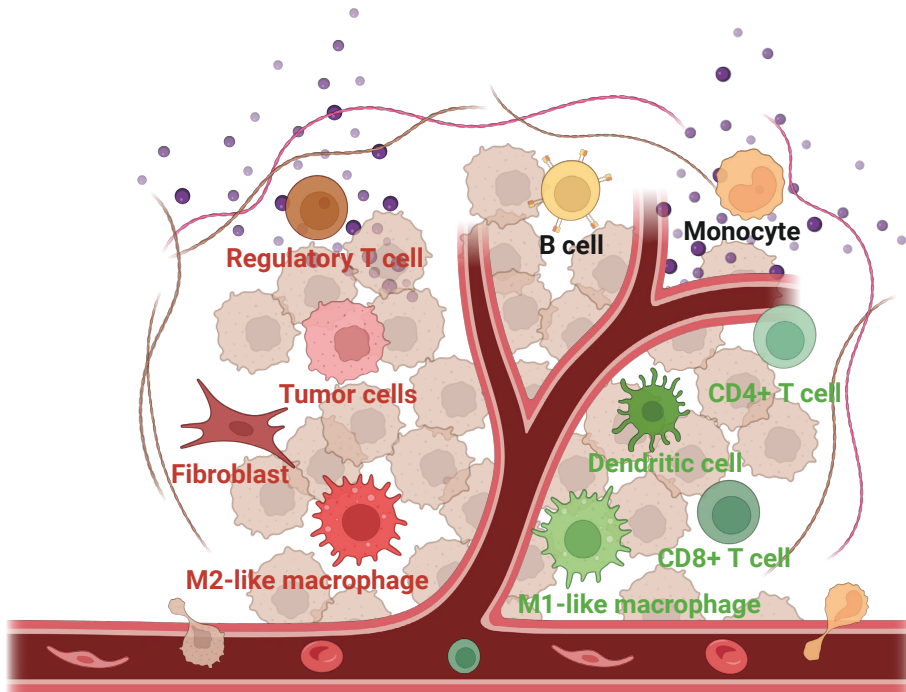


Figure 1. Cellular composition of the tumor microenvironment.

Cell populations associated with effective tumor control are depicted in green, those that promote tumor progression in red and cell types with context-dependent or dual functions are shown in black. Image created with BioRender.

Beyond these individual cellular and molecular components, their reciprocal spatial interactions play a crucial role in tumor dynamics. For example, the proximity of immune cells to tumor cells determines the likelihood of an effective anti-tumor response.¹ Similarly, fibroblast distribution and ECM density can create physical barriers that hinder drug penetration or promote immune exclusion by limiting lymphocyte infiltration.² Endothelial cells positioned near tumor nests sustain angiogenesis and facilitate metastatic dissemination.³ Recent advances in high-resolution imaging have underscored the importance of these spatial relationships. Elucidating these patterns is essential for understanding tumor biology and for designing more effective therapeutic strategies.

The hallmarks of cancer, as described by Hanahan and Weinberg⁴, provide a conceptual framework for tumor biology, several of which are tightly linked to the TME. Angiogenesis, for instance, is driven by endothelial cells responding to pro-angiogenic cues, while metastasis depends on ECM remodeling

by cancer-associated fibroblasts (CAFs). Immune evasion is mediated by immunosuppressive populations such as regulatory T cells (Tregs) and tumor-associated myeloid cells (TAMs), often perpetuated by chronic inflammation. Moreover, metabolic reprogramming within the TME allows tumor adaptation to hypoxia and nutrient deprivation. This underscores the TME's centrality in cancer progression and elements therein as promising targets for therapeutic innovation.

Factors shaping the TME

The etiology of a tumor profoundly influences the composition and function of its TME. Tumors arising from carcinogen exposure, such as tobacco-induced lung cancer or ultraviolet light-induced melanoma, typically exhibit a high mutational burden resulting from cumulative DNA damage. This mutational load enhances neoantigen generation and immunogenicity, thereby increasing responsiveness to immunotherapy.⁵

Virus-driven cancers, such as HPV-induced oropharyngeal or genital cancers, EBV-induced nasopharyngeal cancer or Merkel cell polyomavirus-induced skin cancer, often exhibit unique TMEs influenced by viral oncoproteins. Viral antigens enhance immune recognition and provide targets for T cell mediated immunity, supporting immunotherapeutic approaches. Consequently, virus-driven tumors frequently exhibit more inflamed immune profiles, with abundant lymphocytic infiltrates that contribute to improved prognosis and higher responsiveness to immunotherapy.⁶⁻¹⁰

Tumors developing in the context of chronic inflammation, such as colorectal cancer associated with inflammatory bowel disease, arise within an immunologically altered microenvironment. Persistent inflammation recruits innate immune cells and promotes a pro-tumorigenic cytokine milieu characterized by elevated IL-6, IL-10 and TGF- β levels, which suppress adaptive immunity.¹¹

The oncogenic pathways activated within tumor cells further shape the immune TME. Aberrant WNT/ β -catenin signaling, for instance, suppresses dendritic cell (DC) recruitment and promotes an immune-excluded phenotype. Tumors with active WNT signaling, including colorectal cancer and melanoma, often resist immune checkpoint blockade (ICB) owing to this immune-suppressive milieu.¹²⁻¹³ Similarly, constitutive activation of the MAPK pathway induces VEGF and other immunosuppressive cytokines, reducing T cell infiltration and

impairing immune recognition.¹⁴ Loss of p53 function can result in unchecked tumor growth and immunosuppression through pathways that enhance TGF- β signaling, which recruits Tregs and MDSCs and creates a tumor-promoting environment.¹⁵ Likewise, *PTEN* loss, frequently observed in prostate and breast cancers, enhances PI3K/Akt signaling, leading to diminished T cell infiltration and poor responsiveness to ICB.¹⁶

Lastly, the anatomical site of a tumor significantly influences its immune landscape due to the distinct immunological characteristics of each tissue.¹⁷ This aligns with the classical “seed and soil” theory, in which the tumor cells represent the “seed” and the tissue the “soil”. Increasing evidence indicates that it is often the “soil” rather than the intrinsic properties of the tumor “seed” that determines outcome: tumors of identical origin can display very different immune profiles and therapeutic outcomes depending on their anatomical location.¹⁸ For example, liver metastases often exhibit a highly immunosuppressive TME, with increased recruitment of Tregs and MDSCs, as well as the expression of immunosuppressive cytokines like IL-10 and TGF- β . This immunosuppressive environment is likely due to the liver’s intrinsic tolerogenic properties, which facilitate tolerance to foreign antigens but also hinder effective anti-tumor immunity.¹⁹ Similarly, lung and bone metastases exhibit distinct immune contexts that reflect the primary immune functions of these tissues. Lung metastases may show variable immune infiltration, often influenced by ongoing inflammatory responses in the lung, whereas bone metastases tend to involve immunosuppressive interactions with osteoclasts and other bone-resident cells, limiting effective immune responses.²⁰ These site-specific differences carry significant clinical implications, as metastases in more immunosuppressive organs tend to respond poorly to immunotherapy, underscoring the need for strategies that either modulate the local TME or enhance systemic immune activation.^{17 21}

Impact of the TME on clinical outcome

The TME is a major determinant of clinical outcome in cancer. High infiltration by cytotoxic CD8⁺ T cells consistently correlates with improved survival across tumor types. These T cells directly recognize and kill tumor cells, and their abundance reflects a pre-existing anti-tumor immune response.^{22 23} High intratumoral expression of IFN- γ further enhances anti-tumor activity by upregulating MHC molecules and promoting a pro-inflammatory milieu.²⁴ In addition, macrophage polarization towards the classically activated M1 phenotype correlates with favorable prognosis; these macrophages secrete

IL-12 and TNF- α , stimulate T cell recruitment and suppress tumor growth, in contrast to immunosuppressive M2-like macrophages.²⁵ Conversely, increased numbers of immunosuppressive cell populations, such as Tregs and MDSCs, promote an immunosuppressive TME that favors tumor progression and are associated with poor prognosis in many cancer types.^{26 27}

The stromal component of the TME also plays a vital role in prognosis. CAFs are key stromal components that secrete ECM molecules such as collagen and laminin, along with cytokines that promote tumor growth and invasion. High CAF density is linked to adverse outcomes, as CAFs drive desmoplasia and generate physical barriers that impede immune infiltration and drug delivery, particularly in pancreatic cancer and breast cancer.²⁸

The immune TME is also important in modulating the response to conventional cancer therapies, such as chemotherapy and radiotherapy. Chemotherapy, while primarily targeting rapidly proliferating tumor cells, also exerts profound immunomodulatory effects on the TME, which can either enhance or impede therapeutic efficacy. Anthracyclines, such as doxorubicin, induce immunogenic cell death, characterized by the secretion of danger-associated molecular patterns (DAMPs), which promotes DC recruitment, activation and efficient cross-presentation of tumor antigens. This robust priming of tumor-specific CD8⁺ and CD4⁺ T cells fosters durable anti-tumor immunity.²⁹ In contrast, cyclophosphamide and cisplatin mainly induce conventional apoptosis, releasing limited tumor antigens and DAMPs. Moreover, chemotherapy can deplete critical immune populations through bone marrow suppression, including T cells and dendritic cells, thereby reducing overall immune surveillance.³⁰

Similarly, radiotherapy exerts potent immunomodulatory effects that extend beyond its direct cytotoxicity. Ionizing radiation induces DNA damage and immunogenic tumor cell death, enhancing DC recruitment and priming of T cells.³¹ Concurrently, radiotherapy reshapes the TME by modulating cytokine secretion and immune cell infiltration, while the tumor vasculature (regulated by stromal and endothelial cells) controls immune cell access to tumor nests, influencing both local immune surveillance and overall therapeutic efficacy.³²

Immunotherapy

Immunotherapeutic strategies offer the potential for more precise and effective targeting with fewer side effects compared to the above mentioned conventional treatment strategies.

The cancer-immunity cycle and immunotherapy

The cancer-immunity cycle provides a framework for understanding how the immune system interacts with tumors and offers a rational basis for the development of various immunotherapeutic strategies. This cycle describes how tumor antigens are released, captured by dendritic cells and presented to T cells in draining lymph nodes, leading to the priming of tumor-specific effector T cells. These T cells then traffic to and infiltrate the tumor, recognize malignant cells through peptide-MHC complexes and induce their destruction, which in turn releases additional antigens and perpetuates the cycle. However, tumors can often evade immune surveillance by manipulating this cancer-immunity cycle at multiple points.

Immunotherapies differ in their mode of action yet share the common goal of enhancing tumor-specific immune responses. Immune checkpoint blockade (ICB), such as antibodies targeting PD-1 or PD-L1, functions by releasing inhibitory brakes on the immune system, particularly on T cells. This reactivation enables immune recognition and elimination of tumor cells that exploit checkpoint signaling for immune escape. Adoptive cell therapies, including chimeric antigen receptor T cell therapy (CAR-T) and tumor-infiltrating lymphocyte (TIL) transfer, aim to amplify or engineer patients' own T cells for direct tumor targeting. These approaches have shown remarkable efficacy, particularly CAR-T therapy in hematologic malignancies³³ and TIL therapy in melanoma³⁴. Cancer vaccines represent another major strategy, designed to activate the immune system against specific tumor antigens. These vaccines can either stimulate a systemic immune response or bolster pre-existing immune activity within the TME, improving tumor recognition and destruction. Oncolytic viruses, which selectively infect and lyse cancer cells while simultaneously inducing immune responses, add another layer of therapeutic intervention by directly disrupting the tumor and activating anti-tumor immunity. This therapy is often used in combination with other approaches to boost immune priming.³⁵ Collectively, immunotherapeutic interventions act on distinct phases of the cancer-immunity cycle, aiming to overcome immune suppression, amplify tumor-specific responses and achieve durable tumor control.

Impact of the TME on immunotherapy response

The composition of the TME is a key determinant of response to immunotherapy. Tumors with an immunologically “hot” TME, characterized by high infiltration of effector immune populations such as CD8⁺ T cells, CD4⁺ helper T cells, dendritic cells, NK cells and M1-like macrophages, along with high IFN- γ signaling and low abundance of immunosuppressive subsets such as Tregs, M2-like macrophages and CAFs, are generally more responsive to immunotherapy. This responsiveness largely reflects the presence of pre-existing, tumor-infiltrating, activated T cells. ICB can then reinvigorate these T cells, allowing them to target tumor cells more effectively.³⁶ Moreover, hot tumors, such as melanoma and lung cancer, often exhibit high tumor mutational burden (TMB) and abundant neoantigens, promoting immune infiltration and sensitivity to ICB.^{5 37}

In contrast, “cold” tumors display immune excluded or immune-desert phenotypes, lacking pre-existing immune activation and thus demonstrating limited responsiveness to ICB. Efforts to convert cold tumors to hot ones through combination therapies, such as the use of oncolytic viruses³⁸, CXCR4 inhibitors³⁹, or anti-angiogenic agents⁴⁰, are being explored to enhance the efficacy of immunotherapy. A comprehensive understanding of the TME composition and dynamics is essential for optimizing treatment selection, predicting patient outcomes and developing novel therapeutic strategies.

HPV-induced tumors

Etiology of HPV-induced tumors

This thesis focuses on the immunotherapy of HPV-induced tumors. Therefore, we have studied multiple tumor types that are predominantly caused by HPV. These (pre)cancers originate from three different anatomical sites: the vulva, the cervix and the head and neck region (**Figure 2**). These tumors share overlapping mechanisms of pathogenesis: HPV-driven transformation, mutations in tumor suppressor gene *TP53*, or driven by other oncogenic mutations. Cervical cancer is HPV-driven in approximately 99% of cases, vulvar cancer in 20%⁴¹ and head and neck cancers (HNC), particularly oropharyngeal cancer, in 70-80% of cases⁴². Interestingly, cancers of the vulva and the cervix have a defined premalignant precursor lesion, respectively vulvar and cervical high-grade squamous intraepithelial lesion (HSIL), whereas no equivalent precursor lesion has been clearly identified in HNC. Most HPV infections are transient, with >90% cleared within two years through coordinated innate and adaptive immune responses.⁴³ Persistent infection, however, may lead to (pre)cancer development.

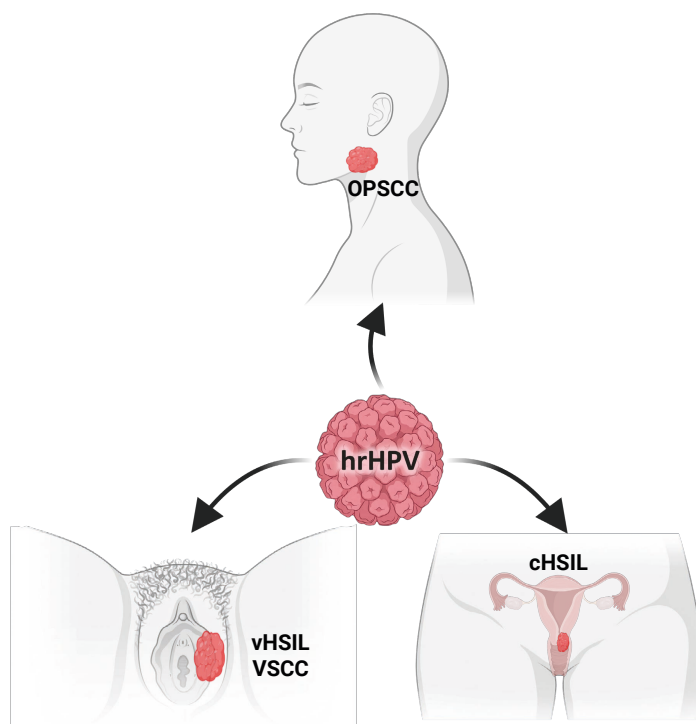


Figure 2. Overview of the HPV-induced tumors studied in this thesis.

High-risk human papillomavirus (hrHPV) is the etiologic driver of cervical high-grade squamous intraepithelial lesion (cHSIL), vulvar high-grade squamous intraepithelial lesion (vHSIL), and the majority of vulvar squamous cell carcinomas (VSCC) and oropharyngeal squamous cell carcinomas (OPSCC). Image created with BioRender.

HPV is a DNA virus that infects basal epithelial cells of stratified squamous epithelium, including the anogenital (cervix, vagina, vulva, penis, anus) and oropharyngeal (tonsils, base of tongue, soft palate, pharynx) regions. It comprises both high- and low-risk genotypes, with HPV16 and HPV18 accounting for over 70% of HPV-associated cancers.⁴⁴ Its oncogenicity arises from the expression of the viral oncoproteins E6 and E7. E6 promotes ubiquitin-mediated degradation of p53, thereby disabling p53-dependent apoptosis in DNA-damaged cells.⁴⁵ E7 binds and induces degradation of the retinoblastoma protein, promoting unchecked cell-cycle progression.⁴⁶ Together, these mechanisms induce genomic instability and malignant transformation.⁴⁷ Since E6 and E7 are expressed in all dysplastic cells, they constitute ideal tumor-specific targets for immunotherapy.

HPV-induced (pre)malignancies offer a uniquely powerful model for studying tumor-immune interactions. Because these tumors are driven by a shared and well-defined oncogenic program (primarily the viral oncoproteins E6 and E7) they exhibit far less genomic and etiological heterogeneity than most solid cancers. This molecular homogeneity minimizes confounding variables and creates a “natural experiment” in which inter-patient differences in immune responses are more likely to reflect variation in host immunity or microenvironmental dynamics, rather than underlying oncogenetics. Furthermore, persistent expression of viral antigens provides a controlled framework to examine chronic antigen stimulation and local immune adaptation, processes central to many cancers.⁴⁸ Additionally, HPV-driven disease spans a well-defined continuum from high-grade intraepithelial lesions to invasive cancer, enabling a rare opportunity to longitudinally study how the TME evolves during malignant progression. As such, HPV-induced tumors serve as a unique platform to understand generalizable principles of tumor immunology and therapeutic response.

Despite their shared viral origin, HPV-induced tumors differ across anatomical sites¹⁷ due to distinct epithelial contexts, immune environments and clinical behaviors. The next sections summarize the key biological and clinical features of the major HPV-induced (pre)cancers addressed in this thesis.

cHSIL

Cervical high-grade squamous intraepithelial lesion (cHSIL), previously termed cervical intraepithelial neoplasia grades 2/3 (CIN2/3), is a premalignant lesion caused by persistent high-risk HPV (hrHPV) infection. The global incidence of cHSIL is increasing, especially among younger women.⁴⁹ Without treatment, up to 50% of cHSIL lesions progress to cervical cancer.^{50 51} Standard management of cHSIL involves surgical excision, typically via large loop excision of the transformation zone (LLETZ) or conization, achieving clearance in approximately 90% of cases.⁵² Nevertheless, recurrence occurs in ~15% of patients within five years.⁵³ LLETZ is associated with an increased risk of preterm delivery in subsequent pregnancies, reflecting a 50-70% higher incidence of cervical insufficiency, particularly in women who undergo repeated procedures.⁵⁴

Cervical cancer

Cervical cancer remains the fourth most common cancer type among women worldwide.⁵⁵ Given that 99% of cervical cancers are hrHPV-induced, it represents a largely preventable disease, prompting the WHO's Global Cervical

Cancer Elimination Initiative, centered on screening and prophylactic HPV vaccination. For early-stage cervical cancer (FIGO stage I-IIA), radical surgical interventions yield excellent outcomes, with >90% cure rates.⁵⁶ Hysterectomy, however, leads to infertility, making fertility-preserving strategies important in younger patients. In locally advanced disease (stage IIB and beyond), cisplatin-based chemoradiation remains the standard of care, achieving five-year survival rates of 66-80%, but at the cost of gastrointestinal and genitourinary toxicities.⁵⁶ Recurrent or metastatic disease carries a poor prognosis, with median survival below 12 months despite systemic chemotherapy.⁵⁷

vHSIL

Vulvar high-grade squamous intraepithelial lesion (vHSIL), formerly termed usual vulvar intraepithelial neoplasia (uVIN), is a premalignant lesion predominantly caused by HPV16. This pleiomorphic dermatosis can cause itching or painful burning sensations. If untreated, approximately 3% of vHSIL cases progress to cancer. Current vHSIL management relies on surgical excision or laser ablation, which achieve lesion clearance in ~80% of patients, but have recurrence rates approaching 30%, especially in immunocompromised individuals. Imiquimod is a first-line nonsurgical option, yielding response rates of about 60%.⁵⁸ Currently there are no biomarkers to predict treatment success.

Vulvar cancer

Vulvar squamous cell carcinoma (VSCC) is the most common pathological subtype of vulvar cancer. VSCC can arise through three distinct molecular pathways: classical *TP53* mutations (66% of cases, poorest survival), other somatic mutations such as *NOTCH1* or *HRAS* (15% of cases, intermediate survival) and persistent hrHPV infection (19% of cases, best prognosis).^{59 60} For early-stage VSCC (FIGO I-II), wide local excision or vulvectomy provides local control in 70-90% of cases depending on disease extent.⁶¹ Advanced (FIGO III-IV) or recurrent VSCC often requires adjuvant chemoradiation, but recurrences remain frequent. In chemoradiation, radiotherapy induces double-strand DNA breaks in rapidly dividing cells (mostly cancer cells). Chemotherapy (cisplatin) acts as a radiosensitizer by forming DNA adducts and crosslinks that distort the DNA helix, which amplifies radiation-induced DNA lesions and slows their repair, thereby increasing the likelihood of cancer cell death.⁶² The significant morbidity of these treatments, including chronic genital and urinary complications, underscores the urgency for more effective, less morbid alternatives.

Head and neck cancer

Head and neck cancer (HNC) represents the sixth most common cancer type worldwide, with squamous cell carcinoma comprising 90% of cases.⁶³ HNC arises from two major etiologic pathways: exposure to carcinogens, such as tobacco and alcohol, leading to oncogenic mutations (e.g. in *TP53*) or persistent hrHPV infection.⁶⁴ Currently, 60% of HNC cases are HPV-driven, and this proportion continues to rise.⁴² Clinically, patients with HPV-positive HNC exhibit improved survival and reduced recurrence following standard treatments compared with HPV-negative cases.⁶⁵ In early-stage HNC, surgery or radiotherapy achieves approximately 75% five-year survival.⁶⁶ Locally advanced disease is typically managed with chemoradiation, achieving five-year survival rates of 60-70% but causing significant treatment-related morbidity, including mucositis and dysphagia.⁶⁷ In recurrent or metastatic HNC, systemic therapies such as cisplatin or cetuximab (an epidermal growth factor receptor inhibitor) provide limited benefit, with response rates near 15% and median survival below 10 months.⁶⁸

Immunotherapy in HPV-induced tumors

Given the strong immunogenic potential of HPV-induced lesions and the central role of viral antigens in driving tumor biology, numerous immunotherapeutic approaches have been explored to boost antitumor immunity. These include local immune stimulants, vaccines designed to prevent or eliminate HPV-infected cells and checkpoint blockade that reinvigorates T cells.

Imiquimod is a topical immune modulator used in dermatology for its immunostimulatory properties. It is approved for the treatment of basal cell carcinoma, actinic keratosis (precursor of squamous cell carcinoma of the skin), as well as for the treatment of external genital warts (condylomas) caused by low-risk HPV. Imiquimod is a toll-like receptor 7 (TLR7) agonist. TLR7 is expressed on dendritic cells and macrophages, stimulating these cells to release pro-inflammatory cytokines such as IFN- α and IL-12.⁶⁹ This cascade triggers local immune activation, recruiting and activating both innate and adaptive immune cells to eradicate dysplastic cells. Clinical studies have reported regression of vHSIL and cHSIL in approximately 60% of patients.^{70 71} Nevertheless, inflammatory side effects such as erythema and ulceration limit patient adherence and nearly half of treated patients fail to respond, suggesting that local immune activation alone may be insufficient in these cases.

Prophylactic HPV vaccines (e.g. Gardasil or Cervarix) are based on virus-like particles composed of the HPV L1 capsid protein. These virus-like particles are taken up by antigen-presenting cells as extracellular antigens and therefore processed via the MHC class II pathway, inducing robust CD4⁺ helper T cell responses that support B cells to produce neutralizing antibodies, which block viral entry into basal epithelial cells. The capsid antigens of prophylactic vaccines are not expressed by infected cells anymore, and therefore these vaccines cannot clear established HPV infections.⁷² In contrast, therapeutic HPV vaccines target viral oncoproteins such as E6 and E7, which are expressed within infected or transformed cells. Intracellular expression of these antigens enables processing via the MHC class I pathway, eliciting CD8⁺ cytotoxic T cell responses, while cross-presentation and professional antigen-presenting cells allow MHC class II presentation to also activate CD4⁺ helper T cells. This coordinated cellular immunity is critical for recognizing and eliminating HPV-expressing cells.⁷² The therapeutic DNA vaccine VGX-3100 targets E6 and E7, and achieved a 50% response rate in cHSIL patients, with complete viral clearance in a subset of responders.⁷³ Another therapeutic vaccine, ISA101, composed of synthetic long peptides derived from E6 and E7, has induced strong T cell responses but demonstrated limited clinical efficacy in established cancers, with response rates around 30%.⁷⁴ This limited efficacy is likely due to the immunosuppressive TME in advanced cancers, where Tregs, MDSCs and cytokines such as TGF- β inhibit cytotoxic T cell activity.⁷⁵ Ongoing efforts therefore focus on combination therapies integrating vaccines with ICB or agents that remodel the TME.

Immune checkpoint blockade (ICB) have transformed the treatment landscape across multiple cancer types, but response rates in HPV-associated malignancies remain variable. These agents target inhibitory immune checkpoints, including PD-1 and CTLA-4, expressed on T cells, whose ligands are frequently upregulated on tumor cells and antigen-presenting cells (APCs), thereby suppressing T cell activation. By blocking these pathways, ICB reinvigorates antitumor T cell activity.

Pembrolizumab (a PD-1 inhibitor) has been approved for PD-L1-positive recurrent or metastatic cervical cancer, following trials demonstrating durable responses in approximately 14% of patients.⁷⁶ The modest efficacy highlights the need for biomarker-guided patient selection and combinatorial strategies. Anti-angiogenic agents such as bevacizumab, targeting vascular endothelial growth factor (VEGF), have further improved survival in advanced cervical

cancer. When combined with chemotherapy, bevacizumab increases both overall survival and response rates, with reported response rates of 48%, versus 36% with chemotherapy alone.⁷⁷ VEGF-targeted therapies may also synergize with immunotherapy by enhancing immune cell infiltration into tumors and mitigating VEGF's immunosuppressive influence in the TME.

For advanced or recurrent VSCC, ICB is under clinical evaluation as an alternative to cytotoxic therapies. Early data show overall response rates around 10%, with median overall survival of six months.⁷⁸ Current trials are testing neoadjuvant pembrolizumab to expand treatment options in this setting.⁷⁹

In recurrent or metastatic HNC, nivolumab (a PD-1 inhibitor) demonstrates an overall response rate of 13% in HPV-positive tumors, with durable remissions in a subset of patients.⁷ Perioperative (neoadjuvant plus adjuvant) pembrolizumab combined with surgery and radiotherapy significantly improves three-year event-free survival (58% versus 46%) in resectable locally advanced HNC.⁸⁰ In addition to ICB, therapeutic HPV vaccines are under evaluation as adjuncts to enhance immune responses in HPV-positive HNC, with early trials showing promising results.⁸¹

Despite the promise of immunotherapy, a notable subset of patients does not respond to current immunotherapeutic approaches. Importantly, a substantial proportion of ICB-nonresponsive patients do harbor neoantigen-specific T cells, indicating that antitumor immunity is present but not effectively engaged by current therapies.⁸² Combination approaches of ICB with radiation or chemotherapy are under investigation to enhance therapeutic efficacy.⁸³ Previous attempts to identify reliable biomarkers for predicting response to immunotherapy, such as PD-L1 expression, tumor mutational burden and the presence of specific tumor-infiltrating lymphocyte subsets have shown limited clinical usability.⁸⁴ Thus, while immunotherapy represents a major step forward in the treatment of cancers, significant challenges remain. The heterogeneity in clinical responses highlights the need for a more comprehensive understanding of the TME and its role in mediating therapeutic efficacy. Identifying predictive biomarkers for patient stratification and developing rational combination strategies are critical steps toward improving response rates and avoiding overtreatment.

Based on prior research linking the TME with survival and immunotherapy response in various cancers, we questioned whether distinct TME features

also contribute to therapeutic outcomes in HPV-induced tumors. Because HPV-induced tumors share the same oncogenic driver, anatomical site and etiological background, they allow precise interrogation of how TME composition shapes immunotherapy response and clinical outcome. Thus, insights gained from studying HPV-induced tumors are broadly informative, revealing principles of TME interactions that may guide treatment strategies across other, more heterogeneous cancer types.

The TME in HPV-induced tumors

The TME of HPV-driven lesions is shaped by a complex interplay of immune and stromal components that influences disease progression and therapeutic response. Immune profiling of cHSIL and cervical cancer has revealed that elevated Treg densities suppress antitumor immunity and correlate with poor prognosis.⁸⁵ M2 macrophages contribute to immune suppression by secreting pro-angiogenic and immunoregulatory cytokines, creating a microenvironment favorable for tumor progression.^{86,87} MDSCs, recruited through tumor-derived inflammatory cues, inhibit T cell activity via IL-10 and TGF- β secretion and are associated with advanced disease.⁸⁸ NK cells in cervical cancer display reduced cytotoxicity and altered receptor expression (e.g. decreased NKG2D), further promoting immune evasion.⁸⁹ Moreover, most cervical cancers have an immunosuppressive cytokine environment, with elevated levels of TGF- β , IL-10 and VEGF, promoting Treg and MDSC expansion while inhibiting CD8⁺ T cell functionality. Concurrently, chronic inflammation driven by cytokines such as IL-6 and TNF- α can support tumor progression by maintaining a pro-tumorigenic environment.⁸⁵

The immune landscape of vHSIL and VSCC has been limitedly explored, as most prior analyses relied on single-marker immunohistochemistry. Available data indicate an increase in CD14⁺ macrophages during progression from vHSIL to VSCC, with M2-like macrophages exceeding M1-like populations. High densities of stromal CD14⁺ macrophages correlate positively with intraepithelial Tregs and negatively with activated CD8⁺ T cells, predictive for reduced recurrence-free survival.⁹⁰ Conversely, dense stromal infiltration by activated CD8⁺TIM3⁺ and CD3⁺NKG2A⁺ T cells associates with absence of recurrence and improved outcomes. Interestingly, in VSCC, high T cell infiltration does not consistently predict survival, potentially reflecting functional exhaustion or anergy of TILs.⁹¹

In HNC, HPV-positive HNC exhibits a more inflamed TME than HPV-negative HNC, characterized by abundant CD8⁺ and CD4⁺ T cell infiltration and higher

immune activation, correlating with improved survival.^{92 93} The presence of HPV-specific TILs predicts excellent survival.⁹² Conversely, HPV-negative HNC, typically associated with tobacco and alcohol use, displays a less inflamed, immunosuppressive TME with reduced lymphocyte infiltration and limited responsiveness to immunotherapy.⁹⁴

Together, these findings highlight that HPV-driven tumors encompass a spectrum of immune microenvironments, ranging from highly inflamed to profoundly immunosuppressive, with significant implications for prognosis and therapy response. Understanding these TME differences is essential for developing more effective immunotherapeutic strategies and thereby improving patient outcomes.

New techniques to high-dimensionally chart the *in situ* TME

Previous in-depth studies of the TME have relied primarily on single-cell analyses of patient-derived materials, such as flow cytometry and single-cell RNA sequencing. These methods have provided key insights into the diversity of cell types and functional cell states within the TME. Nevertheless, a growing body of evidence indicates that the spatial context plays a critical role in shaping clinical outcome.^{22 23} At the start of this PhD, the emergence of high-dimensional spatial profiling technologies enabled detailed *in situ* characterization of the cellular and structural organization of the TME. These techniques reveal interactions between tumor cells, immune cells and stromal components that cannot be captured by fluorescence-activated cell sorting (FACS) or bulk RNA sequencing, which lack the spatial dimension (**Figure 3**). Moreover, they provide a more integrated and comprehensive understanding of the TME than traditional single-marker or low-plex immunohistochemical approaches.

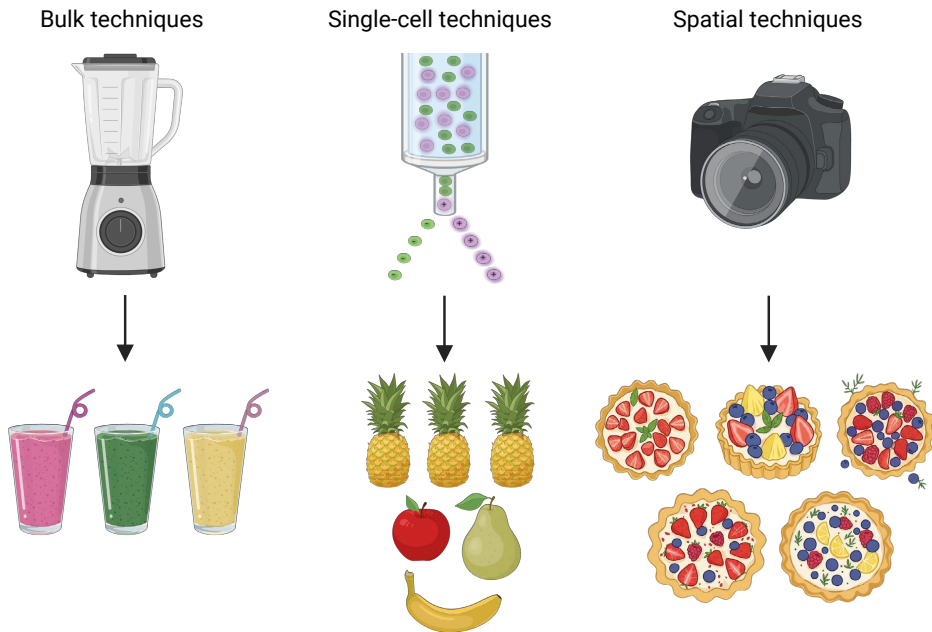


Figure 3. Schematic overview of the techniques to chart the TME.

Bulk techniques generate averaged molecular signatures of the TME. Single-cell technologies disentangle the phenotypic heterogeneity of individual cell types. Spatial techniques localize these cell types within their native tissue architecture, preserving positional context and spatial interactions. Image created with BioRender.

Furthermore, methods such as flow cytometry and RNA sequencing often require fresh tissue, which limits their use for analyzing archival formalin-fixed paraffin-embedded (FFPE) samples. In contrast, technologies including multispectral immunofluorescence (mIF), imaging mass cytometry (IMC) and single-cell spatial transcriptomics enable high-resolution TME analysis directly in FFPE tissue. These approaches facilitate the exploration of archived clinical trial samples, irrespective of size, and allow the discovery of previously unknown cell populations.⁹⁵ The spatial information provided by these methods is essential for understanding how the spatial architecture of the TME influences tumor biology, immune evasion and therapeutic response, particularly for immunotherapy, where the proximity and localization of immune cells relative to tumor cells can determine treatment efficacy.³⁶ Each technique, however, has specific inherent limitations that must be considered.

Multispectral immunofluorescence

At the onset of this PhD, multispectral immunofluorescence (mIF) represented one of the most advanced *in situ* imaging methods available for multiplexed tissue analysis. Today, mIF is a cornerstone technology for studying the spatial organization of immune cells and tumor cells within the TME. Using antibodies conjugated to spectrally distinct fluorophores, mIF enables the simultaneous detection of multiple markers on a single FFPE tissue section. Through spectral unmixing, up to seven fluorescent signals can be quantified, allowing detailed visualization of immune-tumor cell interactions at subcellular resolution (0.5-1 μm per pixel).⁹⁶ Although highly effective for high-resolution spatial mapping, the limited number of markers that can be analyzed per panel constrains its use in studies requiring broader phenotypic coverage. Furthermore, mIF is a medium-throughput technique, which can restrict its application in large-scale or discovery-driven studies where high multiplexing is advantageous.

Imaging mass cytometry

Imaging mass cytometry (IMC) extends spatial proteomic profiling by allowing simultaneous detection of up to 50 markers. In IMC, antibodies are conjugated to heavy-metal isotopes, and tissue is ablated using a laser, ionizing the tags for quantification by mass spectrometry.⁹⁷ This eliminates the spectral overlap inherent to fluorescence-based systems, enabling highly multiplexed spatial analyses. However, IMC's resolution is lower than that of mIF and its throughput is limited by long acquisition times. The integration of spatial data with mass spectrometry-based quantification also requires sophisticated computational workflows for data normalization, visualization and interpretation. Despite these challenges, IMC remains a powerful tool for comprehensive, hypothesis-generating studies of the TME, capable of revealing complex multicellular interactions and signaling networks.⁹⁸

Single-cell spatial transcriptomics

Single-cell spatial transcriptomics represents the next generation of TME profiling by enabling spatially resolved transcriptomic mapping of thousands of genes *in situ*. This approach captures transcriptional heterogeneity within the tissue architecture and links gene expression patterns to specific spatial niches. Platforms such as Visium (10x Genomics), Xenium (10x Genomics) and CosMx (NanoString) preserve spatial integrity while detecting RNA transcripts directly in FFPE tissue sections, generating high-resolution expression maps. While this technology provides an unprecedented view of cellular states, it remains limited by lower throughput and the imperfect correlation between mRNA and

protein expression, which complicates the interpretation of functional immune phenotypes.⁹⁹ Moreover, cellular functions influenced by post-translational modifications or protein-protein interactions require complementary proteomic approaches to achieve a more complete understanding of the underlying biology.

Thesis outline

The immune TME plays a critical role in determining patient survival and response to immunotherapy across multiple cancer types, yet it has not been comprehensively investigated in HPV-induced (pre)cancers. The aim of this thesis is to provide an in-depth characterization of the TME in HPV-driven lesions, extending beyond what was previously feasible, and to relate these findings to clinical outcomes. At the onset of this PhD, available immunotherapeutic strategies elicited clinical responses in only a subset of HPV-associated precancer patients, with no reliable predictive biomarkers. In addition, the impact of the TME on survival in HPV-associated HNC and VSCC was largely unexplored. Addressing these gaps is essential to determine whether, and which types of, immunotherapies should be developed for this patient population. These two overarching questions therefore form the foundation of this thesis.

Part I: immunotherapy and TME of HPV-induced precancer

In part I we explored the impact of the TME on response to immunotherapy in HPV-associated precancers. In **chapter 2** we give an overview of what was known about the immune microenvironment of vulvar (pre)cancer at the time this PhD was started. Previously, it was reported that the clinical response to immunotherapeutic vaccination was related to the strength of the systemic T cell response, yet there was too much overlap between responders and non-responders for it to be the sole determinant.¹⁰⁰ Therefore, in **chapter 3** we investigated whether the pre-existing TME of vHSIL was associated with clinical response to immunotherapeutic vaccination. Not only did we show that there were differences in immune infiltration between non-responders and responders, but our study of post-vaccination samples also revealed that therapeutic vaccination did not convert an immunological cold lesion into hot. The outcomes of this study led to two new studies. The first of these explored whether the TME composition is also important for response to imiquimod immunotherapy. Therefore, we studied pre-imiquimod vHSIL samples of

responders and non-responders to imiquimod in **chapter 4**. This revealed that a pre-existing hot TME was required for responses to both imiquimod and therapeutic vaccination, with imiquimod responses being predominantly driven by high infiltration by inflammatory myeloid cells, and vaccination responses being mainly associated with high infiltration by pro-inflammatory T cells. Since these types of immunological analyses were heavily steered to certain known cell types, we applied single-cell spatial transcriptomics to study the TME of therapeutically vaccinated vHSIL patients in **chapter 5**. This more holistic approach to study the TME revealed that responders had a pre-existing type 1 immune contexture and a distinct spatial ecosystem of well-organized immune cells, in contrast to non- and partial-responders. Finally, in **chapter 6** we analyzed whether the findings that were made in vHSIL regarding the predictive capacity of the TME for response to imiquimod could be extrapolated to cHSIL, and further developed this into a predictive biomarker that is currently prospectively tested.

Part II: survival and TME of HPV-induced cancer patients

In part II we investigated the impact of the TME on survival in VSCC and HNC patients. We for the first time explored the composition and impact of the myeloid landscape of VSCC in **chapter 7**. Here, we found that high monocyte infiltration in the TME forms an independent positive prognostic biomarker for survival in these patients. In addition, we applied combined IMC, single-cell and bulk transcriptomics for an in-depth analyses of the TME in HNC in **chapter 8**. Alike our findings in vHSIL, the presence of specific spatial ecosystems were important for survival. In particular, the presence of intratumoral tumor-specific T cells was associated with the presence of intra-epithelial immune microaggregates, comprising CD4⁺ and CD8⁺ T cells as well as dendritic cells, and these were associated with excellent survival.

The main findings of this thesis are summarized in **chapter 9** (in English) and **chapter 10** (in Dutch), accompanied by a forward-looking outlook that highlights key clinical implications and identifies promising avenues for future research.

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The background of the entire page is a deep space image showing a complex cosmic web. It features a dark blue to black background filled with numerous small, distant stars. Overlaid on this are intricate, glowing filaments of light in various colors including blue, green, yellow, orange, and red. These filaments form a dense, interconnected network that spans the entire frame, resembling the large-scale structure of the universe. The filaments vary in thickness and brightness, with some appearing as thin, wispy lines and others as more substantial, glowing strands. The overall effect is one of vastness and complexity, providing a visually striking backdrop for the text.

PART I

IMMUNOTHERAPY AND TME OF HPV-INDUCED PRECANCER

Chapter 2

The immune microenvironment in vulvar (pre)cancer: review of literature and implications for immunotherapy

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Expert opinion on biological therapy 2018

Abstract

Introduction

Vulvar squamous cell carcinoma (VSCC) develops via two different pathways: *TP53* mutations in a background of lichen sclerosus or a persistent infection with high risk human papilloma virus (HPV). The latter group of tumor responds better to treatment than the non-virally induced VSCC. This may be explained by a difference in the tumor immune microenvironment (TME).

Areas covered

This review summarizes literature on TME of VSCC and its precursors, and extrapolates this to foster the development of new therapeutic strategies.

Expert opinion

Both types of VSCC and their precursors are infiltrated with variable numbers of M2 macrophages, regulatory T cells and CD8+ T cells, indicating that they express targetable tumor antigens. Type 1 T cell immunity in precursor lesions is associated with fewer recurrences and better clinical responses to immunotherapy. Escape of these lesions and progression towards VSCC is associated with the downregulation of HLA class I, increased expression of co-inhibitory molecules, infiltration with immunosuppressive cells and the local production of immunosuppressive enzymes and cytokines.

More in-depth studies of the VSCC TME are required to fully comprehend the impact of the immune system on VSCC, and subsequently to identify patients who will benefit from immunotherapeutic strategies.

Key findings

- Two distinct types of VSCC can be identified: HPV-induced and *TP53* mutated VSCC
- VSCC and its precursor are infiltrated with M2 macrophages, Tregs and CD8+ T cells
- A type 1 immune response correlates with better clinical outcomes in vHSIL
- Analysis of the microenvironment is important for immunotherapeutic strategies in VSCC

Introduction

Vulvar cancer is a rare disease, representing 3-5% of all gynecological malignancies, with an increasing annual incidence of 1-2 per 100.000 women.^{1,2} Around 90% of all vulvar tumors are vulvar squamous cell carcinomas (VSCC).³ Two independent pathways have been identified in the pathogenesis of VSCC (**Figure 1**). Classical development of VSCC is related to *TP53* mutations (HPVnegVSCC) accounting around 80% of all VSCC.⁴ Persistent infection with high risk human papillomavirus (hrHPV) causes the other 20% (HPVposVSCC).^{5,6} The overall incidence of VSCC is rising, likely due to higher life expectancy and the overall increase in HPV infections.⁷ The standard treatment for VSCC consists of surgery and/ or radiotherapy and is associated with high morbidity rates and impaired quality of life.⁸

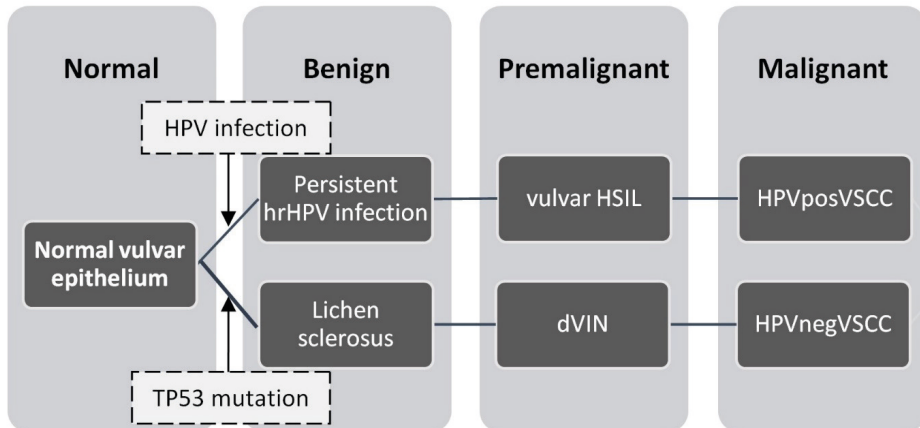


Figure 1. Overview of the multistage evolution of VSCC, depicting its two distinct pathways.

The HPV-driven pathway starts with an HPV infection, of which the lifetime risk for women is 80%. 20% of these infections persist, of which half causes vulvar high-grade squamous intraepithelial lesion (vHSIL). A small percentage of vHSIL develops into HPV positive VSCC. The HPV-independent pathway is associated with lichen sclerosus, which can develop into dVIN, commonly after TP53 mutation. DVIN is considered a direct precursor of HPV negative VSCC, which accounts for 80% of all VSCC cases.

The precursor of HPVnegVSCC is vulvar intraepithelial neoplasia of the differentiated type (dVIN) and often arises in a background of lichen sclerosus (LS).^{5,9} It is suggested that dVIN, rather than LS, is the direct precursor of HPVnegVSCC.¹⁰ HPVnegVSCC is associated with *TP53* mutations, which leads to abnormal expression of p53 protein.^{5,11} Somatic mutations in *TP53* lead to an uncontrolled cell cycle and chromosomal instability, resulting in tumor formation.¹² In HPVposVSCC ~75% of the lesions are caused by HPV type 16,

one of the most dominantly present oncogenic high risk HPV types (hrHPV).⁶ HPVposVSCC's precursor lesion is vulvar high grade squamous intra-epithelial lesions (vHSIL), formerly referred to as vulvar intraepithelial neoplasia of the usual type (uVIN). Cells infected by hrHPV express the viral oncogenes E6 and E7, which disturb the regulation of the cell cycle and promote malignant conversion. The lifetime risk of acquiring an hrHPV infection is approximately 80% for sexually active individuals. About 80% of the infections are cleared by the immune system within 18 months.¹³ Less than 10% of the infections persist and may cause vHSIL, 6.5% of which subsequently progresses towards VSCC.^{9,14} Several retrospective studies suggest that patients with HPVposVSCC display a better progression-free survival when compared to patients with HPVnegVSCC.^{11,15-17} Potentially, this difference is due to a more pronounced type 1 immune response as seen in various other malignancies.¹⁸

The adaptive cellular immune system is known to play a role in the protection against HPV-induced lesions. The group of immunosuppressed individuals (e.g. HIV, transplant patients and patients with leukemia) shows a higher incidence in persistent HPV infections and HPV-related malignancies.¹⁹⁻²¹ In addition, complete regression of HPV-induced lesions is associated with a strong influx of CD4+ and CD8+ T cells.²² Furthermore, healthy individuals display *ex-vivo* measurable frequencies of HPV-specific interferon producing T cells²³, which are largely lacking in patients with HPV-induced cancer.²⁴ Surprisingly though, the immune response in VSCC has not been studied thoroughly. Potential differences based on the distinct etiological pathways leading to HPVposVSCC and HPVnegVSCC have largely been ignored. This could be key to our understanding of the clinical behavior of VSCC. The expressed viral onco-proteins can function as tumor-specific antigens (TSAs) in HPVposVSCC, fueling the induction of a strong local type 1 T cell response and potentially leading to the better clinical outcome of HPVposVSCC when compared to HPVnegVSCC.^{11,17} Probably, the development of VSCC parallels that of oropharyngeal squamous cell carcinoma (OPSCC), which also develops via a non-viral or an hrHPV-driven pathway.²⁵ In this tumor type, the presence of HPV was found to be a strong independent determinant of progression free survival^{26,27} and associated with a dense infiltration of activated CD4+ and CD8+ T cells.^{26,28} Especially the intratumoral presence of type 1 cytokine producing HPV16-specific T cells constituted a type 1 immune contexture and improved clinical outcome. The absence of intratumoral HPV-specific T cells led to worse clinical outcome, indistinguishable from that of most HPVnegOPSCC.²⁹ The recognition that HPVposOPSCC have a better clinical outcome led to new trials

of de-intensified chemo (radio)therapy schedules, testing of novel therapeutics, including immunotherapeutic compounds in OPSCC.³⁰⁻³³ Hence, HPVposVSCC and HPVnegVSCC are different entities and they should be studied separately. Here, we summarize studies on the immune response as measured in the local tumor microenvironment (TME) in VSCC, and correlate this information to what is known about the precursors of VSCC and other HPV-induced malignancies. In addition, we will extrapolate this information to briefly delineate future therapeutic options for VSCC.

Innate immune responses in vulvar lesions

The role of the innate immune system is ambiguous, since it may promote tumor growth as well as prevent tumor progression.^{34,35} For instance, the role of tumor-associated myeloid cells is to foster tumor progression. Myeloid derived suppressor cells and type 2 macrophages can promote initiation and metastasis of tumor cells, and inhibit tumor-specific T cell responses.³⁶ On the other hand, abundantly present M1 macrophages in HPV-driven cervical cancer are an independent prognostic factor for good survival³⁷, and IFN γ -activated macrophages are important for tumor growth control in an HPV-tumor animal model.^{38,39} First, the role of macrophages will be discussed and then the focus is on professional antigen presenting cells (APCs), as these are crucial for the induction of adaptive immune responses.

In one study, myeloid cell infiltration between HPV16 driven primary vHSIL (n=43), 20 recurrent vHSIL, 21 VSCC and 26 normal vulvar tissues was compared.⁴⁰ Progression was associated with an increase in the numbers of CD14+ cells. Both in healthy controls and HPV16+ vHSIL the CD14+CD33-CD163+ mature (activated) M2 macrophages were more abundantly present than the M1 macrophages. Importantly, a higher number of both intraepithelial and stromal mature CD14+ macrophages was positively correlated to the number of intraepithelial regulatory T cells (Tregs, defined as CD4+FOXP3+, CD3+PD1+FOXP3+), negatively correlated to the number of activated CD8 T cells as well as with a decreased recurrence free survival time in vHSIL.⁴¹ The M2 macrophages are immune suppressive as reflected by their production of IL-10 and TGF- β . Potentially, M2 macrophages induce local Treg polarization.⁴² High M2 macrophage counts are associated with poor outcome in a diversity of tumors⁴³⁻⁴⁵, suggesting that they may have a similar negative impact in VSCC. Indeed a large study of 100 VSCC confirmed that these tumors were

infiltrated with variable numbers of CD68+ macrophages and T cells.⁴⁶ It also showed that increased immune infiltration of the tumors was associated with the expression of PD-L1 in 20% of the HPVnegVSCC although this was not observed in the HPVposVSCC.⁴⁶ The expression of PD-L1 was also found in a mouse tumor model³⁸ and may reflect an ongoing antitumor response in those HPVnegVSCC, suggesting that also HPVnegSCC can express antigens that are available for the immune system to react to. Unfortunately, no TME studies have been performed in dVIN, albeit that increased numbers of T cells and CD68+ macrophages were found in LS.⁴⁷

The professional APCs such as Langerhans cells (LCs) and dendritic cells (DCs) connect the innate and adaptive immune system, since they migrate from the epidermis towards the lymph node where they prime naïve T cells. A small immunohistochemistry study on VSCC with unknown HPV status (n=5) showed that VSCC were less infiltrated by CD1a+ LCs than healthy vulvar tissue.⁴⁸ What this means in the context of VSCC is unclear, but already in vHSIL the epithelial cell nests comprise lower numbers of CD1a+ and CD207+ DC than healthy tissue and this corresponds with a lower CD8 T cell infiltrate.^{49 50} The intraepithelial infiltration with CD1a+ DCs is inversely correlated with the grade of VIN.⁵¹ One explanation offered for this was the inhibition CCL20 secretion through E6 and E7 expression.⁵² Interestingly, also the dermis of LS is less infiltrated with CD1a+ DC/LCs, and this lack of infiltration is even more pronounced in VSCC that developed in the background of LS.^{48 53} The decrease in intraepithelial CD1a+ DC/LC numbers is paralleled in overall T cell infiltration.⁵⁴ Thus, non-malignant vulvar lesions and VSCC display low numbers of intraepithelial immature CD1a cells and CD8 T cells.

In addition to the lower number of APCs detected in VSCC, the function of the remaining APCs may have been altered towards a more immune suppressive phenotype. Cancer cells have been shown to inhibit DC maturation *in vitro* via the secretion of IL-6 and PgE2.^{55 56} Furthermore, both subtypes of VSCC are known to secrete receptor activator NF- κ B ligand (RANKL). RANKL can inhibit DC maturation, based on differences in the expression of CD80, CD83, CD86, and HLA-DR as well as their reduced IL-12 production and increased IL-10 production *in vitro*, suggesting that they have become tolerogenic DC. The addition of osteoprotegrin (a RANKL inhibitor) improved DC maturation, suggesting that RANKL could be an interesting therapeutic target to prevent immune escape of VSCC.⁵⁷ Furthermore, VSCC expresses indoleamine 2,3-dioxygenase (IDO).⁵⁸ This molecule not only suppresses T cell responses⁵⁹

but also blocks the maturation of DCs.⁶⁰ Notably, VSCC expressed IDO is an independent prognostic factor for worse overall survival in VSCC⁵⁸ but it remains to be investigated if this holds true for both subtypes of VSCC.

Adaptive immune responses in vulvar lesions

Two studies showed that CD4+ and CD8+ T cells co-infiltrate the cancer cells nests and stroma of primary VSCC tumors. Furthermore, the magnitude of this T cell infiltration positively correlated with the expression of HLA class 1.⁶¹ In many epithelial cancers, including HPVposOPSCC and HPVnegOPSCC, a dense infiltration with CD8+ T cells is associated with improved survival^{18 26 62 63}. However, a strong infiltration with T cells seemed not related to the prognosis of VSCC⁶⁴ which led to the hypothesis that the tumor infiltrating lymphocytes (TILs) had become anergic.⁶⁴ Indeed, the functional status of the T cells could make a difference and the expression of the T cell function suppressing enzyme IDO was shown to have a negative impact on survival in one study of VSCC⁵⁸, but this was not confirmed in another large study.⁶¹ Also the earlier reported expression of PD-L1 may restrain the function of TIL.⁴⁶ Therefore, the functional status of killer lymphocytes was analyzed using single stains for CD56 (expressed by NK/NKT cells) and granzyme B (GrB, for the cytotoxic status). CD56+ lymphocytes were mostly detected in the epithelium whereas GrB+ cells were predominantly found in the stroma. Importantly, the number of activated CD56+GrB+ intraepithelial lymphocytes significantly correlated with longer overall survival in VSCC.⁶⁵ Another study in HPVposVSCC and its precursors corroborated these findings.⁶⁵ Thus, rather than the number of infiltrating lymphocytes, their functional activity is key for clinical impact. Indeed, a detailed study on T cell infiltration, the expression of Tbet (for IFN γ producing T cells) and FOXP3 (for Tregs) and the co-inhibitory molecules programmed cell death 1 (PD1), T cell immunoglobulin mucin 3 (TIM3), and the natural killer cell lectin-like receptor A (NKG2A) to identify activated T cells, confirmed that vHSIL and HPVposVSCC are intensely surveyed by activated CD4+ and CD8+ T cells.⁴⁰ In vHSIL a dense infiltration with stromal activated CD8+TIM3+ T cells and stromal activated CD3+NKG2A+ T cells was related to the absence of recurrences and a prolonged recurrence-free survival.

It is likely that co-infiltration with Tregs blunted the activity of TILs. High numbers of Tregs in vHSIL were negatively associated with time to recurrence, especially when the stromal CD8+TIM3+ infiltration was limited.⁴⁰ In VSCC the

number of Tregs is even higher and outnumber the co-infiltrating activated T cells.⁴⁰ Potentially, these increased numbers of Tregs in VSCC^{40 58 61} are the result of tolerogenic DCs in these tumors that support the differentiation of naïve CD4⁺ T cells into Tregs.⁵⁷ Two studies that analyzed the impact of intraepithelial FOXP3⁺ Tregs in VSCC reported that their number does not influence survival rates.^{58 61} It is not clear if this result was similar for HPVnegVSCC and HPVposVSCC or what the infiltration with activated T cells was. Furthermore, both studies did not report the impact of stromal Tregs^{58 61}, which might be important in view of the presence of activated T cells in the stroma of HPVposVSCC⁴⁰ and the high number of GrB⁺ lymphocytes in the stroma of VSCC, with unknown HPV status.⁶⁵

On top of this, the impact of the infiltrating T cells may be lower due to the loss of HLA class I expression in VSCC.⁶¹ HLA-class I was found to be downregulated in 70% of vHSIL patients, 15% of which was caused by loss of heterozygosity (LOH). In the other cases HLA-class I could still be upregulated by interferon γ .⁶⁶ This will prove more difficult for HPVposVSCC, where HLA-class I downregulation through LOH is found in 25-55% of the cases.⁶⁶

Last but not least, the VSCC-infiltrating T cells may be suppressed following interaction with HLA-E, a non-classical HLA molecule expressed in at least 15% of the HPVposVSCC cases⁶⁷, and found to have a negative effect in other epithelial tumors.^{68 69} HLA-E is the ligand for the co-inhibitory molecule NKG2A, which we know is expressed by T cells in VSCC.⁶⁶ and recently has been shown to function as checkpoint for T cells in several mouse tumor models (van Montfoort et al., *in press*). Furthermore, HLA-E positive tumors show decreased numbers of T cells expressing NKG2A compared to HLA-E negative tumors, indicating a possible negative feedback loop.⁴⁰

In conclusion, the clinical impact of T cell infiltration can't be analysed outside the context of HLA expression, immune suppressive cells and regulatory mechanisms (e.g. expression of PD-1 and NKG2A).

Lichen sclerosis and dVIN

Lichen sclerosis is a chronic inflammatory skin disorder that is histologically characterized by a band-like lymphocytic infiltrate below the dermo-epidermal junction. Until now, the immune microenvironment has scarcely been studied

in LS and not at all in dVIN. In LS, the immune infiltrate is characterized by high numbers CD3+ T cells, consisting of CD4+, CD8+, and FOXP3+ T cells.⁷⁰⁻⁷⁵ The numbers of CD8+ T cells and FOXP3+ Tregs were significantly higher in the dermis of LS lesions than in healthy controls.⁷⁰ A more in depth analysis revealed the presence of high numbers of activated perforin+, GrB+, and type 1 interferon-associated CXCR3+ cytotoxic cells, suggesting that T cell mediated cytotoxicity occurs in LS.^{76,77} Increased numbers of HLA-DR+ cells in the dermal infiltrate indicates that these T cells have been activated following cognate antigen recognition.⁷⁵ Moreover, gene expression arrays showed that significantly more type 1 immune response associated pro-inflammatory cytokines are expressed in LS when compared to healthy tissue.⁷⁰ Together, these data indicate that the microenvironment of LS resembles that of an autoimmune disorder, characterized by a type 1 immune response.

Other indications for a developed T cell response comes from a recent study showing that monoclonal T cell receptor (TCR) rearrangement was identified in 47% of patients with LS.⁷⁸ Interestingly, the infiltrates of the lesions with monoclonal TCR rearrangement were dominated by CD4+ T-cells, B cells, and fascin (a protein involved in antigen presentation)-positive dendritic cells. Similarly, HPV-negative penile cancers, known to be associated with LS, and differentiated penile intraepithelial neoplasia, showed accumulation of T cells with monoclonal TCR rearrangement.⁷⁹ This suggests the presence of a specific antigen in LS and HPVnegVSCC or penile carcinoma to which the immune system can respond.

These findings suggest that LS is an immunologically active lesion, which may give rise to the development of malignancies. Future studies are needed to explore the local immune environment, in particular in relation to clinical outcomes and progression of disease.

Modulation of the local microenvironment

Until now, the cornerstone of the treatment for VSCC, vHSIL and dVIN is surgery. This treatment is associated with significant morbidity due to lymph oedema, wound infections, and psychological and sexual problems.⁸⁰ However, recurrence rates after these treatments occur in up to 50% of patients.⁸¹ Non-surgical treatments for vHSIL include topical creams and gels, HPV vaccines, and photodynamic therapy (PDT). Understanding of the modulation of the local microenvironment is important as this might unveil clues to novel therapy. An overview of previous clinical trials can be found in **Table 1**.

Table 1. Overview immunotherapeutic clinical studies in vHSIL.

Author	Treatment	Study design	Phase trial	Patient number	Primary endpoint	Main immunological outcome	Main clinical outcome
Jayne 2002	Imiquimod	RS	2	13	Clinical response	Not investigated.	8/13 CR, 4/7 75% regression
Van Seters 2002	Imiquimod	PS	2	15	Clinical response	Not investigated.	27% CR, 60% PR
Todd 2002	Imiquimod	PS	2	15	Clinical response	Not investigated.	4/15 showed clinical improvement of which 3 had CR
Wendling 2004	Imiquimod	PS	2	12	Clinical response	Not investigated.	7/12 CR or PR
van Poelgeest 2005* (ref 93)	Imiquimod	PS	2	17	Immunological assay of responders to imiquimod	Systemic HPV16-specific IFN γ -associated CD4+ T cell immunity associated with CR.	Not investigated.
Le 2007	Imiquimod	PS	2	39	Clinical response	Not investigated.	64% showed CR after 16 weeks, 27% had clinically significant shrinkage.
Mathiesen 2007	Imiquimod vs placebo	RCT	2	32	Clinical response	Not investigated.	81% in the imiquimod group showed CR, 10% PR and no responses in the placebo-group
van Seters 2008 (ref 92)	Imiquimod vs placebo	RCT	2	52	Clinical response	Higher numbers of intralesional CD1a+ DCs, CD8+ T cells and CD94+ NK cells with imiquimod.	81% of all patients had >25% reduction of the primary lesion
Terlou 2010 (ref 45)	Imiquimod vs placebo	RCT	2	25	HPV presence (based on p16 IHC)	Lower intralesional CD208+, CD8+, CD4+, CD94+, CD14+ and CD68+ in the HPV cleared patients.	19/25 were HPV+ before treatment, of which 9 became HPV- after treatment.
Westermann 2013	Imiquimod	RS	2	62	Clinical response	Not investigated.	76% CR, 19% PR, 3% weak PR, 1 non-responder. Recurrent disease in 27%.

Author	Treatment	Study design	Phase trial	Patient number	Primary endpoint	Main immunological outcome	Main clinical outcome
Frega 2013	Imiquimod vs cold-knife excision	PS	2	80	Complete response, recurrence rate	Not investigated.	Recurrence rate was lower for imiquimod, relapse rate was higher. The overall CR was inferior in this group.
Kim 2015	Imiquimod	PS	2	9	Clinical response	Not investigated.	4/9 patients had CR, 2/9 PR and 3 were lost to FU.
Winters 2008 (ref 95)	Imiquimod + PDT	PS	2	20	Clinical response	Responders to imiquimod had higher Tregs before treatment, and more CD8+ T cells after treatment.	Overall response rate 55% by intention treat and 64% per protocol.
Kenter 2009 (ref 103)	HPV16 SLP vaccine	PS	2	20	Clinical response	Not investigated.	25% CR and histologic response, 35% PR at 3 months.
Daayana 2010 (ref 94)	Imiquimod + HPV16 vaccine	PS	2	19	Clinical response	After treatment with imiquimod + vaccination more local infiltration of CD4/8+ T cells was seen in the responders with lower Tregs.	32% complete response at week 10, 58% at week 20 and 63% at week 52.
Van Poelgeest 2016 (ref 105)	HPV16 SLP vaccine +/- imiquimod	RCT	2	43	Ex vivo detectable HPV16 specific CD8+ T cell response	84% showed systemic vaccine-induced HPV16-IFN γ associated CD4+ and CD8+ T cell response. No difference was found between the groups.	53% vaccine-induced CR/PR at 3 months. No difference was found between the groups.

An overview of previously performed clinical trials with imiquimod, PDT and/or vaccination in vHSIL.

RCT = randomised controlled trial, PS = prospective cohort study, RS = retrospective cohort study, CR = complete response, PR = partial response. *van Poelgeest 2005 (reference 93) and Terlou 2010 (reference 45) performed immunological analyses on data from the RCT of van Seters 2008 (reference 92).

Imiquimod

Imiquimod activates the innate immune response by interacting with toll-like receptor (TLR)7, expressed by DC, monocytes and macrophages. TLR7 activation induces secretion of proinflammatory cytokines, particularly IFN γ and also enhances DC maturation and antigen presentation.⁸² These cytokines induce polarisation towards a type 1 immune response.⁸³ Imiquimod is FDA approved for the treatment of basal cell carcinoma (BCC) and external genital warts^{84 85} but it can also be effective for the treatment of HPV-induced HSIL of the cervix, vagina and vulva.⁸⁶ In a retrospective study imiquimod was found to be effective, with complete responses between 20-81%, and partial responses of 10-75%, as well as and safe for treatment of vHSIL, although long-term response and progression of disease are unknown. The side effects of imiquimod treatment are mainly local adverse effects (itching, burning), which occur in the majority of patients, and flu-like symptoms.⁸⁷ Importantly, a randomized controlled trial (RCT) showed objective clinical responses in 81% of vHSIL patients compared to none in the placebo group.⁸⁸ In this trial, the clinical response, defined as lesion regression and HPV clearance, was associated with a pre-existing systemic HPV-specific Th1 cell response.⁸⁹ Several studies in vHSIL have shown that imiquimod induced T cell infiltration⁹⁰⁻⁹², including CD8+ T cells.^{90,93} This was associated with expression of CXCR3 and the ubiquitous cytoplasmic protein MxA, indicative of type 1 IFN signalling.⁸⁴ Non-responsiveness to imiquimod was associated with higher levels of intralesional Tregs.⁹² Another study showed that clinical responses after imiquimod were associated with increased numbers of intraepithelial CD1a+ and CD8+ cells and a decrease in Tregs in the stroma.⁹⁰ In addition, the elevated CD14+ cells found in vHSIL lesions returned to control levels in HPV-cleared patients, in contrast to non-cleared patients. A phase II study of imiquimod followed by therapeutic HPV16 E6E7L2 vaccination indicated that increased CD4+ and CD8+ was associated with clinical response to the vaccine and that relatively elevated Treg levels were observed in the group not responding to the vaccine.⁹¹

These findings suggest that a combination therapy in which the number of HPV 16 specific type 1 T cells are amplified by vaccination and the attraction of immune cells to the lesion site is fostered by local administration of imiquimod will be more successful than monotherapy with either one of the treatments.

Photodynamic therapy

Topical photodynamic therapy (PDT) is an effective and safe nonsurgical treatment option⁹⁴ that involves topical application of a non-toxic

photosensitizer, which is absorbed into neoplastic tissue. This leads to the production of reactive oxygen species and tumor cell death.⁹⁵ The acute inflammation following the clearance of destructed cells is known to contribute to prolonged recurrence free survival in advanced cancers.⁹⁵ In addition, the pro-inflammatory effects of PDT might increase DC migration, antigen uptake and maturation. PDT might also increase the immunogenicity of dead tumor cells by exposing or creating new antigens or by the induction of more effective cytotoxic T cells. Clinical studies in vHSIL have shown varying clinical and histological responses from 20-67%^{95 96}, however long-term clinical outcomes are not known.

Immunological studies of PDT in vHSIL have shown a significant CD8+ infiltration in posttreatment vHSIL responders compared to non-responders.⁹⁷ In contrast, loss or downregulation of HLA class I was associated with a lack of response to PDT. A phase II trial of sequential imiquimod and PDT in 20 women with vHSIL showed significantly higher numbers of Tregs in the lesions after imiquimod treatment in non-responders, whereas no differences in the infiltration of CD4+, CD8+, CD68+, and CD1a+ cells were observed between responders and non-responders before or after PDT treatment.⁹² Interestingly, also in this study a systemic pre-existing HPV-specific immune response was significantly correlated with response to PDT. Together, these studies suggest that the efficacy of PDT is related to immune promoting changes in the microenvironment of treated lesions.

Therapeutic vaccination

The aim of therapeutic vaccination against cancer is to launch an immune mediated attack against cancers by amplifying an anti-tumor T cell response. Therapeutic vaccines stimulate T cells against TSA created by oncogenic viruses or DNA mutations.⁹⁸

VHSIL and HPVposVSCC express the viral oncoproteins E6 and E7, and expose therefore ideal TSAs for a therapeutic vaccine.⁹⁹ In two independent clinical trials it was shown that HPV16 synthetic long peptide (SLP) vaccination of patients with HPV16-induced vHSIL resulted in complete and partial regression of the lesion(s), especially in patients who showed a stronger vaccine-induced HPV16-induced T cell response, irrespective of the immune status of these patients. The first study showed a complete response rate of 47% at 3 months.¹⁰⁰ Post hoc analyses suggested that complete responders had significantly stronger type 1 T cell response. Importantly, a subsequent RCT

confirmed that the clinical efficacy of HPV16-SLP vaccination was related to the strength of the vaccine-induced HPV-specific immune response.⁸⁹

Successful treatment of HPV16-induced vHSIL was also achieved with an HPV16 E6E7L2 fusion protein. This phase II study of combination immunotherapy of topical imiquimod followed by HPV16 E6E7L2 vaccine in women with vHSIL showed that there was significantly increased local infiltration of CD4+ and CD8+ T cells in responders, whereas non-responders showed an increased density of Tregs.⁹¹ Similarly, clinical responses after recombinant vaccinia virus encoding HPV16/18 E6E7 vaccination were associated with pre-treatment higher levels of intralesional CD4+, CD8+, and CD1a+ cells compared to non-responders. Although non-responders showed an increase in CD4+ and CD8+, but not CD1a+ cells post-vaccination, their numbers were still lower than the numbers observed in the pre-treatment samples of responders.¹⁰¹ These data suggest that therapeutic vaccines can boost the specific T cell response but are only effective in settings where the lesions have the capacity to attract substantial T cell infiltration. The identification of suitable target antigens in HPVnegVSCC is key when vaccination is considered as treatment option.

Topical treatments for dVIN and LS

Interestingly, topical treatment with pimecrolimus induces a clinical response rate in 80% of the LS patients. The response was associated with a significant downregulation of CD3+, CD8+ T cells, CD57+ NK cells, and CD68+ macrophages.¹⁰²

Intriguingly, a recent study in 507 LS patients showed that regular long-term use of topical glucocorticosteroids reduced the risk of developing dVIN or VSCC.¹⁰³ Glucocorticosteroids are steroid hormones that inhibit the NF- κ B pathway, resulting in decreased numbers of transcription factors which regulate the expression of pro-inflammatory genes (e.g. TNF α and IL-1 β) and anti-apoptotic genes.¹⁰⁴ Glucocorticoids have pro-apoptotic properties in lymphoid cells¹⁰⁵, and can selectively inhibit Tregs. Keeping in mind chronic inflammation as a hallmark of cancer¹⁰⁶, inhibition of chronic inflammation by topical steroids may underlie the reduced risk for malignant transformation in LS.

Conclusion

In this review, we summarized studies that describe the TME in VSCC, and its precursors LS/dVIN and vHSIL (**Figure 2**). The immune infiltrate in VSCC and its precursors is characterized by variable numbers of tumor infiltrating innate and adaptive immune cells. The lesions show an increase in M2 macrophages and Tregs and a decrease in activated CD8+ T cells compared to healthy controls.^{40 41} In patients with vHSIL, the presence of type 1 immunity is associated with fewer recurrences after treatment and also with better response to topical treatment with imiquimod, PDT, or therapeutic vaccination.^{40 89 97 107} Although the number of studies in vHSIL is limited and immune correlates for a treatment response need to be defined, patients with HLA-class I positive lesions, high numbers of infiltrating T cells with low numbers of Tregs, or pre-existing HPV-specific immunity, may help identify patients more likely to respond to immune therapy. Moreover, in LS high numbers of CD4+, CD8+, and Tregs are found indicating that these lesions are immunologically active and suggest that also the HPVnegVSCC might be potential candidate for future immune therapies.⁷⁰⁻⁷⁵

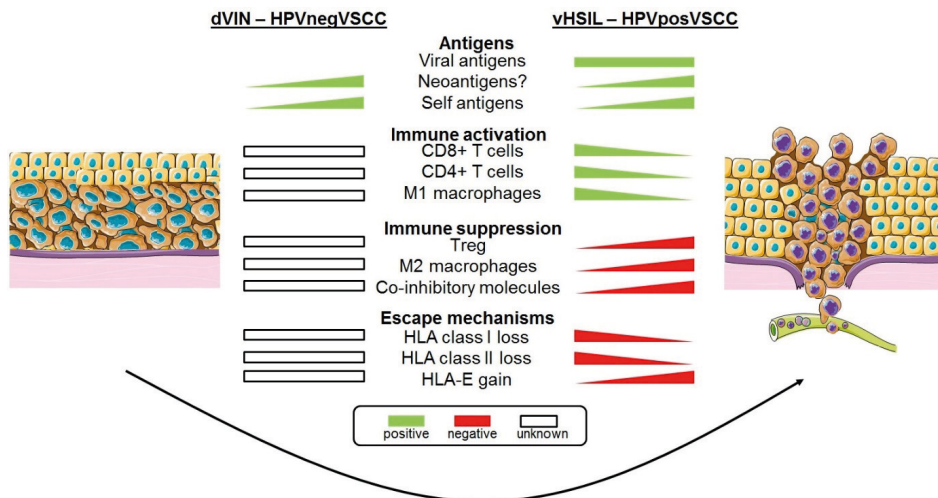


Figure 2. Overview of current literature about tumor microenvironment in VSCC and its precursors.

The immune infiltrate in VSCC and its precursors is characterized by variable numbers of tumor infiltrating innate and adaptive immune cells. HPVposVSCC's precursor lesion is vHSIL, and as progression occurs these lesions show an increase in M2 macrophages, and Tregs and a decrease in activated CD4 and CD8+ T cells. Cancer may escape the immune response due to downregulation of HLA Class I and II molecules on the cell surface and increased expression of co-inhibitory molecules. Literature on HPVnegVSCC and its precursor dVIN is scarce. Illustrations of lesions created from adapted images of Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Unported License.

Expert opinion

Tumors can exploit several immunosuppressive mechanisms to escape from the immune system, by HLA class I loss, or by creating an immune-suppressive TME, by attracting Tregs and suppressive myeloid cells as well as by secretion of immune suppressive cytokines such as TGF- β or IL-10.⁹⁸ In addition, ligation of co-inhibitory molecules expressed by T cells, including cytotoxic T-lymphocyte associated antigen-4 (CTLA-4), programmed death-1 (PD-1) and NKG2a, may occur. Overexpression of the ligand of PD-1 (PD-L1), is associated with poor clinical outcomes in many immunogenic cancers.¹⁰⁸ Monoclonal-antibody-based therapies targeting CTLA-4 and/or PD-1 (checkpoint blockade) have yielded significant clinical benefits including durable responses to patients with different malignancies. Blocking of PD-1/PD-L1 rebalances the host-tumor interaction and is successful in the treatment of many tumor types.¹⁰⁹

Cancers responsive to checkpoint inhibitors may be those with abundant tumor specific target antigens, generated by a high frequency of mutations that are recognized as non-self-peptides by CD4+ and CD8+ T cells.¹¹⁰⁻¹¹² Studies on the expression of immune checkpoint molecules in VSCC are scarce. A recent study in OPSCC showed that the percentage of CTLA-4 and PD1/PD-L1 expressing T cells was higher in TILs compared to peripheral blood mononuclear cells from patients.¹¹³ Recurrent HPV-positive tumors showed a similar composition of T cell subsets and checkpoint molecule expression as found in HPV-negative tumors. Not to our surprise, blockade of PD-1 /PD-L1 gave similar results in HPVpos- and HPVnegOPSCC.³² The absence of a better result in recurrent HPV-induced OPSCC is likely because these are the ones lacking an HPV-specific T cell response.¹¹⁴ Therapeutic HPV16 vaccination induced an immune response which was associated with clinical response in patients with HPV16-induced vHSIL.^{100 107} However, it is unlikely that therapeutic vaccines will be able to overcome the immune-suppressed microenvironment in established and advanced cancers without co-treatment. Indeed, a recent phase I/II study in HPV16-positive cancers showed that HPV16-SLP vaccination in combination with the PD1 antibody nivolumab resulted in a response rate of 36% in OPSCC patients, compared to 16% for nivolumab monotherapy in p16+ OSCC patients.^{32 33} Few studies showed the expression of PD-L1 in a subset of HPVnegVSCC.^{46 115} The presence of CD8+ T cells has been shown to be pivotal for the prediction of response to PD-L1 checkpoint inhibitors in melanoma.¹¹⁶ Although VSCC is highly infiltrated by CD8+ T cells, high numbers of just CD8+ T cells were not associated with survival.⁶¹ However,

three different studies showed that these cells needed to be activated before a positive impact on clinical outcome was detected in early stage VSCC.⁶⁵ PD-L1 expression is also found on cervical cancer cells, but not of prognostic value.¹¹⁷ Besides by tumor cells, PD-L1 is also expressed by M2 macrophages⁵⁶, which are frequently found in HPVposVSCC⁴¹, melanoma and several HPV-positive tumors.¹¹⁸⁻¹²¹ Importantly, PD-L1 positivity of peritumoral immune cells is an independent favorable prognostic factor for survival¹²², probably because it's expression is a telltale for the local production of IFN γ by activated T cells. Further effort must be made to determine the value of this and other checkpoints as predictive biomarkers in both types of VSCC.

As previously mentioned, Tregs are an immunosuppressive cell type in the TME, and some of them are shown to react to tumor antigens.¹²³ Tregs may be inactivated by treatment of patients with cyclophosphamide, or by inhibition of IDO¹²⁴ but also by antibodies against CTLA-4.¹²⁵

Recent clinical data have clearly demonstrated that human cancer cells express antigens that are newly created by mutations in the DNA encoding normal proteins (neoantigens). These mutated cancer proteins can be processed into peptides and presented on the surface of tumor cells leading to immune recognition in vivo as “non-self” or foreign by autologous tumor-specific T cells.

A few studies reported a higher incidence of somatic mutations in HPVnegVSCC compared to HPVposVSCC, correlating with increasing grades of neoplasia.¹²⁶ *TP53*, *CDKN2A* and *HRAS* were most frequently mutated, but the full range of mutations still needs to be established as current studies focused on these known hot-spots.^{11 127} In some cancers, the expression of foreign/mutated antigens correlates with increased numbers of TILs and better with clinical outcome.^{128 129} Moreover, high mutational load in tumors is associated with sensitivity to immune checkpoint inhibition and better clinical outcome.¹³⁰ These developments are highly promising and open the opportunities for precision medicine in patients with VSCC, including vaccination. On the basis of landmark preclinical studies that identified immunogenic mutations and showed survival benefit upon neoantigen vaccination in prophylactic and therapeutic settings¹⁰⁶, clinical phase I/II neoantigen cancer vaccine trials are currently ongoing in metastatic melanoma (NCT 03300843, NCT 01970358, NCT 02897765), glioblastoma (NCT 02287428), and pancreatic cancer (NCT 03122106).¹⁰⁷⁻¹⁰⁹

Combination treatment of vaccination with checkpoint inhibitors, chemotherapy, or modulation of the microenvironment by PDT or radiotherapy has already shown clinical success in different cancer types.¹³¹ Adoptive cell therapy (ACT) makes use of infusion of tumor-specific T cells into patients with advanced cancers. Currently, phase 1 clinical trials have been initiated with neoantigen-based ACT in patients with myelodysplastic syndrome (NCT03258359) or in patients with glioblastoma, non-small cell lung cancer, ovarian, and breast cancer (NCT03412877). In the light of these developments, it is to be expected that HPV16 oncoprotein-based and neoantigen-based vaccination, in combination with chemo-or radiotherapy, checkpoint blockade and/or ACT will become available for the treatment of advanced VSCC.^{132 133}

By unraveling the VSCC mutational and immune landscape, current treatments can be improved, and new immunotherapeutic interventions can be designed for HPVnegVSCC and HPVposVSCC and its precursors. Future studies should aim at whole genome sequencing in combination with in depth analysis of the immune microenvironment, not only with respect to type and numeric distribution of innate and adaptive immune cell types, but also to their functional characteristics.

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Chapter 3

Pre-existing inflammatory immune microenvironment predicts the clinical response of vulvar high-grade squamous intraepithelial lesions to therapeutic HPV16 vaccination

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Journal for ImmunoTherapy of Cancer 2020

Abstract

Background

Vulvar high-grade squamous intraepithelial lesion (vHSIL) is predominantly induced by high-risk Human Papilloma Virus type 16 (HPV16). In two independent trials, therapeutic vaccination against the HPV16 E6 and E7 oncoproteins resulted in objective partial and complete responses in half of the HPV16⁺ vHSIL patients at 12 months follow-up. Here, the pre- and post-vaccination vHSIL immune microenvironment in relation to the vaccine-induced clinical response was investigated.

Methods

Two novel seven-color multiplex immunofluorescence panels to identify T cells (CD3, CD8, Foxp3, Tim3, Tbet, PD-1, DAPI) and myeloid cells (CD14, CD33, CD68, CD163, CD11c, PD-L1, DAPI) were designed and fully optimized for formalin-fixed paraffin-embedded tissue. 29 pre- and 24 post-vaccination biopsies of vHSIL patients, and 27 healthy vulva excisions, were stained, scanned with the Vectra multispectral imaging system, and automatically phenotyped and counted using inForm advanced image analysis software.

Results

Healthy vulvar tissue is strongly infiltrated by CD4 and CD8 T cells expressing Tbet and/or PD-1 and CD14⁺HLA-DR⁺ inflammatory myeloid cells. The presence of such a coordinated pre-existing pro-inflammatory microenvironment in HPV16⁺ vHSIL is associated with complete response after vaccination. In partial responders, a disconnection between T cell and CD14⁺ myeloid cell infiltration was observed, whereas clinical non-responders displayed overall lower immune cell infiltration. Vaccination improved the coordination of local immunity, reflected by increased numbers of CD4⁺Tbet⁺ T cells and HLA-DR⁺CD14⁺ expressing myeloid cells in patients with a PR or CR, but not in NR patients. CD8⁺ T cell infiltration was not increased after vaccination.

Conclusion

A pre-vaccination inflamed type 1 immune contexture is required for stronger vaccine-induced immune infiltration and is associated with better clinical response. Therapeutic vaccination did not overtly increase immune infiltration of cold lesions.

Introduction

The immune system is key to the control of tumor outgrowth. A dense infiltration with type 1 CD4⁺ and CD8⁺ T cells in the tumor microenvironment (TME) is prognostic for longer survival and associated with a favorable response to therapy in many but not all cancers.^{1,2} Several effective strategies, including adoptive cell transfer^{3,4} and immune checkpoint blockade^{5,6} demonstrated the power of immunotherapy in cancer, making it one of the pillars of modern cancer therapy in the clinic. With the widespread testing of these immunotherapies, it has become clear that their success builds on the presence of pre-existing tumor infiltrating T cells, since both their absence and their incapability to infiltrate the tumor cell nests are associated with lack of response after immunotherapy.^{7, 8} Hence, for those patients there is a need for treatment modalities that can stimulate tumor-reactive T cell immunity and/or promote their infiltration into tumors. Here, cancer vaccines come into play, since they can increase the magnitude and width of the tumor-reactive T cell pool by stimulating both the naive T cell repertoire and the existing tumor-specific T cell population. In a recent review at least 18 trials were identified to report regression of premalignant lesions or tumors after vaccination with a series of different antigens over a spectrum of different solid tumors. In general, patients with a clinical response showed a robust or more active type 1 T cell response.⁹ In view of the fact that cancer immunity is influenced by a complex set of host, tumor and environmental factors^{10, 11} it was no surprise that vaccine-induced tumor regression was only observed in a few patients per trial.⁹

About 20% of human cancers are induced by viruses.¹² Human Papillomavirus type 16 (HPV16) is a high-risk DNA virus which causes anogenital and oropharyngeal (pre-)cancers. To increase T cell reactivity against HPV16 caused diseases, two DNA vaccines and one synthetic long peptide (SLP) vaccine have been developed to harness the immune system against the HPV16 encoded oncoproteins E6 and E7.¹³⁻¹⁶ Immunization with these vaccines resulted in the induction of potent CD4⁺ T helper type 1 (Th1) responses and CD8⁺ cytotoxic T cell responses in patients with HPV16-induced precancerous cervical lesions¹⁵⁻¹⁸ and precancerous vulvar lesions (vHSIL)¹⁹⁻²¹. Importantly, these vaccines were able to induce partial and complete regression of the lesions in a substantial number of vaccinated patients.¹⁹⁻²² More recently ISA101, an HPV16 synthetic long peptide (SLP) vaccine, showed strong biological signs of clinical activity in patients with either HPV16-positive oropharyngeal or cervical cancer.²³⁻²⁴ The HPV-specific T cell response after vaccination, as measured in the blood

of patients, was strongly correlated to clinical outcome.^{19-22, 24, 25} Despite these correlations there are still a number of patients who, based on their vaccine-induced immune response, were expected to show a clinical response but failed to do so. There may be a multitude of reasons for this, but inspired by the studies showing that checkpoint blockade is specifically successful in patients with pre-existing T cell inflamed tumors^{10, 11}, we hypothesized that this may also hold true for therapeutic cancer vaccination.

In order to address this question, we studied a group of patients treated with ISA101, about 50% of whom showed an objective regression of their HPV16-induced vHSIL.²¹ Based on our previous studies of the TME in vHSIL, demonstrating the impact of different types of immune cells on the recurrence rate of vHSIL^{26, 27}, two antibody panels for studying T cells and myeloid cells were designed and fully optimized for multispectral immunofluorescence imaging, hereby enabling unprecedented in-depth characterization of the immune composition in the TME of vHSIL biopsies before and after vaccination, and in healthy vulvar tissue for comparison. Our data revealed that complete responders to vaccination had a strong and coordinated pre-existing infiltration with type 1 T helper cells and inflammatory (CD14⁺) myeloid cells, similar to what was found in healthy vulvar tissue. In contrast, clinical non-responders displayed an intrinsic incapacity to attract immune cells as they displayed much lower overall infiltration before vaccination and this was not increased after vaccination.

Materials and Methods

Patient materials

Pre- and post-vaccination formalin-fixed paraffin-embedded (FFPE) biopsies of 29 women of ≥ 18 years old with histologically confirmed HPV16+ vHSIL were taken. These women participated in a phase I/II therapeutic HPV16 SLP vaccination trial with ISA101.²¹ The ISA101 vaccine was injected subcutaneously and consists of 13 SLPs covering the entire amino acid sequence of HPV16 oncoproteins E6 and E7, dissolved in dimethylsulfoxide in 20 mM of phosphate-buffered saline and emulsified with Montanide adjuvant (ISA-51 Seppic). Clinical efficacy assessments in the trial were performed at 3 and 12 months after the fourth and last vaccination. The patients were grouped into the best clinical response at 12 months after vaccination without additional treatment: complete responders (CR) when the lesion completely disappeared, partial responders (PR) when $\geq 50\%$ of the total lesion area had disappeared, and non-responders

(NR) when <50% of the lesion had disappeared. The presence of HPV16 DNA in the lesion was determined by HPV16 PCR analysis. Furthermore, FFPE healthy HPV-negative vulvar tissue from 27 anonymous women who underwent labia reduction surgery was included. All patients gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki and approved by the national Central Committee on Research Involving Human Subjects (CCMO, NL21215.000.08) and the local institutional ethical committee (P08.197).

Multiplex immunofluorescence imaging

The specificity of each primary antibody was first assessed with immunohistochemistry, tonsil slides served as positive and negative control. After selection of the best primary antibodies, the immunodetection conditions for each marker in seven color immunofluorescence were optimized. Dim markers were tyramide signal amplified with Opal to enable their detection by fluorescence microscopy, and primary antibodies with clashing species and isotypes in the panel were directly labeled with fluorochromes or detected with species/isotype-specific antibodies.²⁸ A complete overview of the design of the T cell panel and myeloid cell panel is presented in **Supplemental Files 1 and 2**, respectively. In the seven color immunofluorescent procedure, 4µm FFPE tissue sections were deparaffinized, endogenous peroxidase was blocked with hydrogen peroxide, and heat induced epitope retrieval was performed with citrate (10mM, pH 6.0) in the T cell panel and with tris-EDTA (10mM/1mM, pH 9.0) in the myeloid cell panel. Non-specific binding sites were blocked with SuperBlock (ThermoFisher Scientific). First, the antibodies detected by Opal were applied and subjected to the recommended tyramide signal amplification protocol, followed by the unconjugated antibodies which were incubated overnight. On the second day, after detection of the previous with respective fluorescently labelled secondary antibodies, the directly labeled primary antibodies were incubated for 5 hours and, lastly, DAPI was applied as nuclear counterstain.²⁸

Quantification of immune cells in the TME

Immunofluorescence images were acquired with the Vectra 3.0.5 multispectral imaging microscope (PerkinElmer) at 20x magnification and exposure times were set to avoid spectral overlap. Immune cells in the TME were automatically phenotyped and counted with inForm 2.4 image analysis software (PerkinElmer), after manual training and validation of procedures. The software was trained to segment epithelial and stromal fractions, segment DAPI+ nucleated cells, and to assign a phenotype to each cell (**Supplemental File 3a**). All images

were visually inspected on accurateness in segmenting tissue and phenotyping cells, and if errors were detected the training was further optimized. Given the multitude of possible co-expressed markers, each seven color panel was divided into multiple sub-analyses (**Supplemental File 3b**) to preclude the exclusion of relevant phenotypes, after which the phenotypes of the different sub-analyses were merged per cell based on X,Y-positions to obtain the full seven marker expression profile of each cell. Immune cell counts were normalized for tissue size (cells/mm² epithelium and cells/mm² stroma). After merging all sub-analyses, a threshold of a median cell count ≥ 10 cells/mm² was applied to enable the analysis of relevant phenotypes.

Statistical analysis

Statistical data analysis was performed with SPSS 25.0 (IBM Corporation). Immune counts of two groups were compared with the non-parametric Mann-Whitney U test. Pearson correlation was used to study correlations between the T cell infiltrate and the myeloid cell infiltrate in each group (clinical non-responders, partial responders, complete responders and healthy donor vulva). Two sided p-values <0.05 were marked as significant. GraphPad Prism 8.0.1 (GraphPad Software Inc.) was used to create graphs.

Results

Healthy vulvar tissue is predominantly infiltrated by type 1 activated T cells and inflammatory macrophages

Correct interpretation of the immune infiltration in precancerous vulvar lesions requires a profound understanding of the immune microenvironment in healthy vulvar tissue. In-depth characterization of the immune infiltrate was performed with two panels of antibodies to identify different subsets of T cells and myeloid cells in the epithelium and stroma, using multispectral immunofluorescence imaging (**Figure 1ab and Supplemental File 4**). Analysis of 27 healthy HPV-negative vulvar tissues revealed that normal vulvar tissue is strongly infiltrated with T cells and myeloid cells, albeit at variable levels between individuals (**Figure 2a and Supplemental File 5**). The intraepithelial infiltrate was dominated by type 1 Th cells (CD3⁺CD8⁺Foxp3⁻) expressing Tbet and a smaller fraction of which also expressed PD-1 as well as by inflammatory myeloid cells (CD14⁺ cells). A similar pattern was observed in healthy vulvar stroma, however, here also high numbers of M1 (CD68⁺CD163⁻) and M2 (CD68⁺CD163⁺) macrophages were found and the number of inflammatory

myeloid cells (CD14⁺) superseded any other immune cell count by far. This data suggests that healthy vulvar tissue is a highly inflammatory microenvironment comprising many type 1 (Tbet⁺) and activated (PD-1⁺) T lymphocytes and inflammatory macrophages.

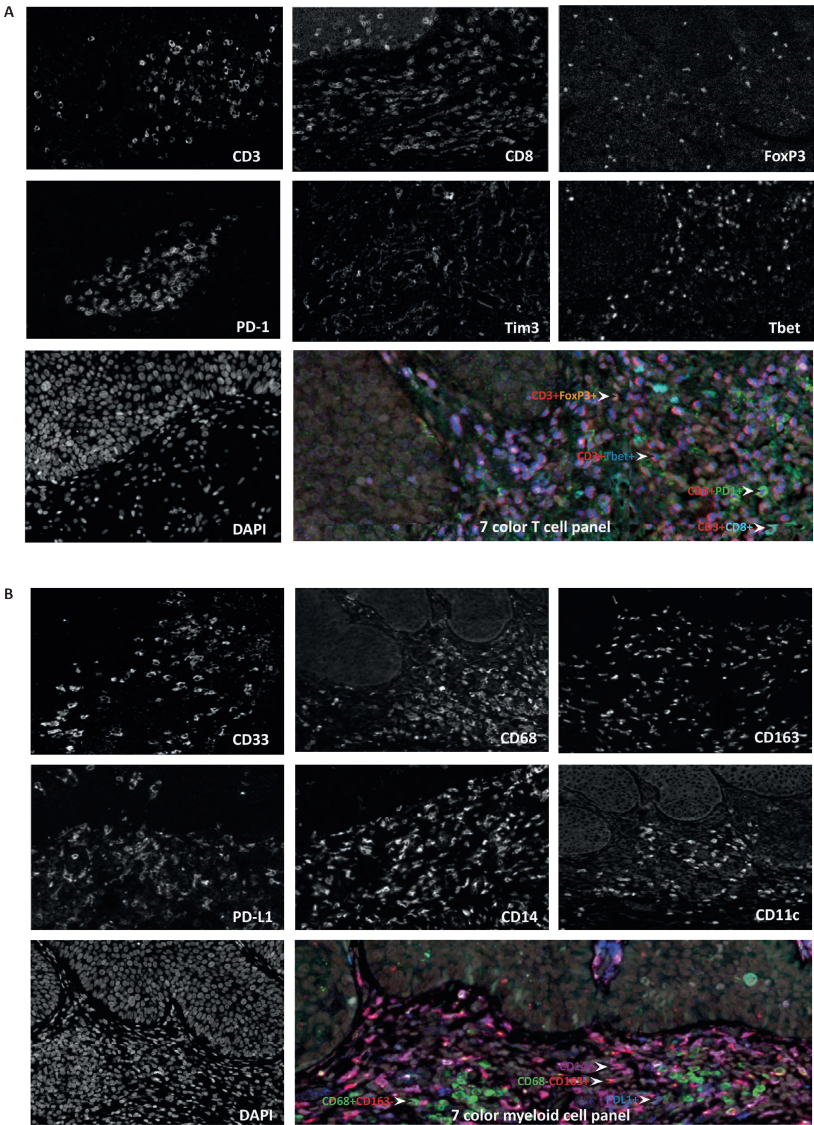


Figure 1. Multiplex immunofluorescence staining to detect T cells and myeloid cells.

Two multiplex panels were developed to detect T cells and myeloid cells in vulvar tissue.

A) The expression of each of the 7 markers for T cells showing CD3, CD8, FoxP3, Tbet, Tim3, PD-1 and DAPI as well as a figure showing all 7 markers simultaneously. Arrows indicate examples of analyzed phenotypes. **B)** The expression of each of the 7 markers for myeloid cells showing CD33, CD68, CD163, PD-L1, CD14, CD11c and DAPI as well as a figure showing all 7 markers simultaneously. Arrows indicate examples of analyzed phenotypes.

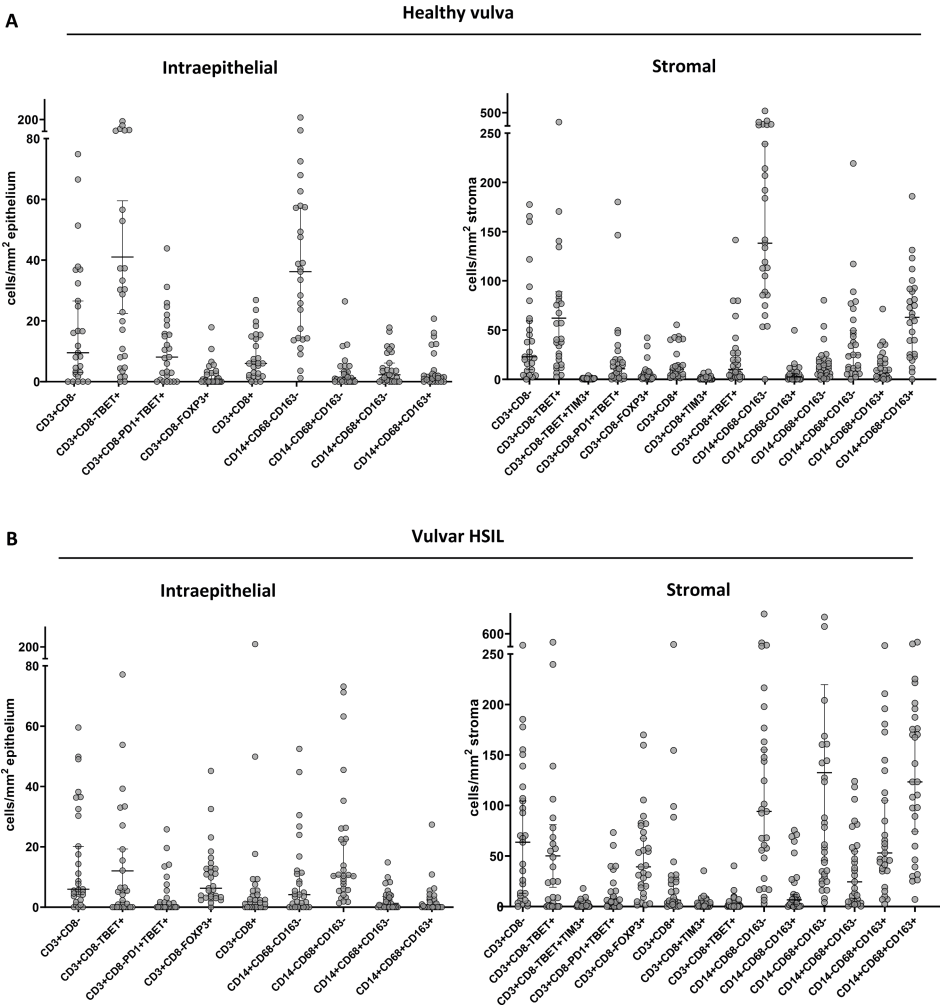


Figure 2. Healthy vulvar tissue is infiltrated by activated type 1 T cells and several types of myeloid cells while the vHSIL immune infiltrate is highly heterogeneous amongst different patients.

The numbers of intraepithelial and stroma infiltrating T cell and myeloid cell subtypes, of which the median cell count exceeded the threshold of ≥ 10 cells/mm², are presented as cells/mm² for **A**) HPV-negative healthy vulva (n=27) and **B**) vHSIL (n=29) before vaccination. Each dot represents an individual sample, the horizontal bars indicate the median cell counts, the vertical bars are the 95% confidence intervals.

Strong variations in the lymphoid and myeloid immune infiltration of pre-vaccination vHSIL

We were able to include 29 of the 34 vaccinated vHSIL patients²¹ for the current analysis (**Table 1**). Two patients could not be included due to unavailability of FFPE tissue and three due to unknown response 12 months after vaccination. Analyses of the multispectral immunofluorescence imaging for T cells and

myeloid cells revealed that despite the same etiology, the 29 vHSIL patients formed a heterogeneous group as the counts of immune cells infiltrating the epithelium and stroma strongly differed per patient (**Figure 2b and Supplemental File 6**). The overall pattern of intraepithelial and stromal immune cell infiltration resembled that found in healthy tissue. However, whilst 55% of the healthy vulvar tissue-infiltrating CD14⁺ myeloid cells expressed HLA-DR, this was only 26% in the vHSIL group (**Supplemental File 7**).

Table 1. Patient characteristics pre-vaccination and best clinical response during 12 months follow-up after therapeutic HPV16 SLP vaccination.

Patient study number	Lesion size (cm ²)	Histology	HPV16	Best response without additional treatment	Pre-vaccination biopsy analyzed	3 months post-vaccination biopsy analyzed
1	15,5	VIN3	+	PR	+	+
3	3,0	VIN2	+	CR	+	.*
6	8,0	VIN3	+	PR	+	+
9	3,5	VIN3	+	CR	+	+
10	4,0	VIN2	+	PR	+	+
11	25,3	VIN3	+	NR	+	+
14	0,3	VIN3	+	CR	+	+
15	2,0	VIN2	+	NR	+	+
18	2,0	VaIN3	+	NR	+	+
19	4,5	VIN3	+	NR	+	+
20	2,0	VIN2	+	PR	+	+
21	6,5	VIN3	+	NR	+	-
22	2,0	VIN3	+	NR	+	+
24	6,2	VIN3	+	NR	+	+
26	1,0	VIN2	+	NR	+	+
28	9,5	VIN3	+	NR	+	+
29	4,2	VIN2	+	PR	+	+
30	10,3	VIN3	+	PR	+	-
32	1,5	VIN3	+	CR	+	.*
51	19,0	VIN2	+	NR	+	+
52	120,0	VIN3	+	NR	+	+
53	42,0	VIN3	+	PR	+	+
55	104,0	VIN3	+	PR	+	+
57	3,0	VIN3	+	NR	+	+
58	44,0	VIN3	+	CR	+	.*
59	8,0	VIN3	+	NR	+	+
61	12,0	VIN3	+	CR	+	+
62	3,0	VaIN3	+	PR	+	+
63	3,0	VIN3	+	PR	+	+

NR=no response (<50% lesion size reduction), PR=partial response (≥50% lesion size reduction), CR=complete response (100% lesion clearance). The data were extracted from our previous report.²¹

*vHSIL already cleared in biopsy

The vHSIL patients were grouped according to their best clinical response obtained without additional treatment during the 12 months follow-up, into CR (n=7), PR (n=10) or NR (n=12). Plots of the data according to clinical outcome revealed a stepwise increase in the median number of intraepithelial and stromal CD8⁺ T cells, CD4⁺ (CD3⁺CD8⁻Foxp3⁻) Tbet⁺ Th cells and CD14⁺ inflammatory myeloid cells as clinical outcome improved, while that of Tregs stepwise decreased (**Figure 3a and Supplemental File 6**). In order to ensure that the CD3⁺CD8⁻ T cells were indeed CD4⁺ T cells, we analyzed a subset of NR, PR and CR patients for the expression of CD3⁺, CD4⁺ and CD8⁺, confirming that on average 99% of the CD3⁺CD8⁻ T cells were CD4⁺ T cells (**Supplemental File 8**). Interestingly, the immune infiltration of pre-vaccination vHSIL in the CR group most closely resembled that of healthy vulvar tissue, whereas this was the opposite for the NR group.

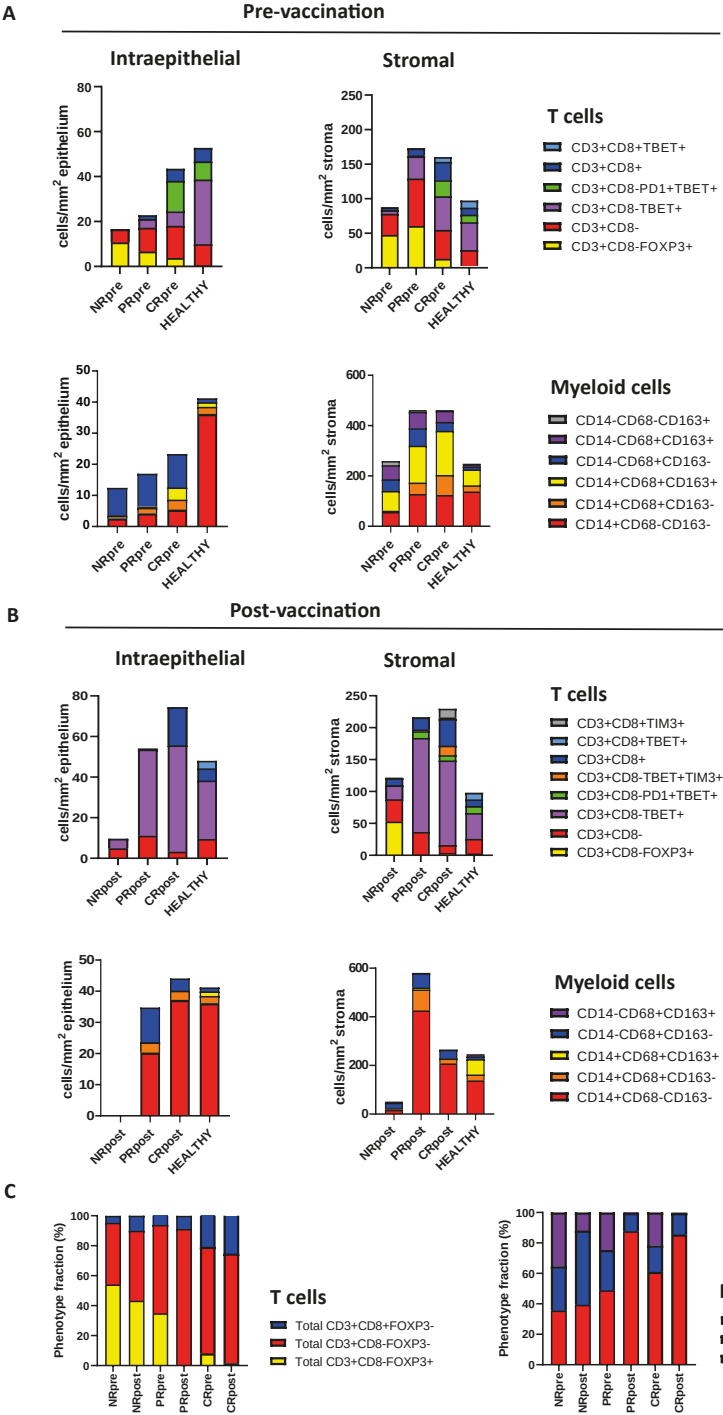


Figure 3. The TME of complete responders displays a similar immune infiltration pattern as found in healthy vulvar tissue.

*The numbers of intraepithelial and stroma infiltrating T cell and myeloid cell subtypes, of which the median cell count exceeded the threshold of ≥ 10 cells/mm², are presented as cells/mm² in the **A)** pre-vaccination (n=29) and **B)** post-vaccination (n=24) vHSIL biopsies of vaccinated patients grouped according to their best clinical response during the 12 months of follow-up, and compared to healthy vulvar tissue (n=27). CR=complete responders (n=7); PR=partial responders (n=10); NR=non-responders (n=12). Statistical differences in the cell types between patient groups are depicted in Supplemental Files 6 and 9. **C)** Highlighted T cell and myeloid cell subtypes that significantly differed amongst different response groups, shown as fractional differences in all CD3⁺ T cells (left) or all CD14⁺ and CD68⁺ myeloid cells (right), pre- and post-vaccination.*

Coordinated high infiltration with CD4 and CD8 type 1 T cells and inflammatory myeloid cells identifies the complete responders to therapeutic vaccination

To gain a better insight in the coordination of the local immune response, we plotted the correlation between the absolute intraepithelial and stromal immune cell counts for the PR, CR and healthy donor tissues (**Figure 4**), and not for the NR group as this would be uninformative due to the overall low cell counts. This analysis shows that there are strong correlations between the number of infiltrating CD8⁺ T cells and Th cells, as well as between the different types of myeloid cells; not only within a tissue segment (epithelium or stroma) but also between both tissue segments in healthy vulva and in complete responders (**Figure 4**). Importantly, there also is a strong correlation between the number of myeloid cells and CD8⁺ T cells (healthy, CR) and Th1 cells (CR), suggesting a coordinated immune infiltration of T cells and myeloid cells in both healthy vulvar tissue and in vHSIL of patients who will respond well to therapeutic vaccination. In contrast, there is no or a negative correlation between the vHSIL-infiltrating T cells and myeloid cells in patients with a therapeutic vaccine-induced PR (**Figure 4**), suggesting that either T cells or myeloid cells are underrepresented in the pre-vaccination vHSIL immune microenvironment of PR patients.

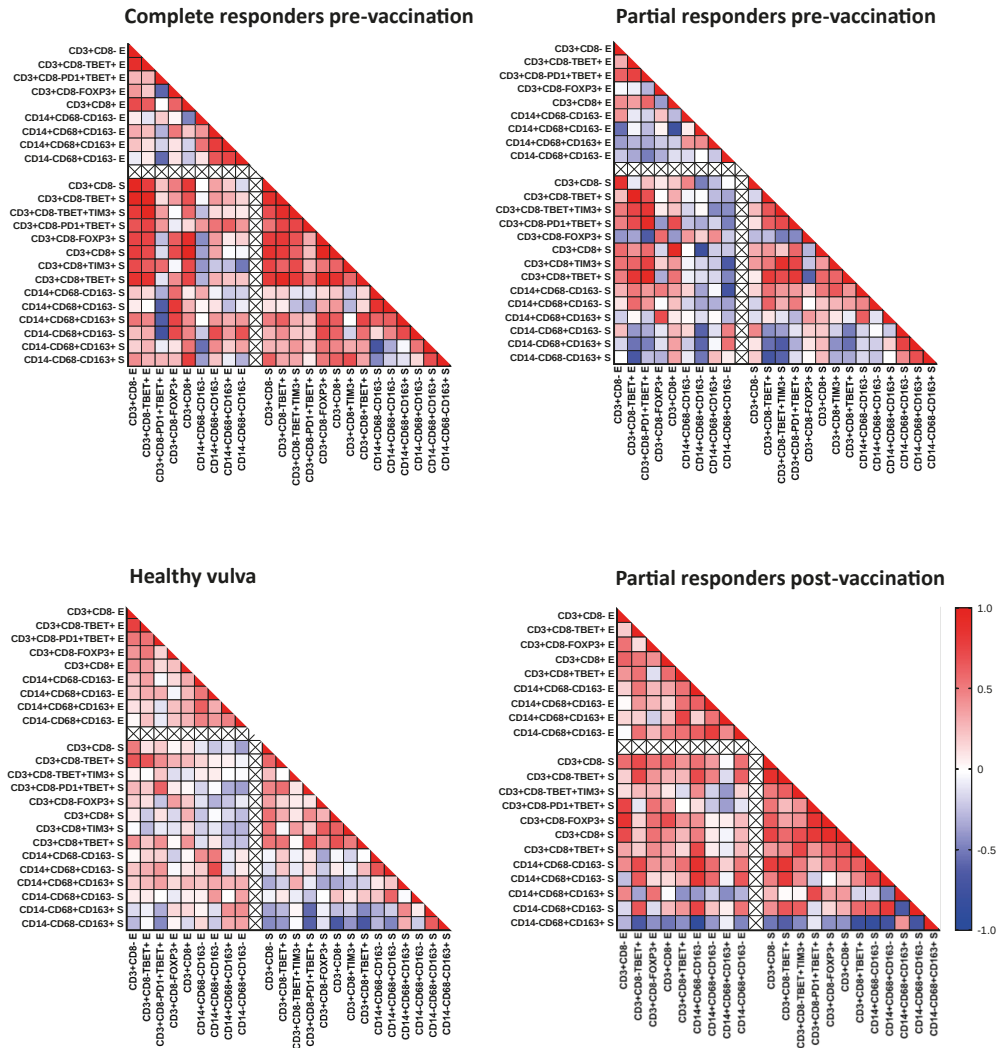


Figure 4. Correlation between the absolute numbers of tissue infiltrating T cells and myeloid cells in the pre- and post-treatment vHSIL biopsies and in healthy vulvar tissue.

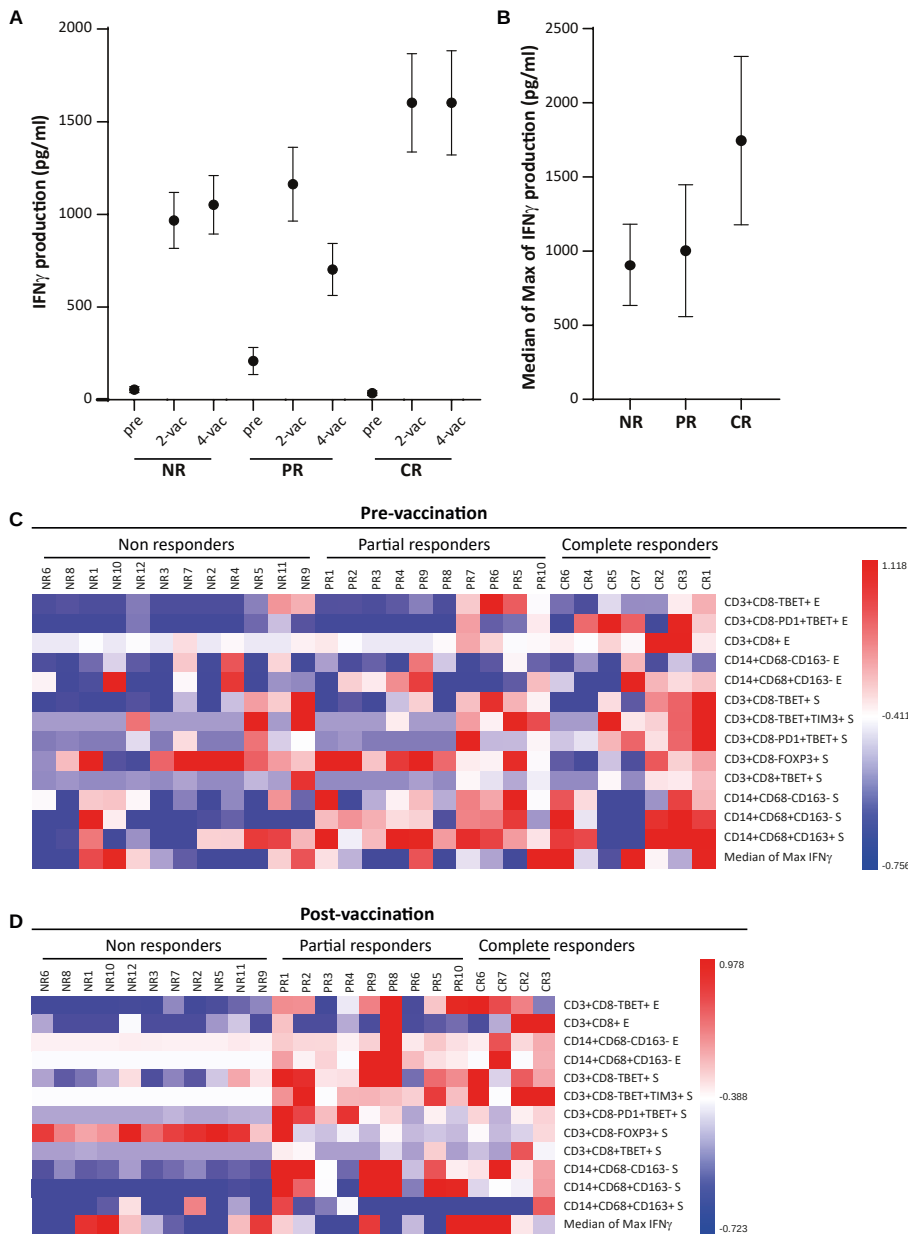
Non-parametric Spearman r correlation analysis (two-tailed) was performed to analyze the co-infiltration of the indicated different immune cell subtypes in the epithelium (E) and stroma (S) and is shown in a heatmap for healthy vulvar tissue ($n=27$), pre-vaccination complete responder vHSIL patients ($n=7$) and partial responder vHSIL patients ($n=10$) as well as post-vaccination partial responder vHSIL patients ($n=9$).

Therapeutic vaccination does not overcome a “cold” low-infiltrated immune microenvironment

Three months post-vaccination all vHSIL patient were re-biopsied, except for 3 CR patients whom already reached complete response at three months follow-up, and for one NR and one PR patient of whom no post-vaccination biopsy material was available. Consequently, we were able to analyze the post-vaccination biopsies of 11 NR patients, 9 PR patients and 4 CR patients in the same manner as the pre-vaccination biopsies were analyzed. This revealed a strong increase in the number of CD3⁺CD8⁺Tbet⁺ Th cells and a decrease in Tregs (**Figures 3b and 3c, Supplemental File 9**), as well as a marked increase in CD14⁺ inflammatory myeloid cells and decreased numbers of M2 macrophages (**Figure 3b and 3c, Supplemental File 9**) in the vHSILs of patients with a PR or CR after vaccination. Notably, not only was the number of CD14⁺ inflammatory myeloid cells increased, but also the proportion of CD14⁺ cells expressing HLA-DR (**Supplemental File 7**), suggesting that these cells provide local help to the activation of the Th1 response. While the vHSILs of NR patients displayed only minor changes in these subsets (**Figure 3b and 3c, Supplemental File 9**), vaccination improved the coordinated immune infiltration of T cells and CD14⁺ myeloid cells in PR patients (**Figure 4**). Last but not least, whereas the vHSIL of CR patients displayed both intraepithelial and stromal CD8⁺ T cell infiltration after vaccination, similar to what is seen in healthy vulvar tissue, the vHSIL of PR patients did not (**Figures 3b and 3c**). Notably, 2 out of 3 CR patients who could not be analyzed because their vHSIL disappeared within 3 months follow-up, already displayed high numbers of Tbet⁺ Th cells (79 respectively 460 cells/mm² stroma), CD8⁺ T cells (44 respectively 107 cells/mm² stroma), CD14⁺ inflammatory myeloid cells (316 respectively 546 cells/mm² stroma).

We have previously shown that patients with a CR displayed an overall stronger systemic IFN γ -associated proliferative response after vaccination²¹, and this was also observed after 2 or 4 vaccinations in the currently selected cohort when the average production of IFN γ to all peptide pools of all patients in the groups was compared (**Figure 5a**), and when the average IFN γ production was depicted as the median of the maximal IFN γ production after either 2 or 4 vaccinations to all 6 E6 and E7 peptide pools per patient (**Figure 5b**). In order to understand the relative differences in immune infiltration and vaccine-induced T cell response between NR, PR and CR patients, we z-transformed the data of those immune cell types of which the counts significantly differed between the patient groups either pre- or post-vaccination, as well as that of the vaccine-specific IFN γ production (**Figure 5cd**). This revealed a propensity

of clinical responders to have pre-existing stromal infiltration with CD4⁺ T cells (CD3⁺CD8⁻) and CD14⁺ inflammatory myeloid cells. Furthermore, it suggests that in case a CD14⁺ inflammatory myeloid cell infiltration is already present a strong vaccine-induced immune response is not required (**Figure 5c**). This profile was even more clear after vaccination, where clinical responsiveness was clearly correlated with the intraepithelial and stromal presence of type 1 CD4⁺ T cells (CD3⁺CD8⁺Tbet⁺) and CD14⁺ inflammatory myeloid cells lacking the expression of CD163 as well as the relative absence of Tregs (**Figure 5d**). The absence of such a coordinated response before vaccination and the failure to alter this by vaccination, explains why some of the patients with a strong vaccine-induced T cell response still displayed a NR. Thus a generally strong and coordinated immune infiltration present already before or after vaccination in the TME by type 1 CD4⁺ T cells and CD14⁺ inflammatory myeloid cells was associated with a better clinical response to vaccination.



tested, before and after 2 and 4 vaccinations; and **B**) the Median of Max calculated by taking the median of the highest responses to each peptide pool after 2 or 4 vaccinations. Shown is the mean $\pm$ SEM of all medians per group. **C and D**) The cell counts of all immune subsets of which the median cell count exceeded the threshold of ≥ 10 cells/mm² and the numbers significantly differed between at least two of the patient groups (NR, PR, CR) either before vaccination (C) or after vaccination (D) as well as the Median of Max of IFN γ production as determined by cytometric bead array are depicted after the data was z-transformed to enable comparison of relative differences.

Discussion

Our aim was to investigate if the immunological make up of HPV16-induced vHSIL was of influence on the clinical response of patients to an HPV16 SLP vaccine, shown to be active in two separate trials.^{19, 21} Our study is the first to show that a coordinated immune infiltration by activated type 1 CD8⁺ and T helper cells as well as HLA-DR⁺CD14⁺ myeloid cells is associated with a complete regression of vHSIL after vaccination. In a previous study, no difference was found in the numbers of pre-treatment vHSIL-infiltrating CD4⁺ and CD8⁺ T cells between patients with or without a complete clinical response after vaccination with an HPV16-E6E7L2 fusion protein vaccine.²⁹ This can be explained by the fact that these patients were pre-treated with imiquimod²⁹, which is known to induce strong local inflammation³⁰ that is reflected by infiltration with CD4⁺ and CD8⁺ T cells, activated DCs and macrophages³¹, thereby recreating the desired immune microenvironment. But also because no functional assessment of the infiltrating T cells was performed, while our data clearly point at the role of CD4⁺ Tbet⁺ (type 1) Th cells. An impact of the pre-existing immune microenvironment on clinical outcome was also not found in cervical HSIL patients vaccinated with the HPV16/18 vaccine VGX-3100, but here only the number of CD8⁺ T cells and Tregs was assessed²⁵ whereas it is known that also CD14⁺ inflammatory myeloid cells are associated with better prognosis in cervical cancer¹⁷ and as such are likely to play a similar role in its precursors. A coordinated response of T cells and inflammatory myeloid cells was also found to be required for the regression of tumors in the HPV16-positive tumor mouse models TC-1 and C3.³² Last but not least, we used software that performed automatic tissue segmentation and cell phenotyping, enabling the analysis of the entire tissue slide thereby precluding selection bias of analyzed areas, and also preventing potential inter-observer variation.

Our analyses revealed that the pre-existing vHSIL-immune infiltrate of complete responders resembled that found in healthy vulvar tissue, whereas the immune infiltrate in vHSIL of PR and NR patients clearly differed. The vHSIL of NR

patients showed an overall low immune cell infiltration except for Tregs which were most abundant in this patient group, indicating that these lesions lacked inflammation and were immune suppressed, hence they can be branded as relatively immune deserted/immunosuppressed.¹⁰ Since healthy vulvar tissue comprises an actively inflamed immune microenvironment, the absence of it in the TME of NR must be labeled as aberrant. The underlying mechanism for this is currently unknown but potentially the answer can be found in the genomic instability of the lesions, which is often found on top of the expression of the HPV16 oncoproteins³³⁻³⁵, and has been related to immune evasion and reduced response to immunotherapy³⁶. Analyses of the post-vaccination samples at 3 months of follow-up made it clear that therapeutic vaccination was incapable of overcoming the “cold” local microenvironment that is present in non-responders. The intrinsic or extrinsic escape mechanism underlying this incapacity to attract immune effector cells is part of future studies.

While the vHSIL of partial responders generally was infiltrated more densely with immune cells, a correlation analysis of the pre-existing immune cell infiltrate revealed that there was either no or a negative correlation between the numbers of vHSIL-infiltrating T cells and myeloid cells in this PR group. Some of them were relatively strongly infiltrated with T cells, a phenotype previously found to be correlated with partial responses of vHSIL after vaccination³⁷, whereas others displayed relatively high levels of myeloid cells only. After vaccination, strong increases in both type 1 Th cells and HLA class II-positive inflammatory macrophages were observed in the vHSIL of patients with a PR or CR at 12 months of follow up. This coincided with lower numbers of Tregs and M2 macrophages. Hence, therapeutic vaccination can incite and modulate inflamed lesions so that the immune infiltration becomes more coordinated and the effector cells increase to similar or higher numbers when compared to healthy vulvar tissue. In this aspect it does not differ from other T cell based approaches, including adoptive T cell transfer³⁸ and checkpoint blockade^{10, 39} all of which have the best possible clinical impact when a tumor is inflamed or hot before start of therapy. The HPV16 SLP vaccine did not increase the numbers of vHSIL-infiltrating CD8⁺ T cells, which were present already in the vHSIL that became a CR but not in vHSIL ending up as PR.

Finally, several trials with therapeutic vaccines against HPV-induced HSIL lesions have reported overall high immunogenicity of the vaccine but complete clinical responses in only part of the treated subjects.^{19-22, 29, 37, 40} The data of this study suggests that the HSIL microenvironment has an impact on the vaccine-

induced clinical outcome and as such calls for the prospective validation of the combination of CD4⁺ cells, Tbet⁺ cells and CD14⁺ myeloid cells as predictive biomarkers for clinical response of HSIL to therapeutic vaccination. These biomarkers can easily be applied in routine diagnostics, as they only require routinely taken FFPE tissue and regular single immunohistochemical stainings. The combination of multiple biomarkers is likely to increase the sensitivity of the prediction tool.⁴¹ Furthermore, our data strongly suggest that the efficacy of therapeutic vaccines in this patient group can be increased when it is combined with other drugs that induce local acute inflammation, to support the infiltration of vaccine-activated T cells and inflammatory myeloid cells. Hence, our findings show that the current paradigm of personalized cancer immunotherapy also applies to therapeutic vaccines for the treatment of patients with virally induced lesions as well as provides a foundation for future combination therapy studies.

Supplemental Files

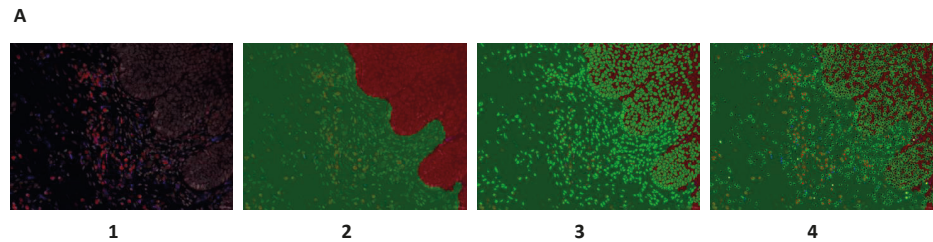
Supplemental File 1. Design T cell panel, antibodies and detection method per marker.

Marker	Primary antibody clone and company	Primary antibody isotype	Secondary antibody	Detection method
PD-1	D4W2J Cell Signaling Technology	Rabbit IgG	Poly-HRP and Opal 570	Opal tyramide signal amplification
Tbet	D6N8B XP Cell Signaling Technology	Rabbit IgG	Poly-HRP and Opal 520	Opal tyramide signal amplification
FoxP3	236A/E7 Invitrogen ThermoFisher	Mouse IgG1	CF goat anti mouse IgG1 633	Secondary antibody fluorochrome labelled
CD8	4B11 Novocastra	Mouse IgG2b	Alexa goat anti mouse IgG2b 647	Secondary antibody fluorochrome labelled
Tim3	D5D5R XP Cell Signaling Technology	Rabbit IgG	Alexa goat anti rabbit IgG 680	Secondary antibody fluorochrome labelled
CD3	D7A6E Cell Signaling Technology, directly labelled with Alexa 594	Rabbit IgG	-	Directly fluorochrome labelled primary antibody
DAPI	ThermoFisher	-	-	Fluorescent probe

Supplemental File 2. Design myeloid cell panel, antibodies and detection method per marker.

Marker	Primary antibody clone and company	Primary antibody isotype	Secondary antibody	Detection method
PD-L1	SP142 Spring Bioscience	Rabbit IgG	Poly-HRP and Opal 520	Opal tyramide signal amplification
CD14	D7A2T Cell Signaling Technology	Rabbit IgG	CF donkey anti rabbit IgG 633	Secondary antibody fluorochrome labelled
CD33	PWS44 Novocastra	Mouse IgG2b	Alexa goat anti mouse IgG2b 647	Secondary antibody fluorochrome labelled
CD163	10D6 Invitrogen ThermoFisher	Mouse IgG1	CF goat anti mouse IgG1 680	Secondary antibody fluorochrome labelled
CD11c	EP1347Y Abcam, directly labelled with Alexa 546	Rabbit IgG	-	Directly fluorochrome labelled primary antibody
CD68	D4B9C XP Cell Signaling Technology, directly labelled with Alexa 594	Rabbit IgG	-	Directly fluorochrome labelled primary antibody
DAPI	ThermoFisher	-	-	Fluorescent probe

Supplemental File 3. Inform analyses.



A) Multispectral immunofluorescence image analysis workflow with inForm , representative example of a subanalysis . InForm uses a stepwise training approach, each step adding a layer of differentiation.

Step 1: Signal extraction	(example subanalysis here shows CD68 in red, CD163 in blue)
Step 2: Tissue segmentation	(epithelium in red, stroma in green)
Step 3: Cell segmentation	(cells are marked in light green)
Step 4: Cell phenotyping	(CD68 CD163 cells are assigned a red dot, CD68 CD163+ cells a blue dot, CD68 CD163- cells a yellow dot, and CD68 CD163- cells a black dot)

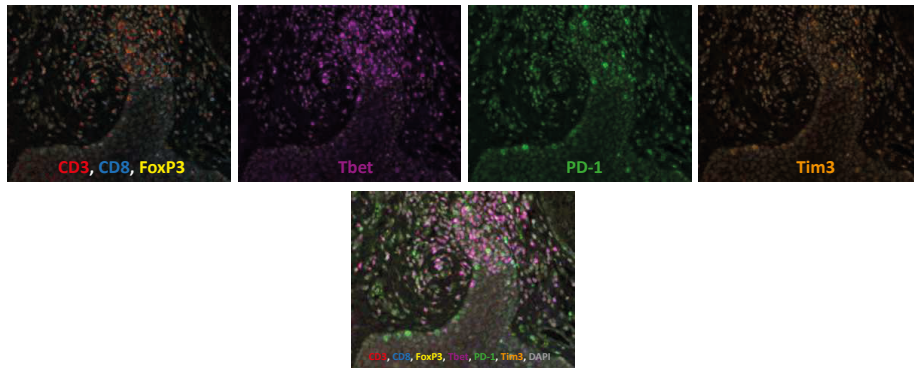
B

T cell panel	Myeloid cell panel
<u>Subanalysis 1:</u> CD3 ⁺ CD8 ⁻ FoxP3 ⁻ (T helper cell) CD3 ⁺ CD8 ⁺ FoxP3 ⁻ (CD8 ⁺ T cell) CD3 ⁺ CD8 ⁻ FoxP3 ⁺ (regulatory T cell) CD3 ⁻ CD8 ⁻ FoxP3 ⁻ (no T cell)	<u>Subanalysis 1:</u> CD68 ⁺ CD163 ⁻ (M1 macrophage) CD68 ⁺ CD163 ⁺ (M2 macrophage) CD68 ⁻ CD163 ⁺ (CD163 ⁺ non-macrophage) CD68 ⁻ CD163 ⁻ (non-macrophage)
<u>Subanalysis 2:</u> Tbet ⁺ (IFN γ producing T cell) Tbet ⁻ (Tbet ⁻ T cell)	<u>Subanalysis 2:</u> CD11c ⁺ CD14 ⁻ (dendritic cell, DC) CD11c ⁺ CD14 ⁺ (inflammatory DC) CD11c ⁻ CD14 ⁺ (inflammatory myeloid cell) CD11c ⁻ CD14 ⁻ (CD11c ⁻ and CD14 ⁻ myeloid cell)
<u>Subanalysis 3:</u> PD1 ⁺ (activated PD1 expressing T cell) PD1 ⁻ (PD1 ⁻ T cell)	<u>Subanalysis 3:</u> CD33 ⁺ PDL1 ⁻ (immature myeloid cell) CD33 ⁻ PDL1 ⁺ (mature myeloid cell expressing PDL1) CD33 ⁻ PDL1 ⁻ (CD33 ⁻ and PDL1 ⁻ myeloid cell)
<u>Subanalysis 4:</u> Tim3 ⁺ (activated Tim3 expressing T cell) Tim3 ⁻ (Tim3 ⁻ T cells)	

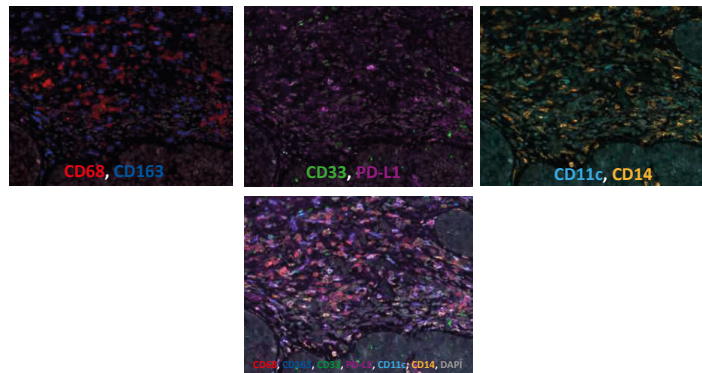
B) Subanalyses of the T cell and myeloid cell panel as used in inForm . The full seven marker panels were divided into multiple subanalyses , to preclude the exclusion of possible relevant phenotypes. Subsequently, the output of the multiple subanalyses were merged per cell based on X,Y positions to obtain the full seven marker expression profile of each cell.

Supplemental File 4. Examples of immune cell subtypes analyzed.

A



B



A) Analysis of the T cell panel consisted of four sub-analyses; first CD3, CD8, FoxP3 positivity was assessed, secondly Tbet, thirdly PD-1, and fourthly Tim3.

B) Analysis of the myeloid cell panel consisted of three sub-analyses: first CD68 and CD163 positivity was assessed, secondly CD33 and PD-L1, and thirdly CD11c and CD14.

Supplemental File 5. Descriptive statistics of immune cells in healthy vulvar tissue.

Immune cell phenotype	Number of cells/mm2 epithelium (median with 95% CI)	Number of cells/mm2 stroma (median with 95% CI)
CD3+CD8-FoxP3-	57.62 (13.81-123)	43.49 (8.67-70.51)
CD3+CD8+FoxP3-	8.52 (3.49-29.13)	17.38 (6.33-21.87)
CD3+CD8-FoxP3+	2.98 (0.81-7.15)	5.23 (1.09-9.90)
Tbet+	100.6 (56.59-176)	106.9 (29.93-139)
PD1+	44.89 (22.53-92.33)	32.10 (23.48-47.29)
Tim3+	14.76 (10.44-19.52)	23.48 (17.31-31.79)
CD68+CD163-	5.20 (3.59-18.59)	52.89 (17.07-106)
CD68-CD163+	0.00 (0.00-1.99)	13.45 (9.53-34.62)
CD68+CD163+	3.78 (1.79-22.32)	96.74 (67.84-140)
CD11c+CD14-	0.54 (0.00-1.73)	11.62 (4.77-20.59)
CD11c-CD14+	50.97 (17.87-99.03)	286.60 (196-526)
CD11c+CD14+	0.00 (0.00-0.00)	0.88 (0.00-3.32)
CD33+PDL1-	16.92 (5.38-35.73)	39.23 (28.22-75.49)
CD33-PDL1+	3.87 (1.20-20.72)	25.33 (9.97-67.06)

N=15 healthy vulvar tissue samples; CI=confidence interval

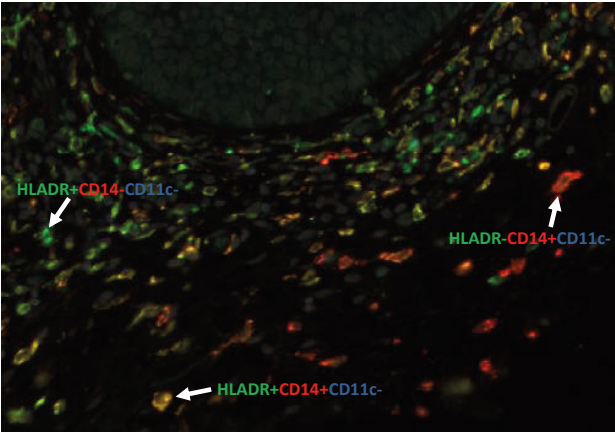
Supplemental File 6. Descriptive statistics of immune cells in vHSIL as one group and based on clinical response after vaccination.

Immune cell phenotype	Number of cells/mm2 epithelium (median with 95% CI)				Number of cells/mm2 stroma (median with 95% CI)			
	vHSIL all	NR	PR	CR	vHSIL all	NR	PR	CR
CD3+CD8-FoxP3-	25.46 (5.94-49.85)	7.45 (5.51-38.53)	28.03 (2.31-97.00)	41.64 (8.28-98.45)	12.39 (4.00-33.32)	8.23 (2.21-20.44)	7.47 (1.74-81.40)	37.60 (11.36-72.40)
CD3+CD8+FoxP3-	2.32 (0.00-5.52)	0.00 (0.00-5.97)	2.32 (0.00-12.85)	5.52 (1.50-22.9)	11.89 (3.91-29.12)	7.55 (3.78-27.31)	13.77 (1.74-35.73)	65.69 (1.34-324)
CD3+CD8-FoxP3+	7.51 (4.47-13.19)	11.30 (4.29-13.86)	8.52 (3.51-23.20)	6.90 (2.86-36.44)	47.93 (23.69-90.40)	51.18 (8.84-112)	69.32 (23.69-102)	22.04 (4.01-99.34)
Tbet+	27.90 (4.14-50.84)	8.66 (0.79-28.16)	33.16 (3.35-90.43)	50.84 (4.14-304.5)	105.20 (37.84-159)	61.33 (12.92-155)	126.6 (22.60-300)	158.70 (14.56-722)
PD1+	20.70 (7.08-50.75)	10.46 (1.19-55.45)	10.21 (0.00-38.78)	150 (20.70-433)	52.15 (34.72-145)	86.10 (24.72-145)	37.03 (9.91-135)	184.90 (29.12-282)
Tim3+	3.70 (2.36-11.59)	3.28 (0.77-15.05)	4.20 (0.91-20.56)	11.46 (0.00-39.21)	28.31 (17.49-98.97)	56.08 (3.59-121)	20.80 (6.95-101)	18.59 (7.66-179)
CD68+CD163-	15.33 (6.88-25.02)	11.22 (3.91-25.02)	17.27 (6.88-26.32)	14.37 (4.60-108)	153.70 (49.05-219)	74.35 (30.01-219)	175.2 (85.51-332)	162.70 (30.05-1562)
CD68-CD163+	0.00 (0.00-0.270)	0.00 (0.00-1.78)	0.00 (0.00-0.46)	0.00 (0.00-6.27)	13.28 (7.14-30.59)	29.73 (1.91-84.54)	12.61 (3.56-30.59)	10.33 (0.00-43.59)
CD68+CD163+	1.19 (0.00-6.90)	1.15 (0.00-6.90)	1.29 (0.00-13.73)	3.89 (0.00-45.73)	253.5 (130-295)	155.50 (65.33-351)	272.2 (142-335)	216.90 (73.41-715)
CD11c+CD14-	1.88 (0.00-4.26)	0.84 (0.00-2.43)	8.67 (0.00-30.03)	3.34 (0.00-9.19)	27.60 (14.90-82.64)	20.58 (12.86-44.05)	96.41 (8.27-224)	51.49 (4.12-246)
CD11c-CD14+	8.36 (3.59-14.71)	3.91 (0.00-27.83)	9.19 (4.53-17.59)	11.68 (0.00-75.81)	228.5 (175-437)	188.60 (122-229)	410.7 (210-579)	496.80 (35.82-648)
CD11c+CD14+	0.00 (0.00-0.54)	0.00 (0.00-3.54)	0.00 (0.00-1.07)	0.00 (0.00-35.78)	10.84 (6.37-16.20)	9.24 (1.26-14.19)	11.98 (6.37-48.39)	12.56 (0.00-133)
CD33+PDL1-	11.73 (6.12-17.72)	13.68 (3.09-23.63)	10.00 (3.43-17.72)	20.22 (1.88-34.10)	63.03 (41.17-92.63)	63.09 (25.73-121)	62.11 (45.73-101)	81.04 (9.25-290)
CD33-PDL1+	5.44 (0.76-25.90)	15.71 (2.20-40.78)	0.38 (0.00-41.69)	5.44 (0.00-100)	53.75 (38.14-80.12)	32.11 (8.58-123)	58.47 (42.92-125)	54.43 (10.33-459)

vHSIL all (n=29); non-responders (NR, n=12); partial responders (PR, n=10); complete responders (CR, n=7); CI=confidence interval

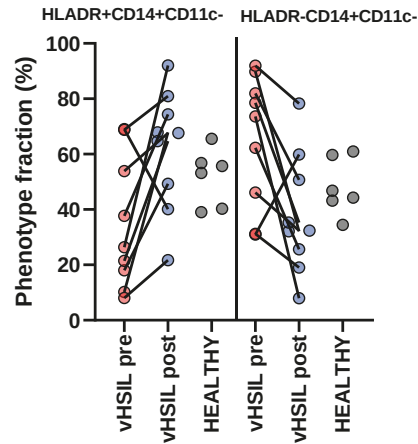
Supplemental File 7. CD14+ inflammatory myeloid cells co-expressing HLA-DR, in pre- and postvaccination vHSIL and in healthy vulva.

A



Example of HLA-DR expressing CD14+ myeloid cells in vHSIL. For this staining, a panel consisting of HLA-DR (clone TAL1B5, directly labelled with Alexa fluor 594), CD14 (clone D7A2T, indirectly detected with CF donkey anti rabbit IgG 633), CD11c (clone EP1347Y, directly labelled with Alexa fluor 546) and DAPI was designed.

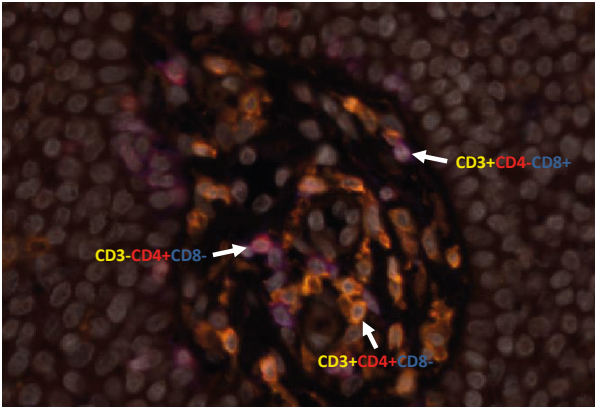
B



6 healthy vulva samples and the pre- and post-vaccination biopsies of 9 vHSIL patients (3 NR, 3 PR, 3 CR) were stained and analyzed with this panel. Samples for staining were selected based on high CD14+ cell counts. Upon vaccination, the fraction of CD14+ cells co-expressing HLA-DR increases.

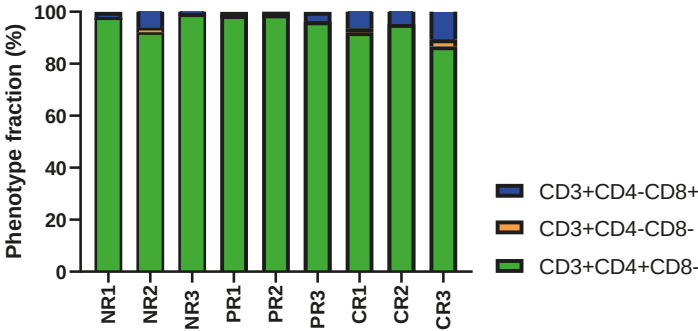
Supplemental File 8. Almost all CD3+CD8-Foxp3- T cells co-express CD4.

A



Example of CD3, CD4 and CD8 expression in a vHSIL biopsy. For this staining, a panel consisting of CD3 (clone D7A6E, directly labelled with Alexa fluor 594), CD4 (clone EPR6855, indirectly detected with Alexa fluor goat anti rabbit IgG 546), CD8 (clone 4B11, indirectly detected with Alexa fluor goat anti mouse IgG2b 647) and DAPI was designed.

B



9 pre-vaccination vHSIL samples (3 NR, 3 PR, 3 CR) were stained and analyzed with the CD3, CD4, CD8, DAPI panel. Samples for staining were selected based on high CD3+CD8-Foxp3- cell counts. Of all CD3+CD8- cells, 99.1% was CD3+CD4+ (median, range 97-100%).

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Chapter 4

A pre-existing coordinated inflammatory microenvironment is associated with complete response of vulvar high-grade squamous intraepithelial lesions to different forms of immunotherapy

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International Journal of Cancer 2020

Abstract

Immunotherapy of vulvar high-grade squamous intraepithelial lesion (vHSIL) is investigated as an alternative for surgery, because of high comorbidity and risk of recurrence. Limited evidence exists on the role and composition of the immune microenvironment in current immunotherapeutic approaches for vHSIL.

The vHSIL of 29 patients biopsied before treatment with imiquimod were analyzed by two multiplex seven-color immunofluorescence panels to investigate the pre-existing T cell and myeloid cell composition in relation to treatment response. The samples were scanned with the Vectra multispectral imaging system. Cells were automatically phenotyped and counted with inForm advanced image analysis software. Cell counts and composition were compared to that of vHSIL patients before therapeutic vaccination (n=29) and to healthy vulva (n=27).

Our data show that the immune microenvironment of complete responders (CR) to imiquimod resembled the coordinated infiltration with type 1 CD4⁺ and CD8⁺ T cells and CD14⁺ inflammatory myeloid cells also found in healthy vulva. However, more CD8⁺ T cells and FoxP3⁺ regulatory T cells were present in CR. The lesions of partial responders (PR) lacked such a coordinated response and displayed an impaired influx of CD14⁺ inflammatory myeloid cells. Importantly, complete responses after imiquimod or therapeutic vaccination showed the same dependency on a pre-existing coordinated type 1 T cell and CD14⁺ myeloid cell infiltration.

In conclusion, a good clinical outcome after two different forms of immunotherapy for vHSIL is associated with the presence of a primary inflammatory process resulting in the coordinated influx of several types of immune cells which is then amplified.

Novelty and impact: This study is the first to show that a complete clinical response of patients with vulvar high-grade squamous intraepithelial lesions is associated with a pre-existent coordinated influx of type 1 T cells and CD14⁺ myeloid cells, irrespective of the type of successful immunotherapy given (topical imiquimod therapy, therapeutic vaccination). This coordinated immune microenvironment closely resembles that of healthy vulvar tissue, suggesting that an impaired primary inflammatory process functions as immune resistance mechanism in non-complete responders.

Introduction

Vulvar High-grade Squamous Intraepithelial Lesion (vHSIL, previously known as usual Vulvar Intraepithelial Neoplasia, uVIN) is a premalignant lesion predominantly induced by high-risk Human Papilloma Virus type 16 (HPV16). The current standard of care is surgery, which is associated with high comorbidity and recurrence, or topical application of the toll like receptor 7 (TLR7) agonist imiquimod to trigger innate immune responses ^{1,2}. In addition, new approaches are being investigated, aiming at either targeting the virus, using cidofovir ¹, or by strengthening the immune system through systemic therapeutic vaccination to increase T cell reactivity against the viral oncoproteins E6 and E7. So far, complete responses have been observed in 35-46% of patients treated with imiquimod ^{1,2}, 46% of patients treated with cidofovir ¹ and 28% of vHSIL patients treated with therapeutic vaccination ³.

Imiquimod is thought to act on macrophages and dendritic cells (DCs), thereby stimulating the local production of pro-inflammatory cytokines such as IFN γ , TNF α and IL-12 which may steer the immune microenvironment towards a milieu that fosters the development of type 1 and cytolytic T cell responses (reviewed in ⁴). In addition, imiquimod may interfere with the adenosine receptor signaling pathways, alleviating local suppression and boosting the pro-inflammatory response (reviewed in ⁵). Last but not least, it may also exert a pro-apoptotic effect on tumor cells, through caspase activation ⁶. Studies on the effects of imiquimod on the immune microenvironment of vHSIL revealed imiquimod-induced consistent increases in the numbers of CD8⁺ T cells, particularly in responding vHSIL patients ^{2,7,8}. Increases in CD4⁺ T cells ⁷ and CD1a⁺ DCs ² were also reported ^{2,7}, but not confirmed ⁸. Non-responsiveness was associated with increased numbers of regulatory T cells (Tregs) after treatment ^{7,8}.

We recently used two multispectral immunofluorescence panels, one for T cells (CD3, CD8, FoxP3, Tim3, Tbet, PD-1, DAPI) and one for myeloid cells (CD14, CD33, CD68, CD11c, CD163, PD-L1, DAPI) to show that a complete regression of vHSIL after therapeutic HPV16 peptide vaccination was associated with a coordinated pre-existing pro-inflammatory immune microenvironment comprising type 1 CD4⁺ and CD8⁺ T cells and CD14⁺ inflammatory macrophages ⁹. In contrast, studies on the pre-existing immune infiltrate before imiquimod treatment showed that the numbers of vHSIL infiltrating CD4⁺ and CD8⁺ T cells as well as CD1a⁺ dendritic cells and CD68⁺ macrophages were not predictive for

responsiveness^{7,8}. In addition, while the number of pre-existing Tregs was a negative predictor in one study⁷, it was not in another⁸. Notably, most studies identified immune cells at the single marker level, and some at a double or triple marker level. This provides only limited insights in the complexity and functional status of the many different immune cells that together compose the immune microenvironment, when compared to the multispectral panels that can be used nowadays⁹.

This prompted the questions if a more in-depth study of the pre-existing immune microenvironment composition can reveal an immune profile related to response to imiquimod therapy and whether such a pre-existing immune profile in vHSIL differs from those vHSIL patients responding to therapeutic vaccination. Therefore, we studied the pre-treatment immune microenvironment of 29 vHSIL patients in relation to the clinical response after imiquimod treatment, and compared this to the pre-existing immune profile found in patients treated with a therapeutic HPV vaccine. Our data showed that a good clinical response to immunotherapy, being either imiquimod or therapeutic vaccination, is associated with a pre-existing pro-inflammatory coordinated immune microenvironment, characterized by CD14⁺ inflammatory cells, CD4⁺Tbet⁺ T cells and CD8⁺ T cells, and suggests that neither therapy is capable of overcoming an immunological cold microenvironment.

Materials and Methods

Patient samples

Pre-treatment formalin-fixed paraffin-embedded (FFPE) biopsies of 29 women of ≥ 18 years old with histologically confirmed vHSIL were retrospectively included. Patient samples were collected from two hospitals in the Netherlands: the Leiden University Medical Center (LUMC, Leiden, The Netherlands), and the Haga Hospital (the Hague, The Netherlands). Clinical complete response (CR, n=14) upon therapy was defined as 100% lesion clearance, partial response (PR, n=12) as $\geq 50\%$ lesion clearance, and no response (NR, n=3) as $< 50\%$ lesion regression. Moreover, FFPE healthy HPV-negative vulvar tissue from 27 anonymized women who underwent labia reduction surgery was included.

Multiplex immunofluorescence imaging

Two seven-color multispectral immunofluorescence panels were applied, one for T cells, consisting of CD3, CD8, FoxP3, Tim3, Tbet, PD-1, DAPI, and

one for myeloid cells, consisting of CD14, CD33, CD68, CD11c, CD163, PD-L1, DAPI, as previously published in ⁹. In these panels a combination of direct detection (primary antibody directly labelled with fluorochrome) and indirect detection (fluorochrome labelled secondary antibody) of markers was used ¹⁰, and dim markers (PD-L1, PD-1 and Tbet) were tyramide signal amplified with Opal (PerkinElmer) to enable their detection by fluorescence microscopy. This combination of different marker detection methods, fully optimized for each marker, enabled the selection of the best primary antibodies without being restricted by clashing isotypes and species. Antibody specificity was first assessed with immunohistochemistry and monoplex immunofluorescence, tonsil slides serving as positive control, and subsequently the immunofluorescence detection conditions for each marker in multiplex immunofluorescence were optimized. A frequently encountered problem in multiplex immunofluorescence is spectral overlap between signals. To overcome this, we designed our panels in such a way that markers with an equally strong signal intensity were put in the same microscope detection filter, we alternated nuclear and membranous markers in the light spectrum when possible, and finally we found that alternating secondary Alexa fluorochromes (ThermoFisher Scientific) with CF dyes (Biotium) when spectral overlap between two consecutive Alexa fluorochromes was detected despite the minimal exposure times used, resolved the cross bleed of signal successfully. An overview of the antibodies and staining methods included in each panel are shown in **Supplemental Table 1**. In short, 4µm FFPE tissue sections were deparaffinized, endogenous peroxidase was blocked with hydrogen peroxide and heat induced epitope retrieval was performed in the T cell panel with citrate (10mM, pH 6.0) and in the myeloid cell panel with tris-EDTA (10mM/1mM, pH 9.0). SuperBlock (ThermoFisher Scientific) was used to block non-specific binding sites. First, the antibodies detected by Opal were applied, followed by the unconjugated antibodies which incubated overnight. On the second day, after binding of the previous with their corresponding fluorescently labelled secondary antibodies, the directly labeled primary antibodies were incubated for 5 hours, and lastly DAPI was applied as nuclear counterstain ⁹.

Quantification of immune cells in the TME

As published in ⁹, immunofluorescence images of the entire tissue sections were acquired with the Vectra 3.0.5 multispectral imaging microscope (PerkinElmer) at 20x magnification. Immune cells in the TME were automatically phenotyped and counted with inForm 2.4 image analysis software (PerkinElmer) after manual training. The software was trained to segment epithelium and stroma, segment

DAPI+ nucleated cells, and assign a phenotype to each cell. All phenotypes were visually inspected on accurateness, and if errors were detected the training was further optimized until all discrepancies were resolved. Given the multitude of possible co-expressed markers and the limited trainability of the inForm software in reliably detecting complex multi-marker expressing cell phenotypes, each seven-color panel was divided into multiple sub-analyses that contained a small number of markers which the inForm software could robustly identify (**Supplemental Table 2**). The phenotypes of all sub-analyses were merged per cell based on its X,Y-positions with an R script, which enabled the description of the full seven marker expression profile for each cell. Immune cell counts were normalized for tissue size (cells/mm² epithelium and cells/mm² stroma). After merging the sub-analyses, a threshold of a median cell count ≥ 10 cells/mm² in at least one group (NR, PR, CR or healthy vulva) was applied to study the changes in biologically common phenotypes.

Statistical analysis

Statistical data analysis was performed with SPSS 25.0 (IBM Corporation). The median immune counts of the different groups (PR, CR and healthy vulva) were compared with the non-parametric Mann-Whitney U test. NRs were not included in the statistical analyses due to their small sample size (n=3). Pearson correlation was used to study correlations between the T cell infiltrate and the myeloid cell infiltrate in each group. Two sided p-values < 0.05 were marked as significant. GraphPad Prism 8.0.1 (GraphPad Software Inc.) was used to create graphs.

Results

The pre-treatment immune microenvironment reveals high heterogeneity between vHSIL patients.

We analyzed the complexity of the vHSIL-infiltrating T cell and myeloid cell populations in 29 patients (**Table 1**), before topical imiquimod treatment, using two seven-color multiplex immunofluorescence panels (**Supplemental Tables 1,2**). Analyses of these images stained for T cells and myeloid cells (**Figure 1; Supplemental Figures 1,2,3**) revealed 6 different phenotypes of T cells and 8 different phenotypes of myeloid cells (**Figure 2A**). The CD3⁺CD8⁻ T cells detected were designated as CD4⁺ T cells, based on our earlier study showing that on average 99% of the CD3⁺CD8⁻ T cells were CD4⁺ T cells ⁹. Moreover, it showed that the vHSIL patients formed a heterogeneous group with high inter-patient variability in the numbers of intraepithelial and stromal immune cells (**Figure 2A; Supplemental Table 3**).

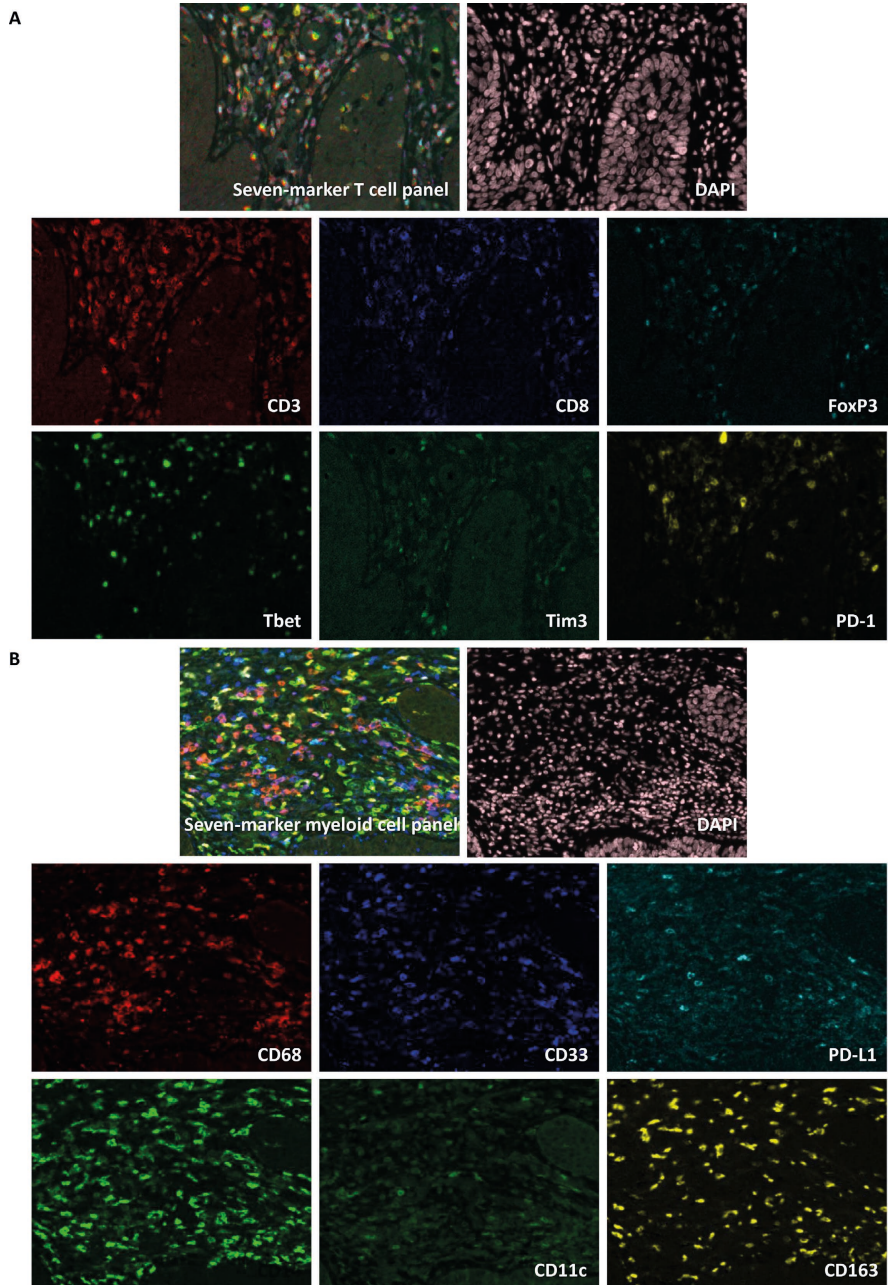
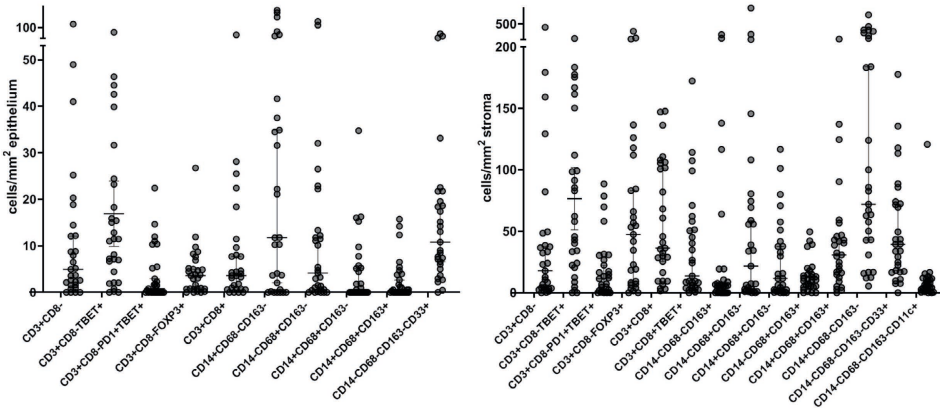


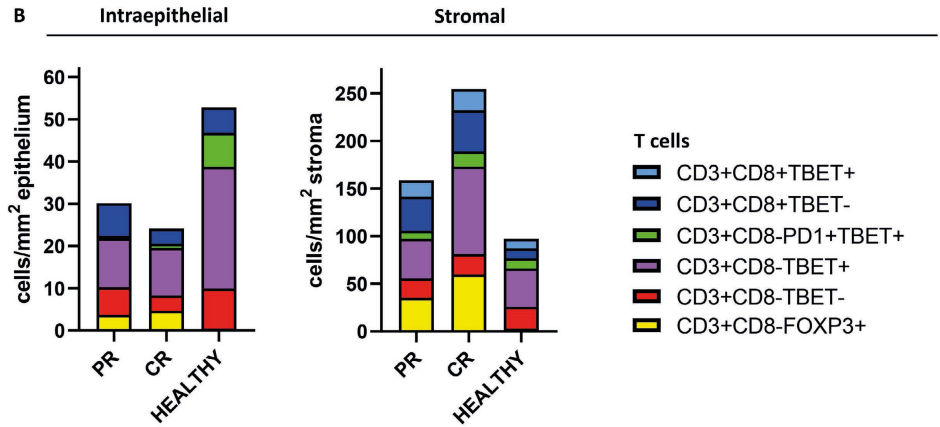
Figure 1. Multiplex immunofluorescence to detect T cells and myeloid cells in vHSIL.

A) Composite image including all individual markers of the seven-color T cell panel staining, consisting of the markers CD3, CD8, FoxP3, Tbet, Tim3, PD-1 and DAPI. **B)** Composite image including all individual markers of the seven-color myeloid cell panel staining, consisting of the markers CD33, CD68, CD163, PD-L1, CD14, CD11c and DAPI.

A



B



C

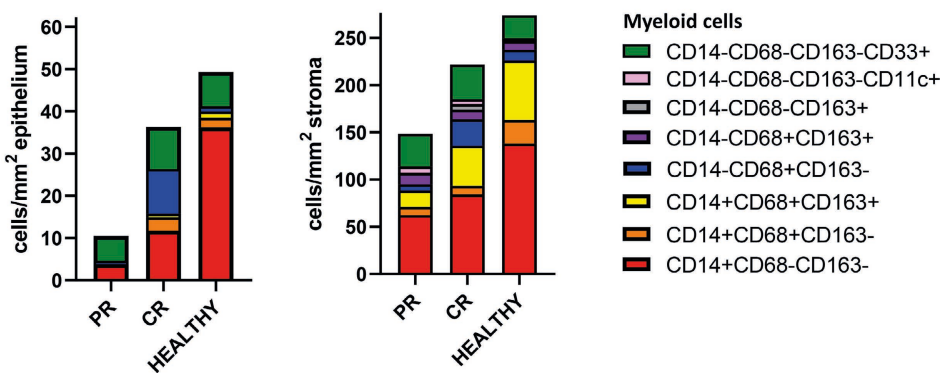


Figure 2. The pre-imiquimod TME differs between the different response groups.

A) The numbers of intraepithelial and stromal infiltrating T cell and myeloid cell subtypes are presented as cells/mm² epithelium and cells/mm² stroma in the pre-imiquimod vHSIL biopsies of 29 patients. Each dot represents an individual sample, the horizontal bars indicate the median cell counts, the vertical bars are

the 95% confidence intervals. The **B)** T cell and **C)** myeloid cell infiltrate of the pre-imiquimod vHSIL patients when grouped according to response (PR, CR), and compared to healthy vulvar tissue (n=27), presented as median cells/mm² epithelium and median cells/mm² stroma. A threshold of a median cell count ≥ 10 cells/mm² in at least one group (NR, PR, CR or healthy vulva) was applied to study the changes in biologically common phenotypes. CR=complete responders (n=14); PR=partial responders (n=12).

Differences in the myeloid cell compartment of vHSIL correlate with response to imiquimod.

The vulva is a highly immune active anatomical location as shown by the high numbers of T cells and myeloid cells infiltrating this healthy tissue (**Figure 2**). To study if quantitative or qualitative differences in immune infiltrate were associated with a different clinical outcome in vHSIL, we grouped the patients by clinical response (PR, CR) and compared them to healthy tissue. This revealed that while the PR and CR patients displayed abundant T cell infiltration in the stroma, with higher numbers of CD8⁺ T cells and CD3⁺CD8⁺FoxP3⁺ Tregs, the intraepithelial T cell infiltration was considerably reduced as compared to healthy tissue, lacking CD3⁺CD8⁺PD1⁺/Tbet⁺ activated type 1 T helper cells in particular (**Figure 2B**; **Supplemental Table 3**). No overt differences in T cell infiltration existed between the PR and CR patients (**Figure 2B**; **Supplemental Table 3**). The myeloid cell population differed the most among the different clinical groups. There was substantial intraepithelial and stromal infiltration by CD33⁺ immature myeloid cells (**Figure 2C**; **Supplemental Table 3**), which most likely are myeloid derived suppressor cells (MDSC) based on our earlier studies ¹¹, but their numbers didn't differ between the groups. Furthermore, a substantial fraction of the myeloid cells in the stroma comprised different populations of CD14⁺ inflammatory myeloid cells, in particular CD14⁺CD68⁺CD163⁺ cells and CD14⁺CD68⁺CD163⁺ M2 macrophages. Whereas the numbers of the different stromal CD14⁺ inflammatory myeloid cell subpopulations between healthy vulva and CR lesions didn't differ much, the numbers of these myeloid cell subpopulations were significantly lower in the lesions of PR patients (**Figure 2C**; **Supplemental Table 3**). Finally, the number of stromal CD11c⁺ DCs was slightly increased in vHSIL when compared to healthy vulva.

Thus while the numbers and composition of infiltrating immune cells of CR patients most closely resembled that of healthy vulva, PR patients showed impaired infiltration with inflammatory myeloid cells (**Figure 2**).

The composition of the vHSIL immune microenvironment in complete responders fits best with the mode of action of the chosen immunotherapeutic treatment.

The correlation between a CR after imiquimod and a pre-existent high infiltration of the lesions with CD4⁺Tbet⁺ T cells and CD14⁺ inflammatory myeloid cells (**Figure 2**) as well as a coordinated overall immune cell infiltration (**Figure 3**) was also observed by us in vHSIL patients treated with a therapeutic vaccine containing synthetic long peptides of HPV16 E6 and E7 oncoproteins⁹. This suggests that the immune composition of the lesions of CR patients is highly similar irrespective of the immunotherapeutic treatment. We, therefore, compared the composition of the stromal T cell and myeloid cell compartment in both groups of treated patients. Interestingly, the composition of the T cell fractions didn't overtly differ between the groups of patients with a different clinical outcome after imiquimod treatment (**Figure 4A**), whereas this was clearly the case between the groups of patients treated with a therapeutic vaccine (**Figure 4B**). Interestingly, the myeloid cell composition didn't differ much between the therapeutically vaccinated patient response groups, contrary to the different response groups to topical imiquimod treatment. In the latter, patients with a PR or CR had a considerably different myeloid cell composition compared to NR patients (**Figure 4**), implying that for response to imiquimod the number and composition of vHSIL infiltrating myeloid cells is of importance. Thus, whereas the composition of the T cell compartment was important for the outcome of a therapy aiming to improve the T cell response by therapeutic vaccination, the numbers of CD14⁺ cells in the myeloid cell compartment were more important for response to a treatment with topical imiquimod, known to stimulate myeloid cells⁴ and CD14⁺ cells in particular¹³ (**Figure 5**).

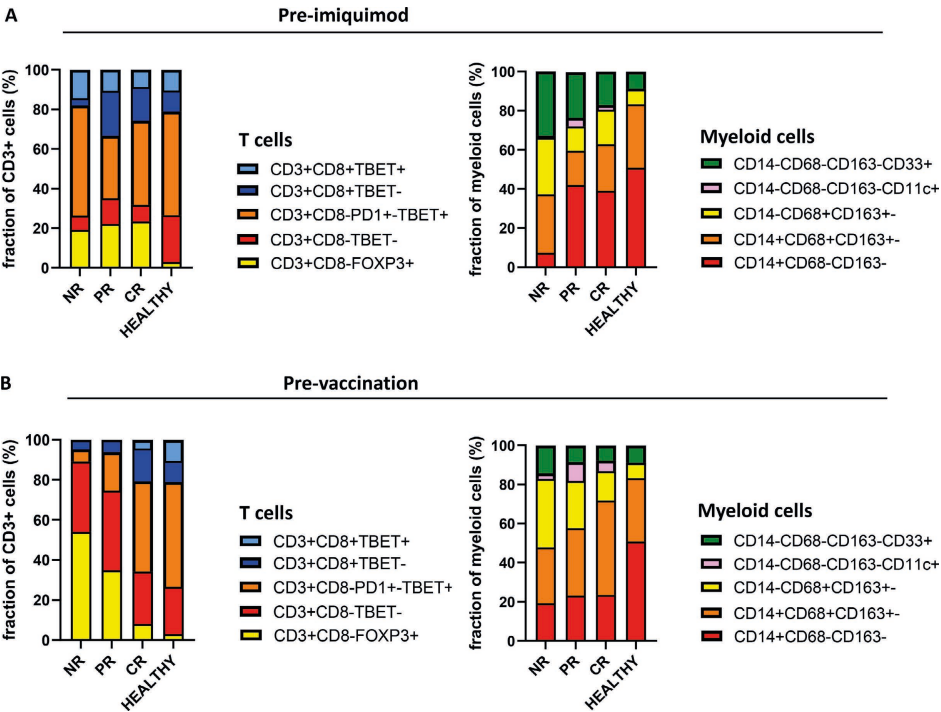


Figure 4. Fractional differences between the pre-imiquimod and pre-vaccination immune microenvironment in vHSIL, divided based on clinical response to the respective therapy.

A) Fractional composition of the T cells and myeloid cells in the pre-imiquimod vHSIL cohort, grouped according to response (NR n=3, PR n=12, CR n=14), and compared to healthy vulva (n=27). **B)** Fractional composition of the T cells and myeloid cells in the pre-therapeutic vaccination vHSIL cohort, grouped according to response (NR n=12, PR n=10, CR n=7), and compared to healthy vulva (n=27).

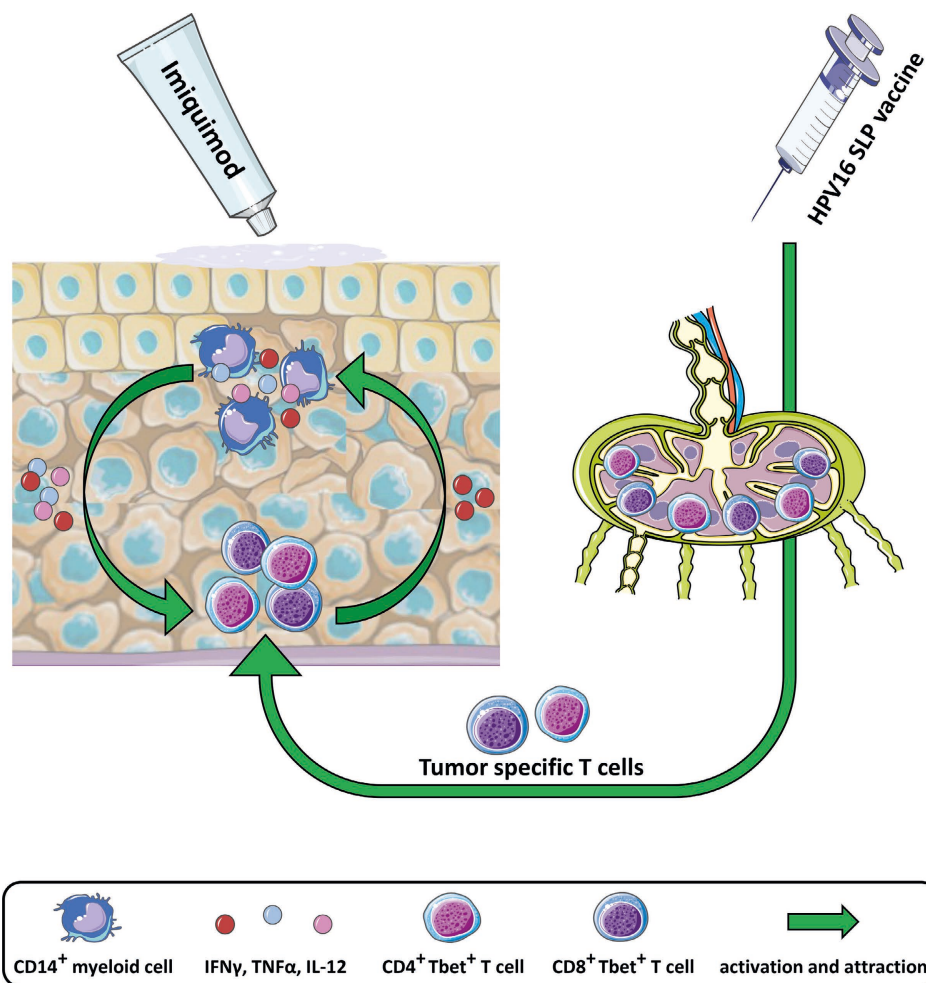


Figure 5. Summary of complete responder's vHSIL immune microenvironment in the context of the mode of action of imiquimod and therapeutic vaccination.

The vHSIL immune microenvironment of complete responders to imiquimod and therapeutic HPV16 synthetic long peptide (SLP) vaccination comprises a coordinated infiltration with type 1 (Tbet⁺) CD4⁺ and CD8⁺ T cells as well as CD14⁺ inflammatory myeloid cells. Imiquimod stimulates CD14⁺ myeloid cells to produce pro-inflammatory cytokines which activate and attract the type 1 T cells¹³. Whereas, therapeutic vaccination stimulates the type 1 T cell response, which in turn produces cytokines that also stimulate the influx of the lesion by CD14⁺ myeloid cells⁹. This figure was created using adapted images of Servier Medical Art, licensed under a creative commons attribution 3.0 unported license.

Discussion

Here we show that a complete regression of vHSIL after imiquimod treatment is associated with a pre-existing well-coordinated overall infiltration between T cells and myeloid cells, and in particular the influx of vast numbers of vHSIL-infiltrating type 1 T cells and CD14⁺ inflammatory myeloid cells. Healthy vulva is richly infiltrated and guarded by pro-inflammatory T cells and myeloid cells. While the immune cell infiltration in the group of patients with a CR didn't differ much from healthy vulva, the myeloid cell infiltration of lesions from PR patients was significantly lower than in healthy vulva. This was also visualized by the clear disconnection between the infiltration with CD8⁺ T cells and myeloid cells in the group of patients with a PR. The requirement for an ongoing T cell response for imiquimod to be effective was also previously reported ¹⁴ but here it is shown that also a strong influx with CD14⁺ myeloid cells should be maintained. This would be in line with the mode of action of imiquimod which is acting predominantly on CD14⁺ cells ¹³, the activation of which leads to the increased infiltration of several types of adaptive immune cells ^{2, 7, 8}. The presence of these CD14⁺ cells at lower numbers and in an unfavorable balance with suppressive immune cells (M2 macrophages, MDSC) may serve as a primary resistance mechanism to imiquimod treatment.

In our recent study on the immune microenvironment of vHSIL completely regressing after therapeutic vaccination, we also found a well-coordinated immune infiltration in CR patients, and a positive association between clinical efficacy and high numbers of vHSIL-infiltrating type 1 T cells and CD14⁺ inflammatory myeloid cells. Moreover, the vHSIL that led to a PR after vaccination displayed a disconnection between CD8⁺ T cell and myeloid cell infiltration ⁹. However, as shown in the current study the clinical outcome after therapeutic vaccination was more related to differences in the composition of the CD4⁺ T cell fraction, fitting with the mode of action of this vaccine ^{3, 15-17}.

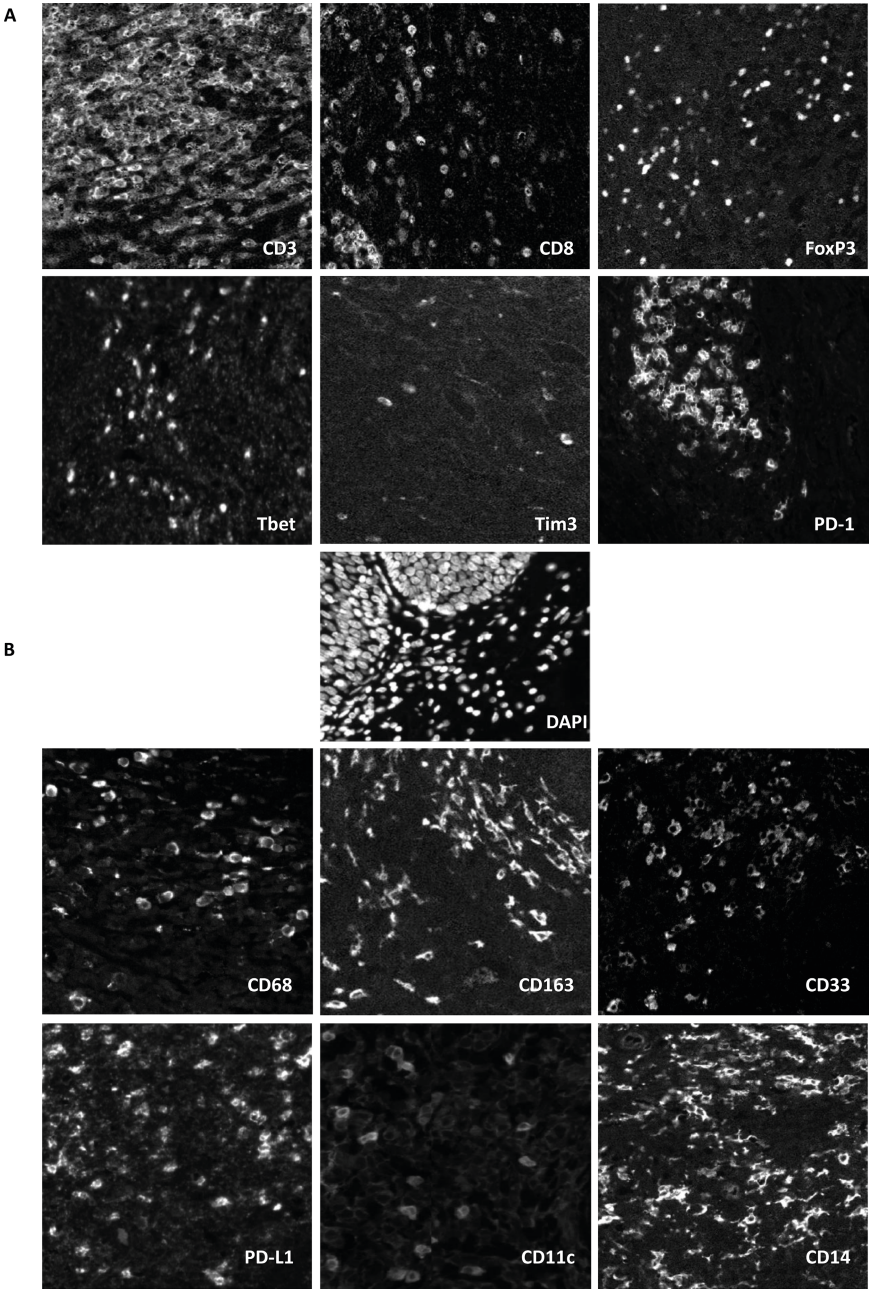
The combination of these two data sets leads to the conclusion that both a coordinated inflammatory immune response comprising type 1 CD4⁺ and CD8⁺ T cells, as well as CD14⁺ myeloid cells are potentially required for optimal immune control and clearance of vHSIL. Previously, it has been demonstrated that the rejection of human tissues (tumors, allografts, autoimmune flares) is associated with an almost identical modular signature and defined as the immunological constant of rejection (ICR) ^{18, 19}. In the context of our observations that complete rejections of vHSIL are associated with the presence of vast

numbers of type 1 T cells and CD14⁺CD68⁺CD163⁺ myeloid cells, it is of interest to know that the ICR is a process that begins with the induction of a primary inflammatory process leading to the chronic infiltration of monocytes and other cells related to innate immune mechanisms but lack effector function. The presence of pro-inflammatory signals such as IFN γ , which is likely to come from the Tbet⁺ T cells in vHSIL, induce the activation of monocytes, which in turn, may start to produce pro-inflammatory cytokines such as IL-12, IL-15, and IL-18, important cytokines to drive and sustain the type 1 immune response required for rejection^{18,19}. Thus, it seems that both imiquimod and therapeutic vaccination will lead to complete rejections when the process of the ICR is already ongoing.

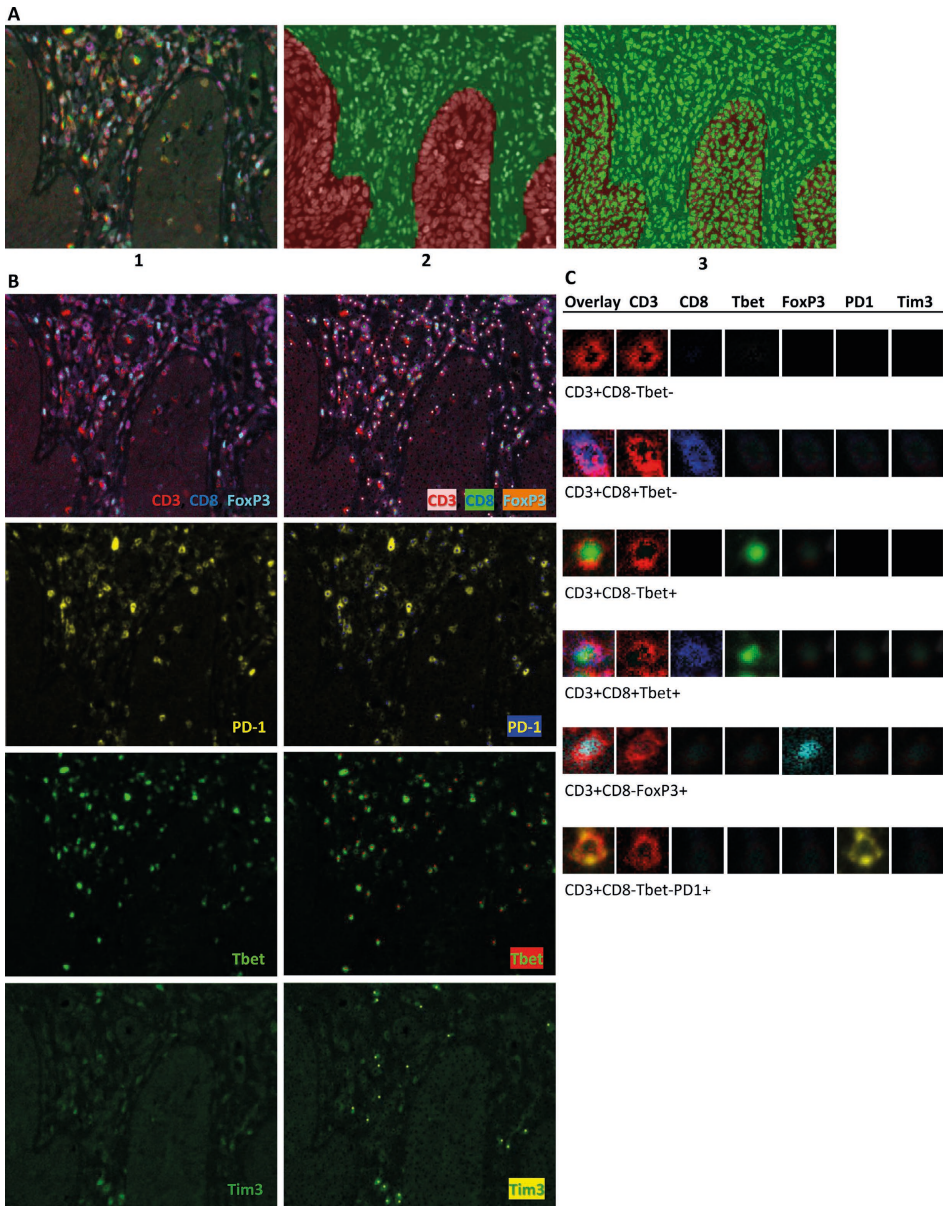
Following this line of reasoning, it also implies that none of the two immunotherapeutic approaches is able to breakdown the barrier existing in cold lesions. We have shown that this is true for therapeutic vaccination⁹. It is likely that this barrier is induced by the infection with HPV as healthy vulva tissue is strongly infiltrated with immune cells. So what could underlie an impaired primary process of inflammation? We and others have shown that HPV exploits several mechanisms to suppress the primary inflammatory process²⁰⁻²². In addition genomic instability in these lesions²³⁻²⁵ may turn these lesions cold. In such cases, vHSIL lesions are better treated with cidofovir based on the fact that cidofovir and imiquimod were reported to be effective in two distinct biologically defined vHSIL groups, reflected by their HPV E2 DNA methylation status²⁶.

Our study shows the benefits of using multispectral imaging in order to obtain a profile of the immune microenvironment, which are correlated to the success or failure of an immunotherapeutic treatment. While previous studies^{2,7,8} were unable to find a relation between the numbers of immune infiltrating T cells or myeloid cells and clinical outcome, this was now possible because additional markers allowed for a better definition of the different subpopulations, some of which were correlated with the response to imiquimod or therapeutic vaccination. For future studies, it would be of interest to study the post-imiquimod immune microenvironment because the imiquimod-induced changes in the different subsets of immune cells will increase our knowledge on what is exactly required for an effective immune control of HPV-induced lesions.

Supplemental Figures

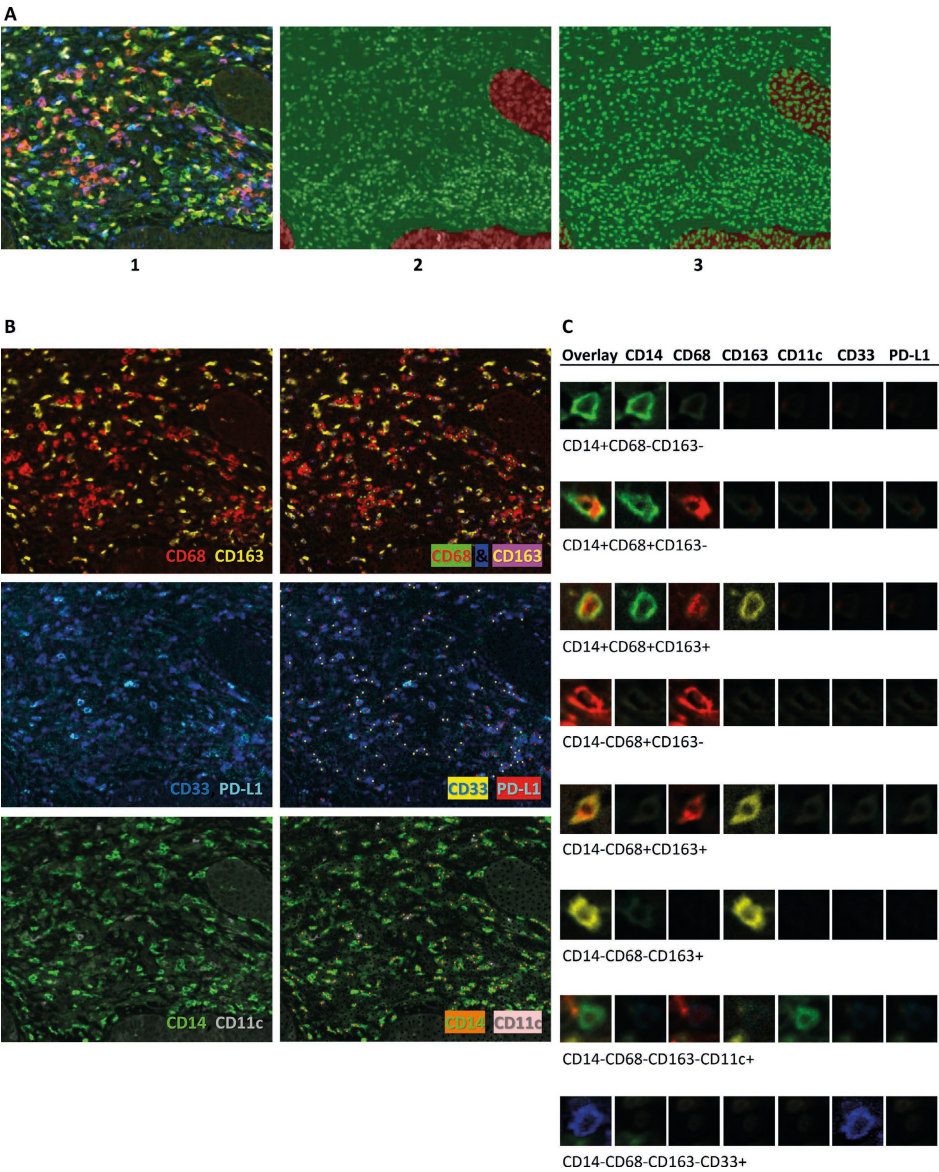


Supplemental Figure 1.
*Single marker photos of the multiplex **A**) T cell panel and **B**) myeloid cell panel in vHSIL*



Supplemental Figure 2.

*InForm training and sub analyses T cell panel. The same tissue section is shown in Figure 1A. **A)** Seven markers signal extraction (step 1), tissue segmentation (step 2) and cell segmentation (step 3). **B)** Division of seven color image in four sub analyses for the detection of complex phenotypes: immunofluorescent images (left image), and respective *inForm* recognition of phenotypes by assigning each phenotype a dot with a specific color (right image). **C)** The full fluorescent spectrum of the six identified T cell phenotypes*



Supplemental Figure 3.

*InForm training and sub analyses myeloid cell panel. The same tissue section is shown in Figure 1B. **A**) Seven markers signal extraction (step 1), tissue segmentation (step 2) and cell segmentation (step 3). **B**) Division of seven color image in three sub analyses for the detection of complex phenotypes: immunofluorescent images (left image), and respective inForm recognition of phenotypes by assigning each phenotype a dot with a specific color (right image). **C**) The full fluorescent spectrum of the eight identified myeloid cell phenotypes*

Supplemental Tables

Supplemental Table 1. Design seven-color T cell panel and myeloid cell panel, antibodies and detection method per marker.

T cell panel				
Marker	Primary antibody clone and company	Primary antibody isotype	Secondary antibody	Detection method
PD-1	D4W2J Cell Signaling Technology	Rabbit IgG	Poly-HRP and Opal 570	Opal tyramide signal amplification
Tbet	D6N8B XP Cell Signaling Technology	Rabbit IgG	Poly-HRP and Opal 520	Opal tyramide signal amplification
FoxP3	236A/E7 Invitrogen ThermoFisher	Mouse IgG1	CF goat anti mouse IgG1 633	Secondary antibody fluorochrome labelled
CD8	4B11 Novocastra	Mouse IgG2b	Alexa goat anti mouse IgG2b 647	Secondary antibody fluorochrome labelled
Tim3	D5D5R XP Cell Signaling Technology	Rabbit IgG	Alexa goat anti rabbit IgG 680	Secondary antibody fluorochrome labelled
CD3	D7A6E Cell Signaling Technology, directly labelled with Alexa 594	Rabbit IgG	-	Directly fluorochrome labelled primary antibody
DAPI	ThermoFisher	-	-	Fluorescent probe
Myeloid cell panel				
Marker	Primary antibody clone and company	Primary antibody isotype	Secondary antibody	Detection method
PD-L1	SP142 Spring Bioscience	Rabbit IgG	Poly-HRP and Opal 520	Opal tyramide signal amplification
CD14	D7A2T Cell Signaling Technology	Rabbit IgG	CF donkey anti rabbit IgG 633	Secondary antibody fluorochrome labelled
CD33	PWS44 Novocastra	Mouse IgG2b	Alexa goat anti mouse IgG2b 647	Secondary antibody fluorochrome labelled
CD163	10D6 Invitrogen ThermoFisher	Mouse IgG1	CF goat anti mouse IgG1 680	Secondary antibody fluorochrome labelled
CD11c	EP1347Y Abcam, directly labelled with Alexa 546	Rabbit IgG	-	Directly fluorochrome labelled primary antibody
CD68	D4B9C XP Cell Signaling Technology, directly labelled with Alexa 594	Rabbit IgG	-	Directly fluorochrome labelled primary antibody
DAPI	ThermoFisher	-	-	Fluorescent probe

Supplemental Table 2. Sub-analyses of the T cell panel and myeloid cell panel as used in InForm.

	T cell panel	Myeloid cell panel
Sub-analysis 1	CD3+CD8- FoxP3- (T helper cell) CD3+CD8+FoxP3- (CD8+ T cell) CD3+CD8- FoxP3+ regulatory T cell) CD3-CD8-FoxP3- (no T cell)	CD68+CD163- (M1 macrophage) CD68+CD163+ (M2 macrophage) CD68-CD163+ (CD163+ non-macrophage) CD68-CD163- (non-macrophage)
Sub-analysis 2	Tbet+ (IFN γ producing T cell) Tbet- (Tbet- cell)	CD11c+CD14- (DC) CD11c+CD14+ (inflammatory DC) CD11c-CD14+ (inflammatory myeloid cell) CD11c-CD14- (CD11c- and CD14- cell)
Sub-analysis 3	PD1+ (activated PD1+ T cell) PD1- (PD1- cell)	CD33+PDL1- (immature myeloid cell) CD33+PDL1+ (immature PDL1+ myeloid cell) CD33-PDL1+ (mature PDL1+ myeloid cell) CD33-PDL1- (CD33- and PDL1- cell)
Sub-analysis 4	Tim3+ (activated Tim3+ T cell) Tim3- (Tim3- cell)	

Supplemental Table 3. Descriptive statistics of immune cells in vHSIL biopsies, divided based on clinical response, and in healthy vulva.

Immune cell phenotype	Number of cells/mm2 epithelium (median with 95% CI)				Number of cells/mm2 stroma (median with 95% CI)				Statistical significant difference
	NR	PR	CR	Healthy	NR	PR	CR	Healthy	
CD3+CD8-	4.97 (0-12.07)	6.56 (0-25.21)	3.62 (1.55-14.45)	9.53 (3.08-26.57)	6.86 (2.49-24.02)	20.46 (1.68-38.59)	21.47 (3.58-159.30)	22.92 (9.76-59.35)	-
CD3+CD8-TBET+	11.47 (0.76-18.29)	11.57 (2.09-24.42)	11.24 (2.06-44.51)	28.74 (8.04-52.88)	45.74 (8.71-166.9)	41.57 (12.47-91.48)	92.05 (22.47-177.6)	40.39 (20.09-76.78)	-
CD3+CD8-PD1+TBET+	1.64 (0-2.93)	0.61 (0-10.31)	0.98 (0-10.39)	8.09 (2.02-15.73)	7.27 (0.41-11.43)	8.43 (0-30.35)	15.70 (0.43-58.20)	10.66 (3.28-20.19)	-
CD3+CD8-FOXP3+	0.73 (0-0.76)	3.68 (0.91-5.17)	4.69 (0.20-8.26)	0.43 (0-2.86)	18.29 (1.45-83.14)	35.18 (7.12-61.34)	59.73 (3.90-211.90)	2.91 (0.98-6.73)	PR****H, CR****H
CD3+CD8+	-	-	-	-	3.43 (1.45-106.2)	35.90 (28.49-110.8)	43.32 (9.42-105.4)	10.29 (5.27-14.30)	PR**H, CR**H
CD3+CD8+TBET+	-	-	-	-	13.72 (0.62-172.3)	16.89 (6.15-50.44)	22.18 (3.77-99.17)	10.21 (3.66-26.75)	-
CD14+CD68-CD163-	22.21 (0.18-82.93)	3.87 (0-63.56)	11.77 (0-41.62)	36.20 (14.35-57.23)	16.71 (16.68-360.3)	62.41 (15.80-183.90)	84.25 (50.19-386.80)	138.10 (88.72-239.10)	PR*H
CD14+CD68+CD163-	-	-	-	-	37.88 (0-70.83)	8.88 (0.37-37.66)	9.04 (1.21-80.05)	24.85 (11.03-49.57)	PR*H
CD14+CD68+CD163+	-	-	-	-	29.60 (1.85-212.5)	17.08 (4.28-32.44)	42.38 (16.96-59.32)	62.99 (25.63-89.09)	PR*CR, PR**H
CD14-CD68+CD163-	4.15 (0-22.21)	0.82 (0-12.21)	10.62 (1.33-32.04)	1.26 (0-3.24)	55.70 (0-108.2)	6.73 (1.10-55.87)	28.09 (1.21-145.6)	11.42 (4.98-18.77)	-

Supplemental Table 3 Continued

Immune cell phenotype	Number of cells/mm2 epithelium (median with 95% CI)				Number of cells/mm2 stroma (median with 95% CI)				Statistical significant difference	
	NR	PR	CR	Healthy	NR	PR	CR	Healthy		
CD14-CD68-CD163+	-	-	-	-	5.56 (0-7.73)	0.72 (0-138.00)	6.07 (0.27-19.45)	2.39 (0.36-5.86)	-	
CD14-CD68+CD163+	-	-	-	-	9.87 (7.41-37.67)	11.49 (0-18.02)	9.95 (4.57-19.99)	9.07 (2.32-18.42)	-	
CD14-CD68-CD163-CD33+	33.14 (15.16-73.72)	5.81 (2.24-22.54)	9.93 (7.15-19.56)	8.08 (2.97-16.08)	-	74.12 (16.55-177.70)	34.48 (16.92-88.76)	36.74 (9.74-72.39)	23.90 (8.84-41.58)	-
CD14-CD68-CD163-CD11c+	-	-	-	-	2.55 (0-6.12)	6.63 (1.44-11.53)	5.26 (0.99-12.07)	0.95 (0.27-4.71)	PR*H, CR*H	

Non-responders (NR, n=3), partial responders (PR, n=12), complete responders (CR, n=14) and healthy vulva (H, n=27). A threshold of a median cell count of ≥ 10 cells/mm2 per tissue compartment was applied, to only select relevant phenotypes for analyses. NRs have not been included in the statistical analyses, due to their small sample size. Differences between two groups were calculated with a Mann-Whitney test, statistical significance is indicated with asterisks: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

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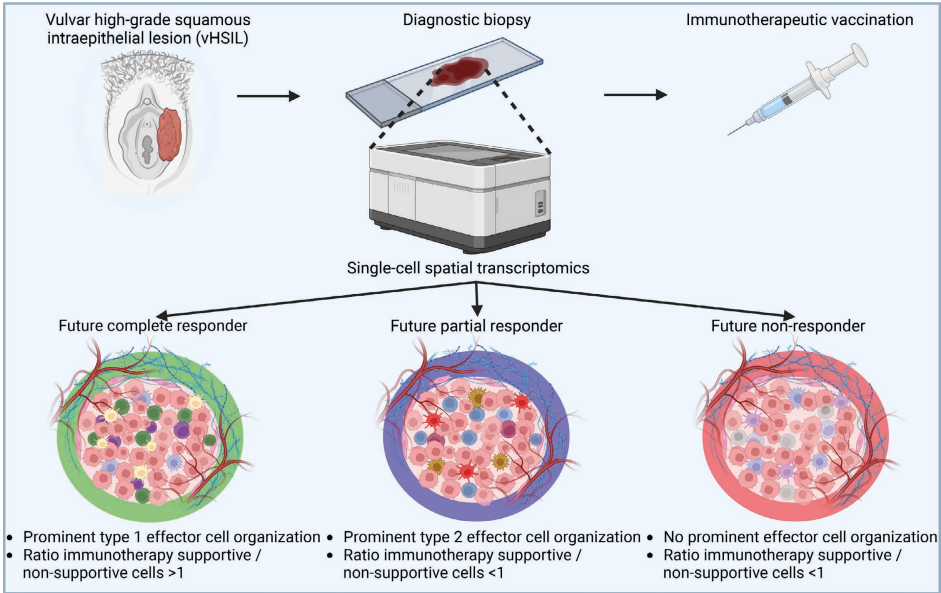
Chapter 5

Single-cell spatial transcriptomics unravels cell states and ecosystems associated with clinical response to immunotherapy

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Journal for ImmunoTherapy of Cancer 2025

Graphical abstract



Key findings

What is already known on this topic

The tumor microenvironment (TME) plays a critical role in determining responses to immunotherapy. Studies focused on predefined cell populations, have identified several cells associated with treatment outcomes but simultaneously underscored the difficulty to use them as reliable biomarkers.

What this study adds

This study confirms the significant heterogeneity in the cellular composition of vulvar high-grade squamous intraepithelial lesions (vHsIL), and demonstrates that in particular the differences in spatial organization of immune cell states is associated with clinical response to immunotherapy.

How this study might affect research, practice, or policy

These findings underscore the importance of spatially resolved single-cell analyses in understanding the TME and suggest that targeting immune-supportive ecosystems could optimize immunotherapeutic strategies across tumor types.

Abstract

Background

The tumor microenvironment (TME) is a complex and dynamic ecosystem that is known to influence responses to immunotherapy. We leveraged single-cell spatial transcriptomics to systematically dissect the intricate complexity of the TME, in particular the cellular heterogeneity and spatial interactions. Their collective impact on immunotherapy efficacy was studied in the context of a homogenous group of patients with vulvar high-grade squamous intraepithelial lesions (vHSIL) treated with an immunotherapeutic tumor-specific peptide vaccine.

Methods

We performed single-cell spatial transcriptomics on 20 pre-treatment vHSIL lesions, stratified by clinical response to immunotherapeutic vaccination into complete responders (CR), partial responders (PR) and non-responders (NR). Using a 1000-gene panel, we mapped over 274,000 single cells in situ, identifying 18 cell clusters and 99 distinct non-epithelial cell states. Findings were validated against public single-cell transcriptomic datasets to assess their broader relevance across tumor types.

Results

Profound heterogeneity within the TME was detected across the response groups. CR lesions exhibited a higher ratio of immune-supportive to immune-suppressive cells—a pattern mirrored in other solid tumors following neoadjuvant checkpoint blockade. Key immune populations enriched in CRs included CD4+CD161+ effector T cells and chemotactic CD4+ and CD8+ T cells. Conversely, PRs were characterized by increased proportions of T helper 2 cells and CCL18-expressing macrophages, which are associated with the recruitment of type 2 T cells and regulatory T cells. NRs displayed preferential infiltration with immunosuppressive fibroblasts. Distinct spatial immune ecosystems further defined response groups. Although a number of immune cells were detected in all patients, type 1 effector cells dominated interactions in CRs, type 2 cells were prominently interacting in PRs, while NRs lacked organized immune cell interactions.

Conclusion

This study underscores the dual importance of both cellular composition and spatial organization in steering clinical response to immunotherapy.

Introduction

Over the past decade, there has been a significant surge in T cell-based immunotherapeutic approaches for cancer treatment, including immune checkpoint blockade, adoptive cell therapy, and therapeutic vaccines.¹⁻³ While these strategies have achieved remarkable success, many patients either fail to respond or experience only transient benefits. This underscores a critical need to deepen our understanding of how patient-specific tumor characteristics influence therapeutic responses. Retrospective analyses, typically focused on predefined markers and cell populations, have identified several tumor microenvironment (TME) features associated with treatment outcomes.⁴⁻⁵ However, the intricate complexity of the TME, particularly the cellular heterogeneity and spatial interactions, remains insufficiently understood, as does their collective influence on immunotherapy efficacy. Deciphering the multifaceted ecosystem of the TME—comprising diverse tumor, immune, and stromal cell populations, their cellular states, and their spatial interactions—could provide valuable insights into its role in modulating responses to specific immunotherapeutic interventions.⁶⁻⁹ Such an understanding has the potential to guide the identification of novel therapeutic targets and optimize patient selection, thereby enhancing the efficacy of immunotherapy.

In two clinical trials, we observed complete regression (CR) or partial regression (PR) in approximately half of the patients with human papillomavirus type 16 (HPV16)-induced vulvar high-grade squamous intraepithelial lesions (vHSIL) following treatment with an immunotherapeutic tumor-specific peptide vaccine comprising HPV16 E6/E7-specific synthetic long peptides.¹⁰⁻¹¹ Importantly, vHSIL patients represent a relatively homogeneous cohort, devoid of confounding factors such as differences in gender¹², stage, oncogenic pathways¹³⁻¹⁵, or tumor location¹⁶. These factors, known to influence TME composition, often complicate studies investigating the relationship between the TME and treatment outcomes. Clinical responsiveness in these trials correlated with the magnitude of the vaccine-induced systemic T cell response¹¹⁻¹⁷ and with a pre-existing abundance of T cells and myeloid cells within the TME prior to vaccination¹⁸. This association highlights the importance of the baseline immune landscape in determining therapeutic success and provides a foundation for further exploration of how TME features shape immunotherapy outcomes.

In this study, we employed single-cell spatial transcriptomics to measure RNA expression at subcellular resolution in formalin-fixed, paraffin-embedded (FFPE) whole tissue samples.¹⁹ This approach combines the high-resolution analytical power of single-cell RNA sequencing with the spatial context provided by *in situ* imaging. Consequently, it enables the interrogation of cellular heterogeneity without relying on predefined marker sets^{19,20-23}, allowing for a comprehensive digital reconstruction of the cellular landscape within these lesions. Furthermore, this methodology facilitated an in-depth analysis of cellular states and spatial interactions within TME in relation to clinical responses to immunotherapy. Key findings from this analysis were validated using publicly available immunotherapy datasets, underscoring the robustness and translational relevance of the results.

Materials and methods

Patient samples

Pre-vaccination formalin-fixed paraffin-embedded (FFPE) biopsies of 20 women of ≥ 18 years old with histologically confirmed HPV16⁺ vHSIL were analyzed. These women participated in a phase I/II therapeutic HPV16 SLP vaccination trial with ISA101.^{10,11} The ISA101 vaccine was injected subcutaneously and consisted of 13 synthetic long overlapping peptides covering the entire amino acid sequence of HPV16 oncoproteins E6 and E7, emulsified with Montanide adjuvant. Clinical efficacy assessments in the trial were performed 3 and 12 months after the last vaccination. The patients were classified based on their best clinical response 12 months after vaccination without additional treatment into: complete responders (CR, n=6) when the lesion completely disappeared, partial responders (PR, n=7) when $\geq 50\%$ of the total lesion area had disappeared, and non-responders (NR, n=7) when $< 50\%$ of the lesion had disappeared. All patients gave written informed consent to participate in this immunotherapeutic trial. The study was conducted in accordance with the Declaration of Helsinki and approved by the national Central Committee on Research Involving Human Subjects (CCMO, NL21215.000.08) and the Leiden University Medical Center institutional ethical committee (P08.197).

Statistical analyses

Statistical data analyses were performed with GraphPad Prism 9.3.1 and with R 4.4.0, which were also used to create graphs to visualize the data. In R, *ggplot2* (v3.5.1) was used to produce plots. The median immune counts of the three

different response groups were compared using the non-parametric Kruskal Wallis test, and of two response groups by the non-parametric Mann-Whitney U test. Bonferroni corrected p-values were marked as statistically significant.

Single-cell spatial transcriptomics

The patient samples, all diagnosed with vHSIL by a specialized pathologist, were analyzed with the CosMx Spatial Molecular Imager (Nanostring), which allowed the visualization of 1000 RNA transcripts at a subcellular resolution *in situ* on FFPE pre-immunotherapy vHSIL tissue, using cyclic fluorescent *in situ* hybridization.¹⁹ This technique allowed both the discovery of new cell types in the tumor microenvironment of vHSIL, as well as the exploration of their spatial interactions. The majority of the patients' vHSIL biopsies could be completely covered with fields of view (FOVs) for spatial transcriptomic analysis. Cell segmentation was performed with CellPose, which has been shown to be one of the most reliable methods.²⁴

Semi-supervised cell type classification

Cell type classification was performed using the semi-supervised cell typing method InSituType.²⁵ An initial round of cell typing was run using an immunoncology based cell type reference profile provided as the default in the R software package, and allowing for 6 unsupervised clusters to characterize epithelial cell types. Subsequently, cells which were classified as an immune cell type were further refined by clustering smoothed averages of marker genes for the cell's nearest neighbors (<https://nanostring-biostats.github.io/CosMx-Analysis-Scratch-Space/posts/marker-gene-smoothing/>). Specifically, a set of 58 genes with known expression specificity in immune cells was pre-selected (**Supplemental Table 1**).

Let $\mathbf{X}_{\text{markers} \times \text{cells}}$ denote a total-count normalized expression matrix for cells classified as non-epithelial and their expression in the pre-selected marker gene set. Let $\mathbf{W}_{\text{cells} \times \text{cells}}$ denote a nearest neighbors matrix where each column indicates the nearest 50 neighbors of the corresponding cell in a uniform manifold approximation and projection (UMAP) space. Further each column was allowed to be standardized such that the sum of each column is 1. The 2D UMAP projection was created using pearson-residual normalized data²⁶ and default UMAP parameters implemented in Seurat software package.²⁷ Then the smoothed expression matrix $\mathbf{S}_{\text{markers} \times \text{cells}}$ takes the form $\mathbf{S} = \mathbf{X} \mathbf{W}$.

This smoothed expression matrix was clustered using k-means and cells were subsequently assigned to an immune cell type based on the expression characteristics of the corresponding k-means cluster.

Plausible gene filtering using a contamination ratio metric

For spatial transcriptomic data, one major challenge is dealing with the impact of cell segmentation uncertainty, where even minor errors can potentially confound or distort downstream analyses. For cell-type specific analyses, we develop a “contamination ratio metric” for pre-emptively excluding genes which may be likely to return spurious results due to imperfect cell segmentation.²⁸ Let $\bar{y}(t)_g$ denote the average expression of a particular gene g for a cell type t of interest. Further denote the average expression per cell of the gene in *neighboring cells* of other cell types by $\bar{y}(t')_g$. Then the ratio $\psi_g = \frac{\bar{y}(t')_g}{\bar{y}(t)_g}$ is a metric by which we quantify the susceptibility to confounding bias from segmentation error. Genes with $\psi_g > 1$ are higher expressed on average in neighboring cells of other cell types than that of cell type t . For cell type subclustering, we filtered the list of genes to be analyzed for a given cell type such that $\psi_g < 1$.

Subcluster identification

To further refine cell types into distinct cell states or subclusters, we first identified for each cell type, a list of genes that are plausibly expressed in that cell type to use for unsupervised subclustering. We defined this list of genes using the contamination ratio metric described above, selecting genes where the ratio metric $\psi_g < 1$. For a given cell type, this corresponds to subclustering using only genes with higher average expression within the cell type than in the spatially neighboring cells of other cell types. Unsupervised Louvain clustering was then performed among each cell type separately using only the filtered list of genes using Seurat.²⁷

Spatial co-localization of cell states

Multinomial logistic regression was used to characterize differential spatial colocalization of cell states between patient groups. First, we identified for each patient all pairs of spatially neighboring cells within a 10 micron radius. Next, we performed multinomial logistic regression²⁴⁹ separately for each possible cell state, estimating the probability of the neighboring cell state identity given patient group. Specifically, let Y_{in} denote the subcluster identity of the n_{th} neighbor to cell i . The regression model estimates $\Pr(Y_{in} = k \mid G_i)$, where G_i denotes the patient group of cell i (non-responder, partial responder, or complete responder), and k denotes cell state category.

For each modeled subcluster, we identified cell state pairs with differential colocalization probability between patient groups using the *emmeans* R package.³⁰

Response group specific ecosystems

First a filter was applied to connections where Estimated Pr(neighbor subcluster) is greater in CR than in both NR and PR. Secondly a filter was applied to connections where at least one of the comparisons (CR vs. NR) or (CR vs. PR) is significant ($p < 5.9e-6$). “Enriched in CR cells” indicates that the subcluster has significantly higher frequency in CR ($P < 0.05/98$ subclusters) compared to NR and PR across all subclusters. The analogous filters were used to describe PR and NR response group ecosystems.

Validation with other single-cell datasets

Single-cell datasets were obtained from public repositories, and an overview of the external datasets and their characteristics is provided in **Supplemental Table 2**. These datasets included an immune cell compendium from Domínguez Conde³¹, colon data from Kong³², breast cancer data from Bassez³³, head and neck cancer data from Franken³⁴ and a large dataset of 30 cancer types and their corresponding normal tissues from Kang³⁵. The transfer functionality from the Seurat package was used to transfer CosMx cell types on these external datasets. Main cell types with a maximal prediction score < 0.4 were considered unassigned. Cell subtypes were also considered unassigned if the parent cell type did not match that of the child cell type. Transfer of cell types was done for each dataset separately. Cell type allocation in the current and external data were compared. For each cell (sub)type the overlap with the cell type in the external data was calculated as percentage of all cells identified as a cell type as identified in the current study.

A paired Wilcoxon rank test was used to identify statistically significant differences between paired pre- and on-treatment samples in the TNBC and HNC cohorts.^{253 254} To identify differences in the ratio of cell types identified as supportive versus non-supportive for immunotherapy, the total number of supportive cells was divided by the total number of non-supportive cells. A positive ratio would indicate a higher fraction of supportive cells versus non-supportive cells. For a direct comparison of supportive cells versus non-supportive cells, we compared the percentages of supportive and non-supportive cells relative to the total number of cells measured in a patient.

Results

Digital reconstruction of a vHSIL cell atlas shows high diversity across patients.

Formalin-fixed paraffin-embedded vHSIL biopsies of 20 patients prior to immunotherapeutic vaccination who displayed a complete response (CR; n=6), partial response (PR; n=7) or no clinical response (NR; n=7)^{10 11} (**Table 1**), were analyzed at the single-cell transcriptomic level with the validated CosMx Spatial Molecular Imager using a 1000-plex RNA panel (**Supplemental Table 3**).¹⁹ A total of 65 fields of view with an accumulated area of >36mm² resulted in the detection of 274,878 cells and a mean number of 413 transcripts detected per cell (**Table 2**). Semi-supervised clustering of cells based on their RNA transcriptome (**Supplemental Table 4**) resulted in the identification of 18 different cell types, including 6 types of epithelial cells, fibroblasts, endothelial cells, and several types of lymphocytes and myeloid cells (**Figure 1A, Supplemental Figure 1A**). Cell type neighborhood clustering revealed three epithelial, one stromal, and one stromal-epithelial interface spatial niches with roughly a similar cell count between all patients (**Figure 1B, Supplemental Figure 1BC**).

Table 1. vHSIL cohort patient characteristics.

CosMx study number	Trial study number	Diagnosis	Number of vaccine doses	Systemic T cell response (MoM)	Clinical response at 3 months	Clinical response at 12 months	Trial reference
S10	206	vHSIL	4	16.5	CR	CR	NEJM
S17	503	vHSIL	4	245.5	CR	CR	CCR
S16	531	vHSIL	2	158.4	CR	CR	CCR
S12	532	vHSIL	2	207.4	CR	CR	CCR
S5	509	vHSIL	4	102	PR	CR	CCR
S11	563	vHSIL	3	178.7	PR	CR	CCR
S8	201	vHSIL	4	96.5	PR	PR	NEJM
S9	510	vHSIL	4	48.7	PR	PR	CCR
S13	512	vHSIL	4	78.2	PR	PR	CCR
S19	529	vHSIL	4	186.4	PR	PR	CCR
S18	530	vHSIL	3	195.3	PR	PR	CCR
S1	203	vHSIL	4	40.8	NR	PR	NEJM
S2	526	vHSIL	2	214	NR	PR	CCR
S15	228	vHSIL	4	61.4	NR	NR	NEJM
S7	519	vHSIL	4	194.5	NR	NR	CCR
S14	522	vHSIL	4	106.6	NR	NR	CCR
S6	524	vHSIL	4	68.1	NR	NR	CCR
S20	528	vHSIL	4	126.5	NR	NR	CCR
S3	202	vHSIL	4	2.9	NR	NR	NEJM
S4	517	vHSIL	4	161.5	NR	NR	CCR

vHSIL = vulvar high-grade squamous intraepithelial lesion
MoM = median of maximum. The MoM value was calculated by taking the median of the highest specific IFNy ELISpot counts against HPV16 E6 and E7 peptides per blood sample obtained during and after immunotherapeutic vaccination, so that one value could be obtained for the systemic T cell response per individual patient. NEJM reference[10]: Kenter et al (2009). Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med*, 361(19), 1838-1847. <https://doi.org/10.1056/NEJMoa0810097>
CCR reference[11]: Van Poelgeest et al (2016). Vaccination against Oncoproteins of HPV16 for Noninvasive Vulvar/Vaginal Lesions: Lesion Clearance Is Related to the Strength of the T-Cell Response. *Clin Cancer Res*, 22(10), 2342-2350. <https://doi.org/10.1158/1078-0432.Ccr-15-2594>

Table 2. CosMx data metrics.

	Complete responders	Partial responders	Non-responders	Full cohort
Number of fields of view (FOVs)	29	17	19	65
Area measured (mm²)	16.43	9.63	10.76	36.82
Number of cells	121,877	62,573	90,428	274,878
Total transcripts	61,739,516	23,399,624	38,913,342	124,052,482
% Of cells with >20 counts	98.08	97.45	98.13	97.95
Mean transcripts per cell	473	323	396	413
Assigned transcripts (%)	93.28	86.34	92.14	91.61
Genes detected above LOD	586	599	609	611
Cellular transcripts per μm^2	3.8	2.4	3.6	3.4
Mean negatives per cell	1.59	1.30	1.43	1.47

The 6 different epithelial cell types were characterized based on their specific keratin expression³⁶ into basal cells, suprabasal cells, activated keratinocytes, contractile keratinocytes, differentiating and fully differentiated keratinocytes (**Supplemental Figure 1DE**). All together this allowed us to digitally reconstruct vHSIL lesions (**Figure 1CD**). Moreover, we validated the concordance between keratin expression at the protein and gene transcript level (**Figure 1E**). The proportion of each epithelial cell type present in the vHSIL lesion differed per patient, sustaining the notion that the disease manifests differently across patients, but no immunotherapy response-specific pattern was found (**Supplemental Figure 1FG**). This was also the case when the proportions of the other cell types were analyzed (**Supplemental Figure 1H**). Thus, these data reveal vast heterogeneity between patients with a tumor that all had the same etiological background and originated in the same anatomical location.

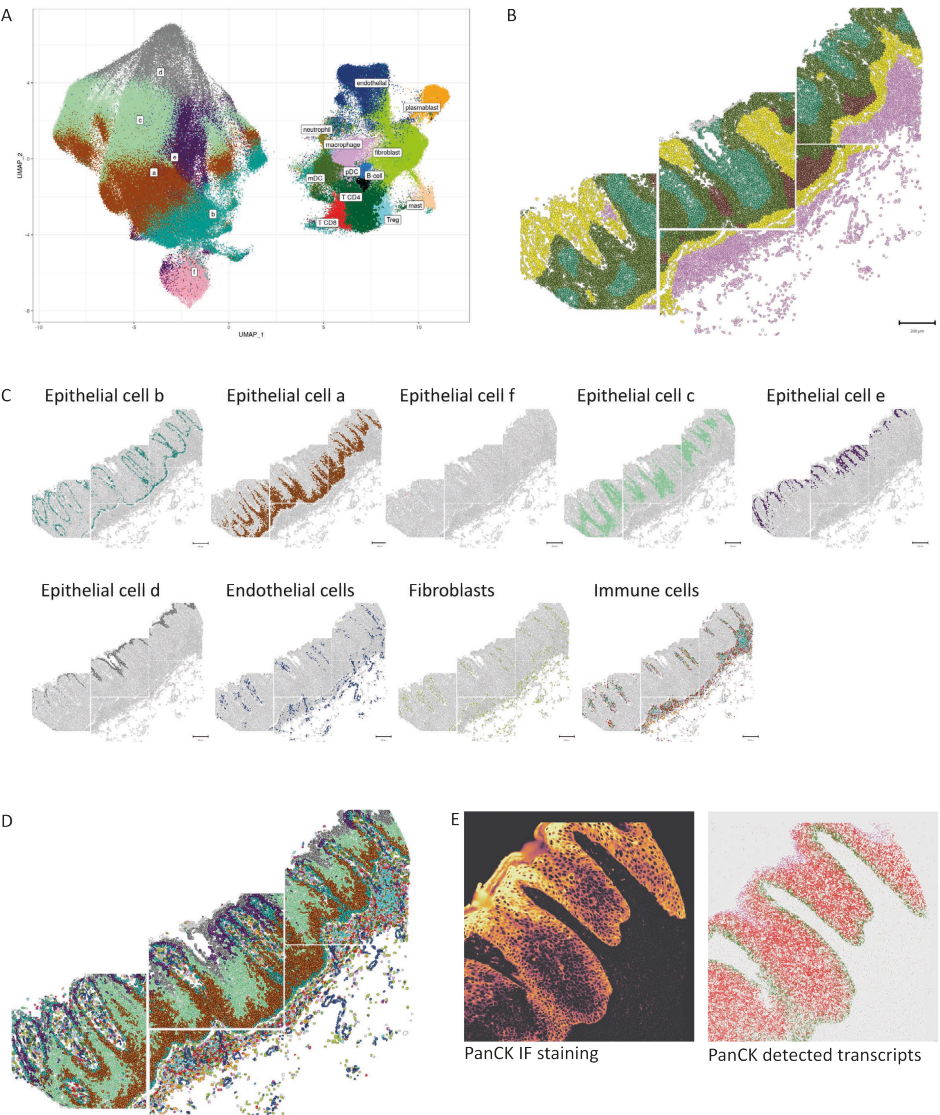


Figure 1. Cellular composition of vHSIL decomposed by spatial single-cell transcriptomics.

Archived FFPE tissue sections obtained from vHSIL patients (n=20) before therapeutic vaccination with an HPV16 synthetic long peptide vaccine, were subjected to single-cell spatial transcriptomics. Upon vaccination, 6 patients showed a complete response (CR), 7 a partial response (PR) and 7 no response (NR). **A)** UMAP plot containing 274,878 cells resulted in the identification of 18 cell clusters.

B) Representative vHSIL CosMx image after cell segmentation and tissue segmentation, indicating the 5 spatial niches (3 epithelial niches, 1 stromal niche, and 1 stroma-epithelium interface niche).

C) Representative images of the spatial location of different types of epithelial cells, fibroblasts, endothelial cells and different immune cells. **D)** Full reconstruction of tissue section by spatially overlaying all cell clusters. **E)** Comparison of pan-cytokeratin (PanCK) protein detection by immunofluorescence (antibody clone NB2-33200AF532, Novus) and RNA detection, showing a good overlay between protein and RNA detection.

Identification and validation of different cell states.

To dissect the diversity of the different cell types within the TME and between patients, the non-epithelial cell types were further subclustered based on the set of genes higher expressed in that specific cell type when compared to all other cells (**Supplemental Table 4**). This resulted in the identification of a total of 99 subclusters among endothelial cells (n=10), fibroblasts (n=10), CD4 T cells (n=11), CD8 T cells (n=8), Tregs (n=8), B cells (n=6), plasma cells (n=9), myeloid DCs (n=9), plasmacytoid DCs (n=6), macrophages (n=10), neutrophils (n=3), and mast cells (n=8) (**Supplemental Figure 2**). To ascertain whether the cell (sub)types identified were specific for vHSIL, their presence was assessed in several publicly available datasets with large numbers of cells analyzed^{32 33 35}, and an immune cell compendium³¹ (**Supplemental Table 2**). This not only successfully verified cell (sub)type identity (**Figure 2A, Supplemental Figure 3**) but also revealed that almost all identified immune cell subtypes in vHSIL, were also present in other tissues, including a series of other healthy tissues and cancer types (**Supplemental Figure 4**). The multiple different functional cell states of the cell types were derived by analysis of the cluster defining genes and literature (**Supplemental Table 5**). Based on the comparison with the other datasets, and marker gene expressions, some cell types were manually corrected (pDC_5, B cell_0 and 4, E_5), also showing that CD4_5 belonged to the Treg subclusters.

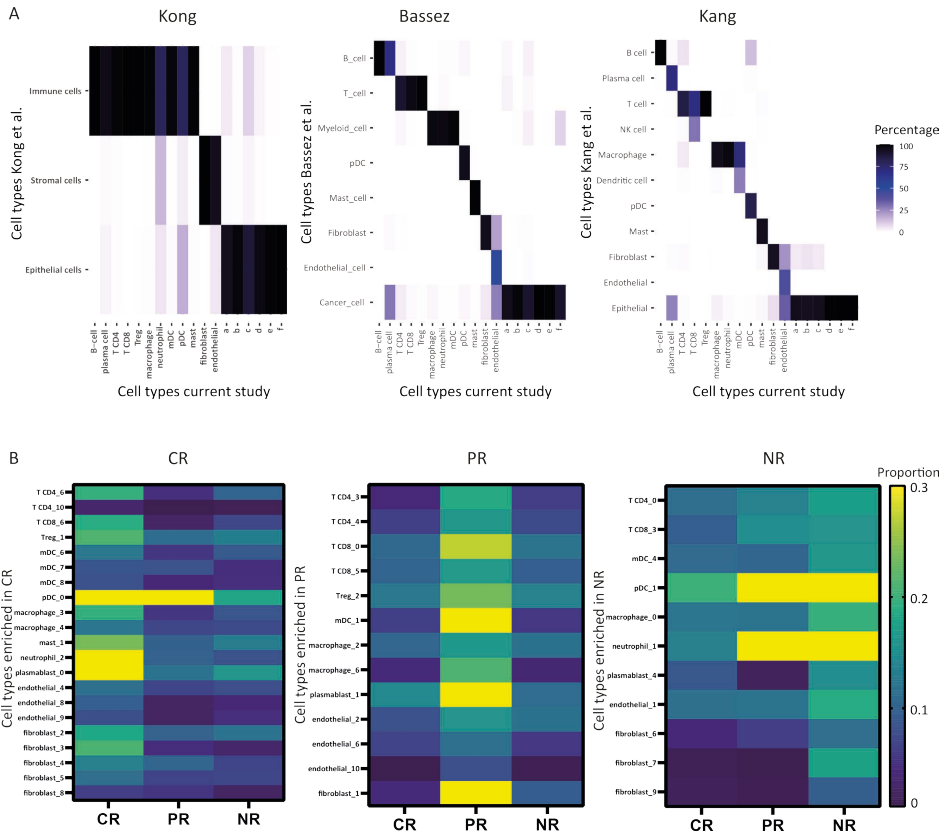


Figure 2. Validation of cell types and cell states specific for clinical response to immunotherapeutic vaccination.

A) To validate the existence of the identified 18 cell clusters, their transcriptome was compared to large numbers of single-cells present in several publicly available datasets: Kong³², Bassez³³ and Kang³⁵, showing high concordance between the identified cell clusters in this study versus that of the public databases.

B) Heatmaps showing the cell states that are specifically enriched (Z-test for difference in proportion, Bonferroni corrected) in complete responders (CR, n=6), partial responders (PR, n=7) and non-responders (NR, n=7) therapeutic vaccination, and compared to their presence in the other response groups. The color legend indicates the proportion of each cell state within the major cell type. The explanation of the cell type abbreviations can be found in Supplemental Table 5.

Balance between vaccine supportive and non-supportive cell states differs per clinical response.

A comparison between the frequencies of the different cell types and their states in the lesions of CR, PR and NR patients was made to assess whether differences in clinical response were associated with the composition of the lesions before immunotherapy. This response group comparison revealed higher proportions of CD8⁺CD103⁺ tissue resident effector cells (CD8_6), CD4⁺CD161⁺ effector cells (CD4_6), phagocytic M1 macrophages (M_3),

classically activated DCs (mDC_7), cDC2s (mDC_8), GZMB⁺, IFN-activated pDC (pDC_0), inflammatory mast cells (mast_1), T cell infiltration supportive *IGFB7*hi endothelial cells³⁷ (E_8/9) and immunity supportive fibroblasts (F_2/3/4/5/8) amongst others in complete responders to immunotherapy (**Figure 2B**).

In contrast, the lesions of partial responders comprised higher proportions of type 2 cytokine CD4⁺ T cells (CD4_3, CD4_4) and *CCL18*-expressing macrophages (M_6), known to attract type 2 T cells and Tregs.³⁸⁻⁴⁰ Non-responding lesions showed preferential infiltration with non-activated immune cells (CD8_3, CD4_0, M_0, mDC_4) and potentially suppressive fibroblasts (F_6/7/9) when compared to the other response groups (**Figure 2B**).

For many subclusters, but not all, their transcriptome allowed to propose a potential positive or negative direction of their effect on immunotherapy, based on our current knowledge of tumor-immunity (**Supplemental Table 5**). The variation in the proportion of each individual cell type and cell state among the patients in each response group (**Supplemental Figure 5**) suggested that rather than a difference in one specific cell type or cell state, it is the overall balance between the anticipated effect of all cell states present in the lesion that may influence clinical outcome. Indeed, while the proportions of cells with a supportive effect on immunotherapy were found in balance with the proportions of non-supportive cells in the CR group, the proportion of cells negatively impacting immunotherapy was higher in the PR and NR patient groups (**Figure 3**). CR lesions exhibited a higher ratio of immune-supportive to immune-suppressive cells, compared to PR and NR lesions (**Figure 3**).

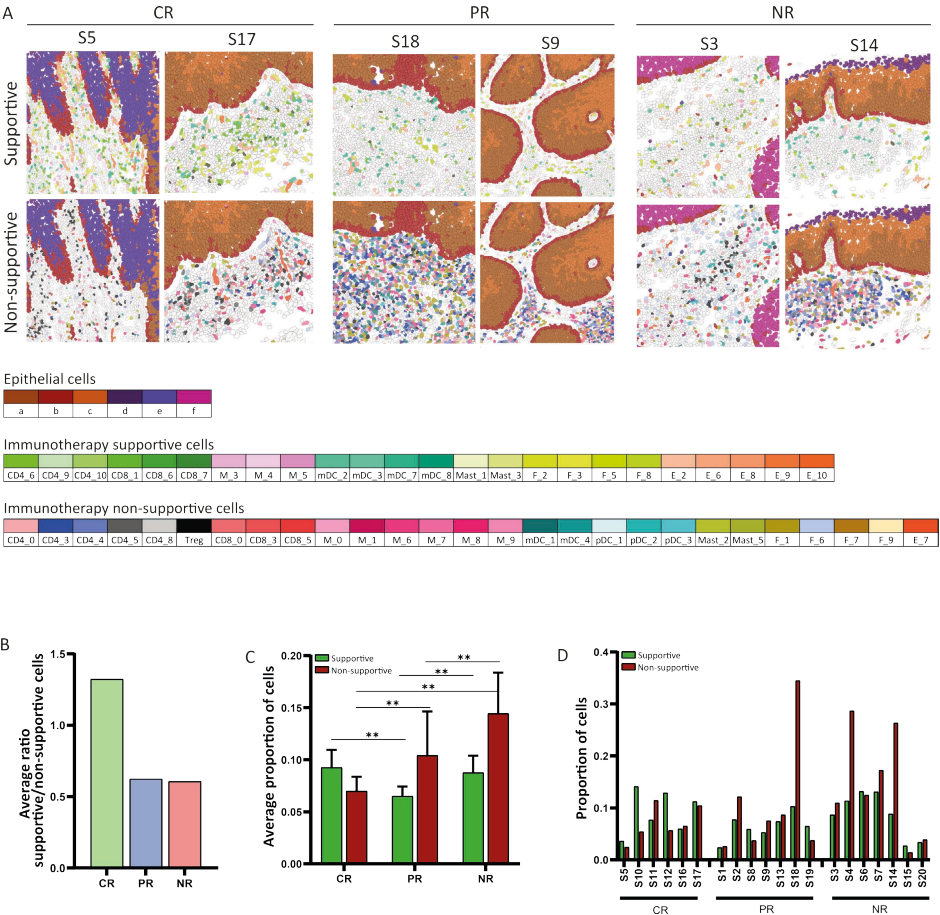


Figure 3. The balance between vaccination supportive and non-supportive cells differs per vaccine response group.

For many subclusters, their transcriptome allowed to identify them as potentially supportive or non-supportive for immunotherapy (Supplemental Table 5). Cells classified as supportive were: CD4_6/9/10; CD8_1/6/7; M_2_1/3/4/5; mDC_2/3/7/8; pDC_4; Mast_1/3; F_2/3/5/8; E_2/6/8/9/10. Cells classified as non-supportive were: CD4_0/3/4; Tregs; CD8_0/3/5; mDC_1_4; M2_0/1/6/7/8/9; pDC_1/2/3; Mast_2/5; F_1/6/7/9; E_7. The explanation of the cell type abbreviations can be found in Supplemental Table 5. **A**) Two representative CosMx images per response group in which the immunotherapy supportive cells (upper image) and the immunotherapy non-supportive cells (lower image) are visualized in situ. Epithelial cells are also shown for reference. **B**) The ratio of therapeutic vaccine supportive versus non-supportive cells was determined in complete responders (CR), partial responders (PR) and non-responders (NR) to therapeutic vaccination. Ratio >1 indicating an excess of supportive cells, <1 indicating an excess of non-supportive cells. **C**) The average proportion of total cells classified either as supportive (green) or non-supportive (red) in the groups of CR, PR and NR patients. Statistical significance is calculated with the Z-test for difference in proportion, and indicated with asterisks: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. **D**) The proportion of total cells classified either as supportive (green) or non-supportive (red), present per individual patient. CR, $n = 6$, PR, $n = 7$, NR, $n = 7$.

Presence and response of detected immune cell states after immune checkpoint blockade.

We identified two patient cohorts who received neoadjuvant checkpoint blockade therapy, of which single-cell transcriptomics data on all cell types present in the TME were available. In the first cohort, patients with triple negative breast cancer (TNBC) were treated with anti-PD1 prior to surgery ³³. In the second cohort, the effect of neoadjuvant anti-PDL1 with or without anti-CTLA4 was studied in advanced resectable head and neck squamous cell carcinoma (HNC) ³⁴. We interrogated these datasets for the presence and response to checkpoint blockade of the immune cell states detected in the vHSIL lesions by a comparison of the frequencies of all these cells in the TME before neoadjuvant checkpoint therapy and at surgery.

In both cohorts, checkpoint blockade resulted in the increased detection of the immune supportive CD4⁺CD161⁺ effector cells (CD4_6) and activated chemotactic CD8⁺ cells (CD8_7), while a decrease was observed in the non-supportive population of suppressive CD8⁺ cells (CD8_0) (**Figure 4AB**). In addition, in both cohorts a decreased presence of non-supportive fibroblasts (F_1, F_6, F_7) was observed (**Figure 4AB**). There were also treatment group specific changes in immune supportive and non-supportive cell states found. In the TNBC cohort an increase in immune supportive proliferating CD4⁺ cells (CD4_9) and chemotactic macrophages (M_5) was observed after checkpoint blockade, while for the immune non-supportive populations an increase in Tregs (Treg_1, Treg_4) and a decrease in CD8_3 cells was found (**Figure 4AB**). In the HNC cohort the decrease of DC-like macrophages (mDC_3) and IGFB7⁺ endothelial cells (E_8) was observed after treatment (**Figure 4AB**).

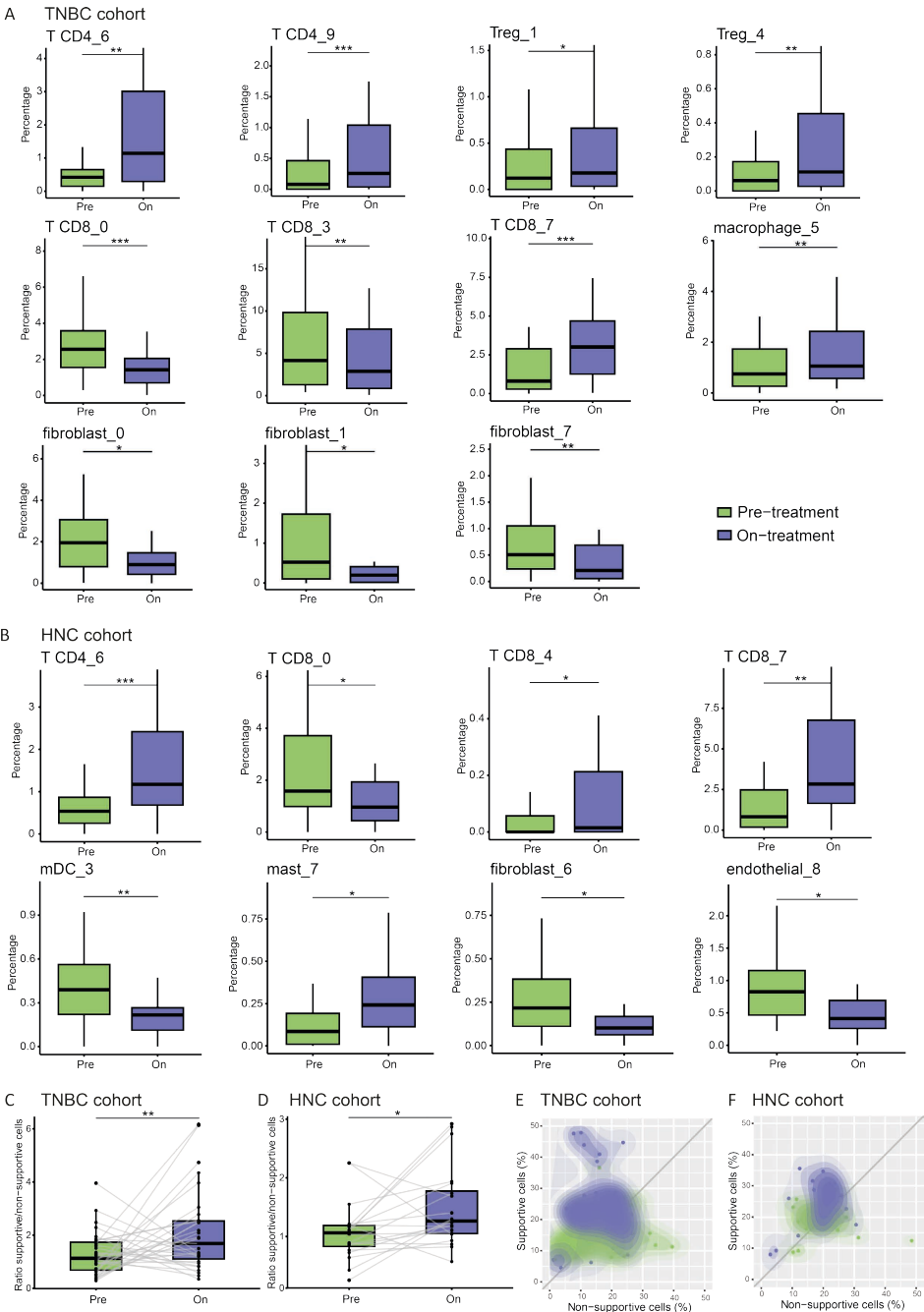


Figure 4. The response of identified cell states upon checkpoint blockade.

*Neoadjuvant checkpoint blockade altered the proportion of several of the identified cell states present in the TME of **A**) patients with triple negative breast cancer (TNBC) ³³, and **B**) patients with advanced resectable head and neck squamous cell carcinoma (HNC) ³⁴. Boxplots show the proportion of those cells that significantly shifted after checkpoint blockade.*

*The ratio of immunotherapy supportive versus non-supportive cells was determined **C**) in the TNBC cohort and **D**) in the HNC cohort, before and after checkpoint blockade therapy. Boxplots depict this ratio, and how it changes upon immunotherapy, with paired patient data (lines connecting the same patient). Statistical significance is calculated with the Wilcoxon rank test, and indicated with asterisks: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. The explanation of the cell type abbreviations can be found in Supplemental Table 5. **E, F**) The shift in the proportion of immunotherapy supportive and non-supportive cells before and after checkpoint blockade was visualized by scatter plots, showing a specific increase in the proportion of immunotherapy supportive cells upon checkpoint blockade therapy (vertical shift of the cloud upon therapy), while the proportion of non-supportive cells did not change upon therapy (no horizontal shift).*

The consistent increase in CD4⁺CD161⁺ effector cells (CD4_6), the activated chemotactic CD8⁺ cells (CD8_7) and decrease in the non-supportive population of suppressive CD8⁺ cells (CD8_0) cells single these cell states out as cells that are particularly being modulated by immune checkpoint therapy, irrespective of the checkpoint blocking antibody used. However, tumor type- and/or therapy-specific changes in immune supportive and non-supportive cell states were also found. To assess if checkpoint therapy displayed an overall positive effect on the proportion of immune supportive cells in the TME, we interrogated the data of these two cohorts with respect to the balance between immunotherapy supportive and non-supportive cells. In both cohorts the ratio between supportive and non-supportive cell states increased, indicating a shift towards a higher presence of immune-supportive cells in the TME after checkpoint blockade (**Figure 4CD**). Scatter plots showing the percentage of all supportive and non-supportive cell states in the TME for the patients before and after checkpoint blockade therapy shows a treatment-associated shift upwards above the diagonal, indicating that the enhanced ratio mostly is due to an increased percentage of immune supportive cell states upon treatment (**Figure 4EF**). Overall, these data indicate that checkpoint blockade is associated with significant alteration of the TME in favor of effective anti-tumor immunity and suggests a more prominent role for the CD4⁺CD161⁺ effector cells (CD4_6) and the activated chemotactic CD8⁺ cells (CD8_7) in this treatment.

Spatial ecosystems prime the patient's clinical response to immunotherapy.

The abundancy of specific cell states from a variety of cell types in each response group can reflect different functional ecosystems in which different cells may play a role to build the ecosystem and/or interact to achieve a particular functional outcome within the ecosystem. We performed a 10µm distance neighborhood analysis to capture potential interactions between

cells that were significantly enriched in one clinical response group when compared to the other two response groups of vHSIL patients. In the CR group a central role was found for CXCL14⁺ reticular fibroblasts (F_3), known to foster immune cell infiltration⁴¹, displaying clustering with many types of T cells and dendritic cells (**Figure 5A, Supplemental Figure 6A**). CXCL14 is a chemokine with both pro-tumor (inducing M2 proliferation of macrophages, stimulating angiogenesis) and anti-tumor (chemotaxis of dendritic cells and natural killer cells) capacities, depending on the context.⁴²⁻⁴⁴ High numbers of CXCL14-expressing fibroblasts have previously been found to be associated with response to immune checkpoint blockade in renal cell carcinoma⁴⁵, and with improved anti-tumor immunity in glioma⁴⁶. Moreover, there was an enrichment of multiple specific cell states, including that of effector T cells (CD4_6; CD8_6), phagocytic macrophages (M_3), pDC_0, Tregs and of a T cell infiltrating supporting endothelial (E_8) subset³⁷ more preferentially present in the CR than in the PR and NR group and with a higher probability to form the ecosystem (**Figure 5**).

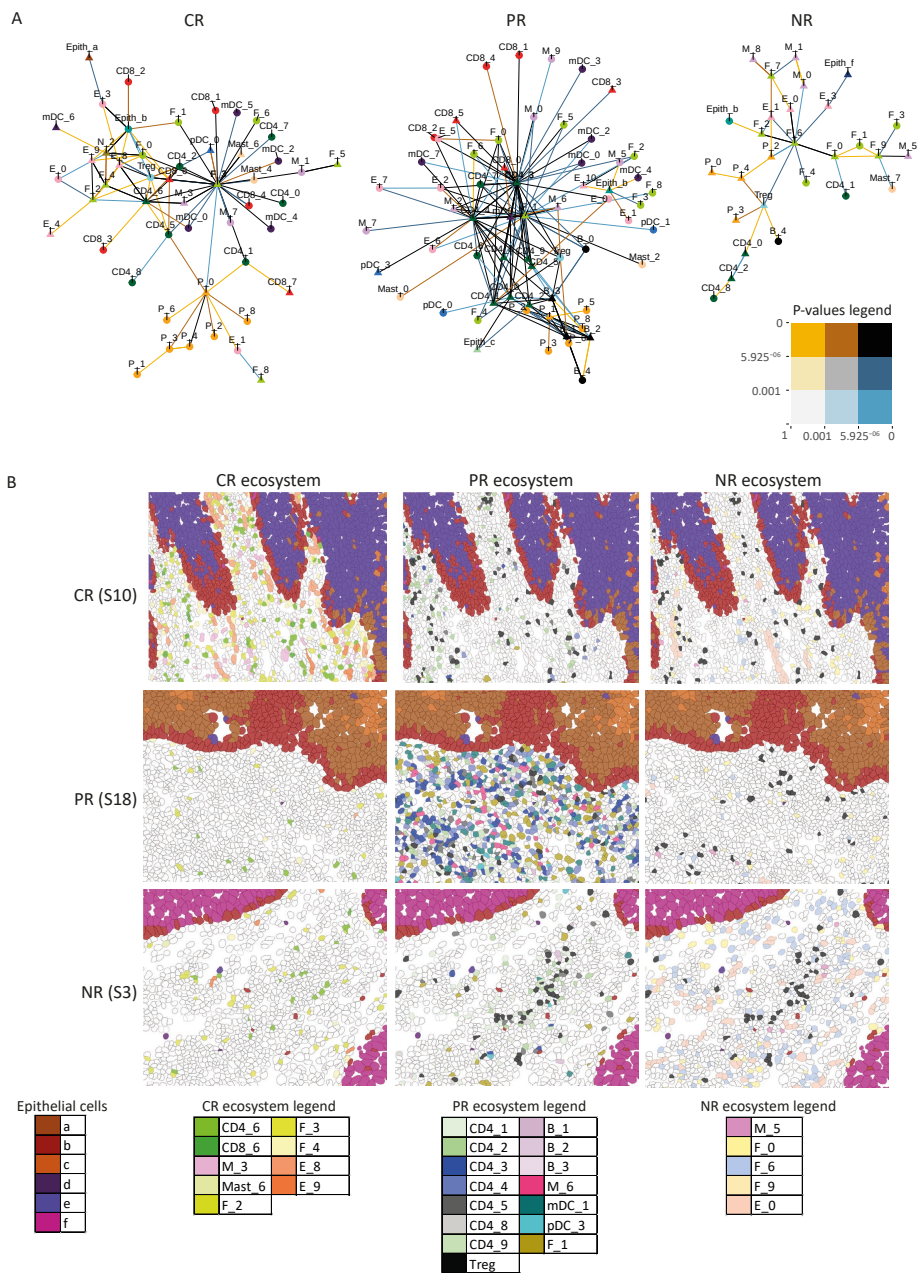


Figure 5. Pre-existing cellular ecosystems are specific for the response to therapeutic vaccination.

To capture potential cellular interactions, a 10 μ m distance neighborhood analysis was performed on the single-cell spatial transcriptomics data of the complete responders (CR, n=6), partial responders (PR, n=7) and non-responders (NR, n=7) in the vHSIL cohort treated with therapeutic vaccination.

A) Spatial cellular interaction networks ('ecosystems') that are specific for therapeutic vaccine-induced CR, PR and NR are shown. A triangle indicates cell types with a cell frequency that is specifically enriched in that response group, a circle indicates those that are not specifically enriched. The color of the connecting line reflects the level of significance and indicates how prominent the spatial interaction is in that response group, calculated by multinomial logistic regression (comparing the connection for pairs of subclusters between groups). The legend for the corresponding p-values is provided as a colored box. For CR: orange line indicates significantly increased compared to PR, blue line indicates significantly increased compared to NR. For PR: orange line indicates significantly increased compared to NR, blue line indicates significantly increased compared to CR. For NR: orange line indicates significantly increased compared to PR, blue line indicates significantly increased compared to CR. **B)** Visualization of the cells forming the CR, PR or NR characteristic ecosystems in a representative CR, PR and NR FFPE vHSIL section. Epithelial cells are also shown for reference. The explanation of the cell type abbreviations can be found in Supplemental Table 5.

In the PR group a central role was found for two CD4 cell states (CD4_3 and CD4_4) interacting with many different states of dendritic cells and macrophages (**Figure 5B**). In particular, two CD4⁺ T cell states associated with type 2 cytokine production clustered at higher probability with CCL18-expressing (M_6) macrophages, fibroblasts_1, suppressive mDC_1 and Tregs (CD4_5 and Tregs) compared to patients with CR or NR (**Figure 5**). As literature shows that especially CCL18⁺ macrophages activate fibroblasts^{47 48}, we investigated the correlation between the CCL18⁺ macrophages (M_6) and fibroblasts. Indeed, a direct relationship was found between the presence of CCL18⁺ macrophages (M_6) and fibroblasts in PR patients ($r = 0.97$, $P = 0.0003$), but not in NR ($r = -0.17$, $P = 0.71$) or CR ($r = -0.24$, $P = 0.65$) (**Supplemental Figure 6B**). Yet, when the correlation between macrophage_6 and the various fibroblast subtypes was examined, we found that the presence of CCL18⁺ macrophages was specifically related to the presence of the fibrosis enhancing subtype fibroblasts_1 in all response groups (PR, $r = 0.95$, $P = 8.7 \times 10^{-4}$; NR, $r = 0.86$, $P = 0.01$; CR, $r = 0.79$, $P = 0.06$) but most pronounced in the PR patient group (**Supplemental Figure 6C**) and fitting with the fact that this patient group displayed the strongest influx with CCL18⁺ macrophages. This confirms that CCL18, but in particular CCL18 expressing macrophages being the most dominant cell type expressing CCL18 (**Supplemental Figure 6D**), induce immunosuppressive fibroblasts.

The CR- and PR-specific spatial ecosystems clearly differed from the one found in the NR group as in the latter group specific enrichment of interactions between non-supportive fibroblasts (F_6) and macrophages (M_0) and Tregs was found (**Figure 5**). These data demonstrate the presence of specific spatial ecosystems in vHSIL, of which the organization of tumor-immunity supporting cell clusters in CR clearly differ from the predominantly type 2

cytokine response-supporting cell clusters organized in PR, and from the absence of specific immune cell clusters in NR. This suggests that the type of cellular ecosystem present in treatment naïve vHSIL supports or restrains the clinical response to immunotherapeutic vaccination. Analysis of two pre- and post-vaccination vHSIL biopsies sustained this notion, showing an increased Th2 and a decreased Th1 outgrowth in a PR patient, but not in a CR patient, despite a similar strong systemic vaccine-induced IFN γ response mediated predominantly by CD4 T cells (**Supplemental Figure 7**).

Discussion

Leveraging single-cell spatial transcriptomics in a clinically driven approach, we addressed a fundamental question in cancer research: the complexity and heterogeneity of the tumor microenvironment and its impact on immunotherapy responsiveness. Our analysis revealed that the complexity and diversity of cellular states in the TME varies significantly between patients, even among those with similar clinical outcomes. Specifically, the proportion of cells potentially supportive or non-supportive to anti-tumor immunity differs across patients and there was no consistent association between specific cell types and clinical response to immunotherapeutic vaccination. Instead, the TME of patients who achieved complete regression displayed a specific ecosystem in which inflammatory cells capable of supporting vaccine-driven anti-tumor responses closely interacted. In partial responders the TME comprised a mixed type 1 and type 2 cellular environment which was specifically dominated by close interactions between type 2 immune cells, generally less effective at supporting anti-tumor immunity.⁴⁹ Our findings also show that neoadjuvant immune checkpoint therapy altered the balance between immune-supportive and non-supportive cell states within the TME, specifically increasing the proportion of immune-supportive cells. These observations underscore the broader clinical relevance of our data.

This study investigates the TME of patients treated with an immunotherapeutic vaccine. Over five decades of research have driven substantial progress in therapeutic cancer vaccine development, including the improved identification of tumor-specific antigens, the engineering of highly immunogenic platforms, and the integration of vaccines with immune checkpoint blockade therapies. These advancements are now bolstered by reliable methods for assessing vaccine pharmacodynamics.^{1 50} Despite these innovations, the identification

of robust predictive biomarkers for clinical success remains a pivotal unmet need. While vaccine-induced immune responses often correlate with clinical activity in trials^{1 17 50-53}, pinpointing specific cellular or molecular biomarkers has proven challenging. This is likely due to the complexity and heterogeneity of the TME, where cell types, cell states, and their spatial interactions exhibit significant interpatient heterogeneity. Our findings underscore the importance of analyzing the TME in its spatial context to unravel this complexity. This spatially informed perspective may transform how we evaluate and stratify patients in immunotherapy, enabling tailored interventions with greater clinical impact.

Our data suggest that the overall balance between immune-supportive and restraining cells in the TME—spanning lymphocytes, myeloid cells, fibroblasts, and endothelial cells—determines vaccine efficacy. Moreover, our findings indicate that distinct cellular ecosystems, rather than isolated cell types, govern the lesion's susceptibility to immunotherapeutic vaccination. An ideal therapeutic vaccine amplifies tumor-specific type 1 cytokine-producing CD4+ and CD8+ T cells.^{1 54} However, PR patients exhibited ecosystems enriched with type 2 cytokine-producing CD4+ T cells (CD4_3, CD4_4), which interacted with Th2-attracting macrophages (M_6), wound-healing fibroblasts (F_1), and certain dendritic cells (mDC_1). This Th2-dominant environment may undermine vaccine-driven anti-tumor responses, despite the presence of robust systemic Th1/Th2 immunity post-vaccination¹⁷.

The presence of distinct ecosystems explaining variable clinical outcomes is consistent with observations in preclinical and clinical models. For example, recent murine studies demonstrated that a spatial triad of CD4+ T cells, CD8+ T cells, and dendritic cells is critical for both anti-tumor immunity and responsiveness to checkpoint inhibitors.⁵⁵ Similarly, Luca et al. identified carcinoma ecosystems (CEs) across 16 human cancers.⁵⁶ Two distinct ecosystems, CE9 and CE10, have been strongly associated with favorable clinical outcomes, including longer overall survival, spontaneous regression of premalignant lung lesions (CE10), and positive responses to checkpoint blockade therapy (CE9). The differential association of CE9 and CE10 with specific clinical outcomes underscores the concept of therapy-specific ecosystems. Consistent with this, the CR-associated ecosystem identified in our study shares several cellular states with CE9 and CE10. For example, DC_S1 and mDC_2 correspond to cDC1; DC_S3 (CD80, CD274) aligns with mDC_7; CD8_S1 (GZMK) matches CD8_3; CD8_S3 (GZMB) is similar to CD8_6; Mast S4 (TPSAB1, TPSB2, CPA3) corresponds to Mast_1; CD4_S1 (IL2RA, CTLA-4, Foxp3, TIGIT) resembles Tregs/

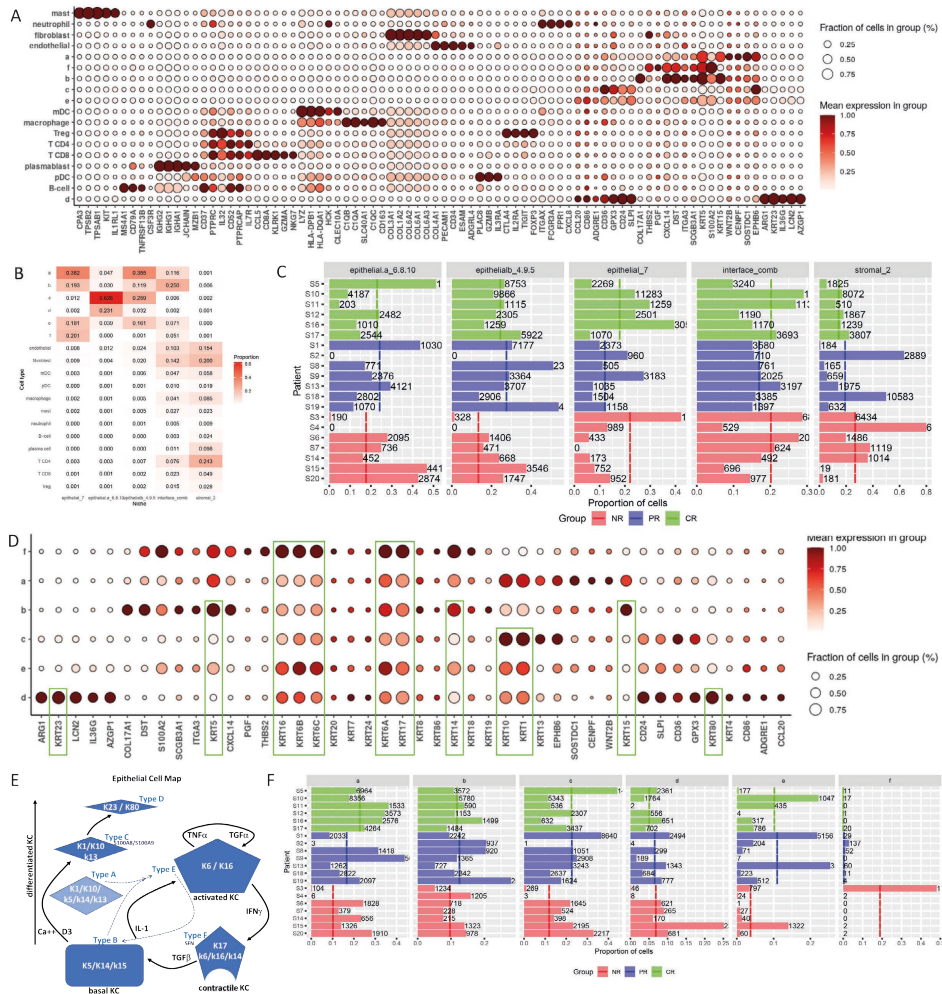
CD4_5; and CD4_S2 (IL7R) aligns with CD4_6. Importantly, our findings also revealed the enriched presence of cDC2 (mDC_8), phagocytic M1 macrophages (M_3), a unique fibroblast state (F_3), and several IGFBP7^{hi} endothelial cell states in CR patients, further illustrating ecosystem-level differences linked to clinical outcomes.

These results extend our understanding of T cell-based immunotherapy, highlighting conserved cellular features across various treatment modalities. One prominent example is the enrichment of CD161(KLRB1)+CD4+ Th1 effector cells in CR patients from our vaccine cohort. This cell population was similarly enriched in responders with triple-negative breast cancer (TNBC) and head and neck cancer (HNC) treated with PD1 or PDL1 checkpoint blockade.^{33 34} Our previous work described this CD161+CD4+ effector memory subset in tumors exhibiting active anti-tumor T cell responses.⁵⁷ These cells are characterized by their ability to produce key effector cytokines, including IFN γ and TNF α ,¹⁶ and their rapid response to antigen recognition compared to CD161-negative counterparts⁵⁸. Moreover, increased numbers of intratumoral CD161+CD4+ effector cells are strongly correlated with improved survival in multiple cancers, including HNC.^{16 58} Collectively, these observations underscore the pivotal role of this CD4+ T cell population in driving effective immunotherapy responses.

Another key finding is the presence of cells producing immune cell homing cytokines, which were detected in both partial and complete responders. Specifically, we identified a subset of CD4+ T cells (CD4_2) expressing IL16, a cytokine known to recruit monocytes, dendritic cells, and T cells into lesions.⁵⁹⁻⁶¹ In vHSIL, CD4_2 cells were spatially interacting with effector cells, including CD4_6 and CD8_6. In checkpoint blockade cohorts, treatment increased the prevalence of CD8+ T cells (CD8_7) producing the chemokines CCL3 and CCL4, which attract various myeloid cell types and T cells.^{62 63} Recent evidence also demonstrates that CCL3 and CCL4-producing CD8+ T cells infiltrate tumors upon therapeutic vaccination, driving the recruitment and activation of phagocytic M1-like macrophages, ultimately leading to complete tumor regression.⁶⁴ Based on their cellular interactions within the CR ecosystem and functional roles described in the literature, several other cell states are likely to positively influence clinical outcomes following immunotherapy. While the contribution of these states remains to be fully elucidated, the importance of CD161+ Th1 cells (CD4_6), chemotactic CD8+ T cells (CD8_7), CD8+CD103+ tissue-resident effector cells (CD8_6), and phagocytic M1 macrophages (M_3) has been firmly established.⁶⁵

The study also identified macrophages expressing CCL18, a marker of alternatively activated macrophages within Th2-dominant environments.⁶⁶ While CCL18 may directly boost tumor growth⁶⁷, its production also leads to the preferential attraction of Th2-like cells and Tregs^{39 40}, both enriched in PR patients. The presence of these macrophages was connected to that of Th2 cells, both of which were predominantly found in patients with a partial response upon therapeutic vaccination. Previously, we have shown that vaccination led to the activation of both Th1 and Th2 CD4+ T cells, and in a vHSIL patient case with a PR we were able to show that only tumor-specific Th2 cells could be cultured from the lesion after vaccination.¹⁷ Our study thus identified CCL18 as a potential target for co-treatment during therapeutic vaccination. A recently developed *in vivo* active CCL18 blocking peptide⁶⁸ may provide such help.

Supplemental Figures



Supplemental Figure 1. Cell clusters, spatial niches and epithelial cells.

Archived FPPE tissue sections obtained from vHSIL patients (n=20) before therapeutic vaccination with an HPV16 synthetic long peptide vaccine, were subjected to single cell spatial transcriptomics. Upon vaccination, 6 patients showed a complete response (CR), 7 a partial response (PR) and 7 no response (NR).

A The 18 identified cell clusters and their top characterizing genes.

B The 5 identified spatial niches (3 epithelial, 1 stromal, 1 stromal-epithelial interface) and the proportions of the various cell types in those spatial niches.

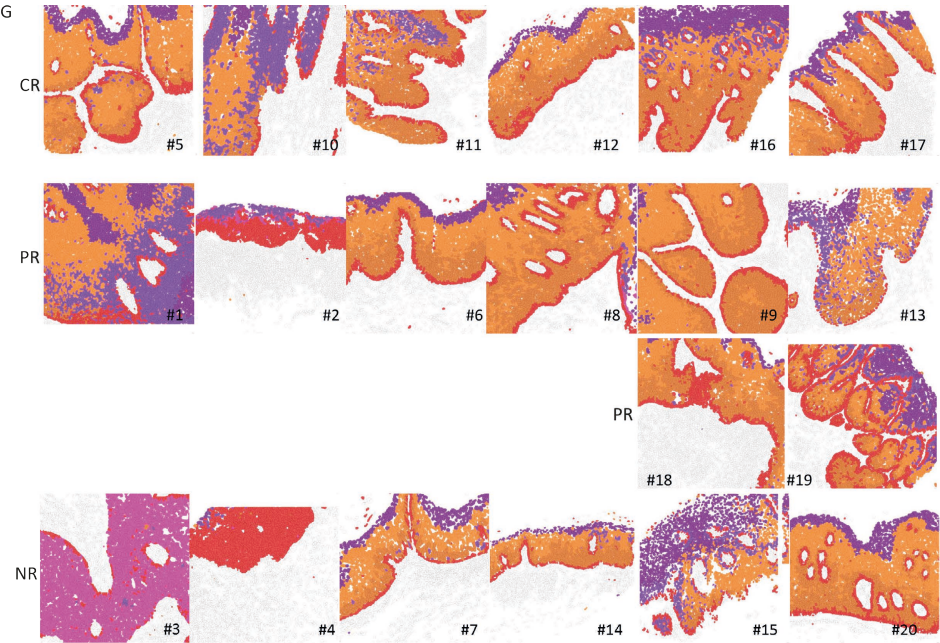
C The number of cells and proportions per spatial niche per patient. The horizontal line indicates the group-wise proportion.

D The 6 epithelial cell clusters, and the top genes expressed in those clusters. Highlighted in green are the keratins.

E Epithelial cell map, showing the various differentiation states of the epithelial cells: basal cells, suprabasal cells, activated keratinocytes, contractile keratinocytes, differentiating and fully differentiated keratinocytes. Adapted from Freedberg et al. J. Invest. Derm. 2001.

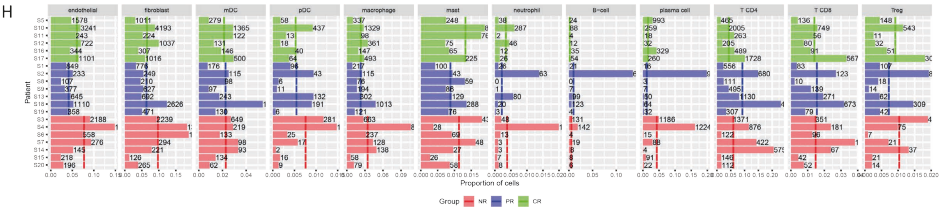
F The number of cells and proportions per epithelial cell cluster per patient.

Chapter 5



Epithelial cells color legend

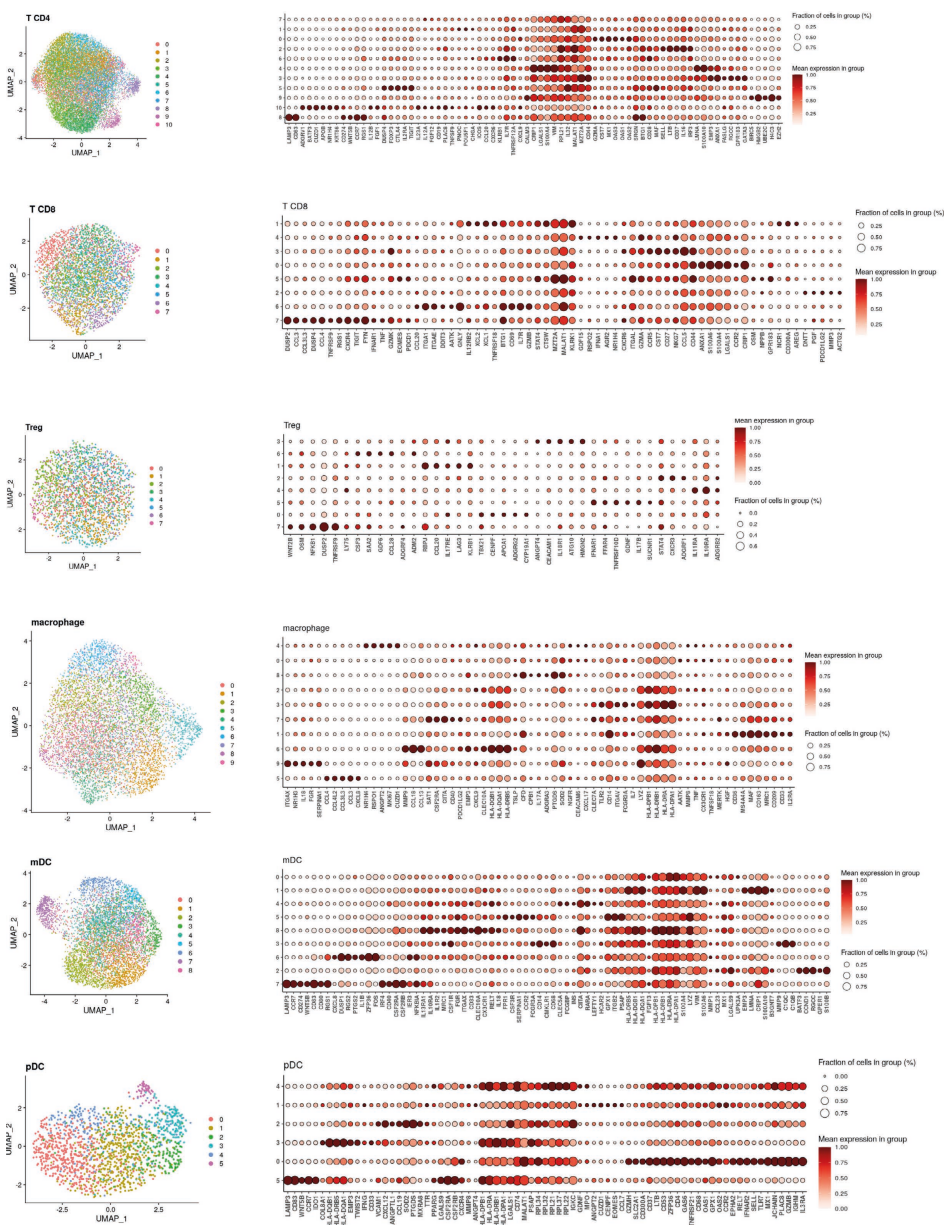
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Supplemental Figure 1. Continued

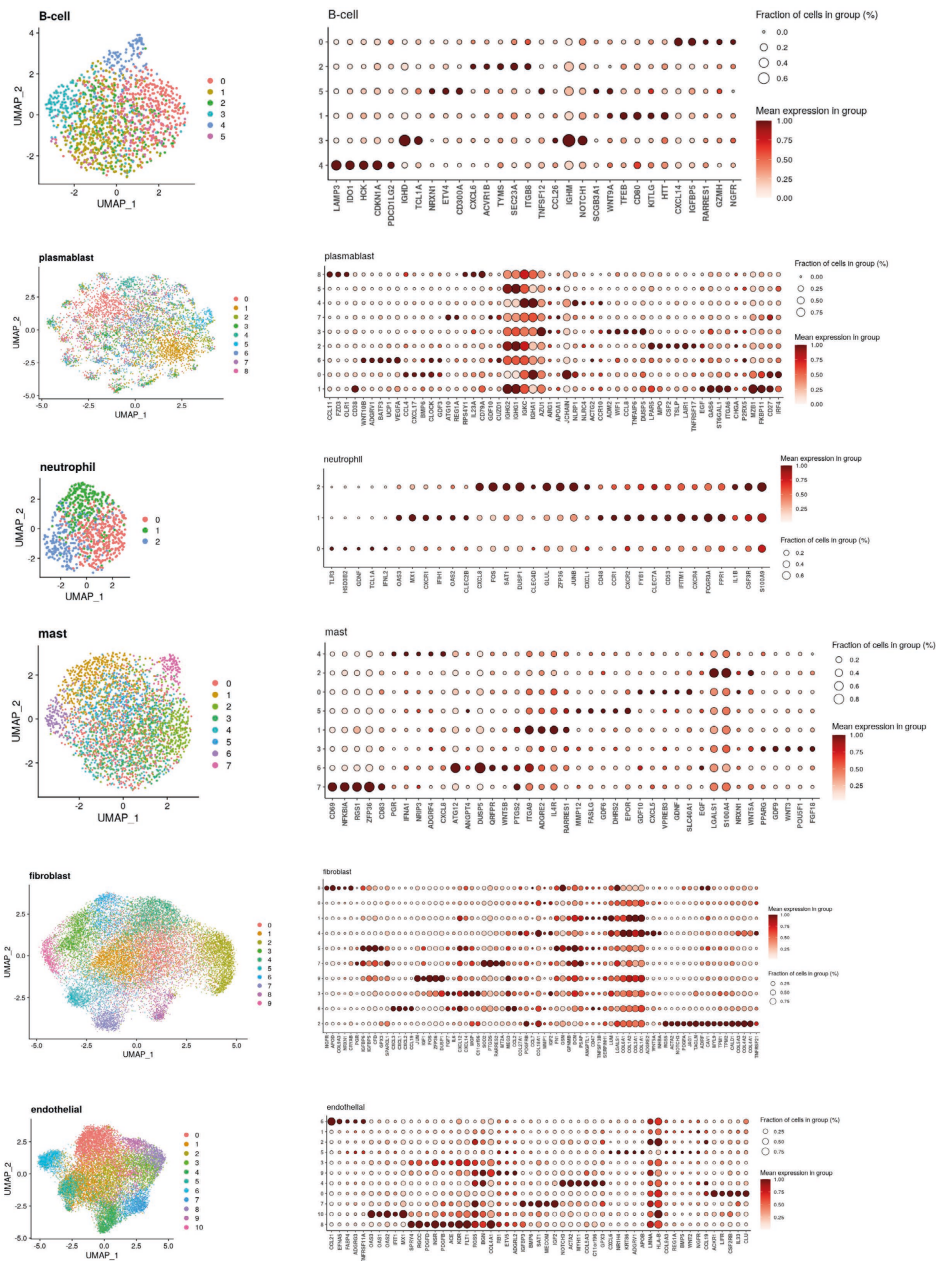
G) CosMx images of the 20 individual patients, and the spatial distribution of the 6 identified epithelial cell clusters.

H) The number of cells and proportions per non-epithelial cell cluster (n = 12) per patient.

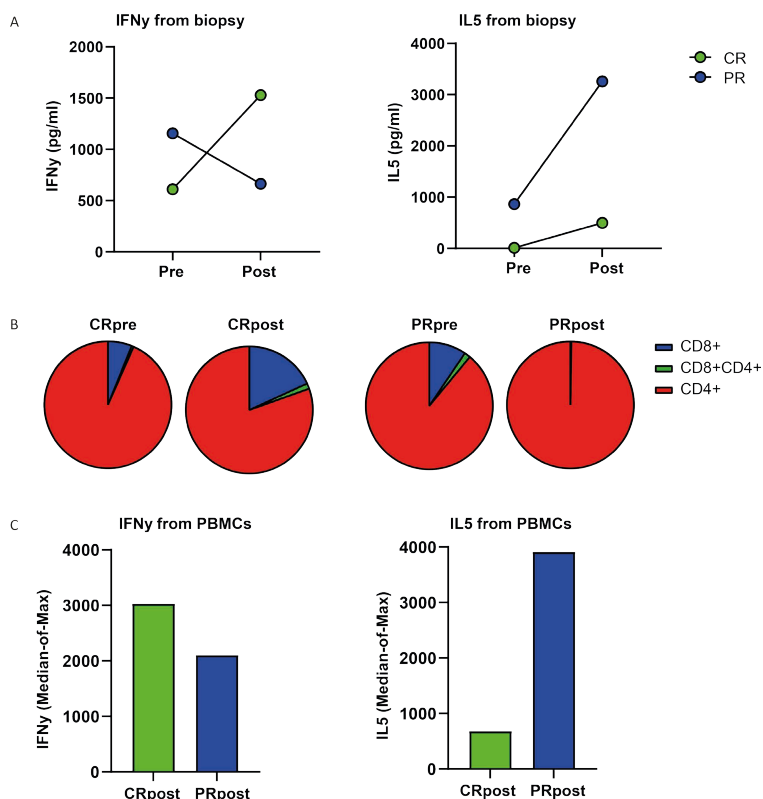


Supplemental Figure 2. All non-epithelial cell clusters and their highly expressed genes.

On the left, the UMAPs of the non-epithelial cell clusters ($n = 12$) and all their cell states ($n = 99$) are shown. Each dot indicates a single cell, derived from the single cell transcriptomics experiment on treatment naive vHSIL patients ($n = 20$). On the right, the top expressed genes per cell state are shown.



Supplemental Figure 2. Continued



Supplemental Figure 3. Cytokine analyses of the immunotherapeutic vaccine-induced T helper profile.

In vitro assays were performed to study the effector T cell response in vHSIL patients upon therapeutic vaccination.

A) Cytokine responses from vHSIL biopsies pre- and post-immunotherapeutic vaccination, in a CR and PR patient (paired samples, connected by a line). On the left IFN γ , indicative for a T helper 1 response, and on the right IL5, indicative for a T helper 2 response

B) Percentage of HPV-specific T cells by T cell subtype, cultured from vHSIL biopsies pre- and post-immunotherapeutic vaccination, indicating that it is predominantly a CD4+ T cell response.

C) Cytokines derived from freshly cultured antigen stimulated PBMCs post-immunotherapeutic vaccination. The Median-of-Max (MoM) value on the y-axis was calculated by taking the median of the highest specific ELISpot counts per blood sample, so that one value could be obtained for the systemic T cell response per individual patient.

Supplemental Tables

Supplemental Table 2. Characterizing gene set per cell type.

Expected cell type	Gene	Expected cell type	Gene
B cell	MS4A1	pDC	IL3RA
	CD19		GZMB
	CD79A	Plasmablast	JCHAIN
	IGKC		IGHG1
Endothelial	VWF		IGHG2
	PECAM1		IGHM
	RGS5		IGHA1
	SPARCL1		MZB1
Epithelial	KRT5		XBP1
	KRT17	T CD4, T CD8	CD3E
	KRT19		CD3D
	EPCAM		CD3G
Fibroblast	COL1A1	T CD4	IL7R
	COL1A2		STAT4
	COL3A1		TBX21
Macrophage	CXCL8	T CD8	CD8A
	CD68		CD8B
	CD163		KLRK1
	C1QA		GNLY
	C1QB	Treg	GZMA
	C1QC		FOXP3
	LYZ		CTLA4
	MMP9		PDCD1
	IL18		IL32
Mast cell	CPA3		TIGIT
	TPSB2		
	TPSAB1		
	KIT		
mDC	CSF3R		
	CLEC10A		
Neutrophil	IL1B		
	ITGAX		
NK cell	NCR1		

Supplemental Table 4. Characteristics of the validation single cell transcriptomics datasets.

Paper	Tissue	Therapy	Number of patients	Number of cells after QC
Kong, Immunity 2023	Crohn's disease + healthy colon	-	85	413877
Kang, Nature Communications 2024	30 cancer types	-	999	4901584
Bassez, Nature Medicine 2021	Breast cancer	Anti PD1 (pembrolizumab)	31	175942
Franken, Immunity 2024	Head and neck cancer	Anti PD1 (durvalumab) +/- anti CTLA4 (tremelimumab)	20	446380

Supplemental Table 5. Cell state defining genes.

Cluster	Cell abbreviation	Cell state	Cell state defining genes and references	Expected immune impact
CD4 T cells				
0	CD4_0	IFN signalling, low cytotoxic	MX1, OAS1-3, CST7[1]	Non-supportive
1	CD4_1	Non-specified CD4 T cell		
2	CD4_2	Homeotypic cytokine producing	IL-16[2], LTB, MALAT-1[3], CD37[4]	
3	CD4_3	Activated Th2	GATA3, CRIP1[5], RGCC[6], CD44[7], LMNA[8], ANXA3[9]	Non-supportive
4	CD4_4	IL-2 stimulated , activated non-Th1 cells	S100A4[10], S100A6, LGALS1, LMNA[8], VIM[11], CRIP1	Non-supportive
5	CD4_5	Treg with relatively lowered Foxp3	IL2RG, CTLA4, TIGIT, MAF, FOXP3 [12]	
6	CD4_6	Th1 effector cell	IL7R[13], CD28[14], KLRB1[15], TNFRSF12A, CD44[7]	Supportive
7	CD4_7	Non-specified CD4 T cell		
8	CD4_8	Activated suppressive, migratory inhibited Th cell	LAMP3[16, 17], CD83[18, 19], IRF4, RGS1[20]	Non-supportive

Supplemental Table 5 *Continued*

Cluster	Cell abbreviation	Cell state	Cell state defining genes and references	Expected immune impact
9	CD4_9	Proliferating Th cells	VIM, TUBB, HMGB2, COTL1, HP90AA1, CALM1 [21]	Supportive
10	CD4_10	Proinflammatory, proliferating effector Th cell	MALAT1, CRIP1, BATF3[22], CUZD1[23], ICOS[24], IL12, IL17, IL20, IL23	Supportive
Tregs				
0	Treg_0	Long term surviving Tregs	BCL2, BCLXL	Non-supportive
1	Treg_1	Effector Tregs	MAF[25, 26], CTLA-4 [12]	Non-supportive
2	Treg_2	Effector Tregs	SRGN, IL2RG, ICOS [12]	Non-supportive
3	Treg_3	Activated Tregs	EMP3 [27]	Non-supportive
4	Treg_4	Non-specified Treg		Non-supportive
5	Treg_5	Non-specified Treg		Non-supportive
6	Treg_6	Homeostatic Tregs	MX1, PX1 [28]	Non-supportive
7	Treg_7	Effector Tregs	IRF4[29], ICOS, GITR, OX40R	Non-supportive
CD8 T cells				
0	CD8_0	Activated, non type 1, suppressive	S100A6[10], S100A4, LGALS1[30], CRIP1, ANXA1[9]	Non-supportive
1	CD8_1	Terminally differentiated, cytotoxic and T cell chemotactic	STAT4[31], CTSW[32], GNLY, XCL1[33, 34]	Supportive
2	CD8_2	Non-specified CD8 T cells		
3	CD8_3	Low cytotoxic, CCL5 producing	GZMK[35], CST7[1], CCL5	Non-supportive
4	CD8_4	Non-specified CD8 T cells		
5	CD8_5	Innate stimuli responding, proliferation Impaired, terminally differentiated	STAT4, IL12R, IL18R, ITGAL[36], CASP8[37], SIGIRR[38], MALAT1[39]	Non-supportive
6	CD8_6	Activated, cytotoxic, terminally differentiated tissue resident	CD69, PD1, GNLY, GZMB, IL7R, ITGAE, MALAT1[39] [40]	Supportive
7	CD8_7	TCR activated, chemotactic	DUSP2[41], DUSP4[41], TIGIT, CCL3[42, 43], CCL4[42]	Supportive

Cluster	Cell abbreviation	Cell state	Cell state defining genes and references	Expected immune impact
Macrophages				
0	M_0	MHC class II-low non-activated	HLA-DRA, HLA-DRB1, HLA-DPA1, SOD2[44]	Non-supportive
1	M_1	CD163 M2c	CD163[45], Selenop[46], PSAP, MAF, CD36, CD14, CD209, MRC1, C1qa[47]	Non-supportive
2	M_2	Non-defined		
3	M_3	Inflammatory MHC class II-high, phagocytic M1-like	TLR2[48], ITGAV[49], IFNGR, FCGR3a[50], CLEC7a[51], HLA-DRA, HLA-DPA1	Supportive
4	M_4	Proliferating	KI67, RSPO[52], CUZD1[23]	Supportive
5	M_5	TLR activated, chemotactic	RGS1, RGS2, CCL2, CCL3, CCL4, CXCL2, CXCL3	Supportive
6	M_6	Th2 attracting and suppressive M2-like	CCL18[53], CCL13, EMP3[54]	Non-supportive
7	M_7	IL10-induced CD163+ suppressive M2-like	IL10R, LAIR1[55], MAF, CD163 [56]	Non-supportive
8	M_8	Prostaglandin and Galectin-3 M2-like macrophages	PTGDS[57], LGALS3[58] [59, 60]	Non-supportive
9	M_9	GPNMB M2-like macrophages	Cd11c, GPNMB[61], IFNGR1	Non-supportive
Neutrophils				Very low numbers of neutrophils across all samples
0	N_0	Non-specified	CSF3R[62]	
1	N_1	CD16hi, IFN sensing, activated	CD16, MX1, IFITM1, FPR1 [63-65]	
2	N_2	Proinflammatory cytokine producing	IL-8, IL1B, PTPRC, FOS, JUN [66, 67]	
pDC				
0	pDC_0	GZMB+ IFN activated pDC	GZMB[68, 69], GPX1[70], MX1	
1	pDC_1	CCR2+ homeostatic pDC	CCR2[71]	Non-supportive
2	pDC_2	PTGDS+ suppressive pDC	PTGDS[59, 60, 72]	Non-supportive
3	pDC_3	Type II cytokine pDC	PSAP[56]	Non-supportive
4	pDC_4	Translationally active pDC	RPL21, RPL37	

Supplemental Table 5 *Continued*

Cluster	Cell abbreviation	Cell state	Cell state defining genes and references	Expected immune impact
5	pDC_5	NOT pDC	lacks: PLAC8, JCHAIN, IL3A [73]	
mDC				
0	mDC_0	Non-specified mDC		
1	mDC_1	Suppressive DC	EMP3[54], CRIP1[5]	Non-supportive
2	mDC_2	Cross presenting cDC1	BATF3[74-77], IDO1	Supportive
3	mDC_3	T cell attracting DC-like macrophages	CD68, CD14, C1q, CXCL9	Supportive
4	mDC_4	Immature suppressive DC	CD33, RELT[78], CLEC5A[79], IL1RN[80]	Non-supportive
5	mDC_5	SerpinA1+ CLEC10A-cDC2	PSAP, LYZ, CLEC12A, CLEC4A, SERPINA1 [81]	
6	mDC_6	IL1B+ DC	IL1B[82]	
7	mDC_7	CCR7+ mature DC	LAMP3[83], CD83, CD80, CD40, PDL1	Supportive
8	mDC_8	CLEC10A+ cDC2	CLEC10A[81, 84]	Supportive
Mast cells				
0	mast_0	Non-specified		
1	mast_1	Mature inflammatory mast cells	CD44[85], IL1RL1, BTK [86]	Supportive
2	mast_2	Suppressive mast cells	LGALS1[87]	Non-supportive
3	mast_3	Inflammatory mast cells	TNFSF10[88]	Supportive
4	mast_4	Non-specified		
5	mast_5	Mature CD37+ non-inflammatory MC	CD44, LGALS3, CD37 [89]	Non-supportive
6	mast_6	Degranulating monocyte attracting mast cells	CTSG, ATG12, AZU, NPPC [90-92]	
7	mast_7	Activated desensitized mast cells	CD69, LIF, NFKBIA, RGS1, RGS2 [93, 94]	
Plasma cells				
0	P_0	IgA dimer producing activated plasma cells, optimized for TLR mediated Ab production	IGKC, JCHAIN, IGHA1, CD27[95], IRF4[96, 97], HSP90B1[98]	
1	P_1	IgG producing plasma cells	IGKC, IGH1, IGH2, XBP1, MZB1, CD38, FKBP11 [99, 100]	
2	P_2	IgG production inhibited plasma cells	LPAR5[101], LAIR1[102], MPO[103]	

Cluster	Cell abbreviation	Cell state	Cell state defining genes and references	Expected immune impact
3	P_3	AZU1+ IgG+ B cells	IGHG1, AZU1[104]	
4	P_4	igA monomeric producing plasma cell	IGKC, IGHA1	
5	P_5	IgG producing plasma cells	IGKC, IGHG1, IRF4	
6	P_6	Class switching B cells (BATF3) low IgG/A	IGKC, BATF3[105]	
7	P_7	Autophagy-related protein 10+ IgA+ memory B cells	IGHA1, CD27, ATG10	
8	P_8	Cytokine producing IgA+ plasma cells	IGKC, IGHA1, CD79A, IL23A[106], CCL11	
B cells				
0	B_0	NOT B cells	marker genes low (MS4A1, CD19, CD79A, CD27, CD24, IGHx)	
1	B_1	Activated B cells	IL27R, CD80, LTB	
2	B_2	Mature B cells 1	MS4A1, CD79A, CD37	
3	B_3	Naive B cells	TCL1A, IL4R, CXCR4,IGHM, IGHD	
4	B_4	NOT B cells	marker genes low (MS4A1, CD19, CD79A, CD27, CD24, IGHx)	
5	B_5	Mature B cells 2	MS4A1, CD79A, CD37	
Endothelial cells				
0	E_0	Post capillary/high endothelial venule Ecs	VWF, ACKR1 [107-110]	
1	E_1	Non-defined ECs		
2	E_2	HLA class I high ECs	HLA-A, HLA-B, HLA-C, IGFBP7[111]	Supportive
3	E_3	Immature angiogenic ECs	FLT1, KDR, GSN, ENG [107-109, 112, 113]	
4	E_4	Late stage cell differentiated arteriolic ECs	TAGLN, ACTA2, MYL9, MYH11 [107-109, 114]	
5	E_5	NOT ECs	marker genes low (PECAM, VIM) [115]	
6	E_6	Lymphatic ECs	CCL21[116], S100A10, ANXA2, CD9, IGFBP7 [107-109, 112]	Supportive

Supplemental Table 5 *Continued*

Cluster	Cell abbreviation	Cell state	Cell state defining genes and references	Expected immune impact
7	E_7	Arteriolic IGF1+ Notch-signtalling ECs	JAG1, SAT1, EFN2, IGF1, ICAM2[117], IGFBP3[118] [107-109, 119]	Non-supportive
8	E_8	Immature IGFBP7-immune supportive angiogenic ECs	FLT1, KDR, ENG, IGFB7[111], RGCC[120] [107-109, 112]	Supportive
9	E_9	CLEC14a+ angiogenic ECs	CLEC14A[121], CD81, CD9 [107-109, 122]	Supportive
10	E_10	HLA class I high, interferon signalling ECs	IFITM1, MX1, IFI27, OAS3, OAS2, IGFB7[111] [107-109, 123]	Supportive
Fibroblasts				
0	F_0	Non-defined fibroblasts		
1	F_1	Fibrosis enhancing fibroblasts	LUM[124]	Non-supportive
2	F_2	Pericytes/vascular smooth muscle cells	TAGLN, ACTA2, PDGFRB, RGS5 [125-128]	Supportive
3	F_3	Type B CXCL14+ reticular fibroblasts	CXCL12, CXCL14[129], MGP [126, 130]	Supportive
4	F_4	Type A fibroblasts	COL1A1, COL6A1, COL6A2, COL18A1 [126]	
5	F_5	Type B immune stimulatory fibroblasts	CFD, CXCL12, CXCL14[129], IGFBP5[131, 132]	Supportive
6	F_6	Type B/S5 fibroblasts driving type 2 cytokine immunity	CXCL12, CCL2, CCL19, CXCL1, CXCL3, VCAN, MEG3, IGF1, PDGFRA [126, 127, 133]	Non-supportive
7	F_7	Type C prostaglandin producing senescent fibroblast	SPARCL1, CFD, RARRES2, PSAP, PTGDS [126, 134-137]	Non-supportive
8	F_8	Adventitial fibroblasts	APOD, GSN, IGFB6 [138]	Supportive
9	F_9	Non-inflammatory fibroblasts	ZFP36[139], DUSP1	Non-supportive

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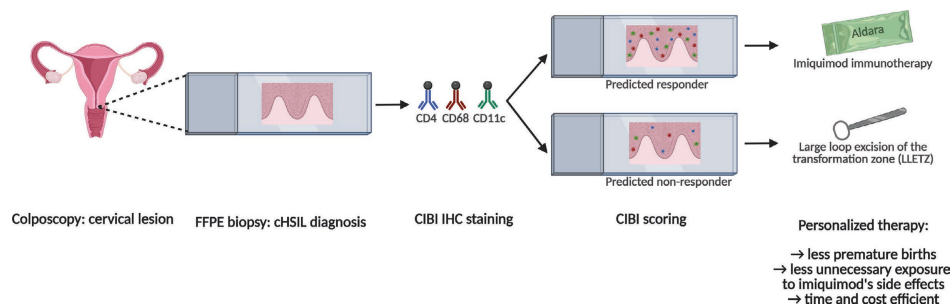
Chapter 6

Immune-based biomarker accurately predicts response to imiquimod immunotherapy in cervical high-grade squamous intraepithelial lesions

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Journal for ImmunoTherapy of Cancer 2022

Graphical abstract



Key findings

- Cervical high-grade squamous intraepithelial lesion (cHSIL) is conventionally treated by surgical large-loop excision, which increases the chance of premature births in following pregnancies, therefore imiquimod immunotherapy is explored, 60% of patients respond to imiquimod.
- Here we developed a new immune-based immunohistochemical biomarker that accurately predicts response to imiquimod, which can be applied on routinely taken pre-therapy biopsies, the cHSIL immune biomarker for imiquimod (CIBI).
- CIBI will enable personalized therapy for cHSIL patients, thereby minimizing side effects and maximizing therapy adherence.

Abstract

Background

The complete response rate of cervical high-grade squamous intraepithelial lesion (cHSIL) patients to imiquimod immunotherapy is approximately 60%. Consequently, many patients are exposed to unnecessary adverse effects of imiquimod. On the other hand, conventional surgical large loop excision therapy is associated with increased risk of premature births in subsequent pregnancies. An in-depth analysis of the cHSIL immune microenvironment was performed in order to identify and develop a predictive biomarker for response to imiquimod, to maximize therapy efficacy and to avoid adverse effects in patients unlikely to respond.

Methods

Biopsies of 35 cHSIL patients, before and 10 weeks on imiquimod treatment, were analyzed by two multispectral seven-color immunofluorescence panels for T cell and myeloid cell composition in relation to treatment response. Based on these results a simplified immunohistochemical detection protocol was developed. Samples were scanned with the Vectra multispectral imaging system and cells were automatically identified using machine learning.

Results

The immune microenvironment of complete responders (CR) is characterized by a strong and coordinated infiltration by T helper cells (activated PD1⁺/type 1 Tbet⁺), M1-like macrophages (CD68⁺CD163⁻) and dendritic cells (CD11c⁺) prior to imiquimod. The lesions of non-responders (NR) displayed a high infiltration of CD3⁺FOXP3⁺ regulatory T cells. At 10 weeks on imiquimod, a strong influx of intraepithelial and stromal CD4⁺ T cells was observed in CR but not NR patients. A steep decrease in macrophages occurred both in CR and NR patients, leveling the pre-existing differences in myeloid cell composition between the two groups. Based on the pre-existing immune composition differences, the sum of intraepithelial CD4 T cell, macrophage and dendritic cell counts was used to develop a quantitative simplified one color immunohistochemical biomarker, the CHSIL Immune Biomarker for Imiquimod (CIBI), which can be automatically and unbiasedly quantified and has an excellent predictive capacity (ROC AUC 0.95, $p < 0.0001$).

Conclusion

The capacity of cHSIL patients to respond to imiquimod is associated with a pre-existing coordinated local immune process, fostering an imiquimod-mediated increase in local T cell infiltration. The CIBI immunohistochemical biomarker has strong potential to select cHSIL patients with a high likelihood to experience a complete response to imiquimod immunotherapy.

Introduction

Cervical high-grade squamous intraepithelial lesion (cHSIL), also referred to as cervical intraepithelial neoplasia (CIN) 2 and 3, is a premalignant lesion induced by a persistent infection with high-risk human papillomavirus (hrHPV). The incidence of cHSIL is rising worldwide, especially among young women.¹ Conventionally cHSIL is treated by large loop excision of the transformation zone (LLETZ), with potential complications such as hemorrhage, infection and an increased risk of premature birth in subsequent pregnancies. Furthermore, surgical treatment strategies are ineffective in 10% of patients, resulting often in difficult to treat recurrences, repeated treatments or even hysterectomy.²

The topical application of immunomodulating imiquimod cream (Aldara/Zyclara), a toll-like receptor 7 (TLR7) agonist, is a new treatment strategy for cHSIL. TLR7 is expressed by antigen presenting cells, such as macrophages and dendritic cells (DCs), and upon binding to imiquimod an intracellular inflammatory cascade is activated. This results in the secretion of pro-inflammatory cytokines such as IFN γ , TNF α and IL-12, the attraction and activation of other immune cells to the lesion, and the polarization of T cells to a type 1 oriented and cytotoxic T cell response, hereby amplifying the local immune response (reviewed in ³). Previous clinical trials have shown that about 60% of cHSIL patients display a clinical response to topical imiquimod therapy.^{4,5} Adverse effects of imiquimod therapy are common and can be extensive, consisting mostly of local inflammation and burning, but also of systemic adverse events (headache and flu-like symptoms).⁶ The adverse effects and long treatment duration of 16 weeks turns therapy adherence into a challenge with up to 20% discontinuation.⁷ Predictive biomarkers to prevent overtreatment and to increase therapy efficacy and adherence are highly warranted.

We have previously shown that the immune microenvironment is of major importance for response to imiquimod in vulvar HSIL (vHSIL), which is also caused by a persistent hrHPV infection, but at a different anatomical site. In vHSIL, a pre-existing coordinated infiltration by CD4⁺ and CD8⁺ T cells as well as inflammatory CD14⁺ myeloid cells is associated with complete response upon imiquimod therapy.⁸ To our knowledge, no studies have been published on the effects of imiquimod on the immune microenvironment in cHSIL, nor if the composition of the pre-existing immune microenvironment can be used as predictive biomarker. Therefore, we performed a comprehensive in-depth characterization of the immune microenvironment using multispectral immunofluorescence on a unique cohort of cHSIL patients treated with topical imiquimod, investigating both pre- and on treatment cHSIL tissue, in the context of their clinical response. Here we show that imiquimod has the strongest effect on immune cell composition in lesions with high pre-existing immunity. In addition, specifically a strong pre-existing infiltration by CD3⁺CD8⁺FOXP3⁺ T cells, CD68⁺CD163⁺ M1-like macrophages and CD11c⁺ dendritic cells accurately distinguished complete responders from non-responders. This observation allowed the development of a simple unbiased predictive and sensitive one color immunohistochemical biomarker for imiquimod response (ROC AUC 0.95, $p < 0.0001$) that is easily applicable in routine diagnostics, the cHSIL Immune Biomarker for Imiquimod (CIBI).

Materials and methods

Patient samples

Pre- and on imiquimod treatment formalin-fixed paraffin-embedded (FFPE) biopsies of 35 women with histologically confirmed cHSIL included in the TOPIC trial (NCT02917746) were analyzed. The TOPIC trial was performed at three hospitals in the Netherlands: Maastricht University Medical Center (which provided medical ethical approval for performing this study in the Netherlands, NL57849.068.16/METC162025), Erasmus University Medical Center Rotterdam and Catharina Hospital Eindhoven, where after providing written informed consent patients were treated with a 16 week imiquimod treatment regimen consisting of 6.25mg (half a sachet) imiquimod 5% cream administered vaginally 3 times a week, instead of standard therapy by LLETZ. Biopsies were 3-5mm diameter in size, and the entire cHSIL region in the biopsies was analysed.⁹ This study was conducted in accordance with the Declaration of Helsinki and in accordance with Dutch law. Biopsies were taken at three time points:

pre-imiquimod (diagnostic), on imiquimod treatment (10 weeks) and after imiquimod treatment (20 weeks). The 20 week biopsies were not analyzed due to the small sample size (3 NR already had received LLETZ treatment due to lesion progression and 10 CR had complete lesion clearance at that time).

HPV genotyping was performed using polymerase chain reaction enzyme immunoassays on either cytological or histological samples. DNA isolation of the samples was performed with the Maxwell 16 kit (Promega). A PCR GP5+/6+ was run with universal HPV primers, and HPV positivity was assessed with agarose gel electrophoresis. HPV positive samples were subsequently subtyped using an enzyme immunoassay for the detection of HPV16, HPV18, or cocktail hrHPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66).

A non-responder (NR) was defined as having a persistent HSIL (HPV⁺CIN2 and CIN3) at 20 weeks, which was an indication for LLETZ. Patients with no dysplasia, CIN1 or HPV-CIN2 at 20 weeks underwent 6 months of follow-up conform standard care, and after 6 months a PAP smear for cytological assessment was taken. Complete response (CR) was defined as cytologically confirmed lesion clearance at 6 months (PAP1), and partial response (PR) was defined as >PAP1 at 6 months. An overview of the study workflow is presented in **Supplemental Figure 1**.

Multispectral immunofluorescence staining

Two previously published seven-color multispectral immunofluorescence panels were applied, one for T cells, consisting of CD3, CD8, FOXP3, TIM3, Tbet, PD-1, DAPI, and one for myeloid cells, consisting of CD14, CD33, CD68, CD11c, CD163, PD-L1, DAPI.^{8 10} In these panels a combination of indirect detection (fluorochrome labelled secondary antibody) and direct detection (primary antibody directly labelled with fluorochrome, for antibodies with clashing species/isotypes) of markers was used. Dim markers (PD-L1, PD-1 and Tbet) were tyramide signal amplified with Opal (PerkinElmer) to enable their detection by fluorescence microscopy. An overview of the antibodies and immunodetection methods included in each panel are shown in **Supplemental Table 1AB**. Optimal antigen retrieval buffers were determined for all individual markers by performing stainings in both citrate and tris-EDTA, the buffer in which all antibodies of a panel performed well was then chosen for that panel. In short, the slides were stained as follows: on the first day, 4 micrometer thick FFPE tissue sections were deparaffinized, endogenous peroxidase was blocked with 0.3% hydrogen peroxide and heat induced epitope retrieval was

performed in the T cell panel with citrate (10mM, pH 6.0) and in the myeloid cell panel with tris-EDTA (10mM/1mM, pH 9.0). SuperBlock (ThermoFisher Scientific) was used to block non-specific binding sites. First, the primary antibodies detected by Opal were applied and amplified according to the manufacturer's protocol. Then the unconjugated primary antibodies were incubated overnight at room temperature. On the second day, the corresponding fluorescently labelled secondary antibodies were applied, followed by 5 hours incubation with the directly labeled primary antibodies. Finally, DAPI was applied as nuclear counterstain and slides were mounted.⁹

Dual immunohistochemical staining

A new dual immunohistochemistry panel was designed, consisting of the markers CD4, CD68 and CD11c (all three rabbit IgG antibodies) stained with DAB (brown), and the markers FOXP3 and CD163 (both mouse IgG antibodies) stained with Vector Red (red), using the ImPRESS Duet double staining polymer kit (Vector laboratories). On the first day, 4 micrometer thick tissue sections were deparaffinized, endogenous peroxidase was blocked with 0.3% hydrogen peroxide, and heat induced epitope retrieval was performed with citrate (10mM, pH 6.0). Non-specific binding sites were blocked with normal horse serum 2.5% for 20 minutes, and the mix of all five primary antibodies (CD4, CD68, CD11c, FOXP3, CD163, panel design included in **Supplemental Table 1C**) was incubated overnight at 4 degrees Celsius. The following day slides were incubated with a mix of horseradish peroxidase (HRP) horse-anti-rabbit IgG and alkaline phosphatase (AP) horse-anti-mouse IgG for 10 minutes, and subsequently antibody binding was detected with DAB (oxidized by HRP to a brown chromogen, 5 minutes incubation) and Vector Red (oxidized by AP to a red chromogen, 30 minutes incubation). Nuclei were counterstained with hematoxylin and slides were dehydrated and mounted.

Quantification of immune cells in the tumor microenvironment (TME)

As published⁸, images of the entire tissue sections stained with the multispectral immunofluorescence or immunohistochemical panels were acquired with the Vectra 3.0.5 multispectral imaging microscope (PerkinElmer) at 20x magnification. Immune cells in the TME of both the immunofluorescence and immunohistochemical images were automatically phenotyped and counted with inForm 2.4 image analysis software (PerkinElmer-Akoya Biosciences) after manual training. The software was trained to segment epithelium and stroma, segment DAPI⁺ or hematoxylin⁺ nucleated cells, and assign a phenotype to each cell. All phenotypes were visually inspected on accurateness, and if

errors were detected the training was further optimized until all discrepancies were resolved. Immune cell counts were normalized for tissue size (cells/mm² epithelium and cells/mm² stroma). Cell phenotypes above the threshold of a median cell count ≥ 10 cells/mm² in at least one response group and at least one time point were included in the analyses, in order to study biologically common cell phenotypes and not to focus on chance findings, as published before.²⁹³

Spatial cellular interaction analyses

Spatial high-dimensional cell-cell interaction analyses were performed using a new version of ImaCyte developed for spatial analyses on multispectral immunofluorescence images (in-house developed program, available from <https://github.com/biovault/ImaCytE>).^{11 12} Similar to others^{13 14}, permutation testing with 1000 iterations was applied to identify spatial interactions that occurred more frequently than expected based on chance, hereby correcting for differences in cell phenotype frequencies among response groups. Only interactions with a permutation based Z-score > 2 (i.e. outside the 95% normal distribution range, so unlikely to be spatially located next to each other by chance) were included for further analyses. To study differences in spatial interactions across two subgroups (NR versus CR), interactions with a permutation based Z-score > 2 (i.e. spatial interaction) in one of the subgroups and a permutation based Z-score < -2 (i.e. spatial separation) in the other subgroup were included in the analyses (thus all opposite spatial interactions).

Statistical analyses

Statistical data analysis was performed with GraphPad Prism 8.0.1 (GraphPad Software Inc.), which was also used to create graphs to visualize the data. The median immune counts of the two different response groups (NR versus CR) were compared with the non-parametric Mann-Whitney U test. PRs were not included in the statistical analyses due to their small sample size (n=3). Differences between pre- and on imiquimod treatment immune cell infiltration were compared with the non-parametric Wilcoxon paired signed rank test. Spearman correlation was used to study correlations between the T cell and myeloid cell infiltrate. Two-sided p-values < 0.05 were marked as statistically significant.

Results

Imiquimod induces a strong intraepithelial influx by T cells and a decrease in macrophages.

To investigate the impact of imiquimod on the immune microenvironment, we compared the lymphoid and myeloid immune cell composition of pre-imiquimod cHSIL biopsies (n=35) versus the on treatment biopsies (n=27, 8 samples were excluded; 6 CR because they had already regressed and only contained healthy cervical tissue, 1 PR whose sample was missing, 1 NR with a sampling error that only contained healthy tissue). An overview of the patient characteristics is provided in **Table 1**. To enable an in-depth immune cell characterization, multispectral immunofluorescence was applied (**Figure 1AB**). Eleven different T cell phenotypes and twelve different myeloid cell phenotypes were identified in cHSIL. The major T cell populations were CD3⁺CD8⁻FOXP3⁻ T helper cells, CD3⁺CD8⁺FOXP3⁻ T cells and CD3⁺CD8⁻FOXP3⁺ regulatory T cells, either activated (TIM3⁺/PD1⁺) or not, and with a type 1 cytokine profile (Tbet⁺) or not. The major myeloid populations were CD68⁺CD163⁻ M1-like macrophages, CD68⁺CD163⁺ M2-like macrophages, CD14⁺CD68⁻CD11c⁻ inflammatory monocytes, CD14⁺CD68⁻CD11c⁺ DCs and CD14⁺CD68⁻CD33⁺ immature myeloid cells (**Supplemental Table 2**).

Table 1. cHSIL imiquimod cohort patient characteristics.

Patient ID	Age	Response	Pre-imiquimod			10 weeks			20 weeks			6 months		
			Histology	HPV PCR		Histology	HPV PCR		Histology	HPV PCR		Cytology	HPV PCR	
ERAS 001	30	CR	CIN2	positive		CIN1	positive		no dysplasia	negative		PAP1	x	
MUMC 001	31	CR	CIN2-3	positive		no dysplasia	negative		dysplasia	negative		PAP1	x	
MUMC 014	36	CR	CIN2	positive		CIN1	negative		no dysplasia	negative		PAP1	negative	
MUMC 018	25	CR	CIN3	positive		CIN2	negative		no dysplasia	negative		PAP1	x	
MUMC 034	30	CR	CIN3	positive		CIN1	negative		no dysplasia	negative		PAP1	negative	
MUMC 040	36	CR	CIN2	positive		CIN1	negative		no dysplasia	negative		PAP1	positive	
MUMC 044	30	CR	CIN2	positive		CIN1	negative		no dysplasia	negative		PAP1	negative	
MUMC 078	36	CR	CIN3	positive		no dysplasia	negative		dysplasia	negative		PAP1	x	
MUMC 081	30	CR	CIN3	positive		CIN2	positive		no dysplasia	negative		PAP1	negative	
MUMC 027	26	CR	CIN2	positive		CIN1	weak +		CIN1	negative		PAP1	positive	
CATH 017	31	CR	CIN3	positive		no dysplasia	negative		CIN1	negative		PAP1	x	
ERAS 002	30	CR	CIN3	positive		CIN1	negative		CIN2	negative		PAP1	x	
ERAS 004	52	CR	CIN3	positive		CIN1	negative		CIN1	negative		PAP1	x	
ERAS 016	32	CR	CIN2	positive		no dysplasia	negative		CIN1	positive		PAP1	negative	

Patient ID	Age	Response	Pre-imiquimod			10 weeks			20 weeks			6 months		
			Histology	HPV PCR	Histology	HPV PCR	Histology	HPV PCR	Histology	HPV PCR	Cytology	HPV PCR	Cytology	HPV PCR
MUMC 010	30	CR	CIN3	positive	no dysplasia	negative	CIN1	positive	CIN1	positive	PAP1	negative	PAP1	negative
MUMC 036	30	CR	CIN3	positive	CIN3	positive	CIN2	negative	CIN2	negative	PAP1	x	PAP1	x
MUMC 049	31	CR	CIN2	positive	CIN1	negative	CIN1	negative	CIN1	negative	PAP1	negative	PAP1	negative
MUMC 062	30	CR	CIN3	positive	no dysplasia	negative	CIN1	negative	CIN1	negative	PAP1	negative	PAP1	negative
MUMC 065	31	CR	CIN3	positive	CIN1	positive	CIN1	positive	CIN1	negative	PAP1	x	PAP1	x
CATH 006	30	CR	CIN3	positive	CIN2-3	positive	x	x	x	x	PAP1	positive	PAP1	positive
MUMC 080	28	CR	CIN3	positive	CIN1	negative	CIN1	negative	CIN1	negative	PAP1	positive	PAP1	positive
CATH 023	30	PR	CIN3	positive	CIN1	negative	CIN1	negative	PAP2	positive	PAP2	positive	PAP2	positive
ERAS 006	33	PR	CIN2	positive	x	negative	CIN1	positive	CIN1	positive	PAP3A2	positive	PAP3A2	positive
MUMC 037	35	PR	CIN3	positive	CIN1	positive	CIN2	negative	CIN2	negative	PAP2	x	PAP2	x
CATH 012	29	NR	CIN2	positive	no dysplasia	negative	CIN3	positive	CIN3	positive	x	x	x	x
ERAS 010	30	NR	CIN2	positive	CIN1	negative	CIN3	negative	CIN3	x	PAP4	positive	PAP4	positive
MUMC 002	30	NR	CIN3	positive	CIN2	negative	CIN3	negative	CIN3	positive	PAP2	negative	PAP2	negative
MUMC 003	29	NR	CIN3	positive	CIN3	positive	x	x	x	x	PAP1	negative	PAP1	negative
MUMC 008	30	NR	CIN3	positive	CIN3	positive	CIN3	positive	CIN3	positive	PAP1	negative	PAP1	negative
MUMC 024	31	NR	CIN3	positive	CIN3	positive	x	x	x	x	PAP1	positive	PAP1	positive
MUMC 038	28	NR	CIN3	positive	CIN3	positive	CIN3	positive	CIN2	positive	PAP1	negative	PAP1	negative
MUMC 045	55	NR	CIN2	positive	CIN1	positive	CIN2	positive	CIN2	positive	PAP3A1	positive	PAP3A1	positive
MUMC 051	59	NR	CIN2	positive	CIN3	positive	x	x	x	x	PAP1	negative	PAP1	negative
MUMC 064	36	NR	CIN3	positive	CIN1	negative	CIN3	positive	CIN3	positive	PAP3A1	positive	PAP3A1	positive
MUMC 079	36	NR	CIN3	positive	CIN2	weak +	CIN2	positive	CIN2	positive	PAP1	x	PAP1	x

CR=complete response, PR=partial response, NR=no response, CIN=cervical intraepithelial neoplasia, PAP=Papanicolaou dysplasia classification for cervix smear cytology, x=sample not available

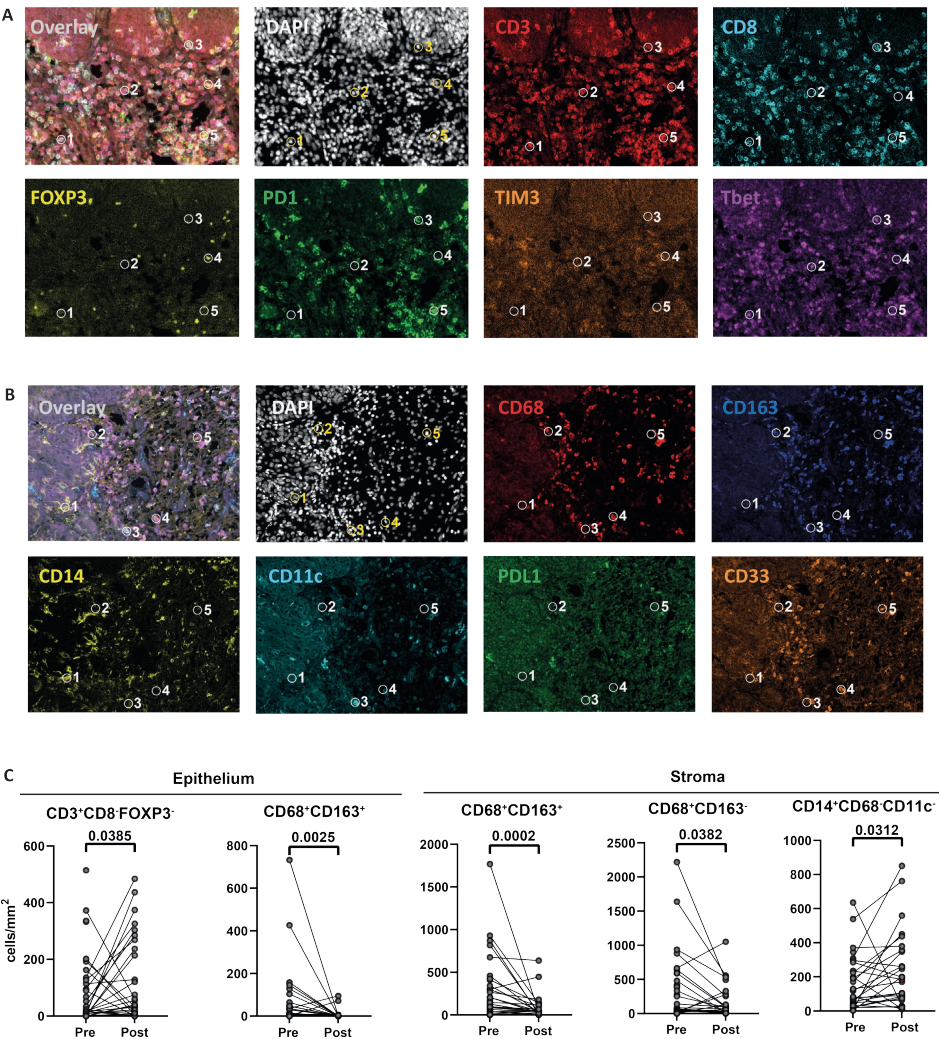


Figure 1. Multispectral imaging of cHSIL's immune tumor microenvironment pre- and on imiquimod treatment.

A) Multispectral immunofluorescence T cell panel, depicting the full seven color panel on a cHSIL biopsy, including all individual markers (DAPI, CD3, CD8, FOXP3, PD1, TIM3, Tbet). The full phenotypes of the encircled cells are: 1) CD3⁺CD8⁺FOXP3⁺Tbet⁺, 2) CD3⁺CD8⁺FOXP3⁺Tbet⁺, 3) CD3⁺CD8⁺FOXP3⁺PD1⁺Tbet⁺, 4) CD3⁺CD8⁺FOXP3⁺ and 5) CD3⁺CD8⁺FOXP3⁺PD1⁺. **B)** Multispectral immunofluorescence myeloid cell panel, depicting the full seven color panel on a cHSIL biopsy, including all individual markers (DAPI, CD68, CD163, CD14, CD11c, PDL1, CD33). The full phenotypes of the encircled cells are: 1) CD14⁺CD68⁺CD163⁺, 2) CD14⁺CD68⁺CD163⁺, 3) CD14⁺CD68⁺CD163⁺CD11c⁺, 4) CD14⁺CD68⁺CD163⁺CD33⁺ and 5) CD14⁺CD68⁺CD163⁺CD33⁺. **C)** Statistically significant changes (Wilcoxon paired signed rank test) in immune cell infiltration upon imiquimod treatment in cHSIL's epithelium and stroma (n=35), as measured pre-treatment and after 10 weeks of imiquimod treatment.

Imiquimod increased the numbers of epithelial CD3⁺CD8⁺FOXP3⁻ T cells and stromal CD14⁺CD68⁺CD11c⁻ monocytes, albeit that this varied per patient. In addition, a steep decrease in macrophage counts, both stromal M1-like (CD68⁺CD163⁻) and epithelial and stromal M2-like (CD68⁺CD163⁺) macrophages, was observed in almost all patients upon imiquimod treatment. The other major cell populations (CD8⁺ T cells, FOXP3⁺ regulatory T cells, CD14⁺ monocytes, CD11c⁺ DCs) did not overtly change upon imiquimod treatment (**Figure 1C**, **Supplemental Figure 2**). Six months after imiquimod treatment, 21 patients had a complete response (CR), 3 patients a partial response (PR) and 11 patients were non-responders (NR). To investigate whether the immunological response to imiquimod was different between CR and NR (PR not included in statistical analyses due to small sample size), the changes in composition of the immune microenvironment were studied (**Supplemental Figure 3AB**). During imiquimod treatment the median total intraepithelial T cell infiltrate was strongly increased in CR patients ($p=0.015$) (**Figure 2A**). In particular there was a higher influx of intraepithelial CD3⁺CD8⁺FOXP3⁻ T cells and both stromal and intraepithelial CD3⁺CD8⁺FOXP3⁺ regulatory T cells in CR patients but not in NR patients (**Supplemental Figure 3A**). The number of intraepithelial and stromal CD3⁺CD8⁺FOXP3⁻ T cells was much higher than their FOXP3⁺ counterparts in these CR lesions (**Supplemental Table 2**). The total number of infiltrating myeloid cells in the epithelium did not change much upon imiquimod treatment in all patients (**Figure 2A**). However, the stromal composition was changed in favor of CD14⁺ myeloid cells (**Figure 2A**), mainly because of a drastic decrease in classical (CD68⁺) macrophages and CD68⁺CD14⁺CD11c⁺ DCs (**Supplemental Figure 3B**). Thus, successful lesion clearance upon imiquimod treatment is associated with the potent accumulation of T cells. A depletion of macrophages was observed in the TME of all patients.

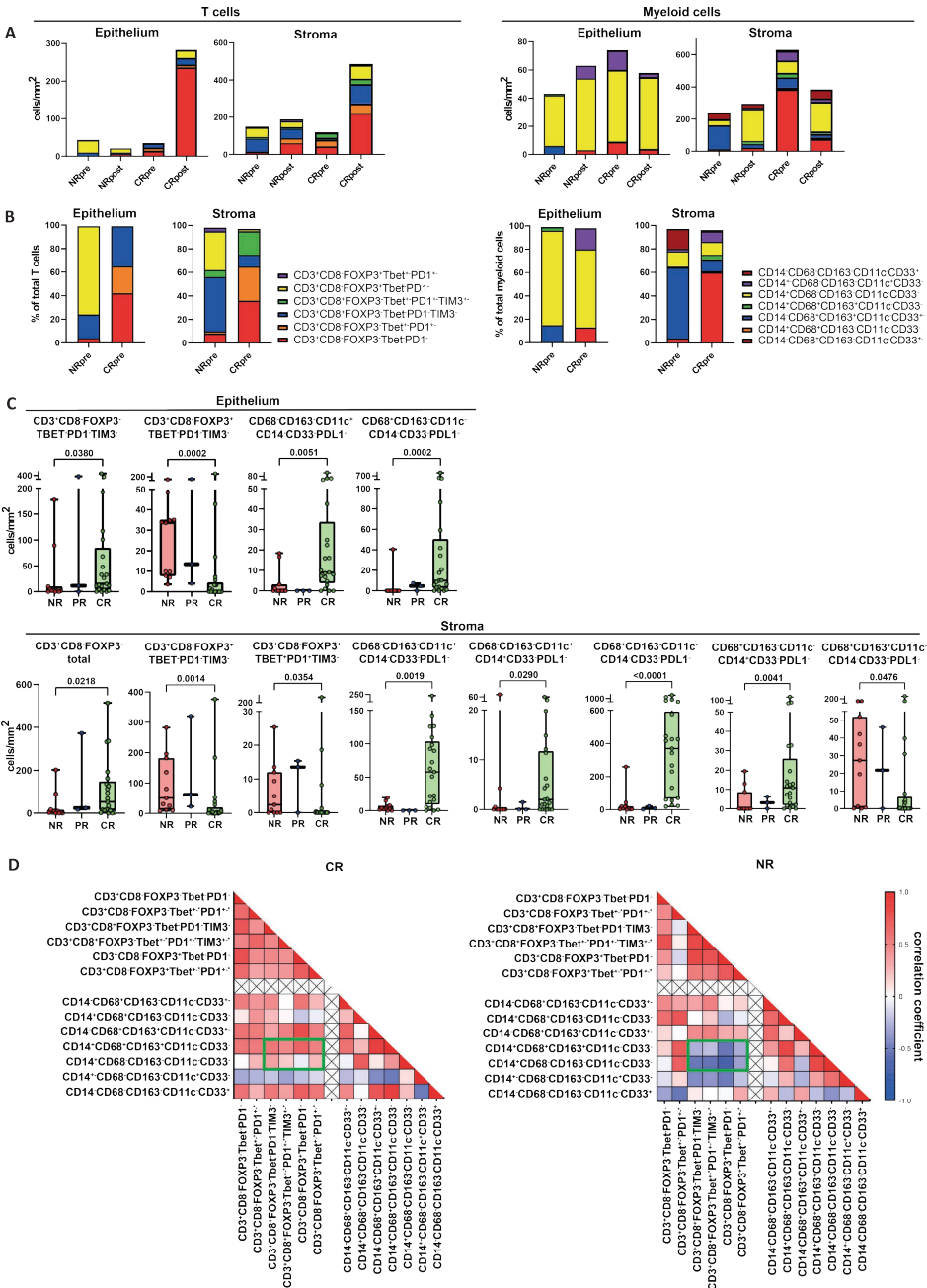


Figure 2. Pre-existing differences in immune cell composition between responders and non-responders to imiquimod.

A) Differences in median T cell and myeloid cell changes pre- and on imiquimod treatment in CR (n=21) and NR (n=11) cHSIL epithelium and stroma. In case of more than one marker indicated by +-, at least one of these markers is positive in that cell phenotype. **B)** Pre-existing differences in composition of T cell and myeloid cell infiltrate in CR (n=21) versus NR (n=11) cHSIL epithelium and stroma. **C)** Statistically significant differences (Mann-Whitney U test) in specific immune cell phenotypes infiltrating CR (n=21) versus NR (n=11) cHSIL epithelium and stroma. PR (n=3) not included in statistical analysis due to small sample size. **D)** Spearman correlation heatmaps of pre-existing stromal T cell and myeloid cell infiltration, lineages separated by the crossed line, in CR (n=21) versus NR (n=11) cHSIL. The green square indicates the most striking differential correlations between CR versus NR.

Quantitative and spatial pre-existing differences in lesion-infiltrating immune cells distinguish responders from non-responders.

In order to develop a biomarker for response prediction, we focused on differences in the pre-existent intralesional immune infiltrate that were present before imiquimod treatment. The T cell and myeloid cell composition and absolute cell numbers were compared between CR versus NR in the pre-treatment samples. The intraepithelial infiltrate was dominated by CD3⁺CD8⁺FOXP3⁻ T cells in CR patients, while CD3⁺CD8⁻FOXP3⁺ regulatory T cells formed the largest fraction of T cells in NR patients (**Figure 2B**). The epithelial region was mostly infiltrated by CD14⁺ cells, however this did not differ between the two patient groups (**Figure 2B**). The fraction of stromal and intraepithelial M1-like macrophages was the largest in CR patients, whereas in NR patients these macrophages were mostly M2-like (**Figure 2B**).

The number of total T cells did not differ between CR and NR, but clearly the total numbers of intraepithelial and stromal CD3⁺CD8⁻FOXP3⁺ T cells were higher in CR patients, while the intraepithelial and stromal numbers of CD3⁺CD8⁺FOXP3⁻ regulatory T cells were higher in NR patients (**Figure 2C, Supplemental Figure 4A**). The median myeloid cell counts were more than two-fold higher in CR compared to NR (**Figure 2A**), with especially more intraepithelial and stromal presence of M1-like macrophages and CD68⁺CD14⁺CD11c⁺ dendritic cells in CR patients (**Figure 2C**). Spearman correlation heatmaps revealed strong positive correlations between various stromal T cells and myeloid cells in CR, that were not found in NR (**Figure 2D**).

An adequate anti-tumor response requires both sufficient numbers of infiltrating immune cells as well as a coordinated organization within the TME.¹⁵ Using a multispectral imaging approach on FFPE tissue, we were able to investigate the *in situ* spatial interactions in cHSIL, and compared those between CR and NR. Clearly, T cells in CR and NR patients displayed different

spatial interactions. In CR lesions different types of type 1 T cells were in close proximity (CD3⁺CD8⁺FOXP3⁺Tbet⁺ T cells with CD8⁺Tbet⁺ T cells). In contrast such type 1 T cells (CD3⁺CD8⁺FOXP3⁺Tbet⁺ T cells, CD8⁺Tbet⁺ T cells) were often directly spatially interacting with CD3⁺CD8⁺FOXP3⁺ regulatory T cells in NR lesions (**Figure 3A**). Notably, the regulatory T cells co-expressed Tbet, indicative for their specific capacity to suppress an ongoing type 1 T cell response.¹⁶ Spatial myeloid cell interaction analysis revealed no distinct interaction pattern between CR and NR, indicating that especially the number and composition of the infiltrating myeloid cell pool rather than their interactions are associated with responsiveness. Altogether, these data suggest that a pre-existing coordinated strong infiltration with T cells and myeloid cells is required for adequate lesion clearance upon imiquimod treatment.

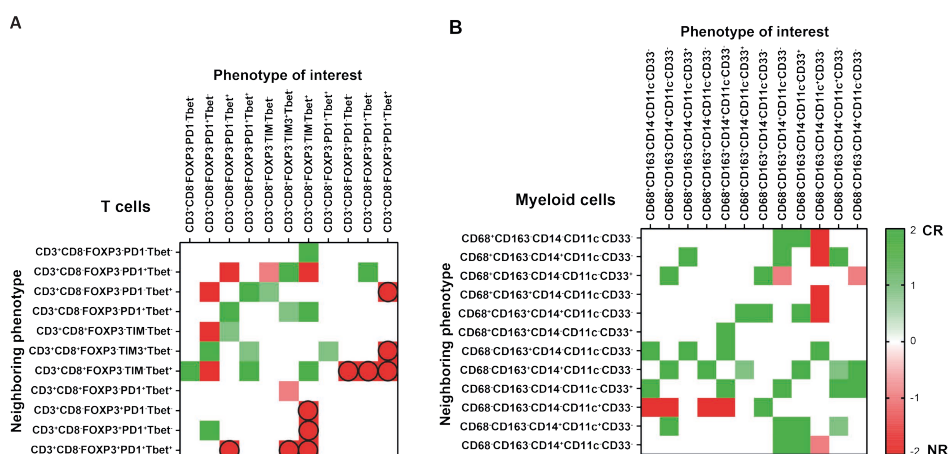


Figure 3. Differential spatial immune cell interactions in responders and non-responders to imiquimod.

Heatmap of pre-existing differential spatial interactions between CR (n=21) and NR (n=11) cHSIL. A red interaction indicates that these two cell phenotypes frequently interact in NR lesions (more than expected based on chance, Z-score>2), and are spatially separated in CR lesions (Z-score<-2). A green interaction indicates that these two cell phenotypes frequently interact in CR lesions (more than expected based on chance, Z-score>2), and are spatially separated in NR lesions (Z-score<-2). **A)** Pre-existing differential T cell spatial interactions, and **B)** pre-existing differential myeloid cell spatial interactions between CR and NR cHSIL.

Immunohistochemical CHSIL Immune Biomarker for Imiquimod (CIBI).

The numbers of epithelium and stroma infiltrating CD3⁺CD8⁺FOXP3⁻ T cells, M1-like macrophages and DCs were higher in CR, while the numbers of infiltrating CD3⁺CD8⁺FOXP3⁺ regulatory T cells were lower compared to NR (**Figure 2C**). This prompted us to examine if CR could be better distinguished from NR when these markers were combined into one quantitative biomarker. To this end, we created a combined biomarker, calculated as the sum of total CD3⁺CD8⁺FOXP3⁻ T cells, total M1-like macrophages and total DCs, minus the total of CD3⁺CD8⁺FOXP3⁺ regulatory T cells, either for the epithelium or for the stroma. Indeed, this biomarker clearly distinguished NR from CR (epithelium $p=0.0001$, stroma $p<0.0001$) (**Figure 4AB**).

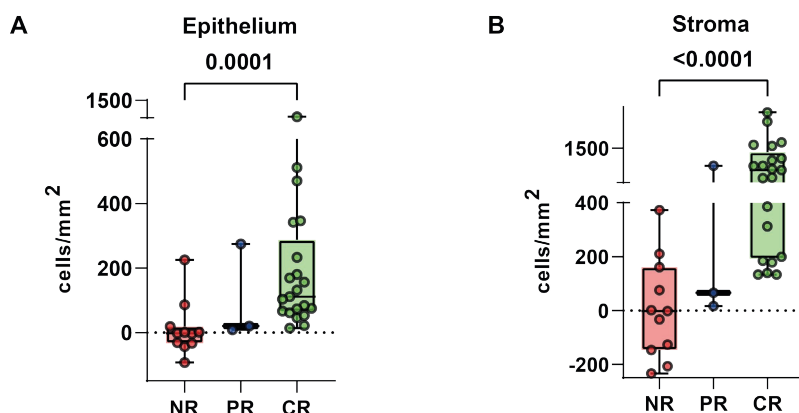


Figure 4. Multispectral immunofluorescence biomarker accurately distinguishes responders from non-responders.

Multispectral immunofluorescence biomarker capability to distinguish CR ($n=21$) from NR ($n=11$) based on pre-imiquimod immunological cHSIL differences (Mann-Whitney U test), consisting of the sum of total CD3⁺CD8⁺FOXP3⁻ T cells, total CD68⁺CD163⁻ M1-like macrophages and total CD11c⁺ dendritic cells, minus total CD3⁺CD8⁺FOXP3⁺ regulatory T cells, in **A**) epithelium and **B**) stroma.

Since the execution of an in-depth multispectral immunofluorescence analysis is not simple, hence not suitable to function as a widely implementable diagnostic biomarker, we explored whether a simplified biomarker could be developed that could be easily applicable in routine diagnostic pathology with a conventional light microscope. We, therefore, optimized a dual immunohistochemistry panel which allowed the separate identification of the immune markers positively (CD4, CD68, CD11c with DAB in brown) and negatively (FOXP3, CD163 with Vector Red in red) associated with clinical outcome in one tissue section (**Figure 5A, Supplemental Table 1C**). We validated the performance of this dual immunohistochemistry approach, by staining the entire cohort ($n=35$)

and calculating the biomarker for both the epithelium and stroma. The overall cell counts in the dual immunohistochemistry biomarker were higher compared to the multispectral immunofluorescence biomarker. This was expected since immunohistochemistry strongly amplifies the signal of each detected marker, resulting in the detection of cells not only with a strong but also with a weaker expression of the markers, whereas direct immunofluorescence only allows the detection of strongly positive cells. Despite the differences in detection method, the biomarker still excellently separated CR from NR, both in the epithelium ($p<0.0001$) (**Figure 5B**) and in the stroma ($p=0.0064$) (**Figure 5C**).

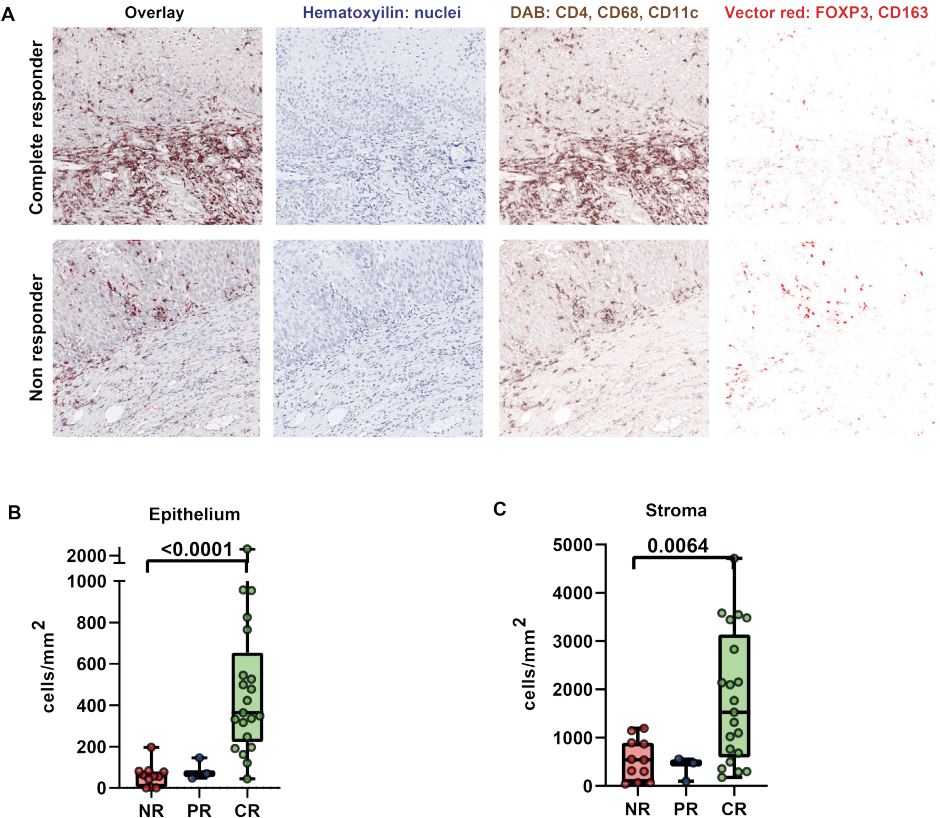


Figure 5. Dual immunohistochemical staining maintains accurate performance of predictive biomarker.

A) Overlay and individual colors of the dual immunohistochemistry panel staining on pre-imiquimod cHSIL of a CR and a NR patient, with the markers CD4, CD68 and CD11c visualized by DAB in brown, the markers FOXP3 and CD163 visualized by Vector Red in red, and hematoxylin as nuclear counterstain. **B)** and **C)** Immunohistochemical biomarker capability to distinguish CR ($n=21$) from NR ($n=11$) based on pre-imiquimod immunological cHSIL differences (Mann-Whitney U test). The biomarker was calculated as the sum of all cells with a completely brown membrane and unstained nucleus ($CD4^+/CD68^+/CD11c^+ FOXP3^- CD163^-$) minus all cells with a brown membrane and red nucleus ($CD4^+ FOXP3^+$), for both the **B)** epithelium and **C)** stroma.

Given the clear demarcation of the epithelial compartment in cHSIL, allowing an easy and objective definition of the area in which the immune cells should be scored without inter-observer variability, we analyzed the performance of the biomarker in the epithelium to distinguish CR from NR patients. The receiver operating characteristic (ROC) curves for the epithelial biomarker calculated with the data of the multispectral immunofluorescence analysis (**Figure 6A**) and of the dual immunohistochemistry analysis (**Figure 6B**) were created. This showed excellent sensitivity and specificity of the predictive biomarker by both techniques, with an area under the curve (AUC) of 0.89 ($p=0.0003$) by immunofluorescence, and an AUC of 0.95 ($p<0.0001$) by immunohistochemistry.

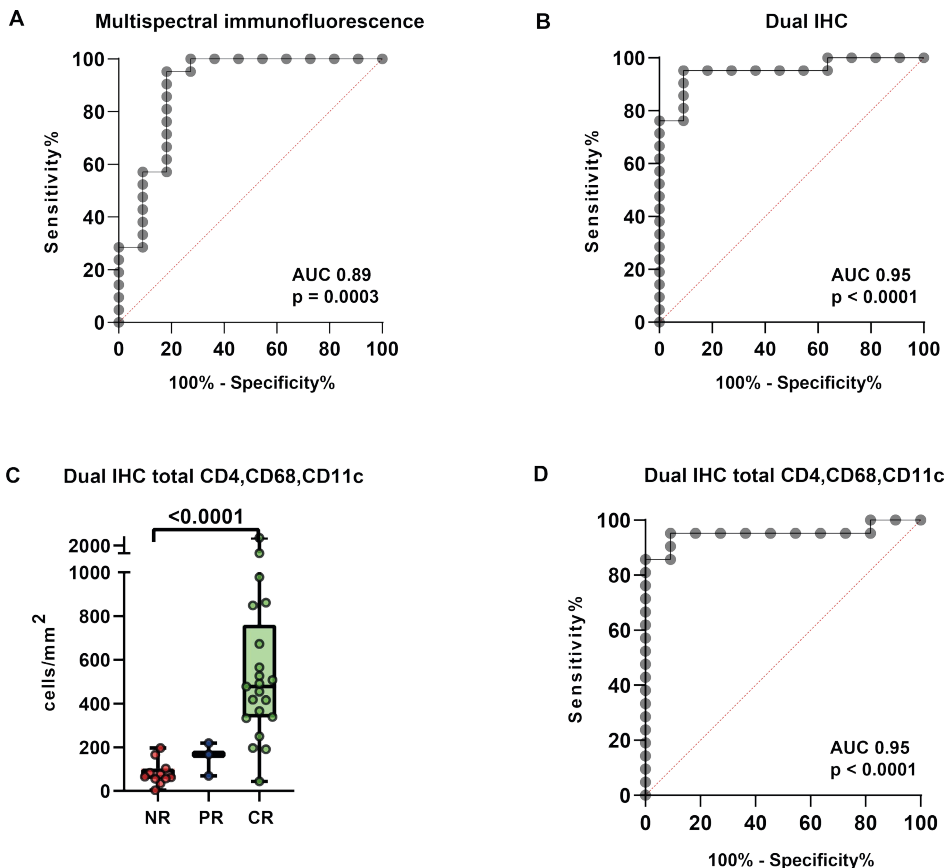


Figure 6. Simplified epithelial immune biomarker is excellent CHSIL immune biomarker for imiquimod (CIBI).

A) Multispectral immunofluorescence biomarker performance, consisting of the sum of total of CD4⁺, CD68⁺CD163⁻ and CD11c⁺ cells minus the total of FOXP3⁺ cells in the epithelium as determined by the receiver operating characteristic (ROC) curve ($n=32$), and the same in **B)** for the dual immunohistochemical biomarker. **C)** Capability of the simplified epithelial dual immunohistochemistry biomarker, the sum of total CD4⁺, CD68⁺ and CD11c⁺ cells, to distinguish CR ($n=21$) from NR ($n=11$) cHSIL (Mann-Whitney U test), and **D)** its performance as determined by the receiver operating characteristic (ROC) curve ($n=32$).

Since the numbers of intraepithelial FOXP3⁺ and CD163⁺ cells were relatively low when compared to their negative counterparts (**Supplemental Figure 5**), we examined whether a further simplification of the dual immunohistochemical biomarker to a single color staining (DAB in brown, as conventionally used in pathology) could be achieved. For this we analyzed the performance of the biomarker without the detection of FOXP3 and CD163 by Vector Red, i.e. only the detection of CD4, CD68 and CD11c by DAB. This simplification did not compromise the performance of the biomarker, as the sum of total intraepithelial CD4, CD68 and CD11c was well capable of distinguishing CR from NR patients ($p < 0.0001$) (**Figure 6C**), and the performance of this one color based biomarker still had an AUC of 0.95 ($p < 0.0001$) (**Figure 6D**). A threshold for this biomarker of intraepithelial CD4, CD68, CD11c cell counts > 178.5 cells/mm² provided the highest likelihood ratio (10.48), as well as sensitivity (95.24%) and specificity (81.82%) scores. In our cohort, this biomarker threshold accurately predicted complete response in 20/21 patients (positive predictive value=95.24%), and no response in 10/11 patients (negative predictive value=90.91%). We designated this intraepithelial immune cell count biomarker the CHSIL Immune Biomarker for Imiquimod (CIBI).

Discussion

This study is the first to identify a strong and easily applicable predictive biomarker for the clinical response of cHSIL patients to local immunotherapy with imiquimod, the CIBI. This biomarker is grounded on a comprehensive in-depth study of the phenotype and spatial composition of immune cells present in cHSIL, showing a strong relation between complete response to therapy and pre-existing CD4⁺ T cell, M1-like macrophage and DC infiltration. These data formed the basis for a simple specific and sensitive single color immunohistochemical detection and scoring method with a positive and negative predictive value $> 90\%$, thereby adding significant value to the current $< 60\%$ a priori chance of clinical response of cHSIL to imiquimod. The use of CIBI may be of great clinical value in the selection of patients responsive to imiquimod, hereby preventing unnecessary exposure to adverse effects of the intensive imiquimod treatment regimen. CIBI-based personalized therapy will improve therapy efficacy in the selected cHSIL patients, can be used to motivate therapy adherence, and may prevent surgical LLETZ treatment, thereby reducing the risk of potential future obstetric complications (i.e. cervical insufficiency and subsequent premature deliveries).

The immune composition of cHSIL in patients with spontaneous regression includes all actors of the immune response required to mediate lesion clearance, and this can be observed in about 20% of cHSIL patients.¹⁷⁻¹⁸ Spontaneous cHSIL regression is associated with a higher CD8⁺ (granzyme B⁺) and CD4⁺ T cell infiltrate, and low infiltration by CD25⁺ regulatory T cells and CD138⁺ B cells, when compared to lesions that persisted.¹⁷⁻²¹ Spontaneous cLSIL regression is associated with reduced CD68⁺ macrophage infiltration compared to lesions that persisted.²² In addition, high numbers of CD4⁺ T cells, Tbet⁺ T cells and CD11c⁺ DCs are associated with the absence of recurrences after surgical therapy.²³ A bias inherent to this type of studies, is that about 20% of the CR patients could have had spontaneous lesion regression and display an immune cell composition, of which not all immune cell types are necessarily associated with full regression after imiquimod treatment. To exclude the possibility that we would identify an immune cell composition associated with spontaneous regression rather than one associated with imiquimod-mediated regression, we focused only on those parameters that differed in the majority of CR patients when compared to NR patients. Interestingly, the pre-existing immune composition of patients with a spontaneous regression or an imiquimod-induced CR display a high level of similarity. Both contain relatively high numbers of CD4⁺ T cells and dendritic cells and low numbers of regulatory T cells.

Patients responding to imiquimod therapy displayed a steep on-treatment increase in infiltrating T cells, most likely activated (PD1⁺) and/or with a type 1 cytokine profile (Tbet⁺), after which the lesion was cleared. This suggests that the mechanism underlying the absence of spontaneous lesion regression is the lack of sufficient such T cell infiltration, which is rescued by imiquimod treatment. In addition, all patients treated with imiquimod displayed a decrease in macrophages, irrespective of their phenotype. One may speculate whether the decrease in M2-like macrophages is required in order for the T cells to infiltrate and execute an effective immune response as shown in other diseases.²⁴ However, imiquimod proved incapable of increasing the infiltration of T cells in NR patients, whose lesions displayed a strong pre-existing but not on-treatment M2-like macrophage infiltrate, precluding M2-like macrophages as a strong mediator of T cell suppression and suggesting that another mechanism prevents this. Potentially, regulatory T cells may have played a role in NR patients as in these lesions the regulatory T cells were not only abundantly present but also displayed direct spatial interactions with the other T cells, suggesting that they locally inhibited these T cells from executing

their effector functions. The data of this study adds unique additional insight to our understanding of which immune cells are pivotal for cHSIL regression. Cell types that were present in low numbers in CR before treatment and did not increase upon imiquimod treatment, i.e. CD8⁺ T cells, are not likely to form key mediators of lesion clearance. In addition, the cell types that are present in high numbers in both spontaneous and imiquimod-mediated complete regressions, and potentially increased by imiquimod, i.e. CD4⁺ T cells and CD11c⁺ DCs, may causally be involved in cHSIL clearance.

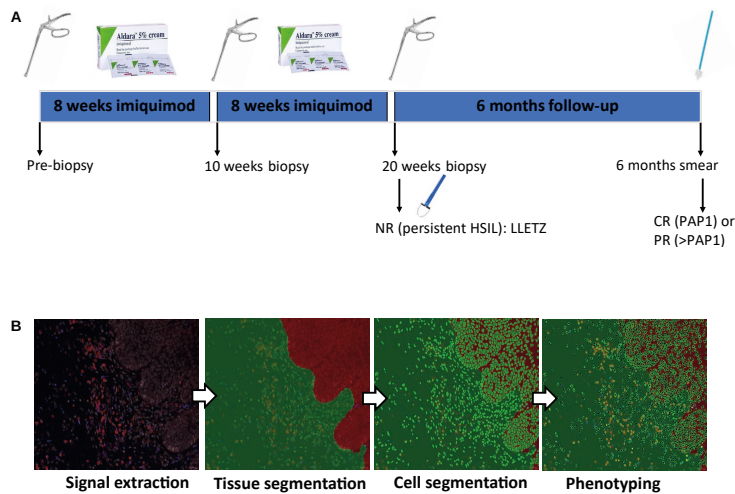
HSIL lesions of the vulva also develop as a consequence of a persistent HPV infection and have been treated with imiquimod. Their immunological composition related to clinical outcome after imiquimod has been extensively studied. Our findings in cHSIL extend earlier observations in vHSIL, where imiquimod was found to increase the number of infiltrating CD8⁺ T cells, CD1a⁺ dendritic cells and CD94⁺ natural killer cells only in responders.²⁵ Both in cHSIL and vHSIL, a pre-existing coordinated high infiltration by pro-inflammatory T cells and myeloid cells is essential for response to imiquimod⁸, and a similar relation was found with response to therapeutic vaccination monotherapy in vHSIL.¹⁰ There are some differences in the cell types associated with imiquimod response, which may reflect the two distinct anatomical sites.²⁶ In vHSIL, responders to imiquimod displayed a high infiltration with both CD4⁺ T cells and CD8⁺ T cells, whereas in cHSIL responders the CD4⁺ T cells dominated. The correlation between a high infiltration of T cells and myeloid cells in responders suggests that cytokines produced by T cells (independent of being CD4⁺ or CD8⁺) play a role in this coordination.¹¹ Moreover, vHSIL responders already presented with high numbers of CD14⁺ inflammatory myeloid cells, whereas in cHSIL mostly M1-like macrophages were present.⁸ Altogether, these data in HSIL suggest that the foundation for the successful treatment of any HSIL by imiquimod, is the pre-existing abundant presence of T cells in combination with pro-inflammatory myeloid cells (either CD14 or M1-like). This fits well with preclinical data showing that the depletion or absence of these type of myeloid cells was associated with reduced lesion clearance.^{27,28} Biologically this represents the onset of a coordinated anti-tumor immune response that is amplified by imiquimod and characterizes the HSIL responders.

There are two overt limitations to this study. First, the clinical response in this study was evaluated after biopsies and imiquimod treatment, so formally we cannot exclude that the clinical response observed is solely due to imiquimod

treatment. Hence, CIBI might be a predictor for the clinical response after biopsy and imiquimod. However, as a biopsy is standard of care in the diagnostic work-up of cHSIL, this distinguishment is clinically irrelevant. Secondly, the number of patients currently analyzed was limited, precluding rigorous statistical compensation, hence our data should be viewed as hypothesis generating. Therefore, a new study (PRedICT-TOPIC, NCT05405270) is currently initiated to validate these findings prospectively in a large cohort and to test the implementation of CIBI in pathology laboratories.

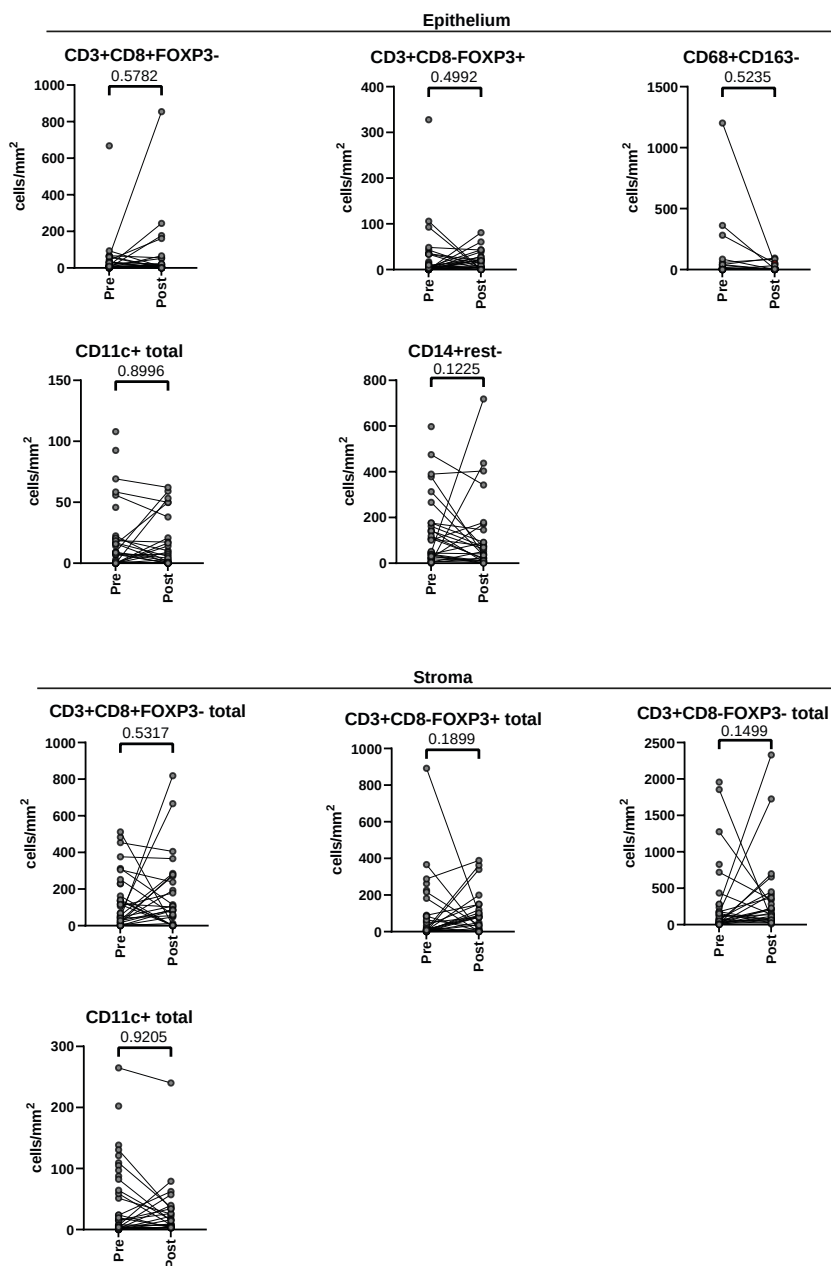
In conclusion, the pre-existing difference in the total number of immune cell subsets found associated with imiquimod-induced regression of cHSIL can be exploited as accurate predictive biomarker in the clinic, based on the intraepithelial expression of CD4, CD68 and CD11c. We propose CIBI to be scored by publicly available open-source digital pathology image analyzers, so that it can be objectively implemented in the clinic.

Supplemental Figures



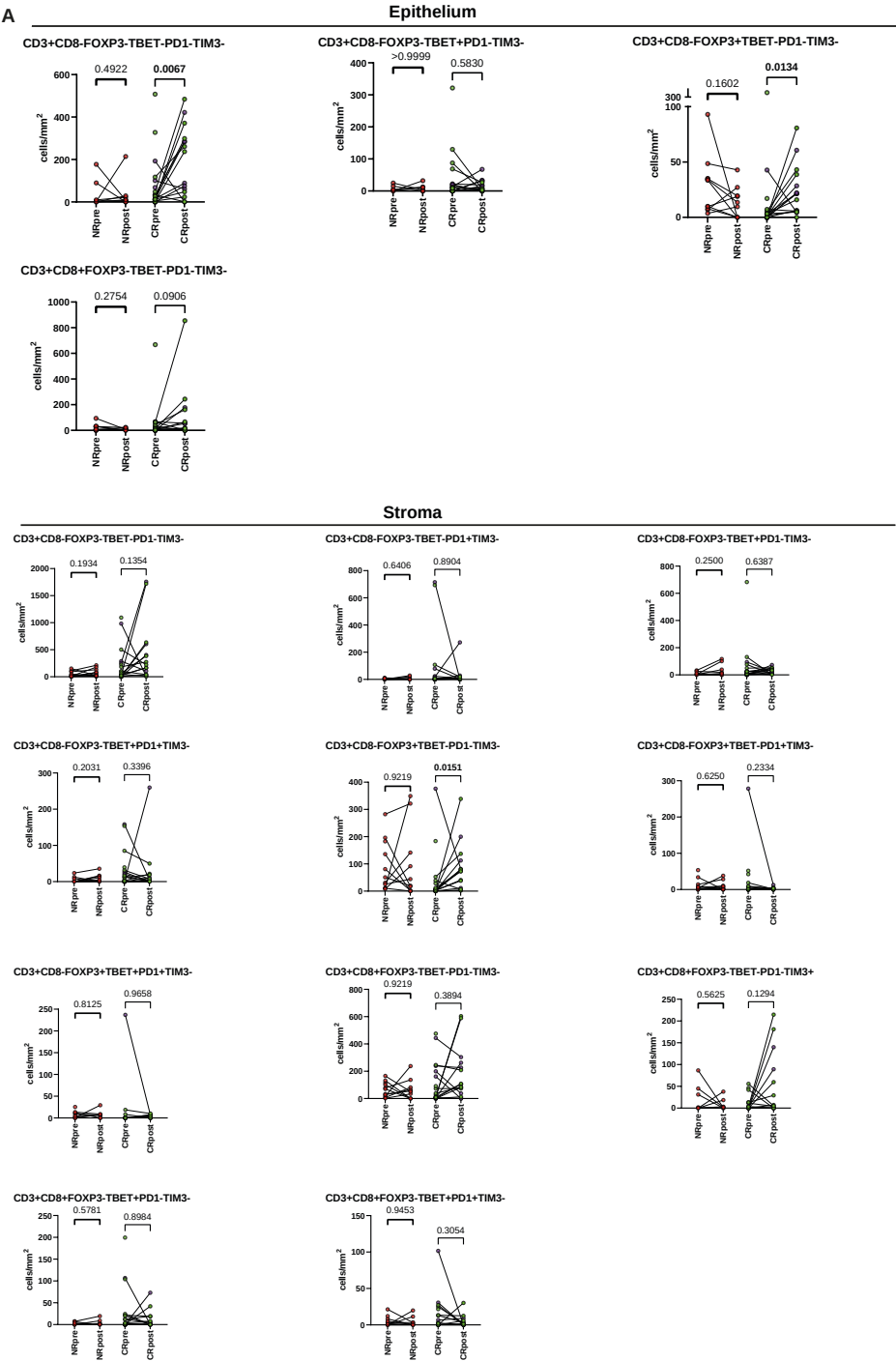
Supplemental Figure 1. Overview study design.

A) Workflow of the cHSIL clinical trial. **B)** Overview of the InForm machine learning based image analysis pipeline.

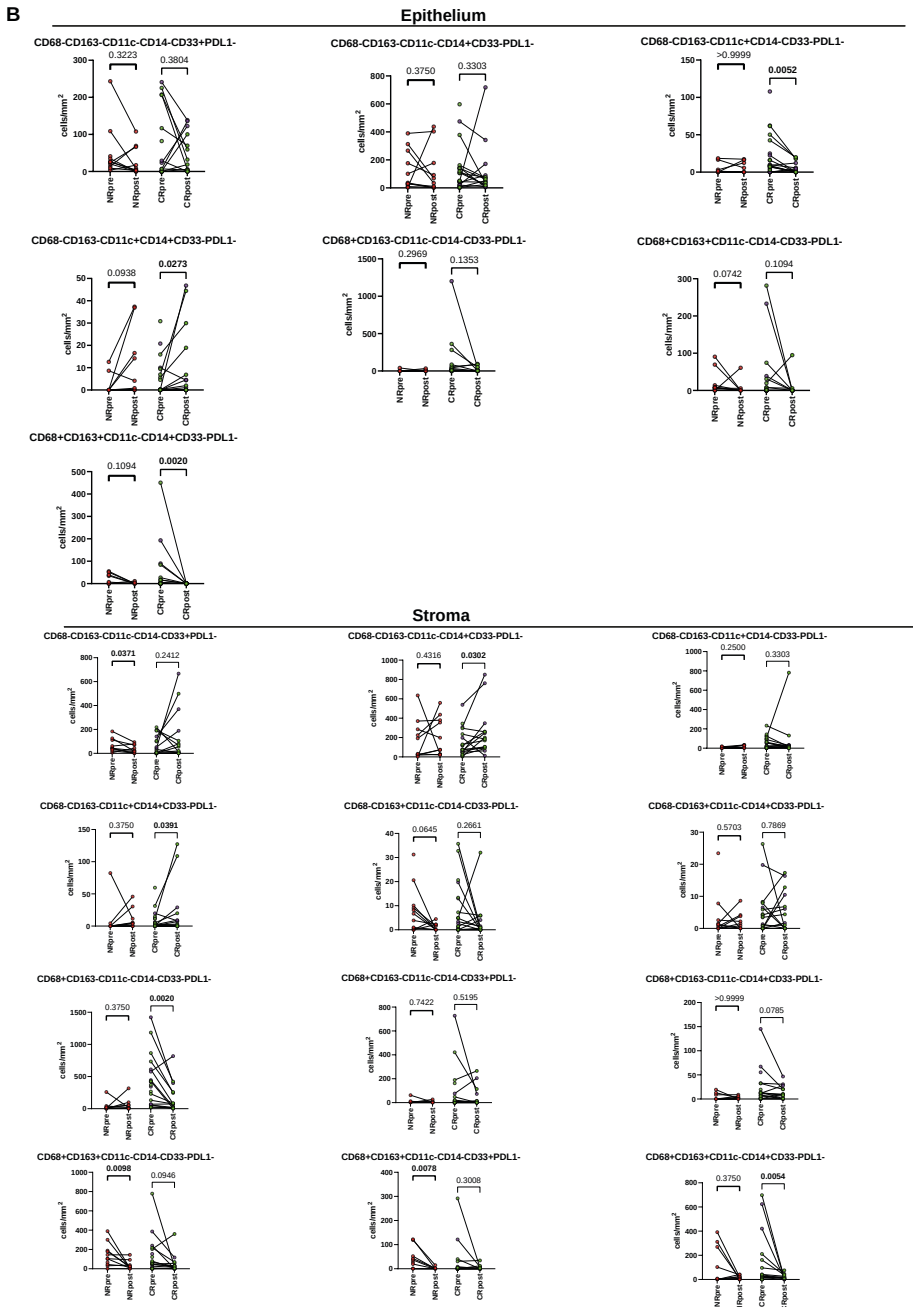


Supplemental Figure 2. Imiquimod-induced changes in the immune tumor microenvironment.

Differences in immune cell infiltration upon imiquimod treatment in cHSIL's epithelium and stroma ($n=35$), as measured pre-treatment and after 10 weeks of imiquimod (not significant, Wilcoxon paired signed rank test).



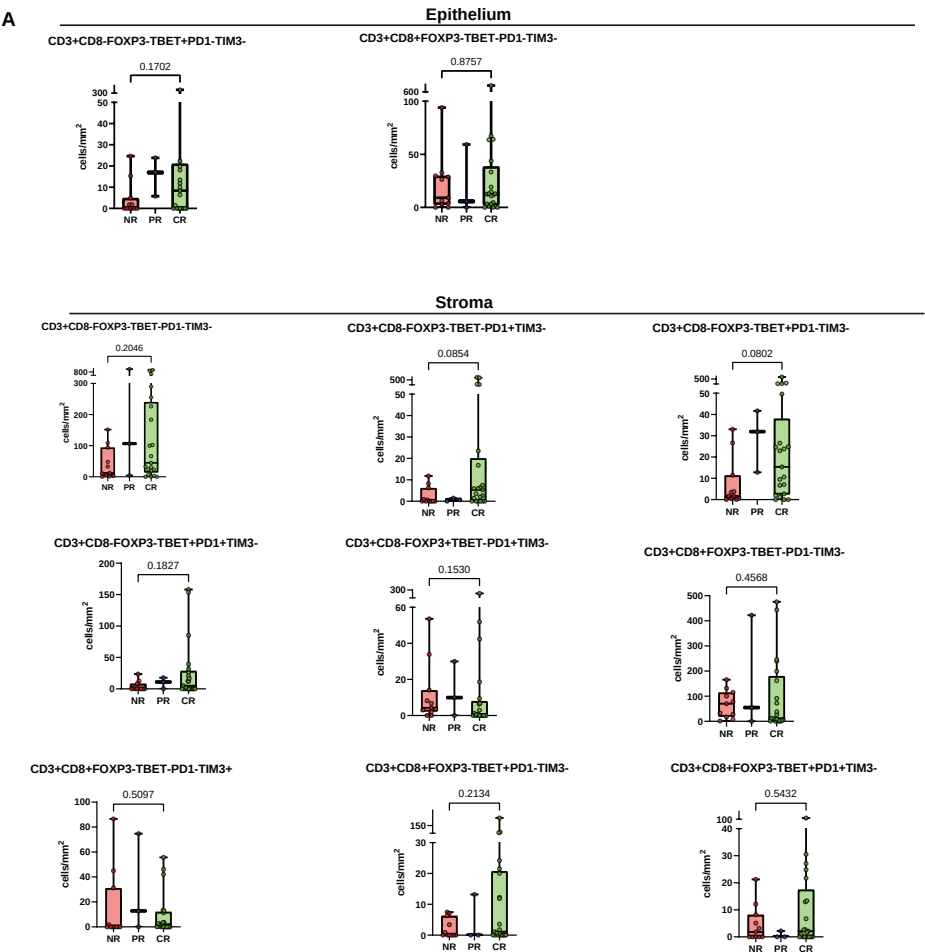
Supplemental Figure 3. Continued



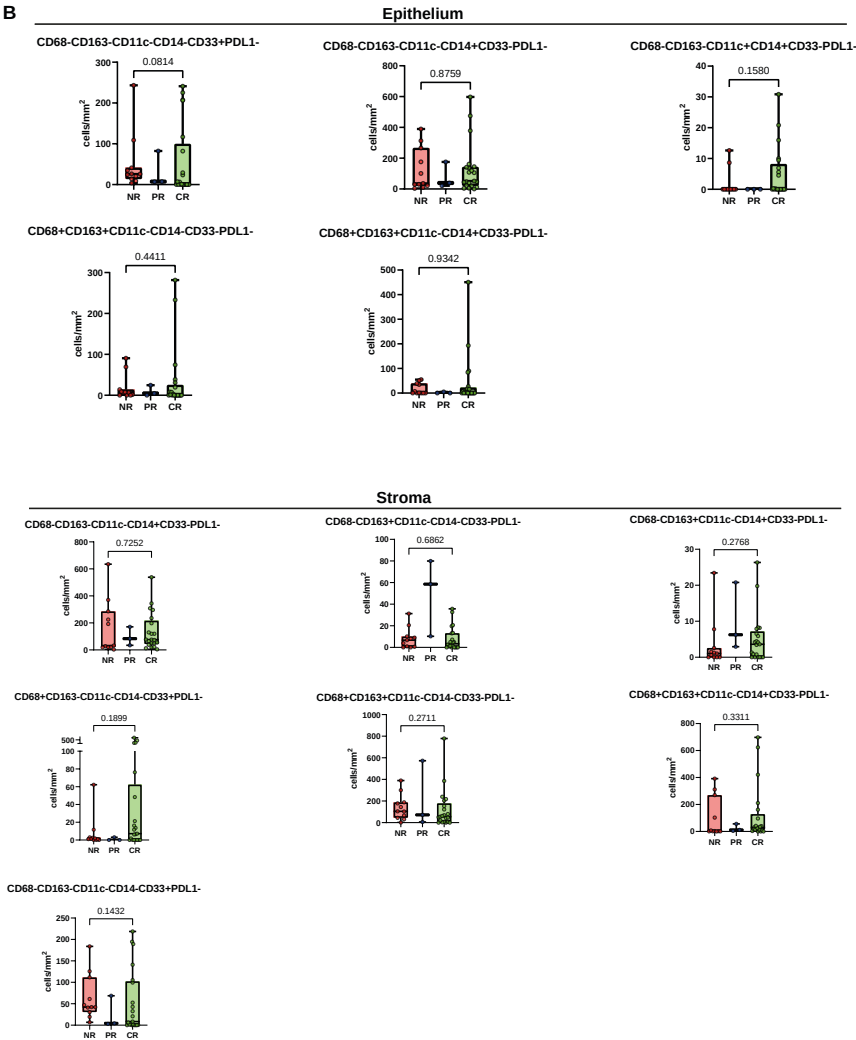
Supplemental Figure 3. Imiquimod-induced changes in immune tumor microenvironment per response group.

Differences between CR (n=21) and NR (n=11) (Wilcoxon paired signed rank test) in immune cell infiltration upon imiquimod treatment in cHSIL's epithelium and stroma, as measured pre-treatment and after 10 weeks of imiquimod, in **A**) T cells and **B**) myeloid cells.

A

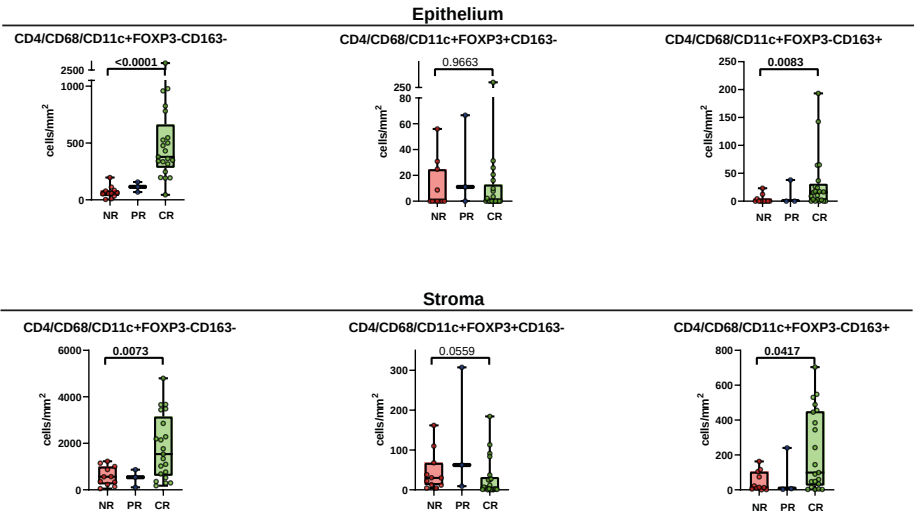


Supplemental Figure 4. Continued



Supplemental Figure 4. Pre-imiquimod differences in immune cell phenotypes in cHSIL per response group.

Pre-existing differences in specific immune cell phenotypes infiltrating CR (n=21) versus NR (n=11) cHSIL **A** T cells and **B** myeloid cells (not significant, Mann-Whitney U test). PR (n=3) not included in statistical analysis due to small sample size.



Supplemental Figure 5. Dual immunohistochemistry cell phenotype counts per response group.

Dual immunohistochemistry counts of all cell phenotypes infiltrating CR (n=21) versus NR (n=11) cHSIL epithelium and stroma (Mann-Whitney U test). PR (n=3) not included in statistical analysis due to small sample size.

Supplemental Tables

Supplemental Table 1. Panel designs

A) Multispectral immunofluorescence T cell panel

Marker	Primary antibody clone and producer	Species and isotype	Dilution	Detection method
PD-1	D4W2J, CST	rabbit IgG	1:1000	Indirect with OPAL 570
Tbet	D6N8B XP, CST	rabbit IgG	1:2000	Indirect with OPAL 520
FOXP3	236A/E7, Invitrogen	mouse IgG1	1:25	Indirect with CF Goat-a-Mouse IgG1 633
CD8	4B11, Novocastra	mouse IgG2b	1:50	Indirect with Alexa Goat-a-Mouse IgG2b 647
TIM3	D5D5R XP, CST	rabbit IgG	1:25	Indirect with Alexa Goat-a-Rabbit IgG 680
CD3	D7A6E, CST	rabbit IgG	1:50	Directly labelled with Alexa 594

B) Multispectral immunofluorescence myeloid cell panel

Marker	Primary antibody clone and producer	Species and isotype	Dilution	Detection method
PD-L1	SP142, Spring Bioscience	rabbit IgG	1:6000	Indirect with OPAL 520
CD14	D7A2T, CST	rabbit IgG	1:25	Indirect with CF Donkey-a-Rabbit IgG 633
CD33	PWS44, Novocastra	mouse IgG2b	1:25	Indirect with Alexa Goat-a-Mouse IgG2b 647
CD163	10D6, Invitrogen	mouse IgG1	1:10	Indirect with CF Goat-a-Mouse IgG1 680
CD11c	EP1347Y, Abcam	rabbit IgG	1:100	Directly labelled with Alexa 546
CD68	D4B9C XP, CST	rabbit IgG	1:50	Directly labelled with Alexa 594

C) Dual immunohistochemistry panel

Marker	Primary antibody clone and producer	Species and isotype	Dilution	Detection method
CD11c	EP1347Y - Abcam	rabbit IgG	1:800	DAB
CD4	EPR6855 - Abcam	rabbit IgG	1:1600	DAB
CD68	D4B9C XP - CST	rabbit IgG	1:800	DAB
FOXP3	236A/E7 - Invitrogen	mouse IgG1	1:200	Vector Red
CD163	10D6 - Invitrogen	mouse IgG1	1:200	Vector Red

Supplemental Table 2. T cell and myeloid cell phenotype counts in cHSIL pre- and on imiquimod treatment.

Pre-imiquimod T cells	Cell type	Median NRpre	Min NRpre	Max NRpre	Median PRpre	Min PRpre	Max PRpre	Median CRpre	Min CRpre	Max CRpre
Epithelium CD3+CD8- FOXP3-TBET-PD1-TIM3-	CD4 helper T cell	1.797633559	0	177.408056	11.56221076	0	355.635188	15.36954535	0	507.624282
Epithelium CD3+CD8- FOXP3-TBET+PD1-TIM3-	type 1 CD4 helper T cell	0	0	24.68286	16.93500894	5.7811054	23.8357715	8.403936908	0	321.638065
Epithelium CD3+CD8- FOXP3+TBET-PD1-TIM3-	regulatory T cell	33.58689403	3.58046926	92.9593161	13.48924589	3.9726286	105.843806	0	0	327.753539
Epithelium CD3+CD8+FOXP3-TBET-PD1- TIM3-	CD8 T cell	9.160062008	0	94.1034036	5.781105382	0	59.2725313	12.51626724	0	668.112984
Stroma CD3+CD8-FOXP3- TBET-PD1-TIM3-	CD4 helper T cell	12.68024098	0.54697899	151.845191	106.7591799	4.563474	1222.52476	44.68460859	0	1093.75499
Stroma CD3+CD8-FOXP3- TBET-PD1+TIM3-	activated CD4 helper T cell	0	0	11.8074488	0.711727866	0	1.46410151	5.376714578	0	714.911665
Stroma CD3+CD8-FOXP3- TBET+PD1-TIM3-	type 1 CD4 helper T cell	1.690871778	0	33.0608568	31.94431832	12.811102	41.7268931	15.37792599	0	682.682575
Stroma CD3+CD8-FOXP3- TBET+PD1+TIM3-	activated type 1 CD4 helper T cell	0	0	23.6148977	10.98076134	0	17.7931967	5.105598455	0	158.033105
Stroma CD3+CD8- FOXP3+TBET-PD1-TIM3-	regulatory T cell	50.75428231	8.19317877	282.482952	61.92032434	22.81737	320.638231	3.359516283	0	376.269297
Stroma CD3+CD8- FOXP3+TBET-PD1+TIM3-	activated regulatory T cell	4.241485769	0	53.6028369	9.964190124	0	30.014081	0.591864468	0	278.43928
Stroma CD3+CD8- FOXP3+TBET+PD1+TIM3-	activated type 1 regulatory T cell	2.361489769	0	25.360482	13.52282945	0	15.3730659	0	0	237.049657

Pre-imitiquimod T cells	Cell type	Median NRpre	Min NRpre	Max NRpre	Median PRpre	Min PRpre	Max PRpre	Median CRpre	Min CRpre	Max CRpre
Stroma CD3+CD8+FOXP3- TBET-PD1-TIM3-	CD8 T cell	70.40866376	1.51933694	165.763231	54.80304568	0	422.393286	12.22526767	0	475.439401
Stroma CD3+CD8+FOXP3- TBET-PD1-TIM3+	activated CD8 T cell	0	0	86.5076863	12.81110159	0	74.6691771	2.10347985	0	55.6928861
Stroma CD3+CD8+FOXP3- TBET+PD1-TIM3-	type 1 CD8 T cell	0	0	7.49286967	0	0	13.1769136	1.119838761	0	199.553368
Stroma CD3+CD8+FOXP3- TBET+PD1+TIM3-	activated type 1 CD8 T cell	1.783444136	0	21.2534079	0	0	2.19615227	2.10347985	0	101.59271

Post-imitiquimod T cells	Cell type	Median NRpost	Min NRpost	Max NRpost	Median PRpost	Min PRpost	Max PRpost	Median CRpost	Min CRpost	Max CRpost
Epithelium CD3+CD8- FOXP3-TBET-PD1-TIM3-	CD4 helper T cell	6.953623372	0	214.012237	27.36013045	0	54.7202609	237.1403068	0	484.386605
Epithelium CD3+CD8- FOXP3-TBET+PD1-TIM3-	type 1 CD4 helper T cell	0	0	32.3128922	3.040014495	0	6.08002899	7.3484418	0	67.3634497
Epithelium CD3+CD8- FOXP3+TBET-PD1-TIM3-	regulatory T cell	11.59520468	0	42.8024474	0	0	0	21.43256707	0	80.7311008
Epithelium CD3+CD8+FOXP3-TBET-PD1- TIM3-	CD8 T cell	4.104246579	0	22.2158784	0	0	0	18.43521524	0	854.925835
Stroma CD3+CD8-FOXP3- TBET-PD1-TIM3-	CD4 helper T cell	61.12478181	6.28607223	212.929742	73.28869852	6.9868361	139.590561	223.0988636	8.3550198	1756.73245
Stroma CD3+CD8-FOXP3- TBET-PD1+TIM3-	activated CD4 helper T cell	3.284812953	0	28.5804675	5.697573916	0	11.3951478	4.308668409	0	272.206243

Supplemental Table 2 Continued

Post-imi quimod T cells	Cell type	Median NRpost	Min NRpost	Max NRpost	Median PRpost	Min PRpost	Max PRpost	Median CRpost	Min CRpost	Max CRpost
Stroma CD3+CD8-FOXP3- TBET+PD1-TIM3-	type 1 CD4 helper T cell	8.197615633	0	116.733057	62.45510345	59.388107	65.5221	26.66650667	0	73.1064235
Stroma CD3+CD8-FOXP3- TBET+PD1+TIM3-	activated type 1 CD4 helper T cell	2.787705281	0	35.668434	32.76105002	0	65.5221	3.520659405	0	259.9214
Stroma CD3+CD8- FOXP3+TBET-PD1-TIM3-	regulatory T cell	31.90646734	0	349.017246	2.848786958	0	5.69757392	73.06248316	2.4184586	338.948582
Stroma CD3+CD8- FOXP3+TBET-PD1+TIM3-	activated regulatory T cell	4.479798066	0	38.2063254	0	0	0	1.132481541	0	12.5325297
Stroma CD3+CD8- FOXP3+TBET+PD1+TIM3-	activated type 1 regulatory T cell	6.385621027	0	29.2166018	2.848786958	0	5.69757392	2.840687969	0	10.1923339
Stroma CD3+CD8+FOXP3- TBET-PD1-TIM3-	CD8 T cell	52.74298083	0	237.720725	1.424393479	0	2.84878696	105.2332187	0	603.931689
Stroma CD3+CD8+FOXP3- TBET-PD1-TIM3+	activated CD8 T cell	0.444295398	0	37.8192063	0	0	0	5.12119146	0	214.715309
Stroma CD3+CD8+FOXP3- TBET+PD1-TIM3-	type 1 CD8 T cell	0	0	19.4555095	0	0	0	0	0	73.1064235
Stroma CD3+CD8+FOXP3- TBET+PD1+TIM3-	activated type 1 CD8 T cell	0.540274375	0	19.8104291	0	0	0	1.461572482	0	30.2150296

Supplemental Table 2 *Continued*

Pre-immiquimod myeloid cells	Cell type	Median NRpre	Min NRpre	Max NRpre	Median PRpre	Min PRpre	Max PRpre	Median CRpre	Min CRpre	Max CRpre
Epithelium CD68-CD163- CD11c-CD14-CD33+PDL1-	immature myeloid cell	25.96801334	3.57560607	243.3711	7.359512427	6.7686165	82.2827975	1.129078259	0	241.179561
Epithelium CD68-CD163- CD11c-CD14+CD33-PDL1-	monocyte	36.29683369	3.70971619	389.845647	39.25073295	21.158434	175.984028	51.39961142	0	597.420466
Epithelium CD68-CD163- CD11c+CD14-CD33-PDL1-	dendritic cell	0	0	18.4086377	0	0	0	9.023853276	0	107.878814
Epithelium CD68-CD163- CD11c+CD14+CD33-PDL1-	inflammatory dendritic cell	0	0	12.6067298	0	0	0	0	0	30.8397669
Epithelium CD68+CD163- CD11c-CD14-CD33-PDL1-	M1-like macrophage	0	0	40.5235355	4.701874144	0	7.35951243	9.998880125	0	1201.87815
Epithelium CD68+CD163+CD11c-CD14- CD33-PDL1-	M2-like macrophage	6.805656317	0	90.5820205	4.701874144	0	24.5317081	0	0	281.587024
Epithelium CD68+CD163+CD11c- CD14+CD33-PDL1-	inflammatory M2-like macrophage	1.62312683	0	54.8259598	0	0	4.90634162	0.209857053	0	450.539239
Stroma CD68-CD163- CD11c-CD14-CD33+PDL1-	immature myeloid cell	42.27601442	6.86742523	183.8591	4.180560935	2.9496537	68.5642765	8.376764531	0	218.565068
Stroma CD68-CD163- CD11c-CD14+CD33-PDL1-	monocyte	32.18024978	2.94651689	635.021308	83.6112187	34.282138	171.079914	76.126306	4.387829	538.354328
Stroma CD68-CD163- CD11c+CD14-CD33-PDL1-	dendritic cell	3.086458334	0	19.4791812	0	0	0	58.18518636	0	233.642663
Stroma CD68-CD163- CD11c+CD14+CD33-PDL1-	inflammatory dendritic cell	0	0	82.2671172	0	0	1.47482684	1.994467745	0	59.7227372
Stroma CD68- CD163+CD11c-CD14-CD33- PDL1-	CD68-CD163+ myeloid cell	6.68732639	0	31.2644232	58.52785309	10.323788	79.9916559	3.37994764	0	35.7160771

Supplemental Table 2 Continued

Pre-immiquimod myeloid cells	Cell type	Median NRpre	Min NRpre	Max NRpre	Median PRpre	Min PRpre	Max PRpre	Median CRpre	Min CRpre	Max CRpre
Stroma CD68-CD163+CD11c-CD14+CD33-PDL1-	inflammatory CD68-CD163+ myeloid cell	1.028819445	0	23.4483174	6.270841403	2.9496537	20.7770535	3.622820611	0	26.3375095
Stroma CD68+CD163-CD11c-CD14-CD33-PDL1-	M1-like macrophage	9.042250821	0	259.561129	7.271968721	1.4748268	20.9028047	369.5357133	18.838667	1422.40066
Stroma CD68+CD163-CD11c-CD14-CD33+PDL1-	immature M1-like macrophage	1.23464983	0	62.2946709	0	0	3.11655802	7.180083883	0	727.487361
Stroma CD68+CD163-CD11c-CD14+CD33-PDL1-	inflammatory M1-like macrophage	0	0	19.4791812	3.116558023	0	6.2708414	10.96251123	0	145.270215
Stroma CD68+CD163+CD11c-CD14-CD33-PDL1-	M2-like macrophage	104.9395833	1.23464983	389.341693	71.68083454	5.8993074	572.736848	55.74358118	0	778.659538
Stroma CD68+CD163+CD11c-CD14-CD33-PDL1-	immature M2-like macrophage	27.35637026	0	122.250869	21.81590616	0	45.9861703	0	0	291.997327
Stroma CD68+CD163+CD11c-CD14-CD33-PDL1-	inflammatory M2-like macrophage	3.014083607	0	391.937305	11.42737942	4.4244805	56.4375726	29.68158339	0	697.549169

Supplemental Table 2 *Continued*

Post-imi quimod myeloid cells	Cell type	Median NRpost	Min NRpost	Max NRpost	Median PRpost	Min PRpost	Max PRpost	Median CRpost	Min CRpost	Max CRpost
Epithelium CD68-CD163- CD11c-CD14-CD33+PDL1-	immature myeloid cell	6.171237564	0	107.949056	9.878684814	9.3421405	10.4152291	18.95468693	0	138.66817
Epithelium CD68-CD163- CD11c-CD14+CD33-PDL1-	monocyte	51.73639583	0	437.831846	77.57767428	9.3421405	145.813208	51.56222639	5.3333912	718.442856
Epithelium CD68-CD163- CD11c+CD14-CD33-PDL1-	dendritic cell	0	0	17.2799314	0	0	0	0.62382596	0	19.9753754
Epithelium CD68-CD163- CD11c+CD14+CD33-PDL1-	inflammatory dendritic cell	2.478069901	0	37.3719543	10.41522915	0	20.8304583	1.983162553	0	46.8107243
Epithelium CD68+CD163- CD11c-CD14-CD33-PDL1-	M1-like macrophage	3.087006169	0	33.1652592	4.67107024	0	9.34214048	4.396415397	0	94.7734346
Epithelium CD68+CD163+CD11c-CD14- CD33-PDL1-	M2-like macrophage	0	0	60.8029751	0	0	0	0	0	94.7734346
Epithelium CD68+CD163+CD11c- CD14+CD33-PDL1-	inflammatory M2-like macrophage	0	0	11.0550864	0	0	0	0	0	0
Stroma CD68-CD163-CD11c- CD14-CD33+PDL1-	immature myeloid cell	21.14267855	0	93.4960605	29.30214646	8.946863	49.6574299	55.45793611	0	666.733651
Stroma CD68-CD163-CD11c- CD14+CD33-PDL1-	monocyte	136.6479181	20.6728389	558.427423	230.4818002	10.736236	450.227365	184.184969	13.010164	850.254868
Stroma CD68-CD163- CD11c+CD14-CD33-PDL1-	dendritic cell	4.1865516	0	34.1641104	4.473431485	0	8.94686297	13.95942581	0	782.217646
Stroma CD68-CD163- CD11c+CD14+CD33-PDL1-	inflammatory dendritic cell	3.993617208	0	45.7925779	23.44171717	7.1574904	39.725944	3.069637175	0	127.337756
Stroma CD68-CD163+CD11c- CD14-CD33-PDL1-	CD68-CD163+ myeloid cell	0	0	4.45618832	26.84058891	0	53.6811778	0.69940923	0	32.0616193

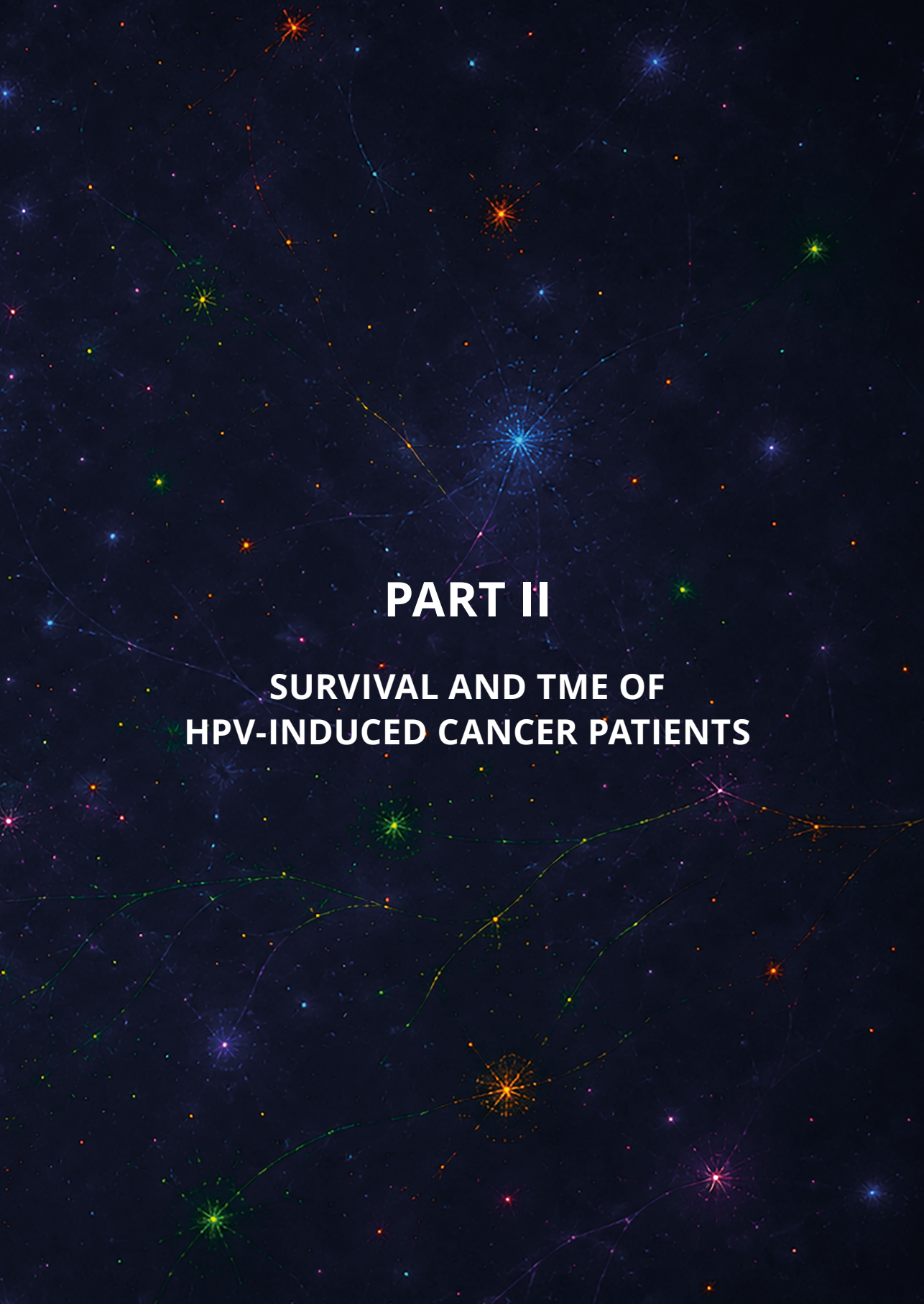
Supplemental Table 2 *Continued*

Post-impliquimod myeloid cells	Cell type	Median NRpost	Min NRpost	Max NRpost	Median PRpost	Min PRpost	Max PRpost	Median CRpost	Min CRpost	Max CRpost
Stroma CD68-CD163+CD11c- CD14+CD33-PDL1-	inflammatory CD68-CD163+ myeloid cell	1.15436377	0	8.64249299	4.473431485	0	8.94686297	1.698684462	0	17.330605
Stroma CD68+CD163-CD11c- CD14-CD33-PDL1-	M1-like macrophage	18.38721201	0	316.629516	138.4081262	6.6209907	270.195262	73.6712922	0	818.63443
Stroma CD68+CD163-CD11c- CD14-CD33+PDL1-	immature M1- like macrophage	2.189160151	0	26.682539	3.578745188	0	7.15749038	2.791885162	0	265.86724
Stroma CD68+CD163-CD11c- CD14+CD33-PDL1-	inflammatory M1-like macrophage	0.39284059	0	8.91237663	3.31049533	0	6.62099066	8.926560296	0	46.7138227
Stroma CD68+CD163+CD11c- CD14-CD33-PDL1-	M2-like macrophage	19.48613654	0	144.565337	303.6568413	13.241981	594.071701	19.51596406	0	360.476585
Stroma CD68+CD163+CD11c- CD14-CD33+PDL1-	immature M2- like macrophage	0	0	14.9279424	9.707424338	3.3104953	16.1043533	0.849342231	0	34.4479148
Stroma CD68+CD163+CD11c- CD14+CD33-PDL1-	inflammatory M2-like macrophage	14.36541575	0	41.9297041	29.9727711	26.840589	33.1049533	16.01063448	0	75.3881319

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The background of the entire page is a deep space image showing a complex cosmic web. It features a dark blue to black background filled with numerous small, distant stars. Overlaid on this are intricate, glowing filaments of gas and dark matter that form a web-like structure. These filaments are primarily in shades of blue, green, and yellow, with some brighter, more concentrated regions that appear as star clusters or galaxies. The overall effect is one of vastness and the interconnectedness of the universe.

PART II

SURVIVAL AND TME OF HPV-INDUCED CANCER PATIENTS

Chapter 7

Monocyte infiltration is an independent positive prognostic biomarker in vulvar squamous cell carcinoma

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Cancer Immunology, Immunotherapy 2024

Key findings

- Vulvar squamous cell carcinoma (VSCC) is an aggressive disease, currently treated with limited success by mutilating therapies. Immunotherapy is studied as an alternative treatment option but its successful application requires in-depth knowledge of the spontaneous immune response in VSCC.
- Here, we identified for the first time that monocyte infiltration forms an independent positive prognostic marker for patient survival.
- Patients of whom their tumor is strongly infiltrated with both monocytes and T cells display the best survival and as such are likely to form excellent candidates for neoadjuvant immunotherapy.

Abstract

Background

Vulvar squamous cell carcinoma (VSCC) arises after an HPV infection or the mutation of p53 or other driver genes, and is treated by mutilating surgery and/or (chemo)radiation, with limited success and high morbidity. In-depth information on the immunological make up of VSCC is pivotal to assess whether immunotherapy may form an alternative treatment.

Methods

A total of 104 patient samples, comprising healthy vulva (n=27) and VSCC (n=77), were analyzed. Multispectral immunofluorescence (15 markers) was used to study both the myeloid and lymphoid immune cell composition and this was linked to differences in transcriptomics (NanoString nCounter, 1258 genes) and in survival (Kaplan Meier analyses).

Results

Healthy vulva and VSCC are both well infiltrated but with different subpopulations of lymphoid and myeloid cells. In contrast to the lymphoid cell infiltrate, the density and composition of the myeloid cell infiltrate strongly differed per VSCC molecular subtype. A relative strong infiltration with epithelial monocytes (HLADR⁺CD11c⁺CD14⁺CD68⁺CD163⁺CD33⁺) was prognostic for improved survival, independent of T cell infiltration, disease stage or molecular subtype. A strong infiltration with T cells and/or monocytes was associated with drastic superior survival: 5-year survival >90% when either one is high, versus 40% when both are low (p<0.001).

Conclusion

A hot myeloid and/or lymphoid infiltrate predicts excellent survival in VSCC. Based on the response of similarly high-infiltrated other tumor types, we have started to explore the potential of neoadjuvant checkpoint blockade in VSCC.

Introduction

Vulvar squamous cell carcinoma (VSCC) can arise due to three distinct etiological pathways, each with differential clinical prognosis: driven by classical oncogenic mutations in TP53 (HPV-p53mut VSCC, poorest clinical outcome), driven by other somatic oncogenic mutations (HPV-p53wt, intermediate clinical outcome), or driven by a persistent infection with the oncogenic human papillomavirus (HPV+ VSCC, best clinical outcome).^{1,2} The current standard of care for VSCC is surgery and/or (chemo)radiation, which is associated with high morbidity due to severe local or systemic side effects. Therefore, novel treatment strategies are urgently needed.

Immunotherapy is increasingly being explored as an alternative for current standard cancer treatments. Studies in other tumor types have shown the importance of a pre-existing well-coordinated immune response for the prognosis and response to immunotherapy of cancer patients.^{3,4} Also in vulvar high-grade squamous intraepithelial lesions (vHSIL), the precursor lesion of HPV+ VSCC, the prerequisite of a pre-existing well-coordinated hot immune microenvironment for response to different forms of immunotherapy has been identified.^{1,5,6}

In VSCC, the presence and impact of lymphoid cells on prognosis has been studied before⁷ and revealed that the presence of high numbers of CD3+CD8+FOXP3+ T helper cells is an independent prognostic marker for survival, regardless of molecular subtype. Furthermore, higher T cell infiltration was associated with the expression of higher myeloid cell associated transcripts.⁸ So far only two studies have analyzed myeloid cells in VSCC, reporting the association between high numbers of CD68+ macrophages and low grade VSCC, and the presence of low CD1a+ dendritic cell numbers with recurrent VSCC.^{9,10} However, these studies were based on single marker immunohistochemistry in small patient cohorts, hence no in-depth insight in the actual myeloid cell composition of VSCC is available.

Here we aimed to fill that gap by an in-depth investigation of the myeloid and lymphoid infiltration in VSCC, using multispectral immunofluorescence and transcriptome analysis. Our study on a large sample set of healthy vulvar tissue and VSCC revealed that the type and number of VSCC infiltrating myeloid cells is linked to the molecular subtype of VSCC. High epithelial infiltration

by CD14⁺CD68⁺CD11c⁻ monocytes formed a strong prognostic biomarker for excellent survival of VSCC patients, independent from CD3⁺ T cell infiltration.

Materials and methods

Patient samples

Formalin-fixed paraffin-embedded (FFPE) tissue of n=104 women of ≥18 years old with histologically confirmed healthy HPV-negative vulva (n=27) or VSCC (n=77) were included. The healthy HPV⁻ vulvar tissue was from anonymized women who underwent labiaplasty. The included VSCC patients had FIGO stage I-IIIb at diagnosis, were treated with standard of care (surgical excision, and if needed adjuvant radiotherapy) at a tertiary hospital in the Netherlands, and all patients underwent 5 years of follow-up. All the tumor material investigated in this study was obtained prior to therapy. The study was in accordance with the Declaration of Helsinki, and approved by the local medical ethical committee of the Leiden University Medical Center (LUMC) and in agreement with Dutch law. HPV, P16 and P53 typing on all formalin-fixed paraffin-embedded (FFPE) tumor sections was performed as described before.⁷ HPV⁺p53mutant VSCC were not observed in our cohort. Routine diagnostic beta-catenin immunohistochemical staining was performed on 10 VSCC patients. A summary of the patient characteristics, treatments and performed tumor analyses is provided in **Supplemental Table 1 and 2**.

Multispectral immunofluorescence staining

A previously developed seven-color multispectral immunofluorescence panel for myeloid cells was applied, consisting of CD14, CD33, CD68, CD11c, CD163, HLA-DR, DAPI.⁶ In this panel a combination of direct detection (primary antibody directly labelled with fluorochrome) and indirect detection (fluorochrome labelled secondary antibody) of markers was used for fluorescence microscopy. An overview of the antibodies and staining methods included in each panel are shown in **Supplemental Table 3**. Briefly, 4µm FFPE tissue sections were deparaffinized, endogenous peroxidase was blocked with hydrogen peroxide and heat induced epitope retrieval was performed with tris-EDTA (10mM/1mM, pH 9.0). SuperBlock (ThermoFisher Scientific) was used to block non-specific binding sites. First, the antibodies detected by Opal were applied, followed by the unconjugated antibodies which were incubated overnight. On the second day the corresponding fluorescently labelled secondary antibodies were applied, followed by 5 hours incubation with the directly labeled primary

antibodies. Finally, DAPI was applied as nuclear counterstain and slides were mounted.⁶

Quantification of immune cells in the TME

As published⁵, images of the entire tissue sections stained with the multispectral immunofluorescence panels were acquired with the Vectra 3.0.5 multispectral imaging microscope (PerkinElmer) at 20x magnification. Immune cells in the TME were automatically phenotyped and counted with inForm 2.4 image analysis software (PerkinElmer-Akoya Biosciences) after manual training. The software was trained to segment epithelium and stroma, segment DAPI⁺ nucleated cells, and assign a phenotype to each cell. All phenotypes were visually inspected on accurateness, and if errors were detected the training was further optimized until all discrepancies were resolved. Immune cell counts were normalized for tissue size (cells/mm² epithelium and cells/mm² stroma). A threshold of a median cell count ≥ 10 cells/mm² in at least one tissue category was applied to study biologically common phenotypes.

Transcriptomic analyses

The archived diagnostic FFPE tissue blocks were used to cut sections of 10 μ m for RNA extraction.⁸ Directly before and after these 10 μ m thick sections, a 4 μ m section was cut for hematoxylin-eosin (HE) staining. A pathologist annotated the tumor regions on the HE slides to ensure that the slides contained tumor. After the sections were deparaffinized, the tumor areas were macro-dissected using the annotated HE slides as reference. RNA was then isolated with the RNA FFPE isolation kit (Qiagen), according to the manufacturer's instructions. The quality and quantity of the RNA was verified with the Agilent 2100 Bioanalyzer System, using the RNA 6000 Nano kit (Agilent). Samples were selected for further processing when $>20\%$ of the RNA fragments were >300 bp and the corrected RNA concentration remained $\geq 60\mu\text{g}/\mu\text{l}$.

To measure the gene expression profiles, 300ng of RNA from each sample was hybridized with the probes of the human PanCancer panel (730 genes) or IO360 panel (750 genes) for 17 hours at 65°C, following the manufacturer's instructions (NanoString). The number of copies of each gene in every sample was counted by scanning 490 Fields Of View using the NanoString nCounter MAX system. The raw data (counts of genes) were uploaded to the Rosalind platform, after which quality checks were performed. The housekeeping genes in the panels facilitated sample-to-sample normalization. Eight negative controls and six synthetic positive controls were included. The data were

reviewed for reliability and validity based on the imaging (>0.75) and binding density ($0.1-2.25$) quality control metrics, and performance check of the positive controls ($0.95-1$). Benjamini-Hochberg adjusted p-values were used to decrease the false-discovery rate. Differentially expressed genes (DEGs) were defined by a log2 fold difference of >1 or ≤ 1 and a p-value <0.05 .

Gene ontology analysis of the differentially expressed genes (DEGs) was performed with ClueGo.¹¹

Gene Set Enrichment Analysis (GSEA) was performed with the Broad Institute GSEA software package and collections for human gene sets, version 4.3.2.^{12,13} 1000 permutations were applied, and small gene sets were excluded by applying a threshold of at least 15 genes. Gene sets with an FDR <0.25 as well as p-value <0.05 were selected.

Statistical analyses

Statistical data analysis was performed with GraphPad Prism 9.3.1, which was also used to create graphs to visualize the data. The median immune counts of three different VSCC molecular subtypes were compared with the non-parametric Kruskal Wallis test, of two subgroups with the non-parametric Mann-Whitney U test. Spearman correlation was used to study correlations between the T cell and myeloid cell infiltrate. Kaplan Meier survival analyses were performed with SPSS 25.0. Differences in survival and hazard ratio (HR) with 95% confidence interval, were calculated with the non-parametric Mantel-Cox test. Two-sided p-values <0.05 were marked as statistically significant.

Results

A diverse myeloid cell infiltration is present in VSCC

An earlier transcriptomic study suggested that stronger T cell infiltration in VSCC was associated with more myeloid cell infiltration⁸, but the actual composition and location of the myeloid cell infiltrate was unknown. Therefore, a multispectral myeloid cell immunofluorescence panel, recently shown to be helpful in understanding the clinical response of vHSIL patients to different forms of immunotherapy^{5,6}, was applied to a large set of 77 VSCC patients (Figure 1A).

Based on the markers CD68, CD163, HLADR, CD11c, CD14 and CD33 used, at least 16 different phenotypes of myeloid cells that infiltrated VSCC were identified. To simplify their notation, the positively stained markers are indicated with a '+', whereas markers that were negative are indicated as 'rest-'. The presence of CD14⁺rest⁻ monocytes, CD68⁺rest⁻ macrophages, and HLADR⁺CD11c⁺rest⁻ dendritic cells was most prominent in the epithelium. The stroma of VSCC contained most of the phenotypes and was richly infiltrated by the three aforementioned myeloid cell subtypes. In addition, CD68⁺rest⁻ macrophages and CD68⁺CD163⁺rest⁻ M2-like macrophages were detected (**Figure 1B**). However, there was a large diversity regarding the numbers of tumor-infiltrating myeloid cells across the 77 patients.

Subgroup analysis based on early stage (FIGO I/II) versus late stage (FIGO III) VSCC showed no differences in epithelial myeloid cell infiltration. However, in the stromal compartment of late stage VSCC, a significant decrease in CD14⁺CD33⁺rest⁻ monocytes, HLADR⁺rest⁻ cells, HLADR⁺CD14⁺rest⁻ monocytes, HLADR⁺CD11c⁺rest⁻ dendritic cells, HLADR⁺CD11c⁺CD14⁺rest⁻ dendritic cells (DCs), CD68⁺CD14⁺rest⁻ inflammatory macrophages, CD68⁺HLADR⁺CD11c⁺rest⁻ cells, CD68⁺HLADR⁺CD11c⁺CD14⁺rest⁻ M1-like macrophages, CD68⁺CD163⁺HLADR⁺/⁻CD14⁺rest⁻ M2-like macrophages, and CD68⁺CD163⁺HLADR⁺CD11c⁺CD14⁺CD33⁺ M2-like macrophages was observed (**Figure 1C**). This indicates that during progression of VSCC from early to late stage, the tumor may become colder in terms of myeloid cell infiltration (**Supplemental Table 4**).

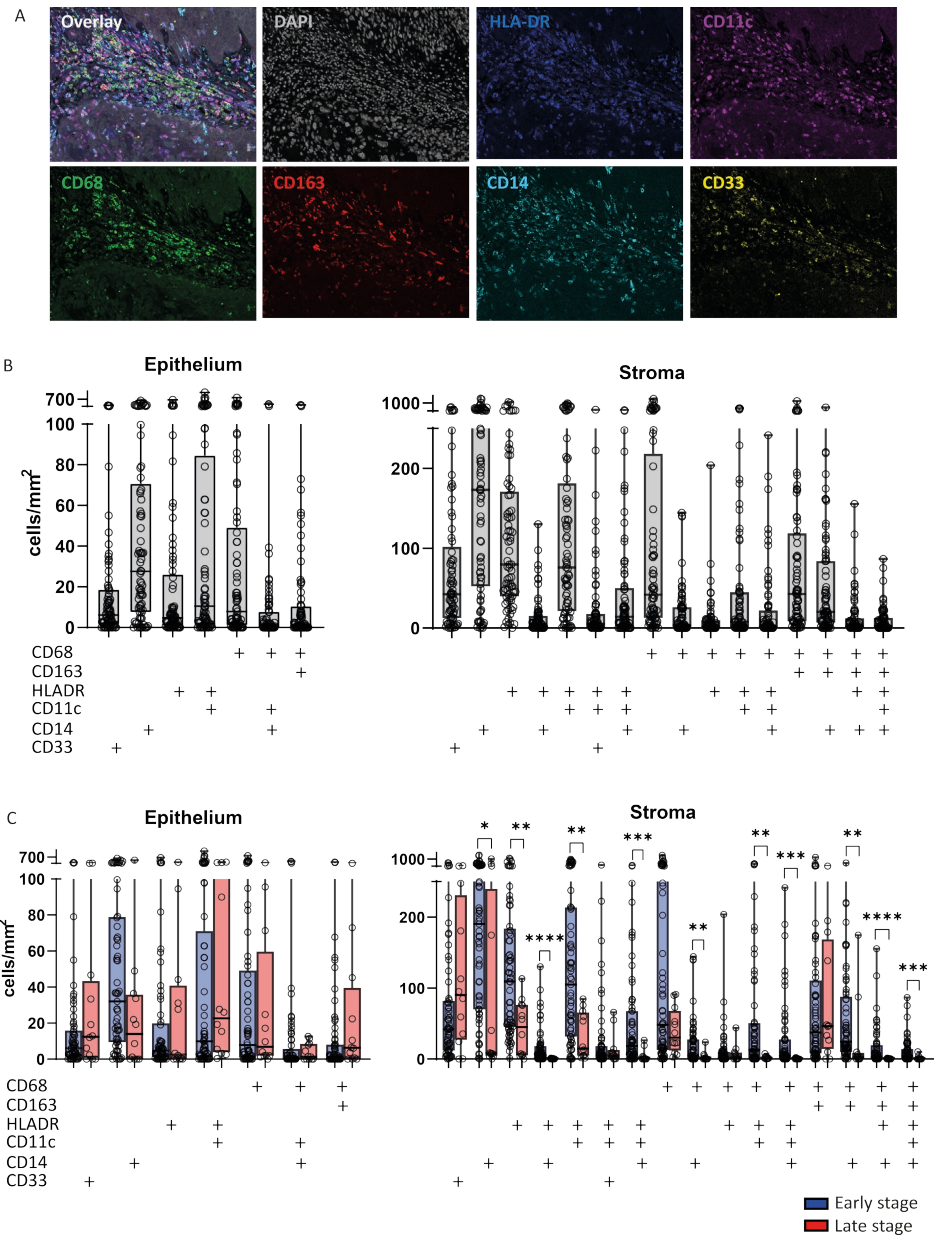


Figure 1. Identification and quantification of myeloid cells in VSCC.

A) Multispectral immunofluorescence staining of myeloid cells in VSCC, single markers staining and overlay. **B)** Myeloid cell phenotypes and quantification in the total VSCC cohort (n=77). **C)** Myeloid cell phenotypes and quantification in early (FIGO I/II in blue, n=65) versus late (FIGO III in red, n=12) stage VSCC. Boxplots with middle line indicating median, colored box indicating interquartile range, vertical lines from minimum to maximum cell count value. Marker+ indicates its expression by that specific cell phenotype. Asterisks indicate statistically significant differences (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

Molecular subtype specific myeloid cell attraction in vulvar cancer

VSCC can be subdivided into three different molecular subtypes which are correlated to survival, based on the presence of HPV and the TP53 status: HPV-p53mutant, HPV-p53wildtype and HPV⁺. To assess if the increased myeloid cell infiltrate in VSCC was a general phenomenon or specific for a molecular subtype, the myeloid cell numbers and composition in each subgroup were studied. HPV⁺ VSCC had a higher number of total infiltrating myeloid cells (both intraepithelial and stromal), with especially more CD14⁺rest⁻ monocytes, HLADR⁺CD11c⁺CD14⁺rest⁻ inflammatory DCs and CD68⁺ rest⁻ macrophages, but less CD68⁺CD163⁺rest⁻ M2-like macrophages when compared to the HPV⁻ subtypes. HPV-p53wt VSCC had more stromal HLADR⁺ CD14⁺rest⁻ cells, HLADR⁺CD11c⁺CD14⁺CD33⁺ dendritic cells, CD68⁺ HLADR⁺ CD14⁺rest⁻ M1-like macrophages and CD68⁺CD163⁺HLADR⁺ CD14⁺rest⁻ M2-like macrophages compared to HPV-p53mut VSCC (**Figure 2AB, Supplemental Table 5A, Supplemental Figure 1**). Twenty out of 23 myeloid subpopulations were statistically significantly different between the VSCC molecular subtypes. In contrast, re-analysis of the number and composition of detected subpopulations of infiltrating T cells⁷ showed that they did not grossly differ across the molecular subtypes of VSCC (**Figure 2C, Supplemental Table 5B, Supplemental Figure 2, Supplemental Figure 3A**).

Since coordination of immunity has been a well-established prognosticator for survival and response to immunotherapy^{3,6,14}, we analyzed the correlations between the lymphoid and myeloid cell infiltrate. No clear correlations were found between lymphoid and myeloid cell infiltration in all VSCC molecular subtypes, except for a weak but significant correlation between stromal CD3⁺PD1⁺ T cells and epithelial CD14⁺rest⁻ myeloid cells in HPV⁺ VSCC (**Supplemental Figure 4**).

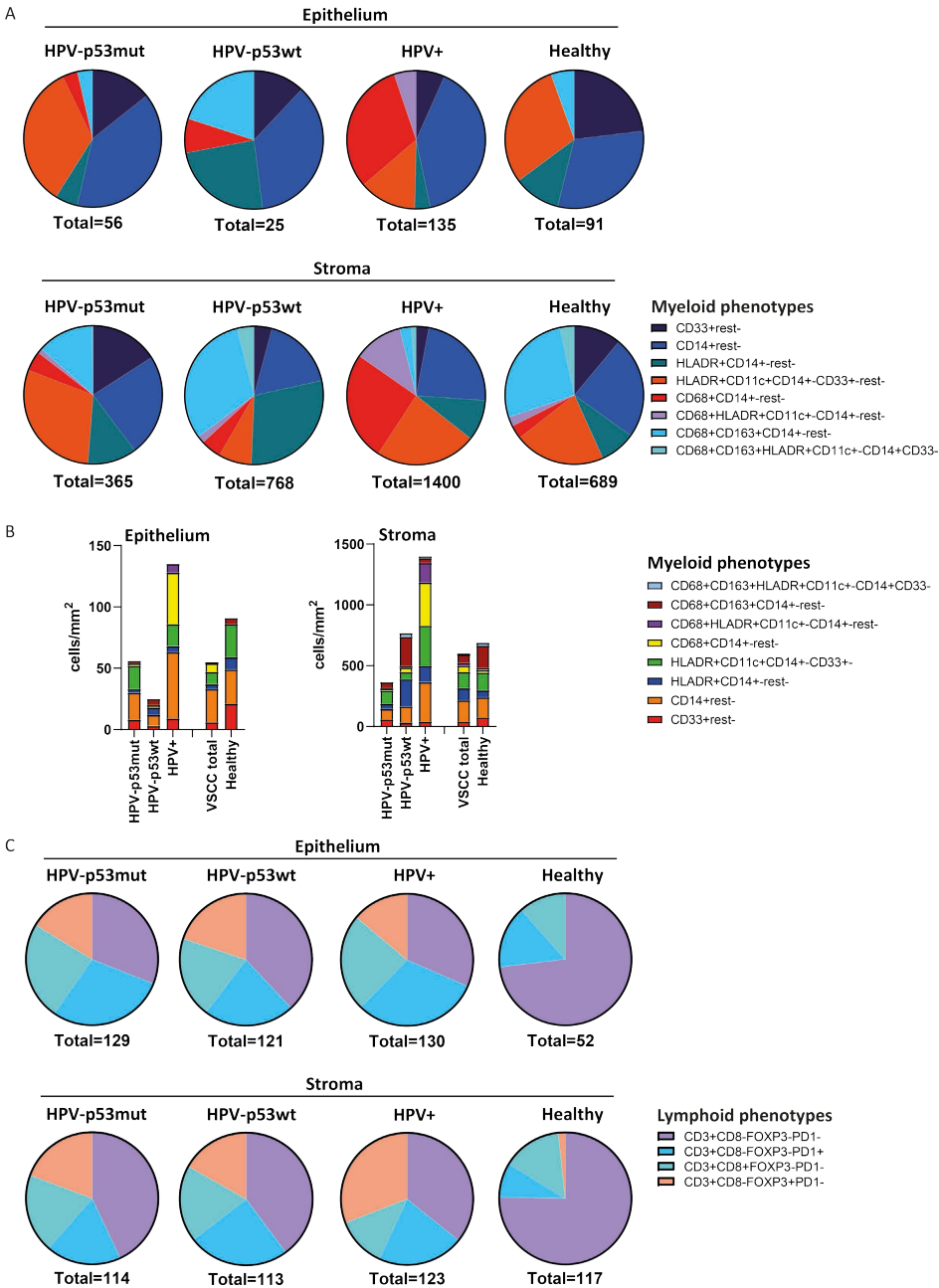


Figure 2. Myeloid and lymphoid immune composition differences per VSCC molecular subtype.

*The myeloid cell composition **A)** fractionally and **B)** quantitatively, as well as **C)** the lymphoid cell composition fractionally in the epithelium and stroma of HPV-p53mut VSCC (n=31), HPV-p53wt VSCC (n=21), HPV+ VSCC (n=25) and healthy HPV+ vulva (n=10). Number below the graphs indicates the median total number of cells/mm². Cell types are indicated by the markers (CD68, CD163, HLADR, CD11c, CD14, CD33) staining positive, markers not stained are indicated as 'Rest'.*

Key myeloid cell phenotypes impacting survival in VSCC

We performed Kaplan Meier survival analyses using the median counts of each myeloid cell phenotype to split the total VSCC cohort into high or low infiltrated. This revealed 3 myeloid cell phenotypes that strongly correlated with survival (**Figure 3A, Supplemental Figure 5**). The strongest separation of the survival curves was observed for epithelial CD14⁺rest⁻ monocytes and stromal CD14⁺CD11c⁺HLADR⁺rest⁻ inflammatory DCs (both p-value = 0.001) (**Figure 3A**). Patients from all three molecular subtypes were represented in these myeloid cell high and low groups (**Figure 3B**). Intriguingly, patients with VSCC in which the function of p53 (in)directly is impaired (HPV⁺ or HPV-p53mut) more often showed high infiltration with epithelial CD14⁺rest⁻ monocytes and stromal CD14⁺CD11c⁺HLADR⁺rest⁻rest⁻ inflammatory DCs, compared to HPV-p53wt VSCC (**Figure 3B**). Functional loss of p53 in cancer cells has previously been linked to enhanced recruitment of myeloid cells to the tumor site.¹⁵⁻¹⁷

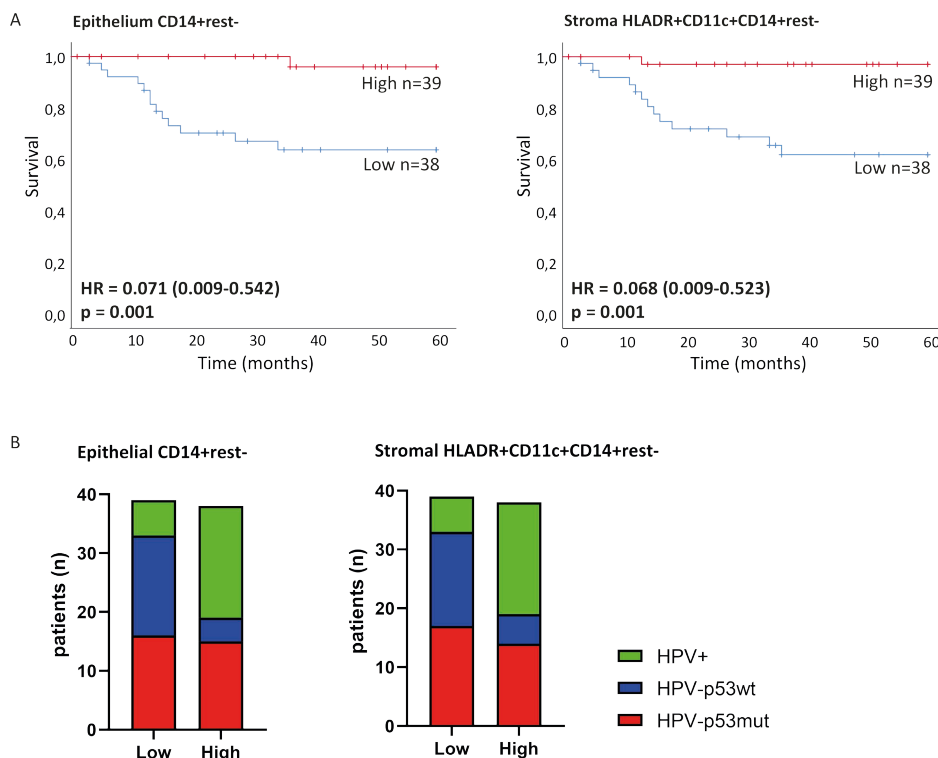


Figure 3. Pro-inflammatory myeloid cell composition predicts excellent survival in VSCC.

A) Kaplan Meier survival curves for VSCC (n=77) with high or low myeloid cell infiltration, including hazard ratio (HR, between brackets the 95% confidence interval of the hazard ratio is provided), and p-value. Total VSCC cohort split by median into high versus low counts. Left survival curve for separation by CD14⁺rest⁻ monocyte infiltration, low infiltration (blue): HPVp53mut n=16, HPVp53wt n=17, HPV⁺ n=6, high infiltration (red): HPVp53mut n=15, HPVp53wt n=4, HPV⁺ n=19. Right survival curve for separation by stromal HLADR⁺CD11c⁺CD14⁺rest⁻ dendritic cell infiltration, low infiltration (blue): HPVp53mut n=17, HPVp53wt n=16, HPV⁺ n=6, high infiltration (red): HPVp53mut n=14, HPVp53wt n=5, HPV⁺ n=19. **B)** Distribution of VSCC molecular subtypes (n=77 total) across low versus high (split by median) epithelial CD14⁺rest⁻ monocyte infiltration (left graph), and stromal HLADR⁺CD11c⁺CD14⁺rest⁻ dendritic cell infiltration (right graph).

Transcriptomic differences in myeloid hot versus cold tumors

In order to gain more insight into what may underly the difference between specific myeloid cell phenotype hot and cold tumors, we combined the transcriptomic data (1258 different individual genes) of a group of 44 VSCC patients, containing both early and late stage patients, and all three molecular subtypes⁸. These patients were divided in two groups based on the median epithelial CD14⁺rest⁻ monocyte cell count or the stromal CD14⁺CD11c⁺HLADR⁺rest⁻ inflammatory DC count, and the differentially expressed genes (DEGs) between the myeloid hot versus cold groups were

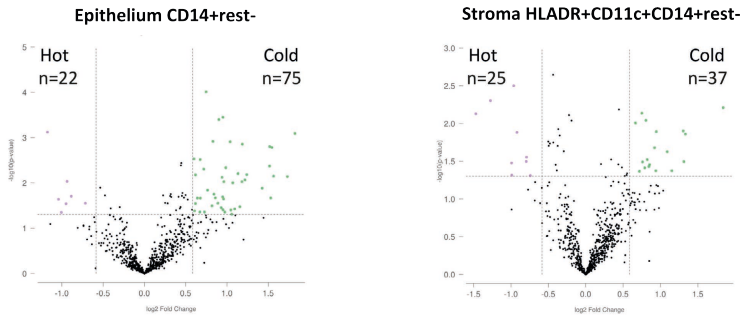
analyzed. This rendered a total of 97 DEGs between the epithelial CD14⁺rest monocyte high versus low group (**Figure 4A, Supplemental Table 6**), and a total of 62 DEGs between the stromal inflammatory DC high versus low group (**Figure 4A, Supplemental Table 7**), using a threshold of fold change difference >1.5 and p-value <0.05. 29 DEGs were present in both comparisons. The great majority of DEGs were higher expressed in myeloid cold tumors (75 and 37, respectively).

In contrast to the stromal region, the epithelial region is unambiguously demarcated and as such, more reproducible to define across studies. Therefore, we focused on the epithelial CD14⁺rest monocytes high/low separation in subsequent analyses. The DEGs between CD14⁺rest monocyte high and low VSCCs comprised a number of cytokines, including increased expression of *CXCL8* and *CCL20*, known to attract monocytes^{18,19}, as well as the inducers of inflammation *IL-1 α* and *IL-1 β* (**Supplemental Table 6**), previously reported to be upregulated in tumors that have lost p53 function¹⁵. The cytokines *IL-34*, *CCL14* and *CCL19* that were overexpressed in the CD14⁺rest monocyte low VSCC (**Supplemental Table 6**) are not known to recruit monocytes. Analysis of the differentially expressed cytokines revealed no overt differences between the VSCC molecular subtypes (**Supplemental Figure 6**).

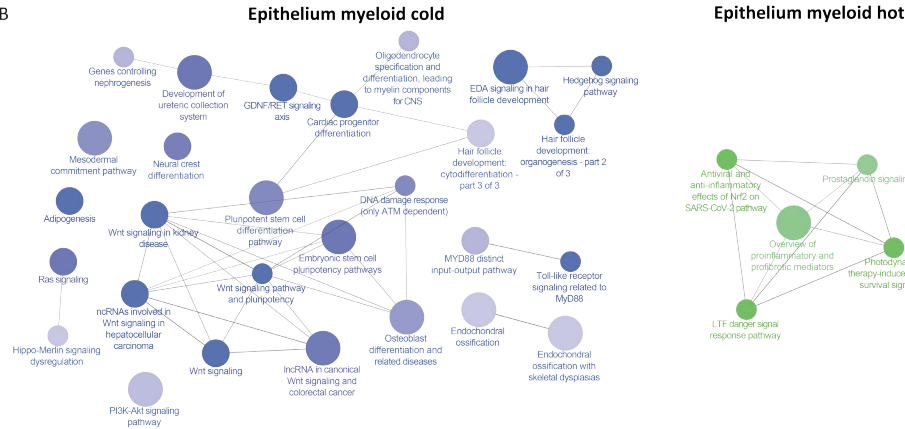
To analyze which underlying signaling pathways were activated in these myeloid hot and cold tumors, ClueGo gene ontology analysis and Hallmark Gene Set Enrichment Analysis (GSEA) was performed. The majority of upregulated genes in CD14⁺rest monocyte cold tumors were involved in organogenesis pathways, including 'pluripotent stem cell differentiation pathway', 'embryonic stem cell pluripotency pathway', 'WNT signaling pathway and pluripotency'. In CD14⁺rest monocyte hot tumors the majority of upregulated genes were involved in pro-inflammatory pathways (**Figure 4B, Supplemental Figure 7**). The GSEA revealed that epithelial monocyte hot VSCCs displayed a gene set upregulated in the response to UV, known to lead to monocyte attraction²⁰. In contrast, epithelial monocyte cold VSCCs were characterized by the upregulation of genes involved in adipogenesis and in the oncogenic KRAS, WNT and TGF β signaling pathways (**Figure 4C**). However, the upregulation of the WNT pathway in monocyte cold VSCC could not be confirmed at the protein level by beta-catenin immunohistochemistry (**Supplemental Figure 8**).

Monocyte infiltration is an independent positive prognostic biomarker in VSCC

A



B



C

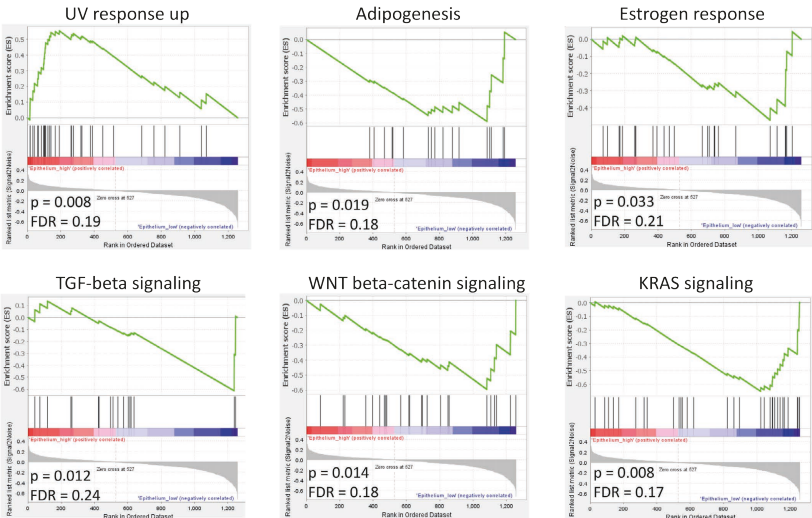


Figure 4. Transcriptomic differences between myeloid hot and cold tumors.

A) Volcano plots of differentially expressed genes (DEGs) between myeloid hot versus cold VSCC lesions (total VSCC n=77, split by median into hot or cold): epithelial CD14⁺rest⁻ monocytes (left graph) and stromal HLADR⁺CD11c⁺CD14⁺rest⁻ dendritic cells (right graph). **B)** ClueGo pathway analysis of identified

DEGs between epithelial CD14⁺rest⁻ monocytes hot versus cold VSCC. **C)** Hallmark gene set enrichment analysis (GSEA), showing gene sets enriched in epithelial CD14⁺rest⁻ hot versus cold VSCC, selected based on FDR<0.25 as well as $p<0.05$.

Myeloid and T cell counts are independent predictors for excellent survival in VSCC

Previously it was described that HPV-p53mut VSCC patients have the poorest survival, HPV-p53wt VSCC patients an intermediate survival, and HPV⁺ VSCC patients an excellent survival², similar to our study cohort (**Supplemental Figure 9**). In addition, epithelial CD3⁺ T cell infiltration was reported to be prognostic in VSCC, irrespective of tumor stage and molecular subtype.^{7,8} As there was no direct correlation between epithelial T cell infiltrate and either identified myeloid cell subpopulation (**Figure 5A**), we explored whether the combination of intraepithelial T cell infiltration with both myeloid cell subpopulations would provide more insight into the prognostic role of either.

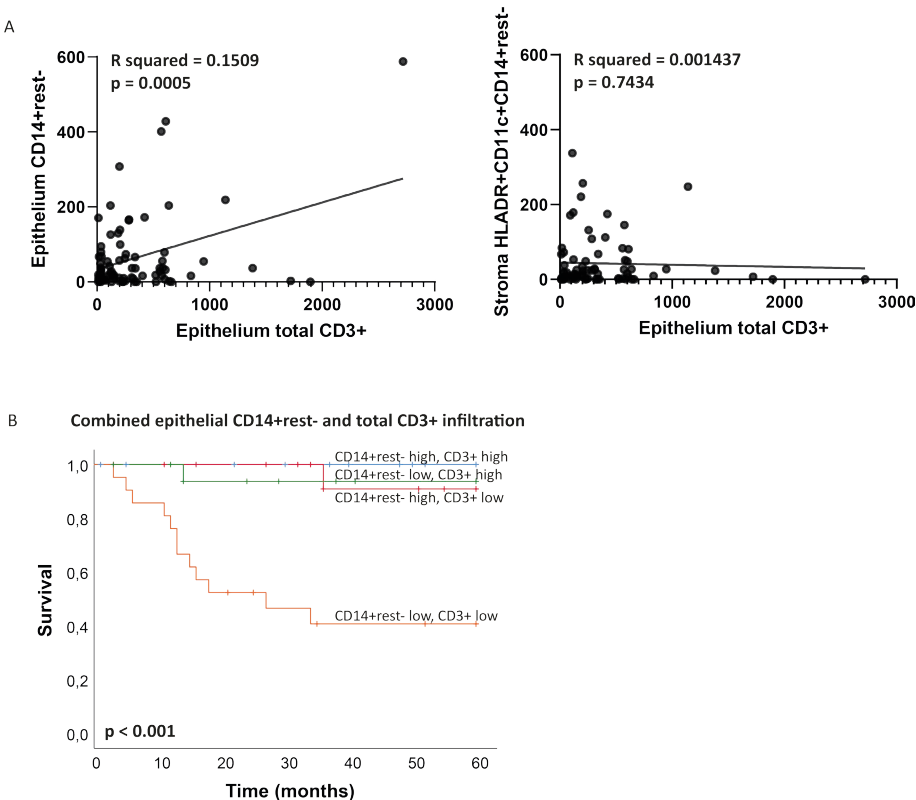


Figure 5. High myeloid cell and T cell infiltration are independent predictors for excellent survival.

A) Pearson correlation plots between myeloid (epithelial CD14⁺rest⁺ or stromal HLADR⁺CD11c⁺CD14⁺rest⁺) and lymphoid cell infiltrate (epithelial total CD3⁺) in VSCC (n=77). **B)** Kaplan Meier survival curves for VSCC patients with differential lymphoid (epithelial total CD3⁺) and myeloid (epithelial CD14⁺rest⁺) cell infiltration (total VSCC n=77, split by respective medians into hot or cold).

Multivariate analyses with tumor stage, molecular subtype, intraepithelial T cell infiltration and either one of the myeloid populations (epithelial monocytes and stromal inflammatory DCs) showed that both myeloid cell types could function as prognostic factors (**Table 1**). However, analysis of all three immune markers simultaneously revealed that both intraepithelial CD3⁺ T cells and the intraepithelial CD14⁺rest⁺ monocytes were the only independent prognostic markers, irrespective of tumor stage and molecular subtype (**Table 1**). An interaction analysis of both latter phenotypes, by splitting the patient group into four based on the median epithelium infiltrating CD3⁺ T cell count (high/low¹⁰¹) and the median epithelium infiltrating CD14⁺rest⁺ monocyte count, revealed that the group of patients with a combined high CD3⁺ T cell and high CD14⁺rest⁺ monocyte infiltration displayed the best survival, similar to the patient groups with a high infiltration of either one of these two immune cell types. The patient group with a low infiltration of both immune cell types displayed poorest survival (**Figure 5B**). This revealed that the presence of a high epithelial CD14⁺rest⁺ monocyte infiltrate is equally important to that of T cells.

Discussion

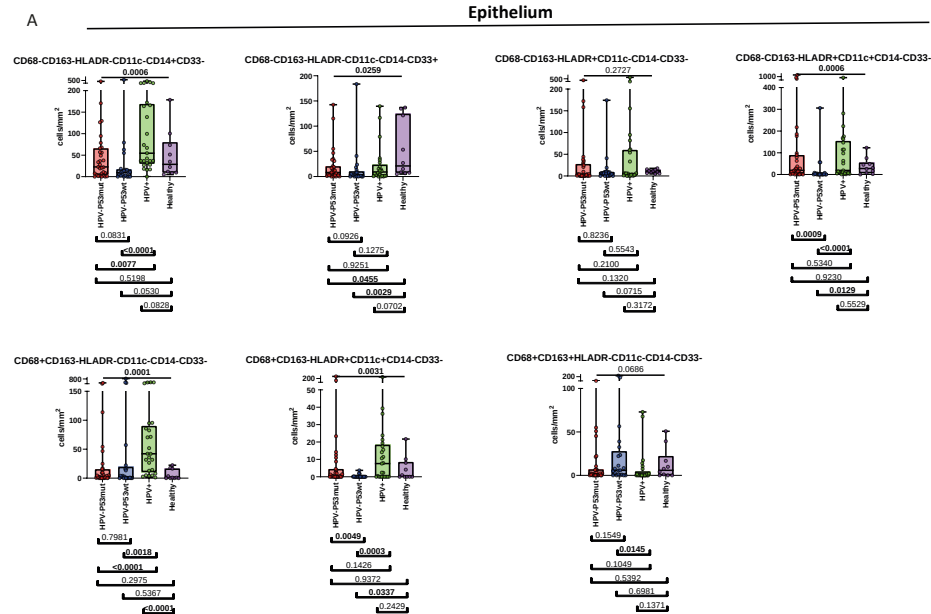
The data from this first in-depth study of myeloid cell infiltration in a large group of VSCCs revealed that the composition of the myeloid cell infiltrate strongly differed per molecular subtype of VSCC, while this is not the case for lymphoid cell infiltrate⁷. Among the sixteen different identified phenotypes, two myeloid cell phenotypes were strongly associated with improved survival: epithelial monocytes and stromal dendritic cells. These myeloid cell subsets were found in higher numbers more often in VSCCs with loss of p53 function, either through interaction with the HPV oncoprotein E6 or via direct TP53 mutation. In comparison to all other known prognostic parameters (tumor stage, molecular subtype, intraepithelial T cell infiltration) only intraepithelial monocytes and T cells were independent prognostic factors in a multivariate analysis. A strong epithelial infiltration with either T cells or monocytes was associated with drastically improved clinical benefit, while low infiltration with both immune cell types was associated with poor survival.

It is well established that molecular features of the tumor may impact the immune composition of the TME.^{15 21-26} While our earlier studies in VSCC revealed no overt impact of the different molecular subtypes on the number and composition of lymphocyte infiltration^{7 9}, our current study suggests that this is different for myeloid cells. There were clear differences with respect to the numbers and phenotypes of infiltrating myeloid cells between molecular subtypes. In particular patients with VSCC not caused by HPV or a TP53 mutation, were less likely to show strong infiltration with intraepithelial monocytes and stromal DCs, immune cell types both shown to be strongly associated with patient survival. Earlier studies have shown that loss of p53 function specifically resulted in the production of cytokines and attraction of innate leukocytes, the specific cytokine and innate cell types depending on the mouse models used^{17 24}, and included monocytes¹⁶. Our data suggest that in VSCC, functional loss of p53 may lead to the enhanced attraction of intraepithelial monocytes and stromal DCs.

At first sight it is surprising to find that the epithelial infiltration of VSCC with monocytes forms such a strong independent prognostic factor. However, monocytes attracted by CCL20 were shown to be essential for efficient cross-priming of T cells after mucosal or skin immunization, and preceded the sequential accumulation of CD11c⁺ MHC class II⁺ DCs in dermis and epithelium¹⁹. While there is evidence that monocytes may present the antigen directly to T cells¹⁹, others have found that they were highly effective in transferring antigens to DCs in lymphoid tissue²⁷, in line with current concepts of antigen cross-priming²⁸. Also in VSCC the increased presence of monocytes is associated with increased expression of CCL20 in tumors, making it a highly likely scenario that in VSCC monocytes may have a similar role. Interestingly, in esophageal cancer high 'tumor monocyte content' was recently found to be a strong predictor for response to immunotherapy and long survival, outperforming tumor lymphocyte content.³³⁰

In conclusion, a dense myeloid and/or lymphoid infiltrate predicts excellent survival in VSCC patients. A hot tumor immune microenvironment generally is associated with better clinical outcome and improved response to different forms of immunotherapy.^{4 30 31} Although many patients with late stage VSCC displayed infiltration by multiple immune cell types, overall this group of patients showed less infiltrating myeloid cells and lymphocytes^{7 9} than patients with early stage VSCC. For the clinical application of immunotherapy, this suggests that treatment should be provided as early as possible. Based on these findings a clinical trial exploring the potential of neoadjuvant checkpoint blockade in primary VSCC has started this year (NCT05761132).

Supplemental Figures

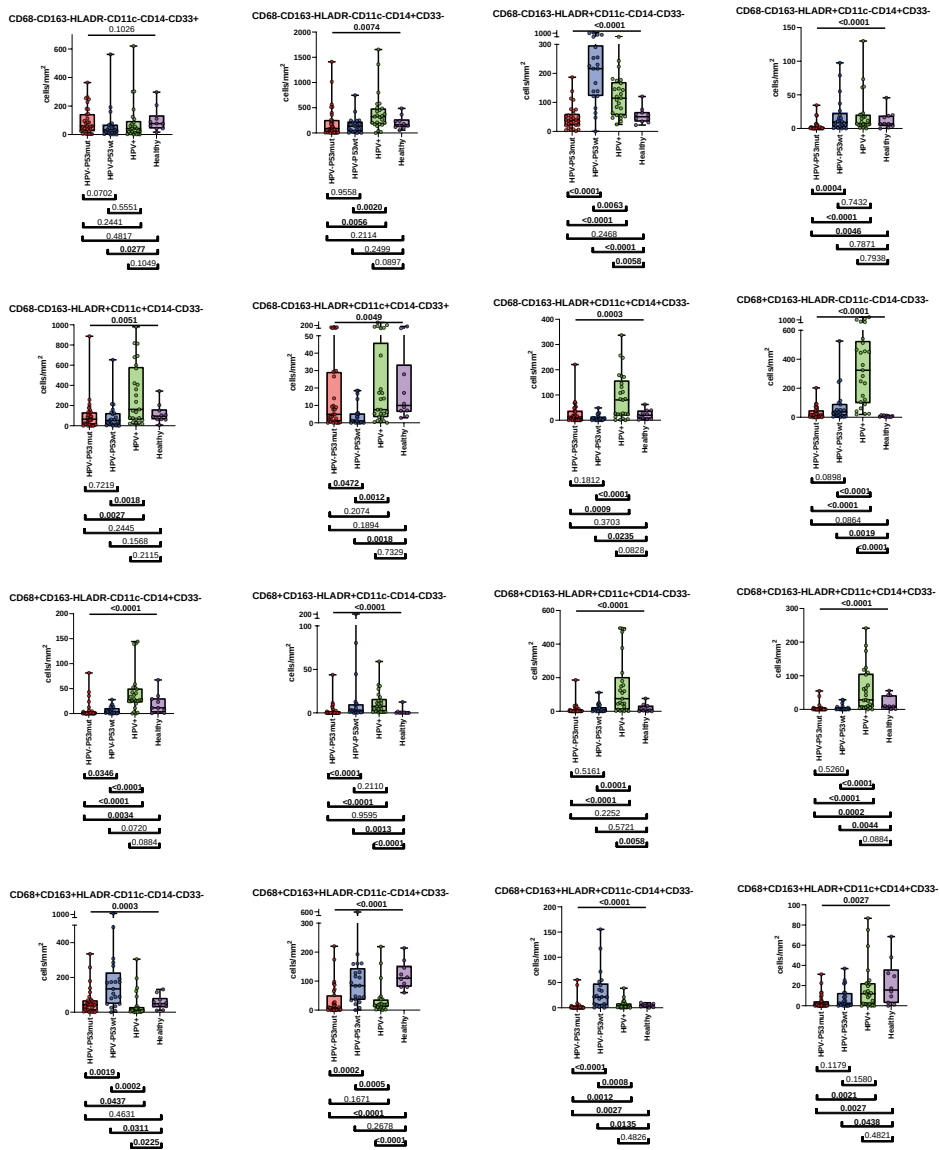


Supplemental Figure 1. Myeloid infiltrate per VSCC molecular subgroup and in healthy vulva.

A) Myeloid infiltrate in the epithelium. HPV-P53mut VSCC n=31, HPV-P53wt VSCC n=21, HPV+ VSCC n=25, healthy HPV- vulva n=10. Boxplots with middle line indicating median, colored box indicating interquartile range, vertical lines from minimum to maximum. Kruskal Wallis test derived p-value depicted on top of the boxplots, indicating a significant difference across the four groups. Mann-Whitney U test derived p-values depicted below the boxplots, indicating a significant difference between two groups.

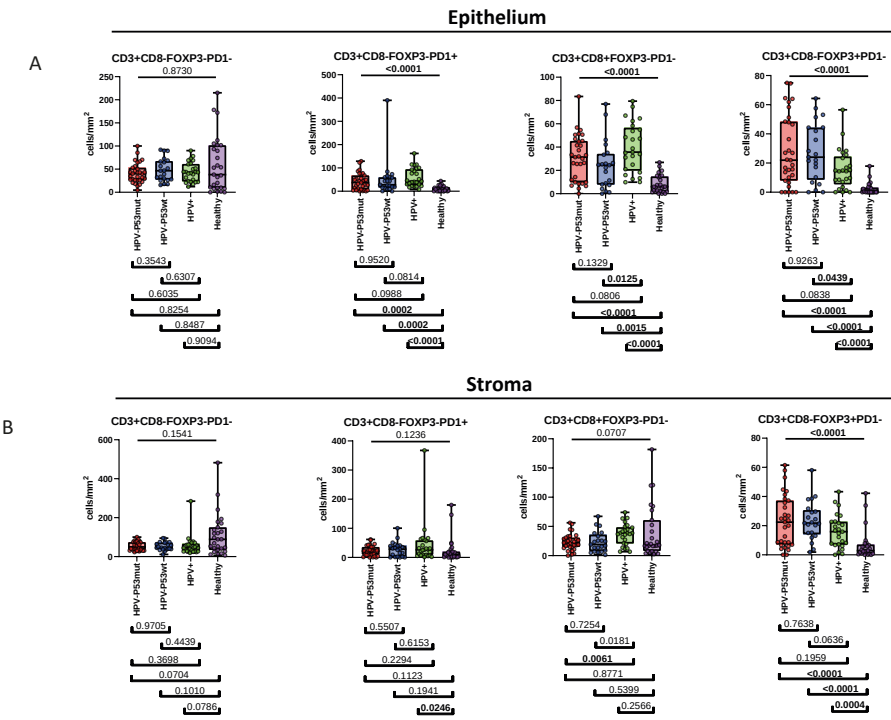
B

Stroma



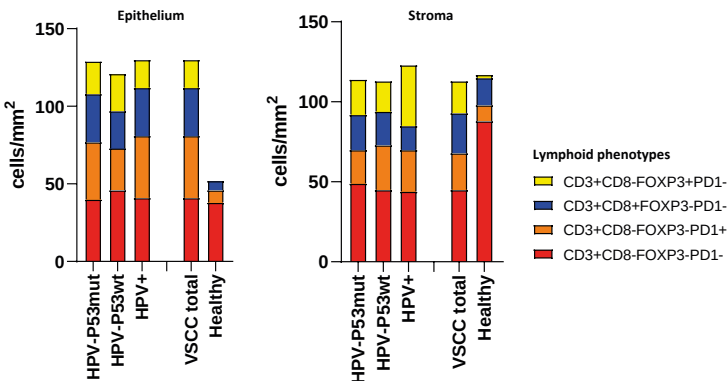
Supplemental Figure 1. Myeloid infiltrate per VSCC molecular subgroup and in healthy vulva.

B) Myeloid infiltrate in the stroma. HPV-P53mut VSCC $n=31$, HPV-P53wt VSCC $n=21$, HPV+ VSCC $n=25$, healthy HPV- vulva $n=10$. Boxplots with middle line indicating median, colored box indicating interquartile range, vertical lines from minimum to maximum. Kruskal Wallis test derived p-value depicted on top of the boxplots, indicating a significant difference across the four groups. Mann-Whitney U test derived p-values depicted below the boxplots, indicating a significant difference between two groups.



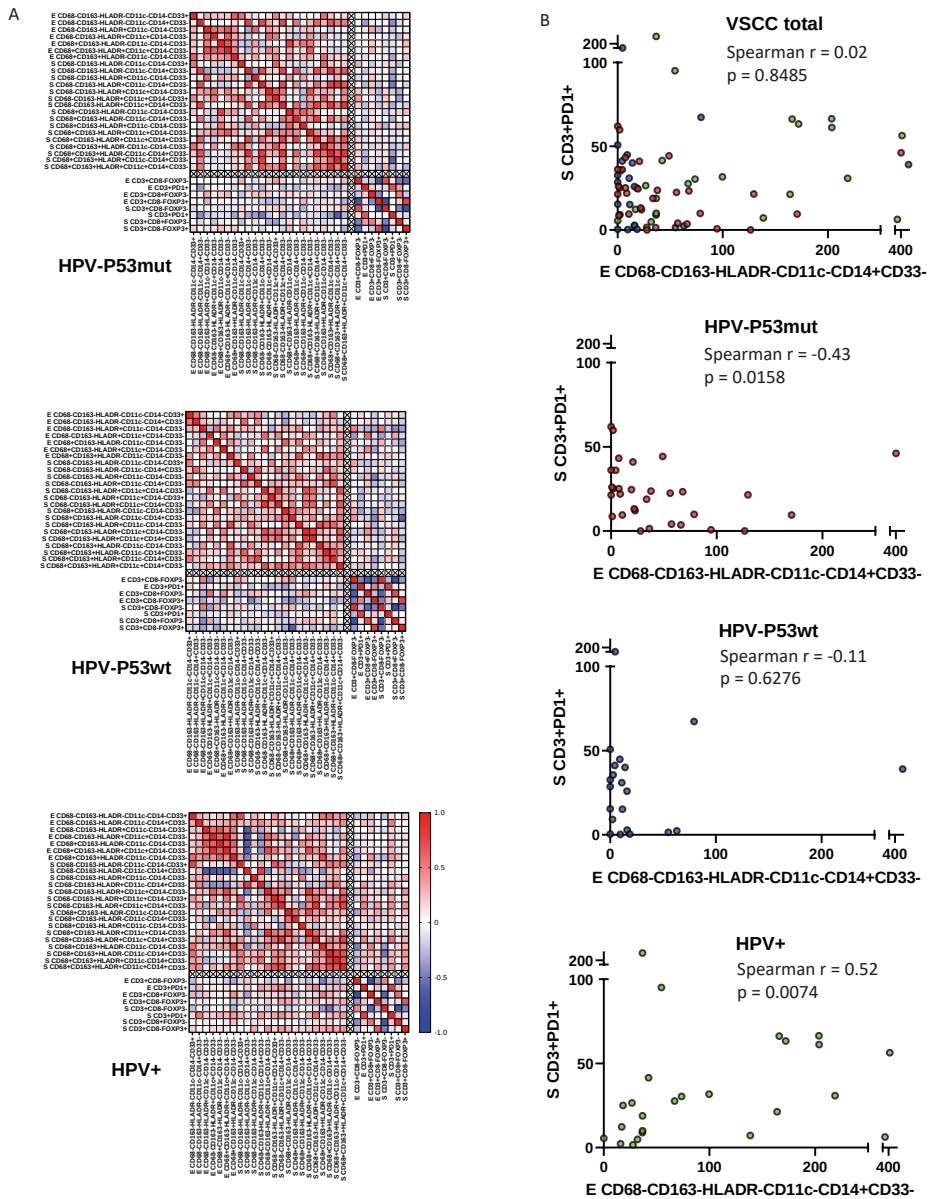
Supplemental Figure 2. Lymphoid infiltrate per VSCC molecular subgroup and in healthy vulva.

A) Lymphoid infiltrate in the epithelium. **B)** lymphoid infiltrate in the stroma. HPV-P53mut VSCC n=31, HPV-P53wt VSCC n=21, HPV+ VSCC n=25, healthy HPV- vulva n=27. Boxplots with middle line indicating median, colored box indicating interquartile range, vertical lines from minimum to maximum. Kruskal Wallis test derived p-value depicted on top of the boxplots, indicating a significant difference across the four groups. Mann-Whitney U test derived p-values depicted below the boxplots, indicating a significant difference between two groups.



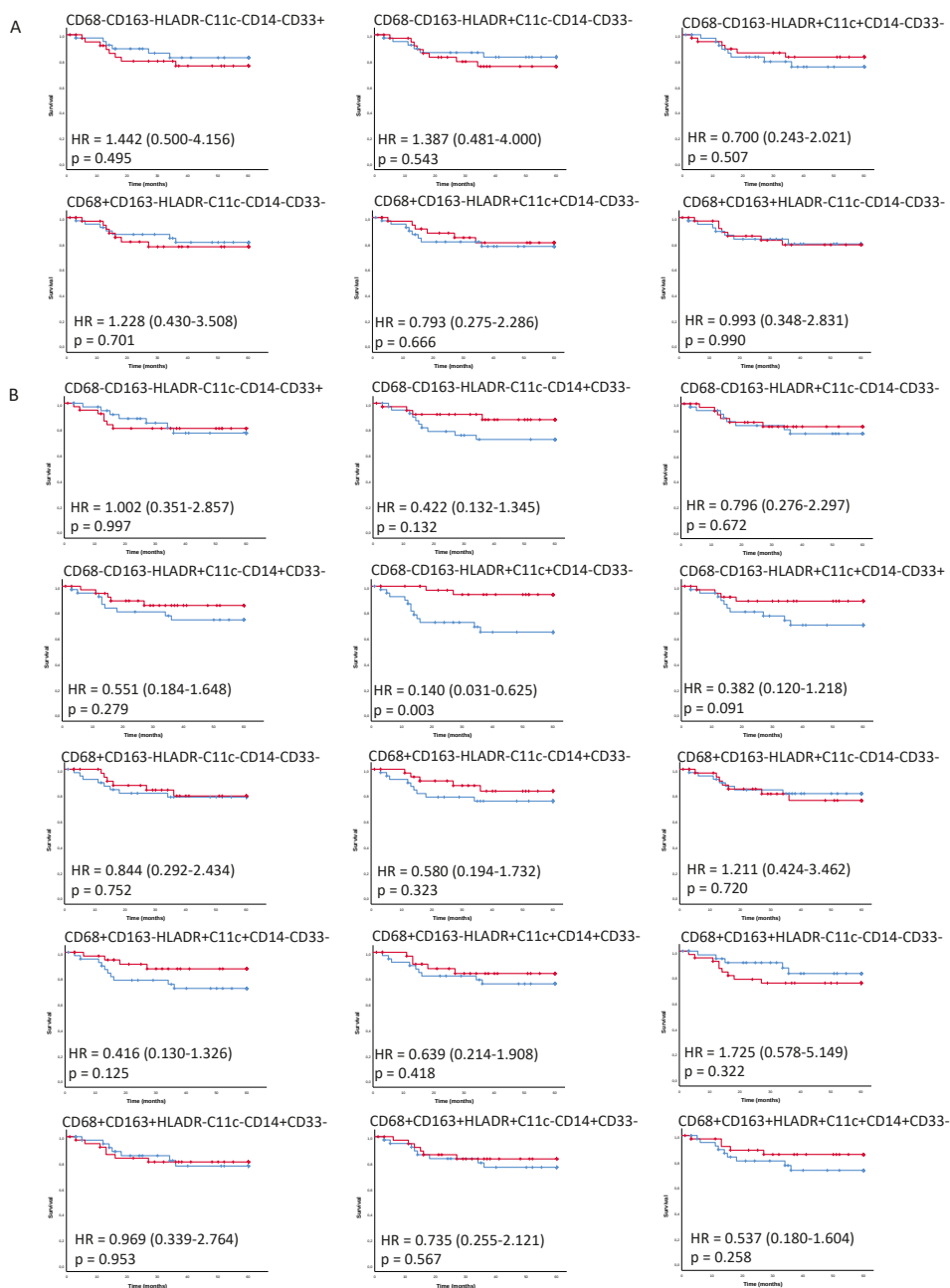
Supplemental Figure 3. No lymphoid immune composition differences across VSCC molecular subgroups.

HPV-P53mut VSCC n=31, HPV-P53wt VSCC n=21, HPV+ VSCC n=25, healthy HPV- vulva n=10.



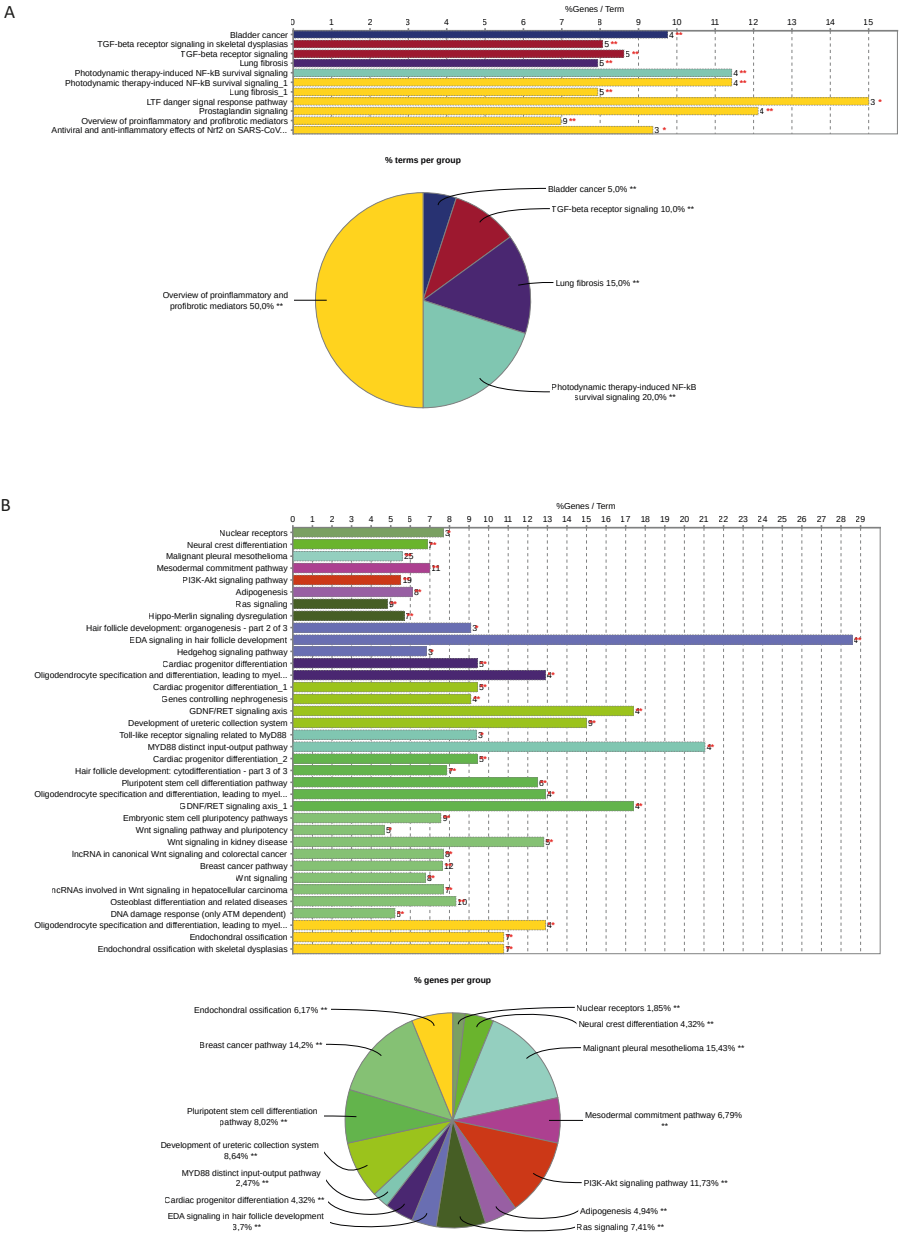
Supplemental Figure 4. Correlation of myeloid and lymphoid immune infiltrate per VSCC molecular subgroup.

A) Spearman correlation heatmap between myeloid and lymphoid immune phenotypes, per VSCC molecular subgroup. HPV-P53mut VSCC $n=31$, HPV-P53wt VSCC $n=21$, HPV+ VSCC $n=25$. **B)** Correlation dot plots between stromal CD3+PD1+ T cells and epithelial CD68-HLADR-CD11c-CD14+CD33- monocytes. Visualized for the total VSCC cohort $n=77$, and per molecular subgroup separately: HPV-P53mut VSCC (red) $n=31$, HPV-P53wt VSCC (blue) $n=21$, HPV+ VSCC (green) $n=25$.



Supplemental Figure 5. Impact of myeloid cell phenotypes presence on survival of VSCC patients.

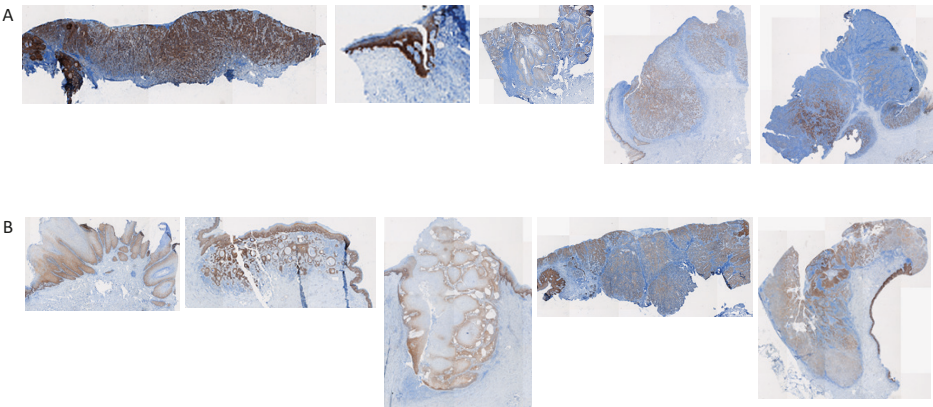
The median counts of each myeloid cell phenotype were used to split the total VSCC cohort (n=77) into high (red) or low (blue) infiltrated. The respective Kaplan Meier curves are depicted in **A**) for the epithelial phenotypes and in **B**) for the stromal phenotypes, including the hazard ratio (HR, between brackets the 95% confidence interval of the hazard ratio is provided) and p-value.



Supplemental Figure 6. Upregulated signaling pathways in monocyte hot and cold VSCC.

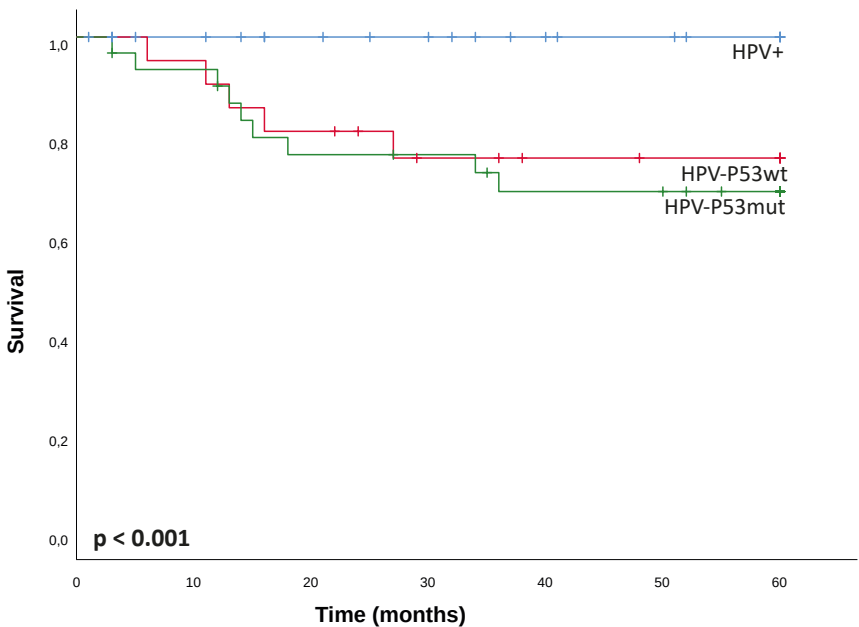
A) Epithelium monocyte hot (n=39) and in B) Epithelium monocyte cold (n=38) VSCC.

Monocyte infiltration is an independent positive prognostic biomarker in VSCC



Supplemental Figure 8. Beta-catenin immunohistochemistry on myeloid hot versus cold VSCC.

10 representative cases of beta-catenin immunohistochemistry in **A**) epithelium myeloid hot (n=5) and **B**) epithelium myeloid cold (n=5) VSCC.



Supplemental Figure 7. Kaplan Meier survival curves for VSCC, split per molecular subgroup.

VSCC total n=77. HPV-P53mut n=31, HPV-P53wt n=21, HPV+ n=25.

Supplemental Tables

Supplemental Table 1. Patient characteristics

	Healthy HPV- vulva	Early stage (FIGO I/II)	Late stage (FIGO III)
Patients (n)	27	65	12
Age (average, min-max)	-	67 (40-87)	73 (56-93)
Molecular subgroups	-		
HPV-p53mut		22	9
HPV-p53wt		20	1
HPV+		23	2
Therapy (n)	-		
Surgery		64	6
Surgery+radiotherapy		1	6

Supplemental Table 2. VSCC cohort analysed by multispectral immunofluorescence myeloid cell analysis and Nanostring transcriptomic analysis.

	Vectra myeloid panel (104)	Nanostring IO360 (44)	Nanostring PanCancer (34)
HPV-p53mut VSCC	31	17	10
HPV-p53wt VSCC	21	10	9
HPV+ VSCC	25	11	9
Healthy HPV- vulva	27	6	6

Supplemental Table 3. Multispectral immunofluorescence myeloid cell panel design.

Marker	Primary antibody clone and producer	Species and isotype	Dilution	Detection method
HLA-DR	TAL1B5 - Abcam	mouse IgG1	1_50	Directly labelled with Alexa 488
CD11c	EP1347Y - Abcam	rabbit IgG	1_100	Directly labelled with Alexa 546
CD68	D4B9C XP - CST	rabbit IgG	1_50	Directly labelled with Alexa 594
CD14	D7A2T - CST	rabbit IgG	1_25	Indirect with CF Donkey-a-Rabbit IgG 633
CD33	PWS44 - Novocastra	mouse IgG2b	1_25	Indirect with Alexa Goat-a- Mouse IgG2b 647
CD163	10D6 - Invitrogen	mouse IgG1	1_10	Indirect with CF Goat-a-Mouse IgG1 680
DAPI	-	-	1_500	Fluorescent probe

Supplemental Table 4. Myeloid cell phenotype counts in VSCC per stage

Full phenotype	VSCC total				Early stage VSCC				Late stage VSCC				Early vs late stage VSCC	Short phenotype	Cell type name
	Median	Min	Max		Median	Min	Max		Median	Min	Max				
Epithelium CD68-CD163-HLADR-CD11c-CD14-CD33+	6.16	0.00	183.61		6.05	0.00	183.61		12.44	0.00	116.69		0.4335	Epithelium CD33+rest-	Immature myeloid cell
Epithelium CD68-CD163-HLADR-CD11c-CD14+CD33-	27.66	0.00	587.97		32.00	0.00	587.97		14.00	0.00	401.28		0.058	Epithelium CD14+rest-	Monocyte
Epithelium CD68-CD163-HLADR+CD11c-CD14-CD33-	4.66	0.00	659.03		4.76	0.00	659.03		2.68	0.00	205.80		0.6117	Epithelium HLADR+rest-	HLADR+ cell
Epithelium CD68-CD163-HLADR+CD11c+CD14-CD33-	10.56	0.00	1289.44		9.87	0.00	1289.44		22.68	0.31	217.46		0.2845	Epithelium HLADR+CD11c+rest-	Dendritic cell
Epithelium CD68+CD163-HLADR-CD11c-CD14-CD33-	7.83	0.00	846.63		7.83	0.00	846.63		6.88	0.00	152.25		0.9529	Epithelium CD68+rest-	Macrophage
Epithelium CD68+CD163-HLADR+CD11c+CD14-CD33-	0.00	0.00	285.62		0.00	0.00	285.62		1.39	0.00	12.62		0.6519	Epithelium CD68+HLADR+CD11c+rest-	M1-like macrophage
Epithelium CD68+CD163+HLADR-CD11c-CD14-CD33-	1.35	0.00	224.67		1.17	0.00	224.67		6.30	0.00	102.55		0.1387	Epithelium CD68+CD163+rest-	M2-like macrophage
Stroma CD68-CD163-HLADR-CD11c-CD14-CD33+	42.30	0.00	620.08		41.72	0.00	620.08		90.18	0.22	300.44		0.1477	Stroma CD33+rest-	Immature myeloid cell
Stroma CD68-CD163-HLADR-CD11c-CD14+CD33-	173.14	0.00	1653.71		190.14	0.00	1653.71		8.46	0.00	1013.98		0.0212	Stroma CD14+rest-	Monocyte
Stroma CD68-CD163-HLADR+CD11c-CD14-CD33-	79.59	0.00	1093.08		109.25	1.15	1093.08		45.06	0.00	113.28		0.0029	Stroma HLADR+rest-	HLADR+ cell
Stroma CD68-CD163-HLADR+CD11c-CD14+CD33-	5.29	0.00	130.08		6.26	0.00	130.08		0.25	0.00	1.22		<0.0001	Stroma HLADR+CD14+rest-	Monocyte

Supplemental Table 4 Continued

Full phenotype	VSCC total			Early stage VSCC			Late stage VSCC			Early vs late stage VSCC	p-value	
	Median	Min	Max	Median	Min	Max	Median	Min	Max		Short phenotype	Cell type name
Stroma CD68-CD163-HLADR+CD11c+CD14-CD33-	75.95	0.00	980.41	104.98	0.00	980.41	14.90	1.22	84.59	0.0025	Stroma HLADR+CD11c+rest-	Dendritic cell
Stroma CD68-CD163-HLADR+CD11c+CD14-CD33+	5.20	0.00	369.37	5.20	0.00	369.37	5.03	0.00	65.71	0.9502	Stroma HLADR+CD11c+CD33+rest-	Immature dendritic cell
Stroma CD68-CD163-HLADR+CD11c+CD14+CD33-	14.04	0.00	337.17	18.45	0.00	337.17	0.11	0.00	26.44	0.0003	Stroma HLADR+CD11c+CD14+rest-	Dendritic cell
Stroma CD68+CD163-HLADR-CD11c-CD14-CD33-	41.92	0.00	1390.00	48.20	0.00	1390.00	30.07	2.70	91.05	0.3061	Stroma CD68+rest-	Macrophage
Stroma CD68+CD163-HLADR-CD11c-CD14+CD33-	3.63	0.00	144.26	5.21	0.00	144.26	0.11	0.00	23.75	0.0033	Stroma CD68+CD14+rest-	Inflammatory macrophage
Stroma CD68+CD163-HLADR+CD11c-CD14-CD33-	1.68	0.00	203.82	1.68	0.00	203.82	1.74	0.00	43.85	0.7848	Stroma CD68+HLADR+rest-	M1-like macrophage
Stroma CD68+CD163-HLADR+CD11c+CD14-CD33-	8.13	0.00	494.83	13.12	0.00	494.83	1.36	0.00	7.58	0.0083	Stroma CD68+HLADR+CD11c+rest-	M1-like macrophage
Stroma CD68+CD163-HLADR+CD11c+CD14+CD33-	1.89	0.00	241.36	4.63	0.00	241.36	0.00	0.00	1.90	0.0004	Stroma CD68+HLADR+CD11c+CD14+rest-	M1-like macrophage
Stroma CD68+CD163+HLADR-CD11c-CD14-CD33-	42.57	0.00	1171.33	37.38	0.00	1171.33	46.35	0.00	257.84	0.7423	Stroma CD68+CD163+rest-	M2-like macrophage
Stroma CD68+CD163+HLADR-CD11c-CD14+CD33-	20.49	0.00	580.58	24.49	0.00	580.58	2.55	0.00	174.41	0.0014	Stroma CD68+CD163+CD14+rest-	M2-like macrophage
Stroma CD68+CD163+HLADR+CD11c-CD14+CD33-	2.09	0.00	155.49	3.13	0.00	155.49	0.14	0.00	1.22	<0.0001	Stroma CD68+CD163+HLADR+CD14+rest-	M2-like macrophage
Stroma CD68+CD163+HLADR+CD11c+CD14+CD33-	2.28	0.00	86.67	4.57	0.00	86.67	0.00	0.00	10.29	0.0001	Stroma CD68+CD163+HLADR+CD11c+CD14+CD33-	M2-like macrophage

Supplemental Table 5. Lymphoid and myeloid cell counts per VSCC molecular subgroup

A. Myeloid cell counts

	HPV-p53mut VSCC			HPV-p53wt VSCC			HPV+ VSCC			Healthy vulva			p-value			Cell type name				
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max	HPV- p53mut vs HPV-p53wt VSCC	HPV- p53wt vs HPV+ VSCC	HPV- p53wt VSCC vs VSCC vs healthy vulva		HPV+ p53wt VSCC vs healthy vulva			
Epithelium CD68-CD163- HLADR-CD11c-CD14-CD33+	8.08	0.00	142.63	3.55	0.00	183.61	9.02	0.00	139.52	21.01	4.97	136.88	0.0926	0.9251	0.1275	0.0455	0.0702	Epithelium CD33+rest-	Immature myeloid cell	
Epithelium CD68-CD163- HLADR-CD11c-CD14-CD33-	22.67	0.00	401.28	9.59	0.00	587.97	54.61	0.00	428.19	28.70	4.97	178.72	0.0831	0.0077	<0.0001	0.5198	0.053	0.0828	Monocyte	
Epithelium CD68-CD163- HLADR-CD11c-CD14-CD33-	3.30	0.00	339.91	6.16	0.00	174.26	5.78	0.00	659.03	10.09	4.78	17.71	0.8236	0.21	0.5543	0.132	0.0715	0.3172	Epithelium HLADR+rest-	HLADR+ cell
Epithelium CD68-CD163- HLADR-CD11c-CD14-CD33-	19.32	0.00	1289.44	0.74	0.00	306.06	18.72	0.98	815.47	27.76	0.00	123.28	0.0009	0.534	<0.0001	0.923	0.0129	0.5529	Epithelium HLADR+CD11c+ rest-	Dendritic cell
Epithelium CD68+CD163- HLADR-CD11c-CD14-CD33-	2.75	0.00	267.33	2.60	0.00	846.63	42.11	1.20	343.11	0.97	0.00	22.46	0.7981	<0.0001	0.0018	0.2975	0.5367	<0.0001	Epithelium CD68+rest-	Macrophage
Epithelium CD68+CD163- HLADR-CD11c-CD14-CD33-	0.57	0.00	285.62	0.00	0.00	3.69	7.63	0.00	260.93	0.48	0.00	21.71	0.0049	0.1426	0.0003	0.9372	0.0337	0.2429	Epithelium CD68+HLADR+ CD11c+rest-	M1-like macrophage
Epithelium CD68+CD163+ HLADR-CD11c-CD14-CD33-	2.00	0.00	102.55	5.88	0.00	224.67	0.00	0.00	73.01	5.85	0.00	50.77	0.1549	0.1049	0.0145	0.5392	0.6981	0.1371	Epithelium CD68+CD163+ rest-	M2-like macrophage
Stroma CD68-CD163- HLADR-CD11c-CD14-CD33+	58.43	3.46	364.29	32.68	0.00	562.04	41.72	0.22	620.08	76.65	16.73	297.47	0.0702	0.2441	0.5551	0.4817	0.0277	0.1049	Stroma CD33+ rest-	Immature myeloid cell
Stroma CD68-CD163- HLADR-CD11c-CD14+CD33-	87.47	0.00	1411.62	133.82	1.03	747.34	324.88	0.00	1653.71	164.19	56.46	488.17	0.9558	0.0056	0.002	0.2114	0.2499	0.0897	Stroma CD14+ rest-	Monocyte
Stroma CD68-CD163- HLADR-CD11c-CD14-CD33-	39.13	1.15	186.94	216.50	0.00	1093.08	114.71	24.29	303.58	50.65	21.88	120.50	<0.0001	<0.0001	0.0063	0.2468	<0.0001	0.0058	Stroma HLADR+ rest-	HLADR+ cell
Stroma CD68-CD163- HLADR-CD11c-CD14+CD33-	0.88	0.00	34.54	9.25	0.00	97.51	7.01	0.00	130.08	6.95	0.00	45.71	0.0004	<0.0001	0.7432	0.0046	0.7871	0.7938	Stroma HLADR+ CD14+rest-	Monocyte
Stroma CD68-CD163- HLADR-CD11c-CD14-CD33-	65.52	0.00	886.84	52.47	0.00	653.51	161.71	16.97	980.41	95.69	6.87	343.71	0.7219	0.0027	0.0018	0.2445	0.1568	0.2115	Stroma HLADR+ CD11c+rest-	Dendritic cell
Stroma CD68-CD163- HLADR-CD11c-CD14-CD33+	4.90	0.00	121.74	0.97	0.00	18.47	7.56	0.00	369.37	9.99	2.86	151.17	0.0472	0.2074	0.0012	0.1894	0.0018	0.7329	Stroma HLADR+ CD11c+CD33+rest-	Immature dendritic cell

B. Lymphoid cell counts

	HPV-p53mut VSCC				HPV-p53wt VSCC				HPV+ VSCC				Healthy vulva				p-value			
	Median		Min		Max		Median		Min		Max		Median		Min		Max		Median	
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min
Epithelium CD3+CD8-FOXP3-	40.07	4.64	92.02	40.38	18.29	100	43.13	12.27	90.12	38.27	0	215.12	0.3543	0.6035	0.6307	0.8254	0.8487	0.9094		
Epithelium CD3+PD1+	43.45	0	390.36	32.05	0	122.92	42.84	7.29	162.33	8.09	0	43.85	0.0926	0.9251	0.1275	0.0455	0.0029	0.0702		
Epithelium CD3+CD8+FOXP3-	22.02	0	74.66	22.32	0	74.99	35.71	9.88	79.45	6	0	26.86	0.8236	0.21	0.5543	0.132	0.0715	0.3172		
Epithelium CD3+CD8-FOXP3+	25.18	0	83.48	30.14	0	54.64	14.37	0	56.5	0.43	0	17.9	0.0009	0.534	<0.0001	0.923	0.0129	0.5529		
Stroma CD3+CD8-FOXP3-	49.66	11.05	94.95	40.67	27.35	100	44.89	19.16	284.75	88.97	6.46	482.3	0.9705	0.3698	0.4439	0.0704	0.101	0.0786		
Stroma CD3+PD1+	21.47	0	100.59	24.62	0	61.95	26.53	1.61	367.48	10.66	0	180.15	0.5507	0.2294	0.6153	0.1123	0.1941	0.0246		
Stroma CD3+CD8+FOXP3-	21.78	1.93	58.02	24.85	0	61.5	38.51	6.82	74.1	17.91	2.58	181.7	0.7254	0.0061	0.0181	0.8771	0.5399	0.2566		
Stroma CD3+CD8-FOXP3+	23.55	1.37	67.17	18.71	0	54.13	15.88	0	43.28	2.91	0	42.2	0.7638	0.1959	0.0636	<0.0001	<0.0001	0.0004		

Supplemental Table 6. DEG list epithelium CD14+rest- low versus high

Gene	Full name	Panel	Upregulated in	Fold change	Log fold change	p-value	p-adjusted
WNT16	wingless-type MMTV integration site family, member 16	PanCancer	epithelium_low	5.02158	2.32814	0.002986	0.187512
RXRG	retinoid X receptor, gamma	PanCancer	epithelium_low	4.74662	2.2469	0.000282	0.086675
PRLR	prolactin receptor	IO360	epithelium_low	3.53672	1.82241	0.000808	0.114686
PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)	IO360	epithelium_low	3.31905	1.73077	0.007248	0.231926
EYA1	EYA transcriptional coactivator and phosphatase 1	PanCancer	epithelium_low	3.11424	1.63888	0.005493	0.187512
NTRK2	neurotrophic tyrosine kinase, receptor, type 2	PanCancer	epithelium_low	2.977	1.57386	0.003532	0.187512
PNOC	prepronociceptin	IO360	epithelium_low	2.95625	1.56377	0.007053	0.231926
MAGEB2	melanoma antigen family B2	IO360	epithelium_low	2.91578	1.54388	0.001649	0.117099
SFRP4	secreted frizzled-related protein 4	IO360	epithelium_low	2.8868	1.52947	0.021551	0.32077
C7	complement component 7	IO360	epithelium_low	2.86066	1.51635	0.001577	0.117099
CCL19	chemokine (C-C motif) ligand 19	IO360	epithelium_low	2.85765	1.51483	0.00423	0.200228
MYB	v-myb avian myeloblastosis viral oncogene homolog	PanCancer	epithelium_low	2.68555	1.42522	0.035954	0.275286
DKK1	dickkopf WNT signaling pathway inhibitor 1	IO360	epithelium_low	2.68522	1.42504	0.013088	0.309756
TSPAN7	tetraspanin 7	PanCancer	epithelium_low	2.56534	1.35915	0.001339	0.168602
GN7	guanine nucleotide binding protein (G protein), gamma 7	PanCancer	epithelium_low	2.56506	1.35899	0.001863	0.168602
CACNA2D3	calcium channel, voltage-dependent, alpha 2/delta subunit 3	PanCancer	epithelium_low	2.46077	1.29911	0.030215	0.25632
WNT3	wingless-type MMTV integration site family, member 3	PanCancer	epithelium_low	2.41955	1.27474	0.00909	0.187512
RELN	reelin	IO360	epithelium_low	2.36089	1.23933	0.00655	0.231926
HOXA11	homeobox A11	PanCancer	epithelium_low	2.34891	1.23199	0.001922	0.168602
LIFR	leukemia inhibitory factor receptor alpha	PanCancer	epithelium_low	2.33202	1.22158	0.000196	0.086675
MAPK10	mitogen-activated protein kinase 10	PanCancer	epithelium_low	2.32805	1.21912	0.01757	0.196143
CD70	CD70 molecule	IO360	epithelium_low	2.32487	1.21715	0.008576	0.246126
CX3CR1	chemokine (CX3-C motif) receptor 1	IO360	epithelium_low	2.27442	1.1855	0.001393	0.117099
FGF13	fibroblast growth factor 13	IO360	epithelium_low	2.26704	1.18081	0.00936	0.246126

Gene	Full name	Panel	Upregulated in	Fold change	Log fold change	p-value	p-adjusted
CD19	CD19 molecule	IO360	epithelium_low	2.2274	1.15536	0.033519	0.397624
CCL14	chemokine (C-C motif) ligand 14	IO360	epithelium_low	2.1925	1.13258	0.006223	0.231926
SOX10	SRY (sex determining region Y)-box 10	IO360	epithelium_low	2.13245	1.09251	0.037547	0.410131
GPC4	glypican 4	IO360	epithelium_low	2.09836	1.06926	0.009957	0.25248
ESR1	estrogen receptor 1	IO360	epithelium_low	2.08012	1.05667	0.049051	0.447525
CLEC4E	C-type lectin domain family 4, member E	IO360	epithelium_low	2.06521	1.04629	0.039517	0.410661
IL34	interleukin 34	IO360	epithelium_low	2.05396	1.03841	0.001227	0.117099
AR	androgen receptor	PanCancer	epithelium_low	2.05294	1.03769	0.022308	0.225853
BMP6	bone morphogenetic protein 6	PanCancer	epithelium_low	2.03489	1.02495	0.008948	0.187512
IGF1	insulin-like growth factor 1 (somatomedin C)	PanCancer	epithelium_low	1.98393	0.988358	0.028215	0.254764
TNFSF8	tumor necrosis factor (ligand) superfamily, member 8	IO360	epithelium_low	1.97829	0.984252	0.004608	0.204497
SPIB	Spi-B transcription factor (Spi-1/PU.1 related)	IO360	epithelium_low	1.96382	0.973666	0.043945	0.426813
RASGRP2	RAS guanyl releasing protein 2 (calcium and DAG-regulated)	PanCancer	epithelium_low	1.94941	0.963035	0.0117	0.195634
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	IO360	epithelium_low	1.94787	0.9619	0.009347	0.246126
DTX1	deltex 1, E3 ubiquitin ligase	PanCancer	epithelium_low	1.94582	0.960377	0.017233	0.196143
GATA3	GATA binding protein 3	PanCancer	epithelium_low	1.94306	0.958328	0.024358	0.23009
NLRP3	NLR family, pyrin domain containing 3	IO360	epithelium_low	1.94305	0.95832	0.022571	0.326021
PDGFD	platelet derived growth factor D	PanCancer	epithelium_low	1.94122	0.956967	0.012457	0.196126
CD45RA		IO360	epithelium_low	1.93371	0.951371	0.039196	0.410661
LILRA1	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 1	IO360	epithelium_low	1.93152	0.94974	0.000355	0.094293
TLR9	tol-l-like receptor 9	IO360	epithelium_low	1.9281	0.947177	0.020351	0.32077
GLI1	GLI family zinc finger 1	IO360	epithelium_low	1.91837	0.939879	0.007513	0.231926
WNT2B	wingless-type MMTV integration site family, member 2B	IO360	epithelium_low	1.90456	0.929457	0.035289	0.40411
PGPEP1	pyroglutamyl-peptidase I	IO360	epithelium_low	1.86752	0.90112	0.000398	0.094293
PBX1	pre-B-cell leukemia homeobox 1	PanCancer	epithelium_low	1.86136	0.896356	0.008956	0.187512

Supplemental Table 6 Continued

Gene	Full name	Panel	Upregulated in	Fold change	Log fold change	p-value	p-adjusted
CD27	CD27 molecule	IO360	epithelium_low	1.85276	0.889677	0.027904	0.360444
TNFRSF10C	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	IO360	epithelium_low	1.81427	0.859392	0.021437	0.32077
BCL2	B-cell CLL/lymphoma 2	IO360	epithelium_low	1.7926	0.84205	0.017753	0.32077
BMP7	bone morphogenetic protein 7	PanCancer	epithelium_low	1.7804	0.832204	0.029409	0.25632
FGFR1	fibroblast growth factor receptor 1	IO360	epithelium_low	1.7776	0.829932	0.001207	0.117099
ITGA8	integrin, alpha 8	PanCancer	epithelium_low	1.7633	0.818275	0.008345	0.187512
HLA-DQB	major histocompatibility complex, class II, DQ beta	IO360	epithelium_low	1.76238	0.817529	0.032085	0.392761
DUSP8	dual specificity phosphatase 8	PanCancer	epithelium_low	1.73225	0.792647	0.001471	0.168602
PTCH1	patched 1	PanCancer	epithelium_low	1.71463	0.777899	0.005152	0.187512
PLCE1	phospholipase C, epsilon 1	PanCancer	epithelium_low	1.70623	0.770814	0.014797	0.196143
TWIST1	twist family bHLH transcription factor 1	IO360	epithelium_low	1.69899	0.764677	0.014475	0.32077
IL11RA	interleukin 11 receptor, alpha	IO360	epithelium_low	1.67842	0.747103	9.74E-05	0.069174
CXCR2	chemokine (C-X-C motif) receptor 2	IO360	epithelium_low	1.6553	0.727095	0.043767	0.426813
BMP4	bone morphogenetic protein 4	PanCancer	epithelium_low	1.65359	0.725604	0.015807	0.196143
FZD8	frizzled class receptor 8	IO360	epithelium_low	1.64641	0.719323	0.004969	0.207544
INHBB	inhibin, beta B	PanCancer	epithelium_low	1.62932	0.704271	0.041457	0.291147
ANGPT1	angiotensinogen 1	IO360	epithelium_low	1.59881	0.676996	0.021686	0.32077
GMIP	GEM interacting protein	IO360	epithelium_low	1.59689	0.675266	0.003052	0.180592
TNFRSF18	tumor necrosis factor receptor superfamily, member 18	IO360	epithelium_low	1.59218	0.671002	0.043784	0.426813
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	PanCancer	epithelium_low	1.58235	0.662067	0.043329	0.291147
TLR7	toll-like receptor 7	IO360	epithelium_low	1.55965	0.641225	0.021642	0.32077
SOX17	SOX (sex determining region Y)-box 17	PanCancer	epithelium_low	1.54548	0.628055	0.037764	0.275286
TLR5	toll-like receptor 5	IO360	epithelium_low	1.53542	0.618629	0.00665	0.231926
ITGAM	integrin, alpha M (complement component 3 receptor 3 subunit)	IO360	epithelium_low	1.53516	0.618388	0.028609	0.360444
FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32)	IO360	epithelium_low	1.52322	0.607128	0.041387	0.41978

Gene	Full name	Panel	Upregulated in	Fold change	Log fold change	p-value	p-adjusted
DTX4	deltex 4, E3 ubiquitin ligase	IO360	epithelium_low	1.5161	0.600369	0.002935	0.180592
LTPB1	latent transforming growth factor beta binding protein 1	PanCancer	epithelium_high	-1.5031	-0.587941	0.044903	0.293304
CBLC	Cbl proto-oncogene C, E3 ubiquitin protein ligase	PanCancer	epithelium_high	-1.54841	-0.630788	0.010018	0.187512
THBS1	thrombospondin 1	IO360	epithelium_high	-1.64234	-0.715756	0.027971	0.360444
ITGA3	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	PanCancer	epithelium_high	-1.67056	-0.74033	0.010078	0.187512
DLL1	delta-like 1 (Drosophila)	PanCancer	epithelium_high	-1.67788	-0.746641	0.003482	0.187512
HMGA2	high mobility group AT-hook 2	PanCancer	epithelium_high	-1.71497	-0.778185	0.040439	0.288715
HEY1	hes-related family bHLH transcription factor with YRPW motif 1	IO360	epithelium_high	-1.84831	-0.88621	0.019721	0.32077
IL1B	interleukin 1, beta	PanCancer	epithelium_high	-1.88886	-0.917519	0.011787	0.195634
IL1R2	interleukin 1 receptor, type II	IO360	epithelium_high	-1.91402	-0.936605	0.009227	0.246126
CCL20	chemokine (C-C motif) ligand 20	IO360	epithelium_high	-1.93099	-0.94934	0.028937	0.360444
CDKN2A	cyclin-dependent kinase inhibitor 2A	IO360	epithelium_high	-2.00863	-1.00621	0.044485	0.426813
EGFR	epidermal growth factor receptor	IO360	epithelium_high	-2.05695	-1.04051	0.022959	0.326021
LAMA3	laminin, alpha 3	PanCancer	epithelium_high	-2.12408	-1.08684	0.017454	0.196143
CXCL8	chemokine (C-X-C motif) ligand 8	PanCancer	epithelium_high	-2.24165	-1.16456	0.013784	0.196143
ATF3	activating transcription factor 3	IO360	epithelium_high	-2.25702	-1.17442	0.000754	0.114686
IL1A	interleukin 1, alpha	PanCancer	epithelium_high	-2.36712	-1.24313	0.013596	0.196143
SPP1	secreted phosphoprotein 1	PanCancer	epithelium_high	-2.42351	-1.2771	0.036716	0.275286
FST	folistatin	PanCancer	epithelium_high	-2.54741	-1.34903	0.006988	0.187512
PLAT	plasminogen activator, tissue	PanCancer	epithelium_high	-2.79442	-1.48255	0.002283	0.175205
MMP3	matrix metalloproteinase 3	PanCancer	epithelium_high	-2.97995	-1.57529	0.015173	0.196143
IL19	interleukin 19	PanCancer	epithelium_high	-3.05106	-1.60931	0.011831	0.195634
COL11A1	collagen, type XI, alpha 1	PanCancer	epithelium_high	-3.07056	-1.6185	0.006294	0.187512

Supplemental Table 7. DEG list stroma CD11c+CD14+HLADR+ DC low versus high

Gene	Full name	Panel	Upregulated in	Fold change	Log fold change	p-value	p-adjusted
MMP7	matrix metalloproteinase 7	IO360	stroma_low	3.57759	1.83899	0.006168	0.600719
PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)	PanCancer	stroma_low	3.34884	1.74366	0.02539	0.358192
WNT16	wingless-type MMTV integration site family, member 16	PanCancer	stroma_low	3.14858	1.6547	0.040496	0.408286
NTRK2	neurotrophic tyrosine kinase, receptor, type 2	PanCancer	stroma_low	2.76701	1.46833	0.00779	0.301901
THBS4	thrombospondin 4	PanCancer	stroma_low	2.60774	1.3828	0.043355	0.415933
CD19	CD19 molecule	IO360	stroma_low	2.51996	1.3334	0.013796	0.600719
MAPK10	mitogen-activated protein kinase 10	PanCancer	stroma_low	2.49267	1.31769	0.011159	0.301901
SPP1	secreted phosphoprotein 1	IO360	stroma_low	2.48485	1.31316	0.032011	0.6933
VTCN1	V-set domain containing T cell activation inhibitor 1	IO360	stroma_low	2.46969	1.30433	0.012562	0.600719
TSPAN7	tetraspanin 7	PanCancer	stroma_low	2.45649	1.2966	0.002776	0.298128
KIAA0125	KIAA0125	IO360	stroma_low	2.22003	1.15058	0.042317	0.710285
PPP2R2B	protein phosphatase 2, regulatory subunit B, beta	PanCancer	stroma_low	2.13149	1.09186	0.037946	0.408286
SPIB	Spi-B transcription factor (Spi-1/PU.1 related)	IO360	stroma_low	2.12818	1.08962	0.023691	0.672819
BMP6	bone morphogenetic protein 6	PanCancer	stroma_low	2.03799	1.02715	0.010117	0.301901
GNF7	guanine nucleotide binding protein (G protein), gamma 7	PanCancer	stroma_low	2.02677	1.01918	0.025321	0.358192
RASGRP2	RAS guanyl releasing protein 2 (calcium and DAG-regulated)	PanCancer	stroma_low	1.92994	0.948555	0.014962	0.316773
HEY1	hes-related family bHLH transcription factor with YRPW motif 1	IO360	stroma_low	1.92359	0.943805	0.012766	0.600719
CD45RA		IO360	stroma_low	1.91453	0.936987	0.042347	0.710285
COL4A4	collagen, type IV, alpha 4	PanCancer	stroma_low	1.89996	0.925967	0.041919	0.408548
CD3E	CD3e molecule, epsilon (CD3-TCR complex)	IO360	stroma_low	1.89295	0.920637	0.020958	0.646967
ITGA8	integrin, alpha 8	PanCancer	stroma_low	1.81354	0.858811	0.00627	0.301901
XCL1/2		IO360	stroma_low	1.80668	0.853343	0.034999	0.6933
CD27	CD27 molecule	IO360	stroma_low	1.79559	0.844459	0.037232	0.702875
CCND1	cyclin D1	PanCancer	stroma_low	1.78016	0.832007	0.048953	0.429809

Gene	Full name	Panel	Upregulated in	Fold change	Log fold change	p-value	p-adjusted
CX3CR1	chemokine (C-X3-C motif) receptor 1	IO360	stroma_low	1.76914	0.823046	0.030131	0.6933
ACVR1C	activin A receptor, type IC	IO360	stroma_low	1.74801	0.80571	0.009069	0.600719
HLA-DOB	major histocompatibility complex, class II, DO beta	IO360	stroma_low	1.72889	0.789844	0.038609	0.702875
CD2	CD2 molecule	IO360	stroma_low	1.69351	0.760015	0.032307	0.6933
GPR160	G protein-coupled receptor 160	IO360	stroma_low	1.683	0.751031	0.007286	0.600719
PLCG2	phospholipase C, gamma 2 (phosphatidylinositol-specific)	PanCancer	stroma_low	1.66029	0.731432	0.021809	0.347309
IL11	interleukin 11	IO360	stroma_low	1.6441	0.717302	0.043017	0.710285
MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	PanCancer	stroma_low	1.62676	0.702003	0.010627	0.301901
ANGPT1	angiotensinogen 1	PanCancer	stroma_low	1.62663	0.701889	0.026252	0.358192
CSF1R	colony stimulating factor 1 receptor	PanCancer	stroma_low	1.61971	0.695736	0.041228	0.408286
FZD8	frizzled class receptor 8	IO360	stroma_low	1.58569	0.665115	0.009855	0.600719
ATM	ATM serine/threonine kinase	PanCancer	stroma_low	1.58538	0.664832	0.00877	0.301901
FUT8	fucosyltransferase 8 (alpha (1,6) fucosyltransferase)	PanCancer	stroma_low	1.55277	0.634845	0.040619	0.408286
EPHA2	EPH receptor A2	PanCancer	stroma_high	-1.50484	-0.589607	0.028638	0.374125
WEE1	WEE1 G2 checkpoint kinase	PanCancer	stroma_high	-1.53782	-0.620886	0.020026	0.347309
CBLC	Cbl proto-oncogene C, E3 ubiquitin protein ligase	PanCancer	stroma_high	-1.53998	-0.622911	0.013276	0.301901
POLD1	polymerase (DNA directed), delta 1, catalytic subunit	PanCancer	stroma_high	-1.55377	-0.635769	0.000623	0.293842
MGMT	O-6-methylguanine-DNA methyltransferase	PanCancer	stroma_high	-1.57061	-0.651326	0.00468	0.301901
POLE2	polymerase (DNA directed), epsilon 2, accessory subunit	PanCancer	stroma_high	-1.62282	-0.698502	0.007173	0.301901
ST100A9	ST100 calcium binding protein A9	IO360	stroma_high	-1.67235	-0.741878	0.048933	0.731821
RORC	RAR-related orphan receptor C	IO360	stroma_high	-1.73198	-0.792424	0.027904	0.6933
GNG4	guanine nucleotide binding protein (G protein), gamma 4	IO360	stroma_high	-1.73616	-0.795897	0.031855	0.6933
STMN1	stathmin 1	PanCancer	stroma_high	-1.73656	-0.796236	0.022402	0.347309
CDC25C	cell division cycle 25C	PanCancer	stroma_high	-1.76092	-0.816328	0.00305	0.298128
CCNE2	cyclin E2	PanCancer	stroma_high	-1.85641	-0.892515	0.003884	0.298128
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	IO360	stroma_high	-1.89242	-0.920229	0.013107	0.600719

Supplemental Table 7 Continued

Gene	Full name	Panel	Upregulated in	Fold change	Log fold change	p-value	p-adjusted
E2F1	E2F transcription factor 1	PanCancer	stroma_high	-1.912	-0.995084	0.002847	0.298128
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	IO360	stroma_high	-1.95001	-0.963484	0.003156	0.600719
CDKN2A	cyclin-dependent kinase inhibitor 2A	IO360	stroma_high	-1.9848	-0.988995	0.048343	0.731821
CD70	CD70 molecule	IO360	stroma_high	-1.98893	-0.991991	0.033433	0.6933
PLAT	plasminogen activator, tissue	PanCancer	stroma_high	-2.40789	-1.26777	0.01893	0.301901
EGFR	epidermal growth factor receptor	IO360	stroma_high	-2.41922	-1.27454	0.004965	0.600719
GPC4	glypican 4	PanCancer	stroma_high	-2.47737	-1.30881	0.021889	0.347309
IL23A	interleukin 23, alpha subunit p19	PanCancer	stroma_high	-2.6175	-1.38819	0.008496	0.301901
IL19	interleukin 19	PanCancer	stroma_high	-2.61777	-1.38834	0.035111	0.401402
CNTFR	ciliary neurotrophic factor receptor	PanCancer	stroma_high	-2.73642	-1.45229	0.033622	0.401402
PRLR	prolactin receptor	IO360	stroma_high	-2.76995	-1.46986	0.007426	0.600719
MYB	v-myb avian myeloblastosis viral oncogene homolog	PanCancer	stroma_high	-3.02551	-1.59718	0.02188	0.347309

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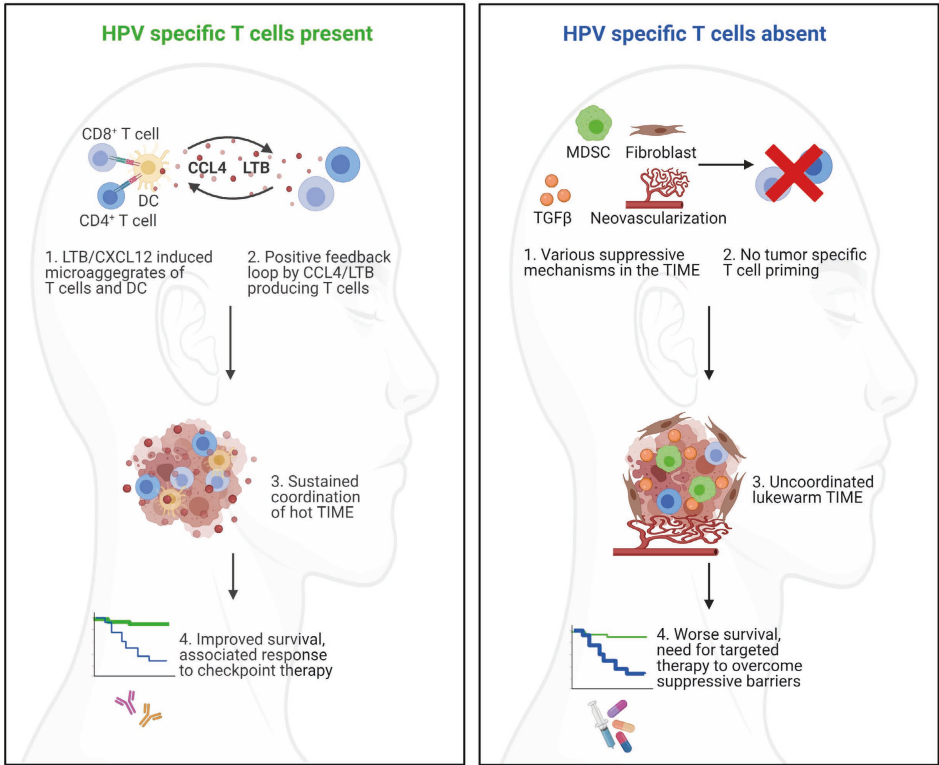
Chapter 8

Tumor-specific T cells support chemokine-driven spatial organization of intratumoral immune microaggregates needed for long survival

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Journal for ImmunoTherapy of Cancer 2022

Graphical abstract



Key findings

- A specific spatial phenotypic signature (SPS) portrays long surviving oropharyngeal cancer patients.
- The SPS consists of intratumoral T cell and dendritic cell microaggregates.
- Chemokines produced by tumor-specific T cells sustain the orchestration of the SPS.
- Patients lacking this SPS express multiple targetable immunosuppressive mechanisms.

Abstract

Background

The composition of the tumor immune microenvironment (TIME) associated with good prognosis generally also predicts the success of immunotherapy, and both entail the presence of pre-existing tumor-specific T cells. Here, the blueprint of the TIME associated with such an ongoing tumor-specific T cell response was dissected in a unique prospective oropharyngeal squamous cell carcinoma (OPSCC) cohort, in which tumor-specific tumor-infiltrating T cells were detected (IR⁺) or not (IR⁻).

Methods

A comprehensive multi-modal, high-dimensional strategy was applied to dissect the TIME of treatment naive IR⁺ and IR⁻ OPSCC tissue, including bulk RNA sequencing by NanoString, imaging mass cytometry (Hyperion) for phenotyping and spatial interaction analyses of immune cells, and combined single-cell gene expression profiling and T cell receptor sequencing (scRNAseq) to characterize the transcriptional states of clonally expanded tumor-infiltrating T cells.

Results

IR⁺ patients had an excellent survival during >10 years follow-up. The tumors of IR⁺ patients expressed higher levels of genes strongly related to IFN γ signaling, T-cell activation, T-cell receptor signaling, and mononuclear cell differentiation as well as genes involved in several immune signaling pathways, than IR⁻ patients. The top differently overexpressed genes included CXCL12 and LTB, involved in ectopic lymphoid structure development. Moreover, scRNAseq not only revealed that CD4⁺ T cells were the main producers of LTB, but also identified a subset of clonally expanded CD8⁺ T cells, dominantly present in IR⁺ tumors, which secreted the T cell and dendritic cell (DC) attracting chemokine CCL4. Indeed, immune cell infiltration in IR⁺ tumors is stronger, highly coordinated and has a distinct spatial phenotypic signature, characterized by intratumoral microaggregates of CD8⁺CD103⁺ and CD4⁺ T cells with DCs. In contrast, the IR⁻ TIME comprised spatial interactions between lymphocytes and various immunosuppressive myeloid cell populations. The impact of these chemokines on local immunity and clinical outcome was confirmed in an independent The Cancer Genome Atlas OPSCC cohort.

Conclusion

The production of lymphoid cell attracting and organizing chemokines by tumor-specific T cells in IR⁺ tumors constitutes a positive feedback loop to sustain the formation of the DC-T-cell microaggregates and identifies patients with excellent survival after standard therapy.

Introduction

Head and neck cancer is the sixth most prevalent cancer type and predominantly comprises squamous cell carcinoma (90%).¹ Head and neck tumors can develop in the pharynx, larynx, oral cavity and sinonasal tract following genetic alterations driven by carcinogen exposure or high-risk human papillomavirus (HPV) infection. The majority of oropharyngeal squamous cell carcinomas (OPSCC), which include tumors of the tonsils and the base of tongue, are induced by HPV type 16 (HPV16).² Patients with HPV16⁺ OPSCC display a longer overall survival (OS) and a lower recurrence rate after surgery and/or chemoradiation than patients with HPV⁻ OPSCC.³ Head and neck squamous cell carcinomas (HNSCC) are generally treated with surgery and/or chemoradiation⁴, associated with high comorbidities and thereby severely impacting patients' quality of life. Immune checkpoint inhibition (ICI) is nowadays used in recurrent or metastatic HNSCC after failure of first line treatment, and is only successful in 20-30% of patients.^{5,6} The low response rate to ICI highlights the need to better understand the complex biology underlying the state of the tumor immune microenvironment (TIME) in HNSCC, in order to improve the efficacy of ICI and to provide a rationale for new immunotherapy design.

Biomarker studies of the TIME suggest that parameters associated with good prognosis also predict the efficacy of ICI, and both entail the strong presence of pre-existing CD4⁺ and CD8⁺ tumor-reactive T cells.⁷⁻¹⁰ In most types of tumors it is difficult to assess the presence of tumor-reactive tumor infiltrating lymphocytes (TILs) as it requires the identification of tumor (neo)antigens recognized by T cells for each individual patient. Consequently, it will be tough to identify the immunological blueprint of a productive – i.e. a tumor-reactive T cell-infiltrated – TIME, and subsequently to pinpoint what issues should be addressed by novel immunotherapy approaches. In the case of HPV16-induced OPSCC all tumors have the HPV neoantigens in common, providing a unique setting to unravel the make-up of a productive TIME.

Here, we made use of a unique prospective cohort of OPSCC patients to address these questions. We recently showed that the improved survival of HPV16⁺ OPSCC was strongly associated with the presence of HPV16 E6- and/or E7-reactive tumor-specific CD4⁺ and CD8⁺ TILs ², which were detected in 57% of the HPV16⁺ OPSCC patients. The dichotomy of HPV16-induced OPSCC with or without the presence of tumor-specific TILs provides an exceptional opportunity to identify TIME features associated with immune responsiveness (IR⁺) or lack thereof (IR⁻) using a multi-modal high-dimensional approach. Our studies in treatment naive tumor tissues revealed distinct interactions of CD8⁺ and CD4⁺ T cells with different myeloid cells between HPV16⁺IR⁺ and HPV16⁺IR⁻ OPSCC patients. These differences were associated with higher expression of *CXCL12* and *LTB*, as well as the clonal expansion of T cells producing CCL4 in HPV16⁺IR⁺ OPSCC compared to its IR⁻ counterpart. Clinical and bulk mRNA sequencing data from The Cancer Genome Atlas (TCGA) ¹¹ confirmed our findings in an independent HPV16⁺ OPSCC cohort.

Materials and methods

Patients

Patients included in this study were part of a larger prospective observational study in OPSCC. Patients with histologically confirmed OPSCC were included in the P07-112 study investigating the circulating and local immune response in patients with head and neck cancer.^{2,12} Patients were included after signing informed consent. The study was in accordance with the Declaration of Helsinki, and approved by the local medical ethical committee of the Leiden University Medical Center (LUMC) and in agreement with Dutch law. All the tumor material investigated in this study was obtained prior to therapy. Patients were treated with standard of care therapy consisting of surgery, radiotherapy, chemotherapy or combinations thereof. HPV typing on formalin-fixed paraffin-embedded (FFPE) tumor sections was performed as described.^{2,12} Tumor staging was performed according to the National Comprehensive Cancer Network (<https://www.nccn.org/professionals>). An overview of the study design is shown in **Supplemental Figure 1A**. A summary of the OPSCC patient characteristics and performed tumor analyses is provided in **Supplemental Table 1**. The overall survival (OS) of the patients was updated until February 2021.

Blood and tumor cell isolation

Venous blood samples (54ml) were drawn in sodium heparin tubes (BD Bioscience) prior to surgery and processed within 6 hours. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll density centrifugation and stored in the vapor phase of liquid nitrogen until use. Tumor material was obtained from the operation theater or from the outpatient clinic. One part of the tumor was fixed in formalin and embedded in paraffin for routine diagnostics at the pathology department, whereas the other part was used in parallel for single-cell dissociation and culture of tumor infiltrating lymphocytes (TIL), as described.^{2 12} In brief, OPSCC tumors were cut into small pieces. One third of the tumor pieces were put in culture in IMDM supplemented with 10% human AB serum (Capricorn Scientific, Ebsdorfergrund, Germany), 100IU/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine (Lonza, Breda, Netherlands; IMDM complete) and 1000IU/ml human recombinant IL-2 (Aldesleukin, Novartis, Arnhem, the Netherlands). Cultures were replenished every 2-3 days until sufficient cells were obtained. Two third of the tumor pieces was incubated for 15 minutes at 37°C in IMDM dissociation mixture containing 10% human AB serum, high dose of antibiotics (as above) and 0.38mg/ml Liberase (Roche Liberase TL research grade, Sigma Aldrich Chemie N.V., Zwijndrecht, the Netherlands). Following incubation, the cell suspension was passed through a 70µm cell strainer (Falcon, Durham, NC, USA) to obtain a single-cell suspension, counted using trypan blue exclusion (Sigma Aldrich Chemie N.V.), and cryopreserved at approximately 2 million cells/vial. All cells were stored in the vapor phase of liquid nitrogen until further use.

Tumor-specific T cell reactivity analysis

Cultured TIL batches were analyzed for the presence of HPV16-specific T cells as described previously.² To this end, reactivity of the cultured TIL was tested using autologous monocytes loaded with 5µg/ml HPV16 E6/E7 synthetic long peptides (SLP; 22-mers with 14 amino acids overlap) in a 5-day [3H]-thymidine-based proliferation and cytokine production assay. PHA (0.5µg/ml, HA16 Remel, Thermo Fisher Scientific, Bleiswijk, the Netherlands) served as a positive control, while unloaded monocytes served as negative controls. At day 1.5 and 4, supernatant (50µl/well) was harvested for cytokine analyses. Antigen-specific IFN γ , TNF α , IL-10 and IL-5 production was measured by using a cytometric bead array (CBA, Th1/Th2 kit, BD Bioscience, Breda, the Netherlands). The cut-off value for cytokine production was 20pg/ml, except for IFN γ for which it was 100pg/ml. Antigen-specific CCL4 and CXCL13 production was assessed by a custom-made 2-plex cytokine assay (Luminex; ProcartaPlex Immunoassay, Thermo Fisher

Scientific). Positive cytokine production was defined as at least twice above that of the unstimulated cells. HPV16 reactivity was also determined by intracellular cytokine staining (ICS). ICS was performed with antibodies for the markers CD3, CD4, CD8, CD137, CD154, IFN γ , TNF α and CCL4 following stimulation with HPV16 E6/E7 synthetic long peptide-loaded EBV-transformed B-lymphoblastoid cell lines (BLCL) as described.¹² Unloaded BLCL served as negative control and PHA as positive control. A positive response was defined as at least two times the value of the negative control, and at least 10 events in the gate. Acquisition was done on a LSRII Fortessa (BD Biosciences, LUMC flow cytometer core facility) and data analysis with FlowJo software (version 10.7.1).

NanoString PanCancer IO360 panel analysis

Archived diagnostic FFPE histological specimens were used to cut ten consecutive sections of 10 μ m for RNA extraction. Directly before and after these 10 μ m thick sections, a 4 μ m section was cut for hematoxylin-eosin (HE) staining. An OPSCC-specialized pathologist annotated tumor regions on these two HE slides to ensure that all 10 μ m slides contained tumor. After the sections were deparaffinized, the tumor areas were macro-dissected using the annotated HE slides as reference and placed in Buffer PKD of the RNA FFPE isolation kit (Qiagen, Hilden, Germany). RNA was isolated according to the manufacturer's instruction. The quality and quantity of the RNA was verified with the Agilent 2100 Bioanalyzer System, making use of the RNA 6000 Nano kit (Agilent, Santa Clara, CA, USA). Samples were selected for further processing when >15% of the RNA fragments were >300bp and the corrected RNA concentration remained $\geq 60\mu\text{g}/\mu\text{l}$. To measure the gene expression profiles, 300ng of RNA from each sample was hybridized with the probes of the human PanCancer IO360 panel for 17 hours at 65°C, following the manufacturer's instructions (NanoString Technologies Inc., Seattle, WA, USA). The number of copies of each gene in every sample was counted by scanning 490 Field Of View using the nCounter[®] MAX system (NanoString Technologies). The raw data (counts of genes) were uploaded to the nSolver[™] 4.0 software (NanoString Technologies), after which a quality check was performed in the gene expression analysis. The 20 housekeeping genes in the IO360 panel facilitated sample-to-sample normalization. Eight negative controls and six synthetic positive controls were included. The data were reviewed for reliability and validity based on the imaging (>0.75) and binding density (0.1-2.25) quality control metrics, and performance check of the positive controls (0.95-1). Samples with less than 8 housekeeping genes with >100 counts were removed during quality control (n=3). The data were analyzed using nCounter Advanced Analysis module 2.0 software (NanoString Technologies), in which automated cell type profiling and

pathway analysis based on the expression of pre-defined genes was performed (**Supplemental Table 2**). Benjamini-Hochberg adjusted *P*-values were used to decrease the false-discovery rate. Differentially expressed genes (DEGs) were defined by a log2 fold difference of >1 or ≤ 1 and an adjusted *P*-value < 0.05 .

Imaging mass cytometry and imaging processing analysis pipeline

Imaging mass cytometry (IMC) was performed using a previously optimized 33-marker panel.¹³ FFPE whole tumor sections of 4 μm thick were deparaffinized. Heat induced epitope retrieval was performed with citrate (10mM, pH6.0), and SuperBlock (Thermo Fisher Scientific) was used to block nonspecific binding sites. The first mix of 16 heavy metal conjugated antibodies was incubated for 5 hours at room temperature, the second mix of 17 heavy metal conjugated antibodies was incubated overnight at 4°C. Finally, an intercalator (DNA binder) was applied. An overview of the antibodies and their conjugated heavy metals can be found in **Supplemental Table 3**. The tissue slides were then analyzed by IMC using the Hyperion system (Fluidigm). Tumor regions were annotated by a pathologist, and two to three 1mm² regions per tumor were analyzed to account for intra-tumoral heterogeneity. The selected regions on the slides were laser ablated and processed through a mass cytometer, after which the detected heavy metals and their corresponding antibody (marker) were linked to the ablated region with a sub-cellular resolution of 1 μm^2 per pixel (overview IMC workflow provided in **Supplemental Figure 1BC**).

The generated high-dimensional output was analyzed with a newly developed imaging processing pipeline, combining multiple previously validated publicly available software programs. The pipeline incorporates 6 automated consecutive steps, of which an overview is presented in **Supplemental Figure 1C**. Firstly, the signal of the markers were normalized, to prevent sample biased clustering as this is frequently encountered when using FFPE tissue. Semi-supervised machine learning was employed to train the software to distinguish signal from background noise for each individual marker, using the software programs Ilastik and CellProfiler¹⁴, creating a binarized mask in which markers with their location on the image were expressed. Secondly, the same semi-supervised machine learning approach was used to segment the tissue into tumor and stroma, as well as to segment the individual cells on the images. These masks with layers of required information (normalized signal for all 33 markers, tissue segmentation, cell segmentation) were then combined in a single FCS file using ImaCyte (in-house developed software program for IMC data analysis, <https://github.com/biovault/ImaCyte>).¹⁵ HSNE-based cell clustering was performed with Cytosplore

¹⁶, and cluster verification and phenotype calling was performed using MCD Viewer and ImaCyte. After these pre-processing steps, quantitative analysis of the identified clusters was performed using ImaCyte. Spatial high-dimensional cell-cell interaction analyses and cellular microaggregate detection (more than three cells directly neighboring each other) were performed using ImaCyte and R. Permutation testing with 1000 iterations was performed to identify spatial interactions that occurred more frequently than expected based on chance, thus correcting for the cell cluster frequency. Only interactions with a permutation based Z-score > 2 (i.e. outside 95% normal distribution range) were included for further analyses. To study differences in spatial interactions across two subgroups (HPV16⁺IR⁻ versus HPV16⁺IR⁺), interactions with a permutation based Z-score > 2, occurring at a frequency of at least 2 fold more in either of the subgroups were included in the comparative analyses.

Single-cell RNA sequencing and data analysis

Single-cell tumor samples were thawed and enriched for viable CD3⁺ T cells and CD56⁺ NK cells using the dead cell removal kit (Miltenyi Biotec), followed by combined CD3/CD56 microbeads-guided magnetic cell enrichment (Miltenyi Biotec) according to the manufacturer's instructions. After isolation viability was more than 70% and percentage CD3/CD56 was 62.5 (range 27.5-97) and percentage B cells was 28.7 (range 1.6-64.8). Single-cell RNA sequencing (scRNAseq) was performed as we described previously.¹⁷ In brief, between 2,108 and 6,107 sorted cells per sample were loaded on a Chromium Single Cell Controller (10x Genomics), lysed and barcoded. Reverse transcription of polyadenylated mRNA from single-cells was performed inside each gel bead emulsion using the Single Cell 5' gene expression library workflow. Next-generation sequencing libraries were prepared in a single bulk reaction, and transcripts were sequenced using the HiSeq4000 System (Illumina). FASTQ files were pre-processed with cell ranger v3.0.0 (10x Genomics) using the GRCh38 reference genome. Quality control and downstream analysis was performed using Scanpy v1.6.0¹⁸ and Scirpy v0.6.1¹⁹. All scripts used to perform the analysis are combined in one fully reproducible Nextflow pipeline and publicly available from GitHub (https://github.com/icbi-lab/abdurahman2021_paper). Briefly, low quality cells were excluded retaining only cells with I) >700 detected genes, II) > 2000 detected reads, and III) <11% mitochondrial reads. Cells with more than one TCR-β or more than two TCR-α chains were discarded as putative doublets, in addition to computational doublet detection using SOLO.²⁰ Ribosomal, mitochondrial and T-cell receptor genes were excluded from downstream transcriptomic analysis. Raw counts were normalized per cell and log-transformed. Finally, the 6000 most

highly-variable genes (HVGs) of 14,242 T cells and 2,820 NK cells were selected and used for unsupervised Leiden clustering.²¹ Embeddings were visualized using UMAP.²² Differential gene expression between clusters was computed using edgeR²³, including the number of detected genes into the linear model.

Statistical analysis

Statistical data analysis was performed with GraphPad Prism 8.0.1 (GraphPad Software Inc., LA Jolla, CA, USA), which was also used to create graphs to visualize the data. The median immune counts of the three different OPSCC subgroups (HPV⁻, HPV16⁺IR⁻, HPV16⁺IR⁺) were compared with the non-parametric Kruskal Wallis test, of two subgroups (HPV16⁺IR⁻ versus HPV16⁺IR⁺) with the non-parametric Mann-Whitney U test. Spearman correlation was used to study correlations between the identified (super)clusters. Differences in survival, HR with 95% confidence interval, were calculated with the non-parametric Mantel-Cox test. Two-sided $P < 0.05$ were marked as statistically significant.

Results

Differences in immune signature between OPSCC patient groups with different clinical outcomes.

The OS data from our prospective cohort of 128 OPSCC patients extended earlier observations³ showing that patients with HPV⁺ OPSCC displayed a better clinical outcome than HPV⁻ OPSCC. Importantly, the TILs of 35 out of 61 tested patients with HPV16⁺ OPSCC displayed reactivity against the E6 and/or E7 viral oncoproteins (IR⁺). The fate of this HPV16⁺IR⁺ patient group, of which >90% had at least 5 years of follow up, was much better as they displayed superior survival when compared to the HPV16⁺IR⁻ group (**Figure 1A**), providing a firm basis for a positive role of local T-cell immunity in OPSCC. Differences in survival based on anatomical tumor location could be excluded (**Supplemental Table 1**), and the influence of smoking, TNM stage and received therapy on survival was excluded earlier².

Differences in gene expression, both at pathway and individual gene levels, were studied in 21 OPSCC patients (n=8 HPV⁻; n=6 HPV16⁺IR⁻; n=7 HPV16⁺IR⁺) using the NanoString human PanCancer IO360 panel, with a particular focus on immune-related pathways including estimation of immune cell abundance. This approach revealed 43 differentially expressed genes (DEGs) between HPV16⁺IR⁺ and HPV16⁺IR⁻ OPSCC patients (**Figure 1B**, **Supplemental Table 2A**). The immune profile of HPV⁻ and HPV16⁺IR⁻ OPSCC patients was highly similar as only one DEG was detected (**Supplemental Figure 2A**, **Supplemental Table 4B**), and 209 DEGs

were detected between HPV⁻ and HPV16⁺IR⁺ OPSCC patients (**Supplemental Figure 2B, Supplemental Table 4C**). A number of the upregulated DEGs ($P < 0.05$) in HPV16⁺IR⁻ OPSCC compared to HPV16⁺IR⁺ OPSCC are associated with suppressive myeloid cells (*CCL20*, *CXCL3*, *CSF2*, *TREM1*, *IL-1B*)²⁴, angiogenesis (*CXCL8*)²⁵, TGF β pathway (*INHBA*, *BMP2*)²⁶ and immune suppression (*IL-11*)²⁷. The top upregulated DEGs in HPV16⁺IR⁺ compared to its IR⁻ counterpart were *BLK*, reflecting B cells (**Supplemental Figure 2C**), and the chemokines *LTB* and *CXCL12* that are involved in ectopic lymphoid structure development, required for the coordination of adaptive immune responses.²⁸ Interestingly, the well-known chemokines for T cell attraction CXCL9, CXCL10 and CXCL11 did not differ.

ClueGO analyses of all DEGs between the two HPV16⁺ patient groups²⁹ indicated a strong relation with immune enrichment processes related to IFN γ signaling, T cell activation, T cell receptor signaling, and mononuclear cell differentiation in HPV16⁺IR⁺ tumors (**Figure 1C**). Gene expression-based cell type profiling (**Supplemental Table 2**) suggested an increased infiltration by immune cells in the HPV16⁺IR⁺ group, including T cells, CD8⁺ T cells, natural killer (NK, CD56dim) cells, cytotoxic cells, and macrophages (**Figure 1D**). This group also displayed a stronger involvement of several immune signaling pathways, including antigen presentation, co-stimulatory signaling, IFN signaling, cytokine and chemokine pathway, cytotoxicity, apoptosis, cell adhesion and migration pathway (**Figure 1E**).

Assessment of the impact of the three top upregulated DEGs in HPV16⁺IR⁺ and HPV16⁺IR⁻ on survival revealed that higher expression of *BLK*, *LTB* and *CXCL12* was associated with longer OS, in our own discovery cohort as well as in a larger independent group of 69 patients with HPV16⁺ OPSCC from the publicly available The Cancer Genome Atlas (TCGA) database¹¹ (**Figure 1F, Supplemental Figure 2D**), whereas there was no clinical impact for the three top upregulated DEGs in HPV16⁺IR⁻ (**Supplemental Figure 2E**). Specifically *LTB* expression was consistently related to the presence of CD4⁺ T cells, CD8⁺ T cells, CD11c⁺ myeloid (*ITGAX*) cells and the expression of Tbet (*TBX21*) (**Figure 1G, Supplemental Figure 2F**). Furthermore, *LTB* expression was strongly related to genes of immune cell activation (*RELA*, *NFKB1*, *NFKB2*, *TNF*) but not with *RELB* and *MET* (**Supplemental Figure 2G**), previously reported in the context of LTB to be associated with migration of tumor cells.³⁰

Overall these data suggest that the OPSCC of HPV16⁺IR⁺ patients displays a hot immune signature with a chemokine-driven coordinated influx of different immune cells, whereas HPV16⁺IR⁻ OPSCC display upregulation of genes related to poor survival. These data fit well with the clinical fate of these groups, showing a long OS for HPV16⁺IR⁺ and shorter OS for HPV16⁺IR⁻ and HPV⁻ OPSCC patients.

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A) Kaplan-Meier survival curves of prospectively followed cohort of HPV⁻ and HPV16⁺ OPSCC patients, the latter who are also subdivided into those with a tumor-specific T cell response (HPV16⁺IR⁺), and those lacking this immune response (HPV16⁺IR⁻). **B)** Volcano plots of upregulated DEGs identified by NanoString Pancancer IO360 between HPV16⁺IR⁺ (right) and HPV16⁺IR⁻ (left). The dotted lines indicate the Benjamini-Hochberg adjusted $P < 0.05$ and $P < 0.1$ thresholds. **C)** ClueGo of DEGs ($P < 0.1$) between HPV16⁺IR⁺ and HPV16⁺IR⁻ patients, visualizing the pathways in which these genes are involved. **D)** Deconvoluted cells by expression of genes predefined for different immune cell types depicted as counts in HPV⁻, HPV16⁺IR⁻ and HPV16⁺IR⁺ OPSCC. **E)** Differential expression of predefined pathway genes in HPV⁻, HPV16⁺IR⁻ and HPV16⁺IR⁺ OPSCC. **F)** Kaplan-Meier survival curves based on high/low LTB expression (classification based on median LTB expression), in all OPSCC patients analyzed by NanoString Pancancer IO360 ($n=21$), in the HPV16⁺ patients within this cohort ($n=13$), and in a large independent TCGA cohort of HPV16⁺ OPSCC ($n=69$). **G)** Linear regression analyses of top upregulated DEG LTB in HPV16⁺IR⁺ compared to HPV16⁺IR⁻ OPSCC versus cell type profiles of CD8 (CD8A), CD4, Tbet⁺ T cells (TBX21) and DC (ITGAX, CD11c) both for this cohort ($n=21$, upper panel) and the TCGA cohort of HPV16⁺ OPSCC ($n=69$, lower panel). HPV⁻ (red), HPV16⁺IR⁻ (blue) and HPV16⁺IR⁺ (green).

Identification and quantification of 51 different cell clusters in the TME of OPSCC.

To provide a detailed inventory of the *in situ* immune landscape of OPSCC we extensively characterized the tumors of 20 patients ($n=5$ HPV⁻; $n=4$ HPV16⁺IR⁻; $n=11$ HPV16⁺IR⁺) using a previously optimized 33-marker panel for imaging mass cytometry¹³, and an imaging processing pipeline (**Supplemental Figure 1C**). A total of 142,868 tumor cells, 46,907 stromal cells and 274,775 immune cells (**Supplemental Figure 1D**), were identified in the images (**Figure 2A**) for cluster quantification and for spatial and cell interaction analyses. These cells formed 51 clusters, comprising 14 tumor, 15 lymphoid, 15 myeloid, and 7 other cell clusters (**Supplemental Figure 3A**), all of which were verified in the raw images using MCD Viewer (**Supplemental Figure 4**). The 51 clusters (c) were then grouped into 18 mutually exclusive superclusters (sc) (**Supplemental Figure 3A**), and in 10 non-mutually exclusive superclusters, based on the expression of a particular lineage marker of interest expressed by multiple clusters (**Supplemental Table 5**).

Quantification of the 51 cell clusters (**Supplemental Figure 5**) and 18 mutually exclusive superclusters immediately revealed differences in tumor composition between the three OPSCC groups studied. The HPV16⁺IR⁺ tumors displayed the highest percentage of immune cells among all counted cells in the TME (**Figure 2B**). They were richer infiltrated by T cells, M1 macrophages and dendritic cells (DCs) (**Figure 2BC, Supplemental Figure 3B**). The OPSCC of HPV16⁺IR⁻ and HPV⁻ patients contained relatively more tumor cells, fibroblasts and blood vessels, indicative for a more tumor-promoting environment. The HPV⁻ OPSCC contained the lowest percentage of immune cells (**Figure 2BC, Supplemental Figure 3B**). The differences observed in the cellular composition of OPSCC among the three patient groups underline the transcriptomic signatures found by NanoString analyses and relate to their corresponding clinical course.

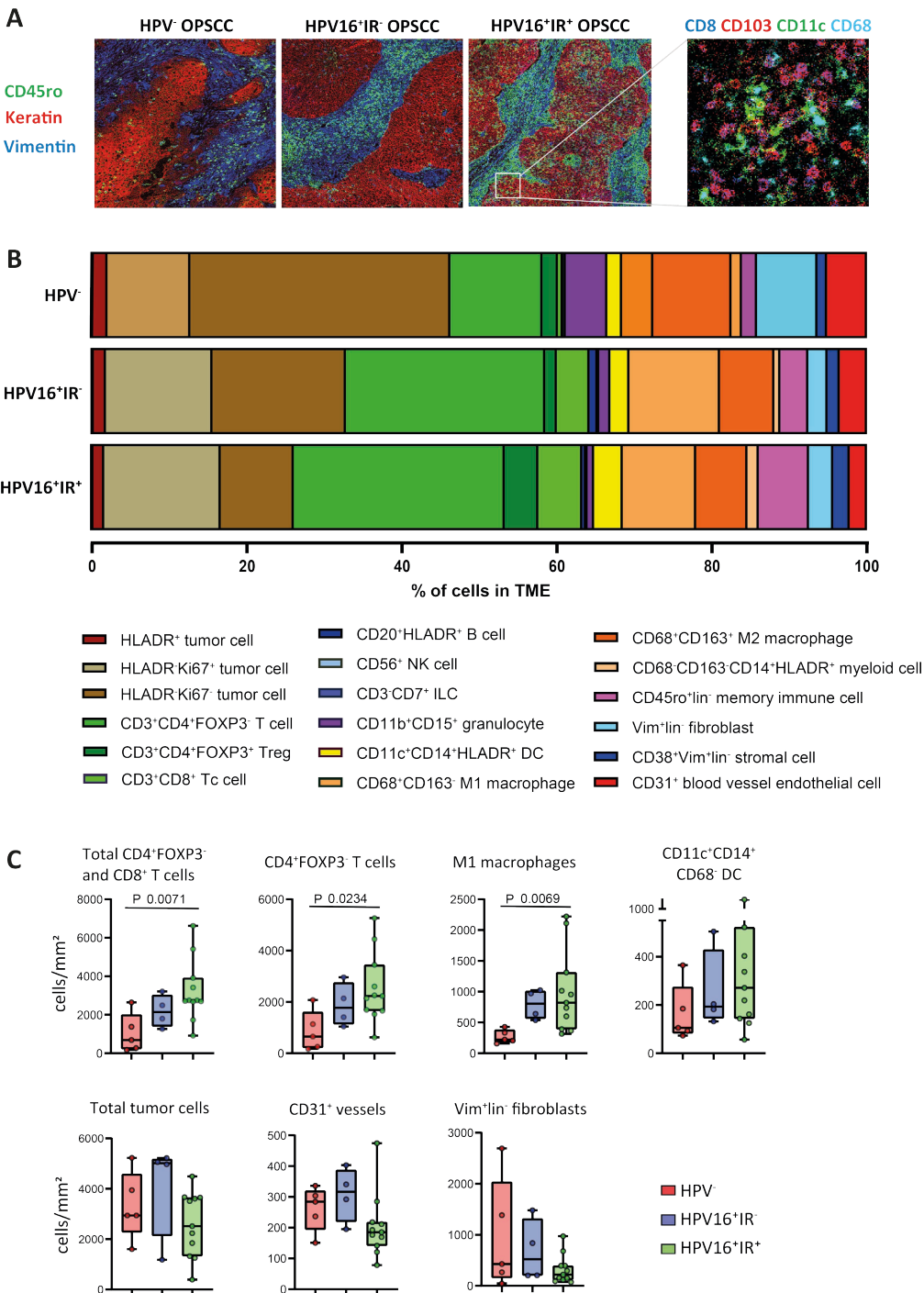


Figure 2. Imaging mass cytometry identifies cell clusters present in different percentages across OPSCC subgroups.

A) Representative images of immune cell infiltrate in the context of the microenvironment of OPSCC, as found in the three distinct subgroups (HPV⁺, HPV16⁺IR⁻, HPV16⁺IR⁺), visualizing the spatial location of immune cells (CD45ro in green) in the tumor epithelium (keratin in red) and stroma (vimentin in blue) region. A selection of both lymphoid and myeloid cell markers in the same image is also shown.

B) Fractional composition of the TME of the three OPSCC subgroups, depicting the median percentage of the 18 superclusters. **C)** Boxplots visualizing the quantitative cell counts in the whole tumor of the most differential superclusters across the three OPSCC subgroups: HPV⁺ red (n=5), HPV16⁺IR⁻ blue (n=4) and HPV16⁺IR⁺ green (n=11).

Principally different immune cell interactions in HPV16⁺IR⁺ versus HPV16⁺IR⁻ patients.

Immune-mediated tumor control requires a coordinated response of several immune cell types in a spatial and dynamic manner.³¹ Spearman correlation heatmaps generated for the 28 superclusters revealed strong positive and negative correlations between the counts of various cell types in HPV16⁺IR⁺ tumors (**Figure 3AB**). Linear regression analyses for those phenotypes with a correlation coefficient >0.6 and $P < 0.01$ were analyzed, showing in particular strong associations between the counts of (CD103⁺) CD8⁺ T cells with DCs and with CD14⁺ inflammatory myeloid cells in HPV16⁺IR⁺ patients (**Figure 3BC**). No significant correlations were detected in HPV16⁺IR⁻ OPSCC (**Supplemental Figure 6A**), emphasizing the lack of uniform immune cell organization in these tumors.

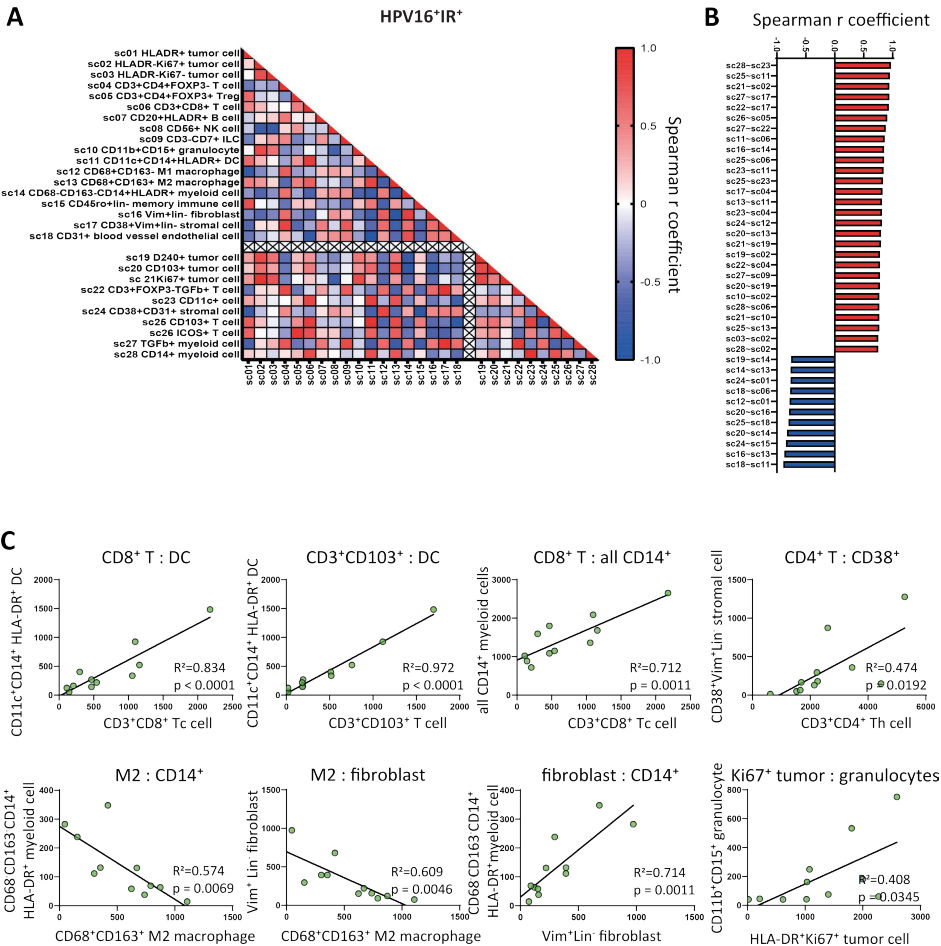


Figure 3. A coordinated and diverse immune infiltrate in HPV16⁺IR⁺ patients.

A) Spearman quantitative correlation heatmaps of the 28 superclusters (18 mutually exclusive, and 10 non-mutually exclusive) in the whole tumor in HPV16⁺IR⁺ OPSCC (n=11). The empty line separates mutually exclusive superclusters (above the crossed line) from the non-mutually exclusive superclusters (below the crossed line). **B)** Waterfall plot of statistically significant (P<0.01) Spearman correlations between the 28 superclusters (sc, defined in Supplemental Table 5) in HPV16⁺IR⁺ OPSCC (n=11), bars indicating their corresponding Spearman r coefficient. **C)** Linear regression analyses of the mutually exclusive superclusters with statistically significant (P<0.01) Spearman correlations in HPV16⁺IR⁺ OPSCC (n=11), including corresponding R squared values and linear regression P-values.

The imaging mass cytometry data enabled us to study which cells are in close vicinity and, therefore, able to interact with each other at the single-cell level. Analysis of the 51 identified cell clusters revealed interactions between immune cells, tumor cells and other cells (**Figure 4A**). As expected³² most cells sojourn in the neighborhood of cells from the same cluster (**Figure 4A**). A clear distinct

spatial phenotypic signature (SPS) became evident when only the interactions specifically occurring at a frequency that a) is higher than would be expected by chance, thereby correcting for differences in cell cluster frequencies by using permutation testing and b) is at least 2-fold higher in one of the subgroups, were visualized (**Figure 4B**). These spatial analyses revealed that in particular DCs and D2-40⁺ (invasive margin) tumor cells were more frequently interacting with both CD8⁺ T cells (c4) and CD4⁺ T cells (c14) in HPV16⁺IR⁺ OPSCC (**Figure 4BC**). T cells (c4, c14) and CD14⁺CD11c⁺HLA-DR⁺CD68⁻ DCs (c12, c13) formed simple immune cell aggregates²⁸ of more than three cells directly neighboring each other, designated as microaggregates, within the tumor cell beds of HPV16⁺IR⁺ OPSCC (**Figure 4C**). Of note, these were not tertiary lymphoid-like structures, of which only one was found in one OPSCC image, and this image was excluded from further analysis as in OPSCC it cannot be differentiated from a normal germinal center. Flow cytometry on the tumor-infiltrating CD14⁺CD11c⁺HLA-DR⁺ cells revealed the co-expression of CD1c, CD32b, CD36, CD141, CD40 and CD86 (**Supplemental Figure 7**).

In HPV16⁺IR⁻ OPSCC lymphocytes were found to interact with immunosuppressive myeloid cells, illustrated by interactions between NK cells (c8) and CD68⁺CD204⁺TGFβ⁺CD14⁻ tumor associated M2 macrophages (c15) as well as CD14⁺CD204⁺ myeloid cells (c16), and interactions between CD4⁺TGFβ⁺ T cells (c34) and CD14⁺HLADR⁺TGFβ⁺ myeloid cells (c17), or CD8⁺CD103⁺ T cells (c8) with CD68⁺CD163⁺ (c51) M2-like macrophages (**Figure 4B**). Notably, PD-L1 expression was not detected on these myeloid cells. The interactions between T cells and myeloid cells varied per patient in accordance with the earlier observed lack in coordination of immune cell infiltration in HPV16⁺IR⁻ OPSCC. A 360° spatial composition analysis of DCs sustained the notion that these DC, CD8⁺ T cell, CD4⁺ T cell and tumor cell microaggregates are scarce in HPV16⁺IR⁻ OPSCC (**Figure 4D**). Finally, visualization of the top 10 complete spatial immune compositions with highest Z-scores indicated dominant interactions with lymphocytes in HPV16⁺IR⁺ OPSCC, while the TME in HPV16⁺IR⁻ OPSCC was dominated by myeloid cell interactions (**Supplemental Figure 8**).

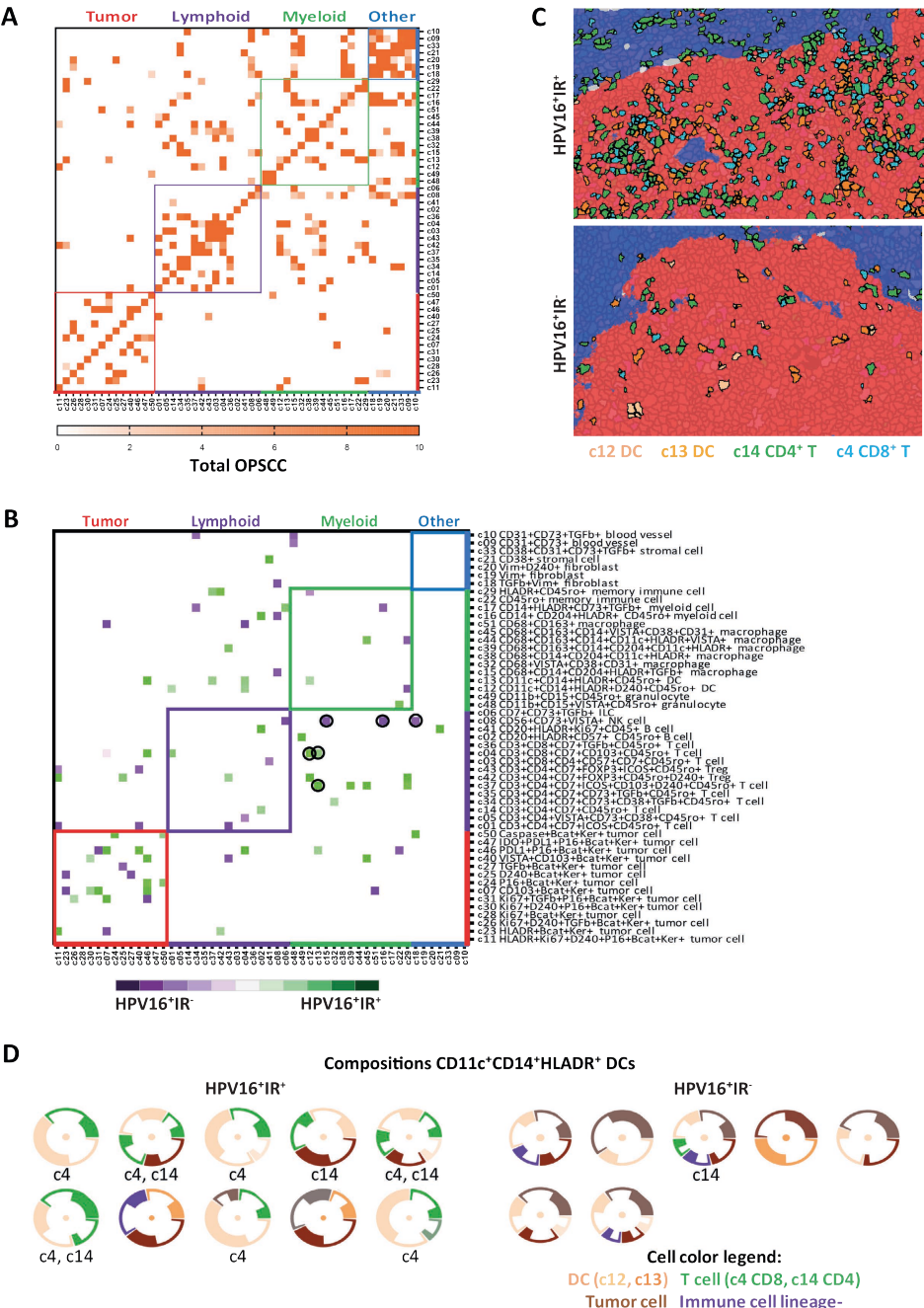


Figure 4. Organized intra-tumoral immune microaggregates specifically in HPV16*IR⁺ OPSCC.

*Spatial interactions heatmap of the 51 identified clusters (Supplemental Table 5), with interaction neighborhood defined as 5µm (<1 cell, direct spatial interaction). Cluster name is based on the markers that are expressed by that cluster, indicating that a cluster is negative for all other tested markers. **A)** Spatial interactions heatmap of the total OPSCC cohort (n=20), showing all spatial interactions with a permutation verified Z-score>2 (i.e. outside the 95% normal distribution range), dark orange indicating highest Z-scores. **B)** Heatmap showing the differences in spatial interactions occurring in HPV16⁺IR⁺ (n=11) versus HPV16⁺IR⁻ (n=4) OPSCC. Only spatial interactions with a permutation verified Z-score>2, which specifically occurred in only one of the subgroups, and had at least a two-fold interaction difference in percentage between the subgroups are visualized. Spatial interactions occurring more frequently in HPV16⁺IR⁺ tumors are indicated in green, and in HPV16⁺IR⁻ tumors in purple. **C)** Hyperion images of the TME of OPSCC, showing the direct spatial interactions between CD8⁺ T cells, CD4⁺FoxP3⁻ T cells and dendritic cells with tumor cells in HPV16⁺IR⁺ OPSCC, and the absence of these microaggregates in HPV16⁺IR⁻ OPSCC. Tumor epithelium is visualized in red, tumor stroma in blue. **D)** 360° spatial interaction compositions of DCs, for HPV16⁺IR⁺ and HPV16⁺IR⁻ tumors. Visualized is the DC (center), surrounded by other clusters (thin circle segment indicates standard deviation). Below each composition the involved T cell cluster numbers are given. Threshold for the 360° compositions: ≥20 occurrences.*

HPV16⁺IR⁺ patients show preferential T cell expansion in CD8⁺ T cell clusters defined by the expression of CCL4.

The profound differences in spatial interactions of T cells and myeloid cells observed between HPV16⁺IR⁺ and HPV16⁺IR⁻ OPSCC is likely to bear impact on the function and phenotype of T cells present in these tumors. This was investigated by a combined single-cell transcriptome (scRNAseq) and T cell receptor (TCR) sequencing analysis on magnetic bead-sorted CD3⁺ and CD56⁺ cells from 13 patients (3 HPV⁻, 4 HPV16⁺IR⁻, 6 HPV16⁺IR⁺), yielding data for 14,242 T cells and 2,820 NK cells. Unsupervised clustering²¹ resulted in the detection of 32 T-cell clusters, comprising twelve CD8⁺, eleven CD4⁺, six FoxP3⁺ (Treg) and three other CD3⁺CD4⁻CD8⁻ T-cell (Tother) clusters, as well as six NK cell clusters (**Figure 5A**). All clusters were found in >1 patient (**Figure 5B and Supplemental Table 6**). Analysis of the distribution of these 38 cell clusters among the three OPSCC groups revealed a significant enrichment of clusters CD8_0 and Treg_0 in HPV16⁺IR⁺ when compared to HPV16⁺IR⁻ OPSCC (**Figure 5CDE and Supplemental Figure 9**). Top 30 DEGs of all clusters are listed in **Supplemental Table 7**.

Integrated single-cell transcriptome and TCR repertoire RNA sequencing analysis was performed on magnetic-bead sorted CD3⁺ T cells and CD56⁺ NK cells from 13 OPSCC samples. Following quality control and doublet-filtering, the 6000 most highly variable genes (HVG) of 14,242 T cells and 2,820 NK cells were selected and unsupervised clustering was performed using the Leiden algorithm ²¹. **A)** A 2-dimensional UMAP plot visualizing the 38 identified clusters. **B)** Bar graphs depicting the number of cells per patient per cluster for CD8⁺ (top), CD4⁺ (middle) T cells and FoxP3⁺ T cells (Treg), Toter and NK cells (bottom; from left to right). **C, D, E)** Box plots depicting the identified CD8 (C), CD4 (D) and Treg (E) clusters, among HPV⁺ (red, n=3), HPV16⁺IR⁺ (blue, n=4) and HPV16⁺IR⁺ (green, n=6) OPSCC tumors. Data is represented as percentage of cluster.

As the TCR is responsible for the specificity and intratumoral TCR clonal expansion may reflect tumor-reactivity³³, analysis of the TCR repertoire yields valuable information that can be connected to the IR status of the patients. Full-length TCR sequences were obtained for 75.4% of all T cells. In total 2,905 of the 10,734 cells (27.1%) were clonally expanded on the basis that at least 2 identical TCRs were found, resulting in a total of 690 of the 8,519 individual clonotypes (8.1%) being expanded. Clonotype network analysis revealed that clonally expanded TCRs are present in all patients and clonal expansion was the strongest and more frequent among CD8⁺ T cells (**Figure 6A, Supplemental Figure 10A**). Moreover, the number of cells and clonotypes with expanded TCRs tended to be higher in HPV16⁺IR⁺ OPSCC among CD8⁺ and CD4⁺ T cells, but not among Treg and Tother cells (**Figure 6B, Supplemental Figure 10B**).

To link the preferentially expanded CD8⁺ and CD4⁺ T cells in HPV16⁺IR⁺ OPSCC to transcriptional profiles, integration of the single-cell transcriptome and TCR repertoire data sets was performed. Strong CD8⁺ T-cell expansion existed in the majority of patients and clusters, whereas expansion of CD4⁺ T cells and Tregs were lower or absent in some patients and clusters (**Supplemental Figure 10CD**). Importantly, the percentage of expanded cluster CD8_0 T cells was clearly higher ($P=0.057$) in HPV16⁺IR⁺ OPSCC than in IR⁻ OPSCC (**Supplemental Figure 11ABC**). The CD8_0 cluster can be defined as a cytokine-producing CD8⁺ T cell subset, in which particularly *CCL4* was highly expressed. This subset differs substantially from cluster CD8_3, which did not significantly differ between our patient groups and is made up of cells that, based on their DEG profile, resemble highly activated tumor-resident cytotoxic effector T cells (**Supplemental Figure 12, Supplemental Table 7**). *CCL4* was also expressed by NK cells (**Figure 6C and Supplemental Figure 13**), but as NK cells were >20 fold less abundant than T cells in these tumors (**Supplemental Figure 3B**), their contribution is much lower. The production of *CCL4* prompted us to re-examine the expression of chemokine receptors in the NanoString data. This revealed that the expression of CCR5 (*CCL4*'s receptor), CCR2 (*CCL2*'s receptor), CXCR3 (*CXCL9,10,11*'s receptor) and CXCR6 (*CXCL16*'s receptor) but not CXCR2, CXCR4 and CX3CR1, CCR4, was increased in HPV16⁺IR⁺ tumors compared to HPV16⁺IR⁻ tumors (**Figure 6D**). Of note, none of the T and NK cell clusters showed expression of *CXCL12*, whereas *LTB* was expressed by the majority of the CD4⁺ T-cell clusters (**Figure 6C and Supplemental Figure 13**). Extensive characterization of all the T and NK cell clusters in terms of tissue-residency, co-stimulatory and co-inhibitory genes is provided in **Supplemental Figure 12**, showing that in contrast to cluster CD8_3, cluster CD8_0 has an overall low expression of immune checkpoints.

To confirm that CCL4 was predominantly produced by T cells in HPV16⁺IR⁺ OPSCC, the supernatants of TIL cultures from HPV16⁺ OPSCC were analyzed for the production of CCL4 and as control CXCL13, as it is expressed by cluster CD8_3 T cells which did not differ between the patient groups. While a similar production of CXCL13 was found in the PHA-stimulated cultures of IR⁺ and IR⁻ patients, CCL4 production was almost exclusively found in HPV16⁺IR⁺ OPSCC, with all nine cultures showing substantial production of CCL4 when stimulated with tumor antigen or PHA (**Figure 6EF, Supplemental Figure 14**). Subsequent flow cytometric analysis showed that all IFN γ -producing CD8⁺ T cells co-produced CCL4, whereas half of the IFN γ -producing CD4⁺ T cell population produced CCL4 when stimulated with cognate antigen or PHA (**Figure 6GH**).

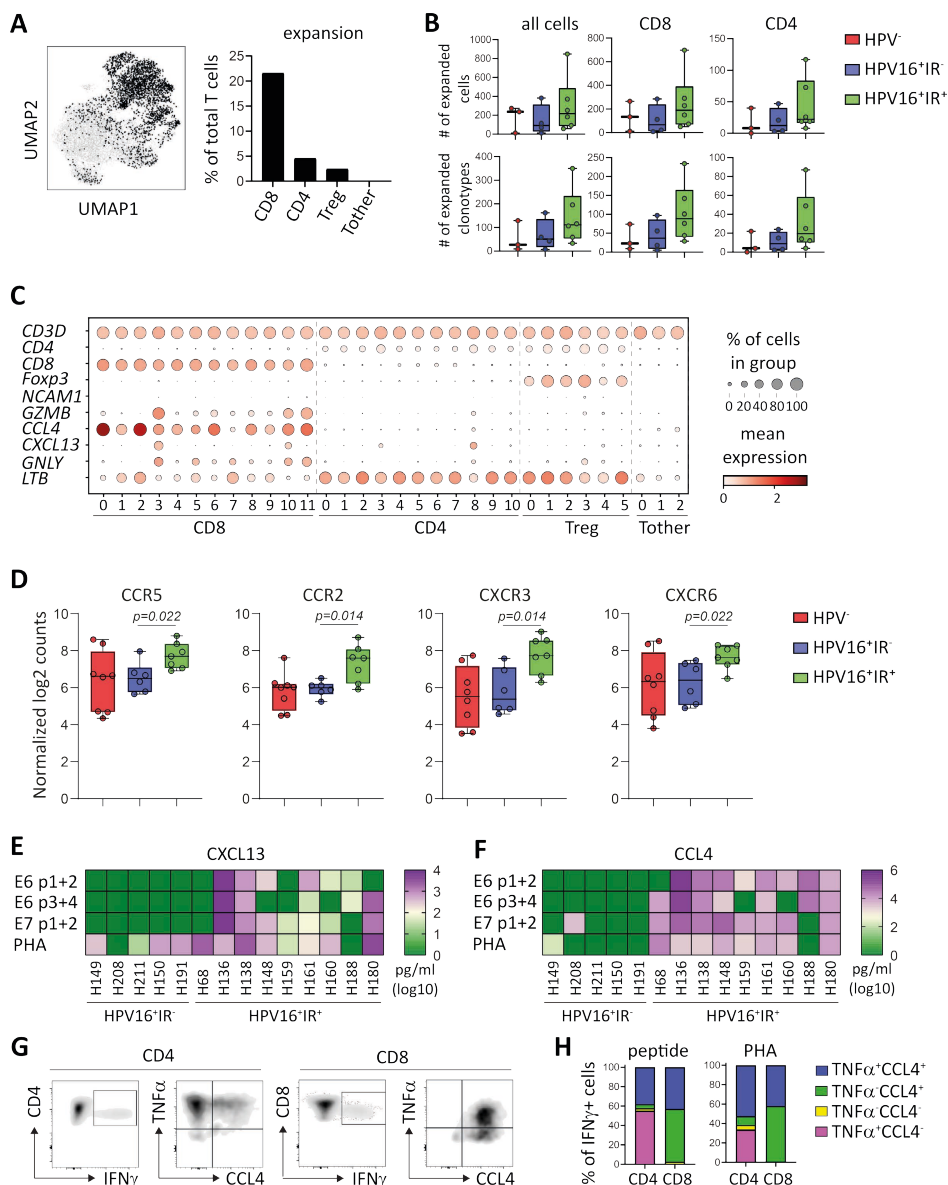


Figure 6. HPV16⁺IR⁺ patients show T cell expansion in clusters particularly defined by the expression of CCL4 or CXCL13.

Integrated single-cell transcriptome and TCR repertoire RNA sequencing analysis was performed on magnetic-bead sorted CD3⁺ T cells and CD56⁺ NK cells from 13 OPSCC samples. **A**) UMAP plot depicting the localization of expanded TCR clonotypes (left) and graph depicting the percentage of expanded TCR clonotypes within CD8, CD4, Treg and Tother cells (right). **B**) Box plots displaying the number of expanded cells (top) and clonotypes (bottom) within all cells (left), CD8 (middle) and CD4 (right) cells detected in HPV⁻ (red, n=3), HPV16⁺IR⁻ (blue, n=4) and HPV16⁺IR⁺ (green, n=6) OPSCC patients. **C**) Dot plots showing the gene expression of selected determining genes in the clusters. **D**) Normalized log2 counts for the expression of chemokine receptors in HPV16⁺IR⁻ (green, n=7) compared to HPV16⁺IR⁺ (blue, n=6) OPSCC (Mann-Whitney

*U test). **E, F** Heatmap presenting specific (E) CXCL13 and (F) CCL4 cytokine production of cultured CD4⁺ and CD8⁺ T cell containing TIL in response to HPV16 E6 peptide (pool 1+2 and 3+4)-, HPV16 E7 peptide (pool 1+2)-loaded autologous monocytes for HPV16⁺IR⁺ (n=9) and HPV16⁺IR⁻ (n=5) OPSCC. PHA served as positive control. Three OPSCC TIL cultures containing HPV-specific T cells were analyzed for HPV16-specific cytokine production by intracellular cytokine staining and flow cytometry following stimulation with BLCL loaded with pools of HPV16 E6/E7 synthetic long peptides. Expression of CD3, CD4, CD8, IFN γ , TNF α and CCL4 following overnight stimulation in the presence of Brefeldin A. **G**) A representative example. The TIL were gated for viable and single cells, and further gated for CD3, CD4 and CD8. The cells producing TNF α and CCL4 within the CD8⁺IFN γ ⁺ (left) and CD4⁺IFN γ ⁺ (right) T-cell population are depicted. **H**) The percentage of TNF α and CCL4 within the total IFN γ -producing CD4⁺ and CD8⁺ T cells is depicted for two patients for HPV16 peptide (left) and three patients for PHA (right).*

CCL4 expression is related to T cell and DC tumor infiltration as well as survival in HPV16⁺ OPSCC TCGA patients.

Our T cell data indicated a role for CCL4 in a productive TIME, hence a gene set enrichment analysis of the TCGA RNA-sequencing data from the 69 patients with HPV16⁺ OPSCC ¹¹ was performed. Our previously published immunogenomic analytic strategy to estimate subpopulations of tumor-infiltrating immune cells ³⁴ was applied to determine which immune cells were relatively enriched or depleted in HPV16⁺ OPSCC with high versus low *CCL4* gene expression (**Figure 7A**). These analyses confirmed a positive relationship between *CCL4* with a larger fraction of activated and effector memory CD8⁺ T cells and activated CD4⁺ T cells as well as with B cells, T follicular helper cells, Tregs, macrophages and eosinophils suggestive for a more effective antitumor inflammatory response. This notion was supported by a strong correlation between the expression of *CCL4*, CD8⁺ T cells (*CD8A*), CD4⁺ T cells (*CD4*), Tbet⁺ T cells (*TBX21*) and CD11c⁺ myeloid cells (*ITGAX*) (**Figure 7B**) consistent with recent literature suggesting that CCL4 functions as a long-range homotypic signaling molecule to attract more T cells and cross-presenting DCs into the TME.^{35 36}

To gain more information on the immune enrichment processes associated with high expression of *CCL4* in the tumor microenvironment, ClueGO analyses ²⁹ were performed. This showed that especially *CCL4* was positively associated with T cell co-stimulation, activation, and proliferation as well as lymphocyte chemotaxis and IFN γ responses (**Figure 7C, Supplemental Figure 15**). Finally, the expression of *CCL4* was associated with superior disease control in patients with HPV16⁺ OPSCC (**Figure 7D**), indicating the significance of the immune response associated with this cytokine.



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enrichment (red) and depletion (blue) of immune cell types in CCL4-high versus CCL4-low HPV16⁺ OPSCC is shown. **B)** The Pearson correlation between the expression of CCL4 with CD8A (for CD8 T cells), TBX21 (for Tbet⁺ T cells) and ITGAX (CD11c for DCs) as well as CD8A and ITGAX in the tumors of the 69 patients with HPV16⁺ OPSCC in the TCGA database. **C)** The correlation between CCL4 with a subset of genes representing immune related functions selected based on correlation analysis (Pearson correlation coefficient (R) > 0.6; adjusted $P < 0.05$) were used for gene ontology (GO) analysis of immune enrichment processes using ClueGO²⁹. **D)** Kaplan-Meier survival plots of the 69 patients with HPV16⁺ OPSCC in the TCGA database grouped according to high and low gene expression using the median value of CCL4 as cut-off.

Discussion

This multi-modal high-dimensional study of the TIME in OPSCC revealed a distinct spatial phenotypic signature in the immune landscape of HPV16⁺IR⁺ and that of HPV16⁺IR⁻ OPSCC. Immune cell infiltration in HPV16⁺IR⁺ tumors is highly coordinated resulting in characteristic CD8⁺(CD103⁺) T cells, CD4⁺ T cells and DCs microaggregates within the tumor cell beds, a perfect place for DCs to ingest tumor antigens and stimulate tumor-specific T cells. In this study T cell:DC interactions are associated with longer OS but they have also been reported to be required for successful anti-PD1 therapy.³⁷ These microaggregates are most likely organized under the influence of the chemokines LTB and CXCL12^{28 38} and sustained by CCL4, known to attract T cells and cross-presenting DCs.^{35 36 39} Indeed an increased expression of CCL4's receptor CCR5 was detected in HPV16⁺IR⁺ tumors compared to HPV16⁺IR⁻ tumors. Previously, it was shown that the production of CCL4 by tumor cells was key to attract DCs and to start tumor immunity.^{9 36} Here we show that CCL4 can also be produced by tumor-specific T cells and lead to a productive TIME. While CCL4 is capable of attracting both tumor supporting (regulatory T cells, M2 macrophages, MDSC) and tumor-rejecting (cytotoxic T cells, M1 macrophages) immune cells, depending on the context in which it is produced⁴⁰, we did not observe differences in counts of regulatory T cells and macrophages, suggesting that in the HPV⁺IR⁺ context the chemotaxis of tumor-rejecting immune cells by CCL4 prevails.

The formation of organized aggregates by tissue infiltrating leukocytes occurs often during inflammation and such regions may exist as the simple aggregates found here inside the tumor cell bed, or as more sophisticated structures in the tumor stroma that resemble tertiary lymphoid structures (excluded in our imaging analyses) and are known to stimulate adaptive immunity.²⁸ Of the chemokines, LTB was produced by multiple CD4⁺ T-cell clusters, indicating that the sheer presence of more activated CD4⁺ T cells was responsible for its overexpression in HPV16⁺IR⁺ tumors. CCL4 was expressed specifically by

clonally expanded, most likely tumor-reactive³³, CD8⁺ T cells that were found more frequently in HPV16⁺IR⁺ tumors. It was also produced by tumor-specific T cells cultured from these tumors. High intratumoral T-cell clonality has previously been positively linked to clinical outcome in melanoma patients treated with ICI.⁸ The detection of more clonally expanded T cells in HPV16⁺IR⁺ tumors is suggestive for the accumulation of intratumoral tumor-specific T cells, fitting well with the detection of type 1 CD4⁺ and CD8⁺ HPV-specific T cells among the TILs in these IR⁺ OPSCC.² Our current study suggests that through the production of the chemokines LTB and CCL4, these two types of tumor-specific T cells act as a positive feedback loop for the spatial organization of DC-T cell aggregates, necessary to maintain effective tumor immunity. The discovery of these intratumoral immune microaggregates are of particular importance as potential biomarker in OPSCC, as in this tumor type TLS are not of diagnostic value since they cannot be differentiated from normal lymphoid tissue in which the tumor arises.

In addition to the significant enrichment of CCL4-expressing expanded CD8_0 T cells in HPV16⁺IR⁺ OPSCC tumors, also the clonally expanded clusters CD8_5 and CD4_5 tended to be higher in HPV16⁺IR⁺ OPSCC tumors. Cluster CD8_5 is characterized by the expression of XCL1, known for its capacity to attract cross-presenting DCs into the TME³⁹, and the clonally expanded CD4⁺ T-cell cluster is characterized by the expression of *LMNA* (lamin A), which is upregulated early after T cell activation to accelerate the formation of the immunological synapse with APCs⁴¹ and critical for their Th1 differentiation.⁴² However, these two clusters also displayed an increased expression of several heat shock protein and stress pathway-related genes. As the expression of such genes may be related to cryopreservation, thawing and dissociation procedures⁴³, the interpretation of data for these clusters should be done with caution.

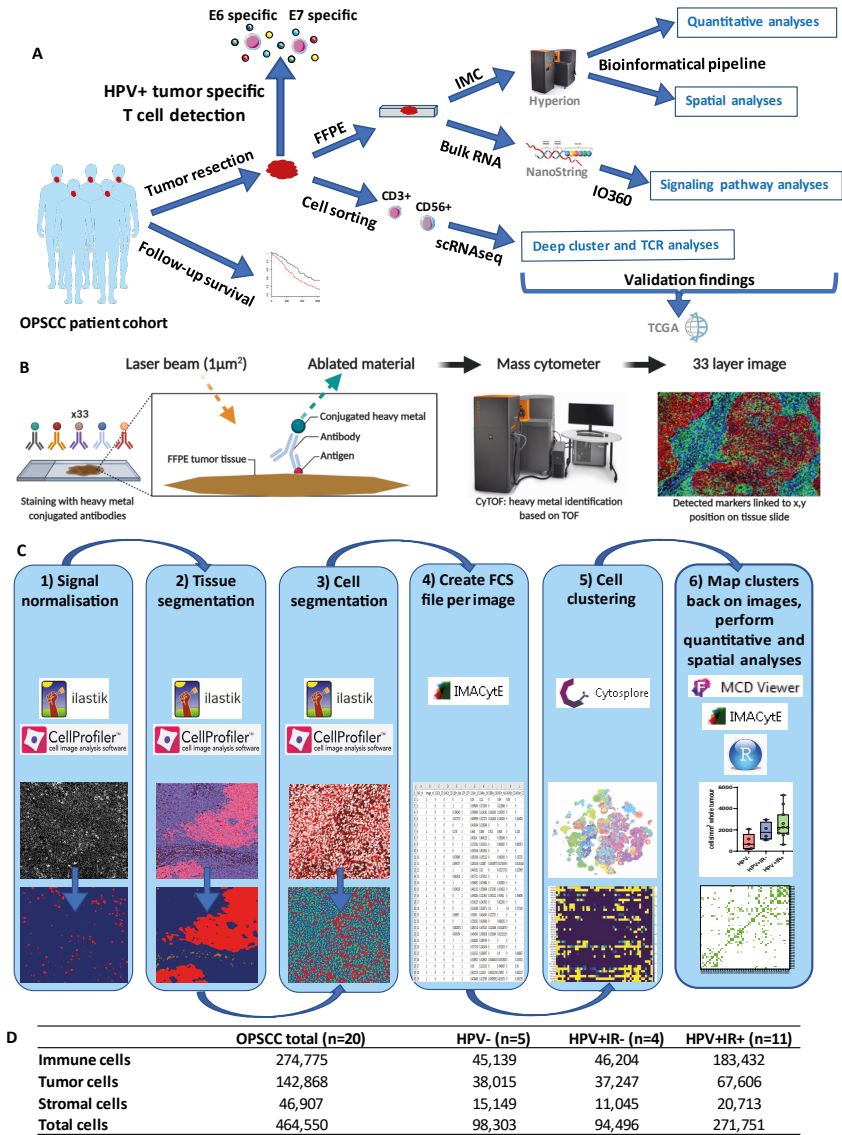
The DCs in these microaggregates were defined by their expression of CD11c, CD14 and HLA-DR but not CD68, and co-expressed CD1c, CD32b, CD36, and CD141. The presence of such inflammatory APCs have been linked to acute inflammatory processes^{44 45}, to the survival of patients with HPV-induced cervical cancer⁴⁶ and to immunotherapy responsiveness in HPV-induced premalignant lesions^{47 48}, while the incapacity to attract these cells constituted a secondary escape mechanism to immunotherapy in a mouse model⁴⁹. Our current findings, showing that these cells are found in higher numbers and organized in immune microaggregates when a tumor-specific T-cell response is present, sustains the notion that these cells are of major

importance in tumor immunity. Currently OPSCC patients are treated by surgery and/or chemoradiation. A therapy associated with high comorbidity and loss in quality of life. The well-organized TIME in HPV16⁺IR⁺ OPSCC is likely to promote the response to immunotherapy.^{50 51} Notably, the current group of patients treated with ICI after failing standard of care therapy, most likely are those that failed to mount a HPV-specific T-cell response (HPV16⁺IR⁻ group) as they will rapidly present with progressive disease.² Hence, they will not display the distinct SPS of HPV16⁺IR⁺ patients that may make them responsive to ICI. The SPS of HPV16⁺IR⁺ OPSCC could be exploited for the identification and selection of this particular patient group, potentially by CD8 and CD11c dual-immunohistochemistry, for neoadjuvant ICI.³⁷ Alternatively, the mRNA expression profile of IR⁺ OPSCC could be developed into a suitable selection tool to this purpose.

Imaging mass cytometry showed that the TME in HPV16⁺IR⁻ tumors comprises relatively higher number of fibroblasts and blood vessels than HPV16⁺IR⁺ OPSCC. Although HPV16⁺IR⁻ OPSCC are infiltrated with T cells, albeit it less than in HPV16⁺IR⁺ OPSCC, the SPS in these lukewarm tumors was different and comprised spatial interactions between lymphocytes and different subpopulations of immunosuppressive myeloid cells, including CD163⁺ macrophages, CD204⁺ macrophages as well as TGFβ⁺ myeloid cells. This varied per patient, consistent with the lack of coordination of immune cell infiltration in this patient group. Increased expression of the TGFβ superfamily members *INHBA* and *BMP2*²⁶ were also found in our transcriptomic analyses. The presence of these immune suppressive cells⁵²⁻⁵⁴ and the increased expression of the immune suppressive cytokine *IL-11*, known to suppress the attraction and functionality of tumor-reactive CD4⁺ T cells²⁷, potentially explain why tumor-specific T cells were not detected in these tumors. It also provides a rational foundation for combinations of T-cell stimulating agents^{6 55 56} with strategies focusing on the identified resistance mechanisms, e.g. co-targeting M2-like macrophages⁵⁷, TGFβ⁵⁸ and/or angiogenesis⁵⁹.

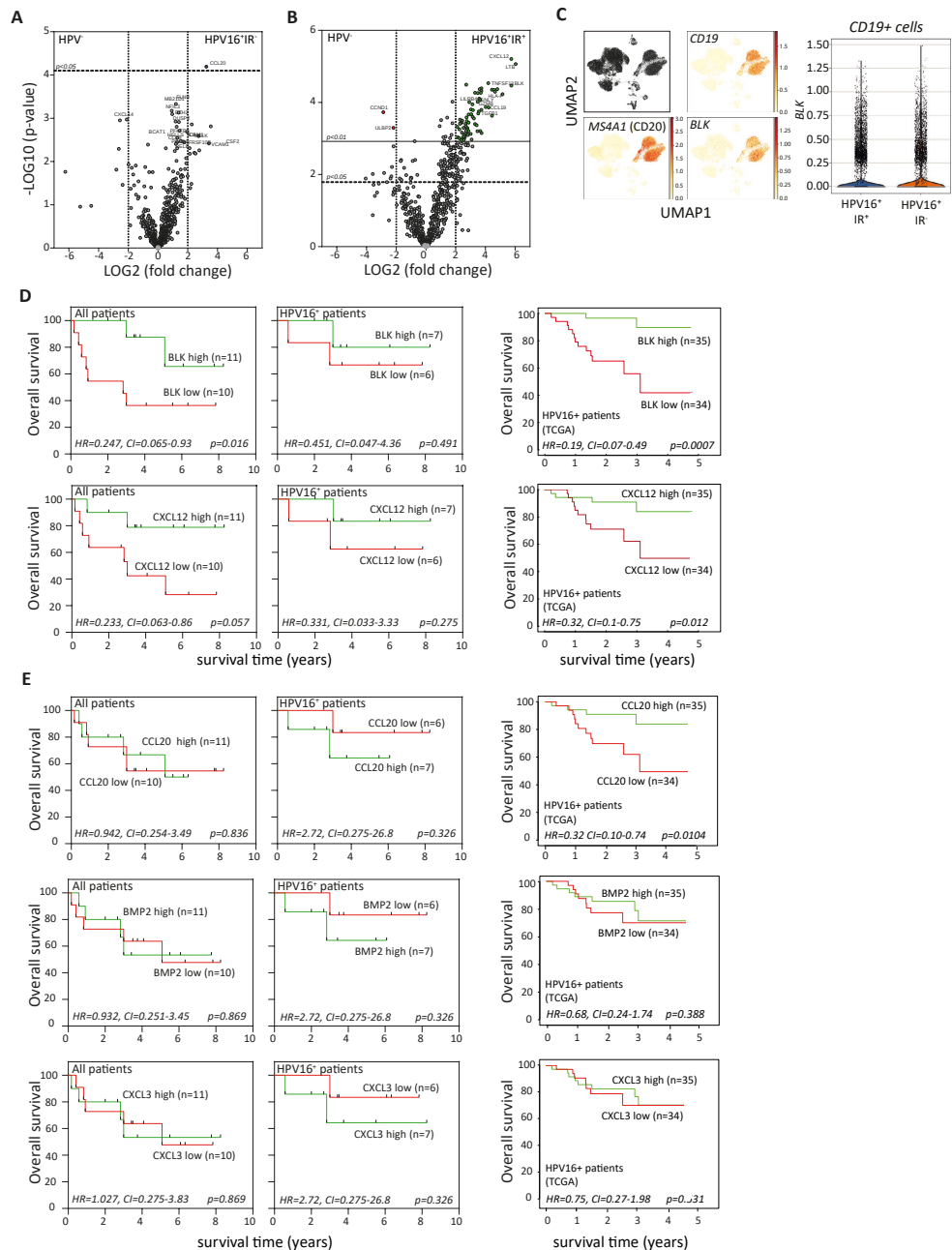
In conclusion, the blueprint of a productive TIME comprises the local production of chemokines that not only attract tumor-reactive CD4⁺ and CD8⁺ T cells as well as DCs, but also organize them into small aggregates within the tumor cell beds where most likely the DCs gobble up antigens from dying tumor cells and stimulate the T cells to exercise their effector function and to amplify the immune response.

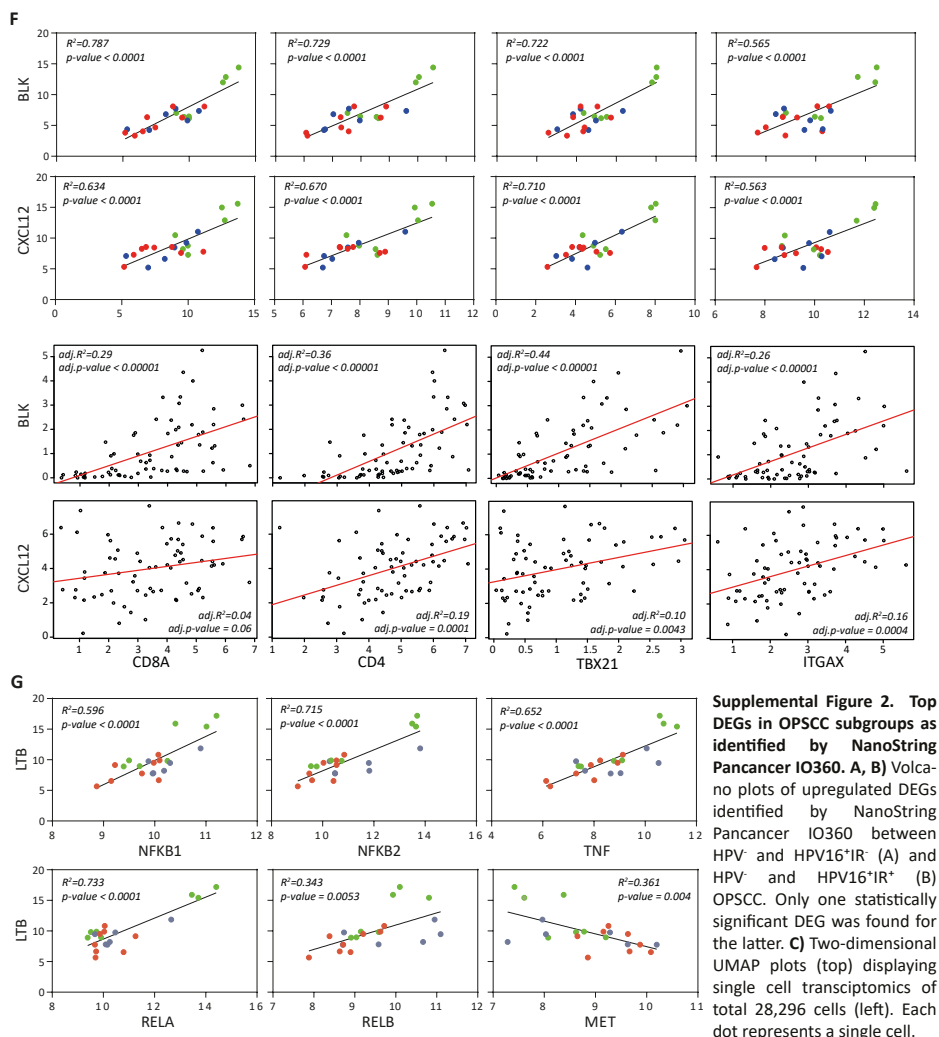
Supplemental Figures



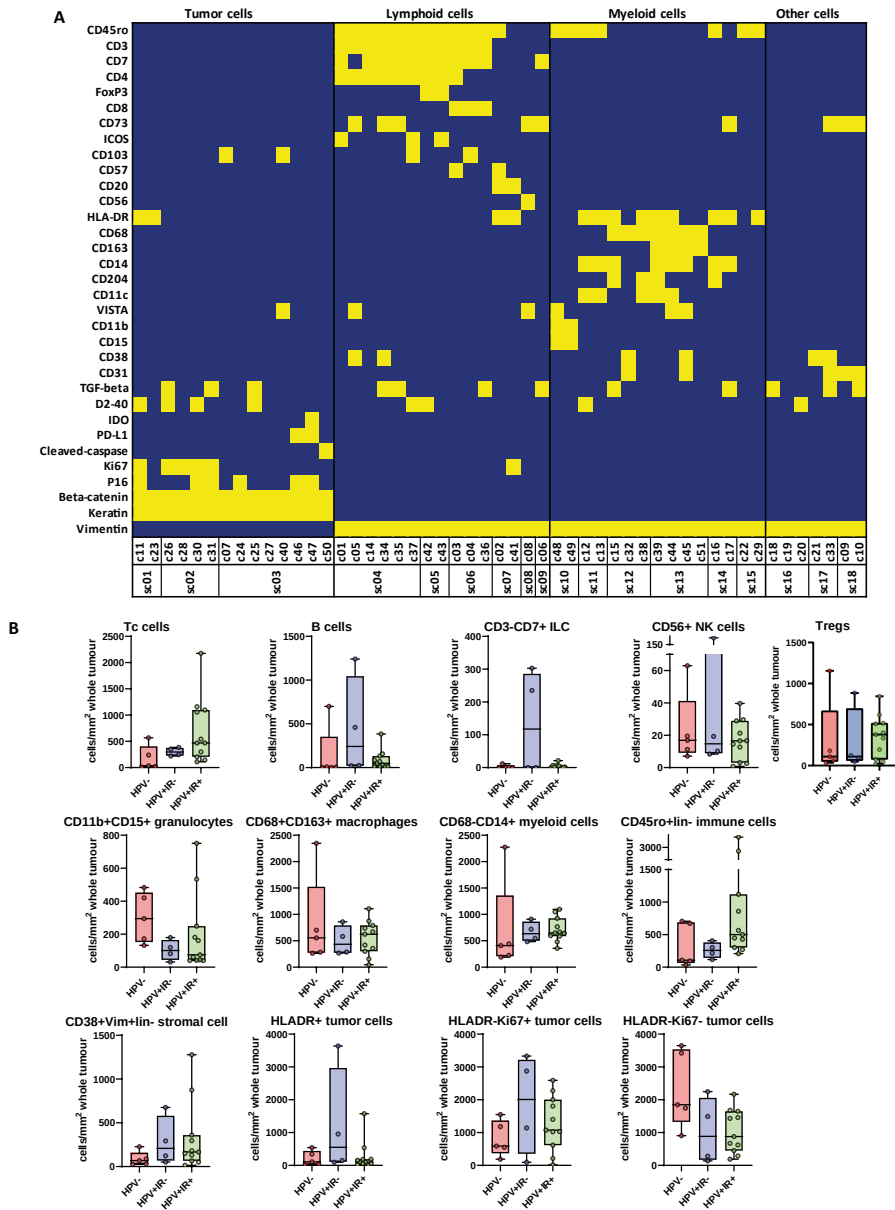
Supplemental Figure 1. Bioinformatical pipeline imaging mass cytometry data analyses.

A) Overview of the multi-dimensional study workflow yielding comprehensive high-dimensional information of the immune landscape of Oropharyngeal Squamous Cell Carcinoma (OPSCC). FFPE=formalin-fixed paraffin-embedded; IMC=imaging mass cytometry; scRNAseq=single cell RNA sequencing; TCR=T cell receptor; TCGA=the cancer genome atlas. **B)** Visualization of the imaging mass cytometry staining and image acquisition workflow. Image created with BioRender. **C)** All analytical steps performed to process the imaging mass cytometry data, including the programs used to perform that analytical step, and visualization of the stepwise image processing. **D)** Counts of cells analyzed by imaging mass cytometry in the total OPSCC cohort and per subgroup, of immune cells, tumor cells, stromal cells and the total of all cells analyzed.





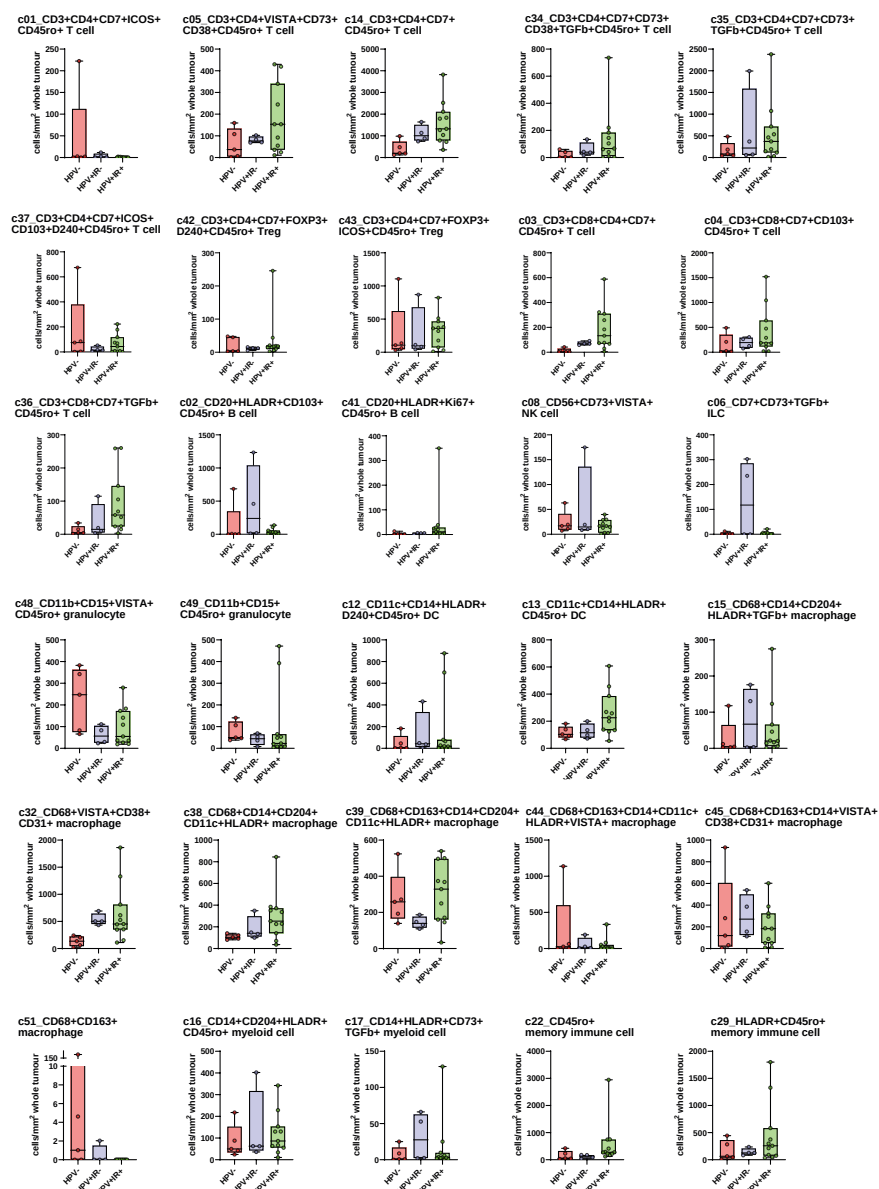
Expression levels of *CD19*, *MS4A1* (*CD20*) and *BLK* are depicted in color code. Violin plot (right) displaying expression of *BLK* within B cells from HPV16⁺IR⁺ (blue; left) and HPV16⁺IR⁻ (orange; right) OPSCC. **D)** Kaplan-Meier survival curves based on high/low *BLK* or *CXCL12* expression (classification based on median *BLK* or *CXCL12* expression) upregulated in HPV16⁺IR⁺ compared to HPV16⁺IR⁻ OPSCC patients for all OPSCC patients analyzed by Nanostring Pancancer IO360 (n=21; left), for the HPV16⁺ patients within this cohort (n=13; middle), and for a large independent TCGA cohort of HPV16⁺ OPSCC (n=69; right). **E)** Kaplan-Meier survival curves based on high/low expression of the top 3 DEGs (*CCL20*, *BMP2* and *CXCL3*) upregulated in HPV16⁺IR⁺ compared to HPV16⁺IR⁻ OPSCC patients for all OPSCC patients analyzed by Nanostring Pancancer IO360 (n=21; left), for the HPV16⁺ patients within this cohort (n=13; middle), and for a large independent TCGA cohort of HPV16⁺ OPSCC (n=69; right). **F)** Linear regression analysis of top upregulated DEGs *BLK* and *CXCL12* in HPV16⁺IR⁺ compared to HPV16⁺IR⁻ OPSCC patients cell type profiles of CD8 (*CD8A*), CD4, Tbet⁺ T cells (*TBX21*) and DC (*ITGAX*, *CD11c*). Upper 2 panels display all OPSCC patients analyzed by Nanostring Pancancer IO360 (n=21). Each patient is represented by a colored dot: HPV⁻ (red), HPV16⁺IR⁻ (blue) and HPV16⁺IR⁺ (green). The lower 2 panels show the regression analysis of the 69 HPV16⁺ OPSCC patients of the independent TCGA cohort. **G)** Linear regression analysis of *LTB* versus indicated genes involved in tumor cell migration (metastases) and cell activation.



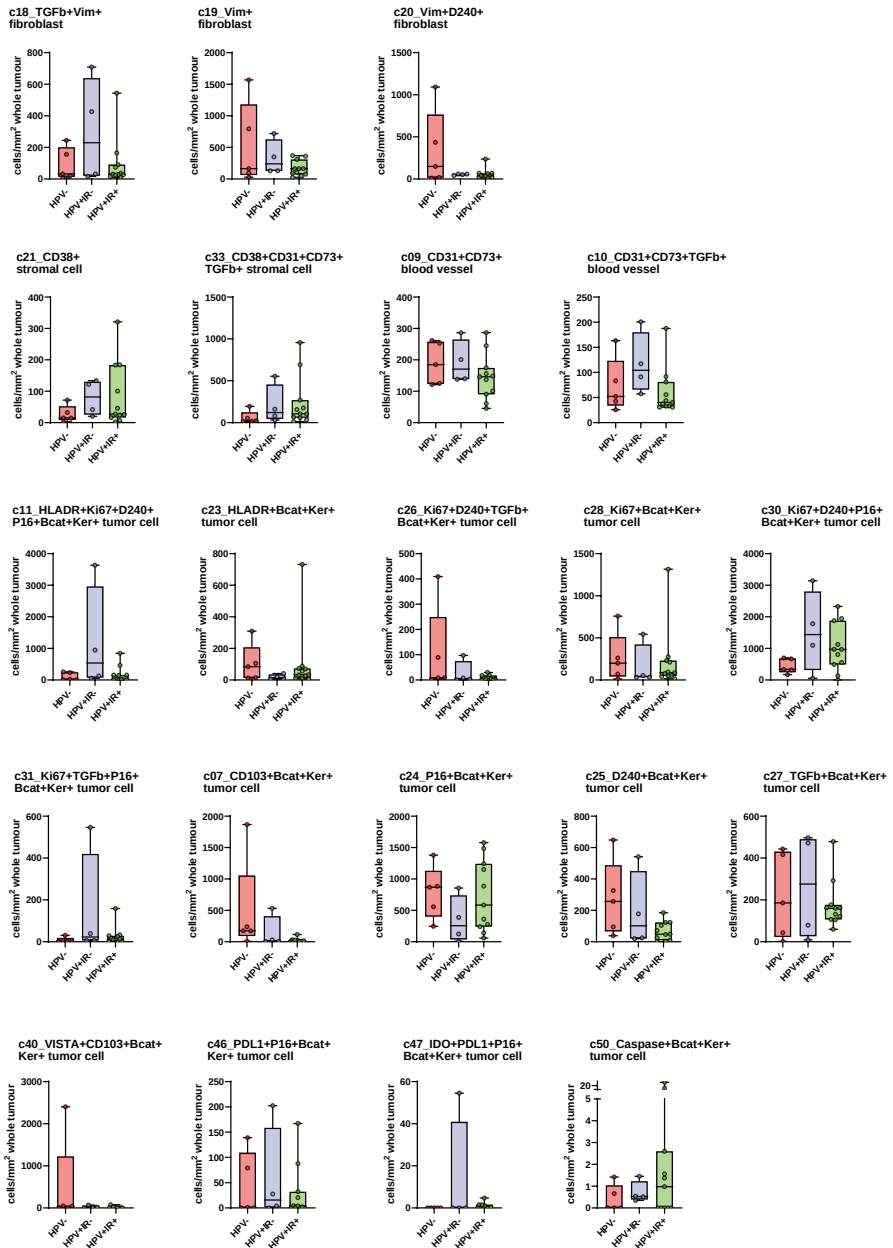
Supplemental Figure 3. Imaging mass cytometry identified (super)clusters in the TME of OPSCC.

Using the unsupervised HSNE-based clustering program Cytosplore, 51 individual cell clusters were identified. **A**) Heatmap of the verified markers expressed in the identified clusters (c01-c51), and the manual grouping into mutually exclusive superclusters (sc01-sc18). **B**) Quantitative comparisons of the remaining 13 of the 18 superclusters present in OPSCC. The three distinct OPSCC subgroups are indicated by different colors: HPV- red (n=5), HPV16+IR- blue (n=4) and HPV16+IR+ green (n=11). Boxplots with bars in box representing the median and interquartile range.

A

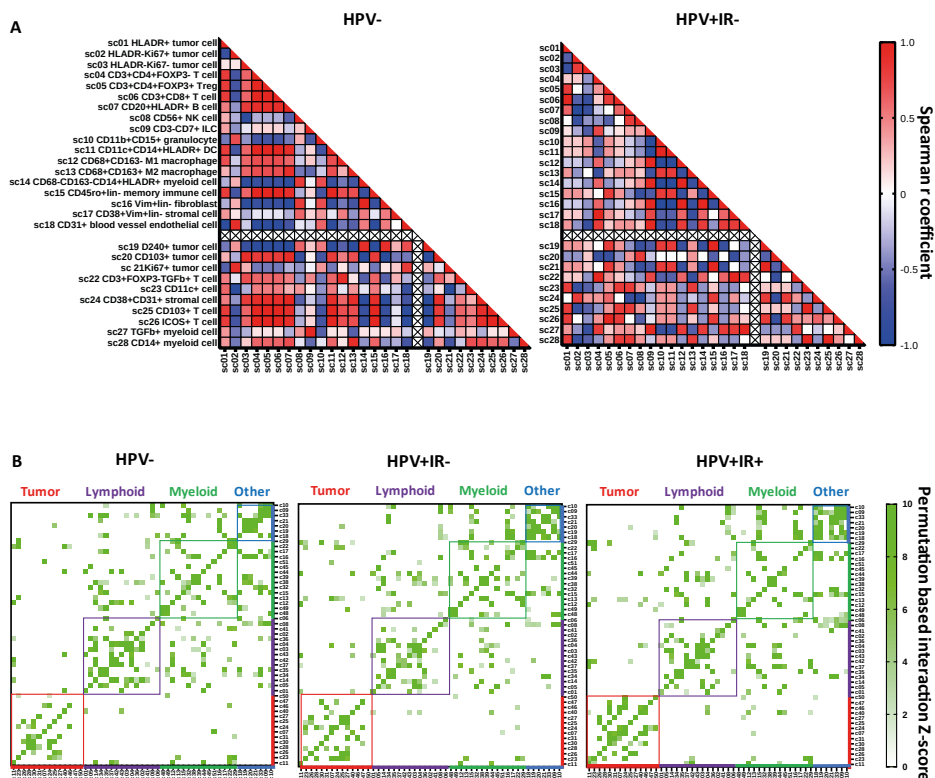


Supplemental Figure 5. Continued

B

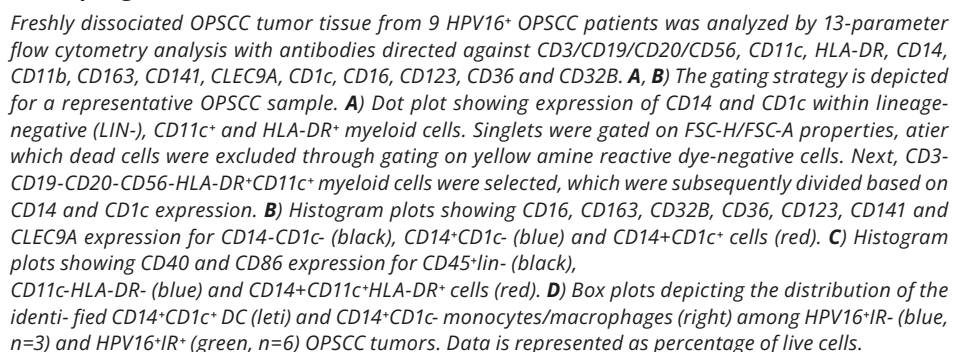
Supplemental Figure 5. Imaging mass cytometry quantitative findings 51 clusters.

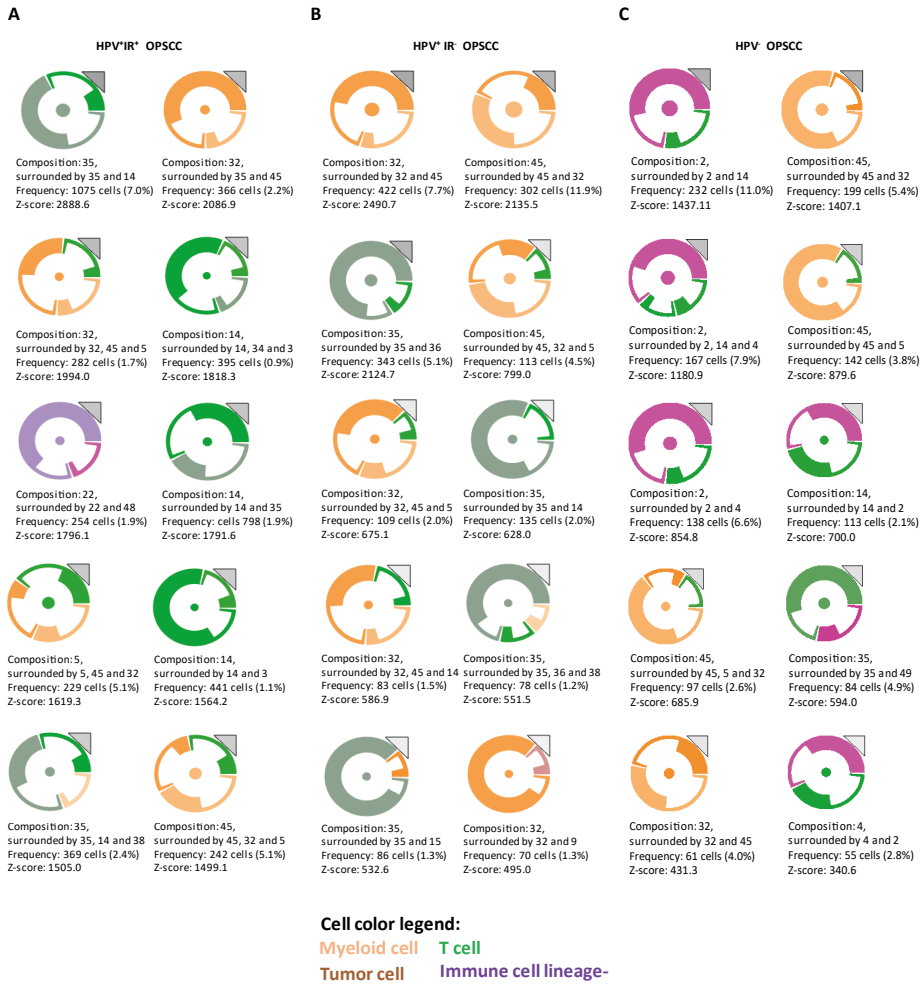
Boxplots showing quantitative comparisons of all 51 identified different cell clusters present in the whole tumor. In **A**) the immune cell clusters and in **B**) the other cell clusters. The three distinct OPSCC subgroups are indicated by different dot colors: HPV- red (n=5), HPV16+IR- blue (n=4) and HPV16+IR+ green (n=11). Boxplots with bars in box representing the median and interquartile range



Supplemental Figure 6. Permutation testing of spatial cellular interactions.

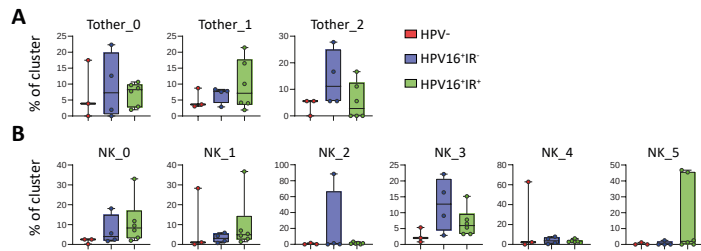
A) Spearman quantitative correlation heatmaps of the 28 superclusters (18 mutually exclusive, and 10 non-mutually exclusive) in the whole tumor for HPV- ($n=5$) and HPV16+IR- ($n=4$) OPSCC. **B)** Heatmap of permutation based Z-scores for spatial interactions between the 51 identified clusters, in HPV- ($n=5$), HPV16+IR- ($n=4$) and HPV16+IR+ ($n=11$) OPSCC. Only interactions with a permutation based Z-score >2 (i.e. outside the 95% normal distribution range) are visualized.





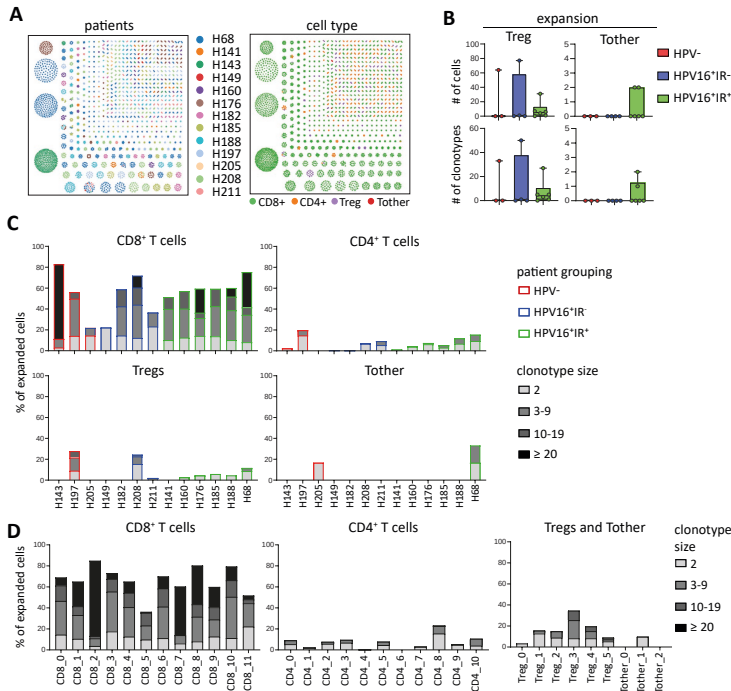
Supplemental Figure 8. Top 10 360° spatial immune compositions per OPSCC subgroup.

Top 10 360° spatial immune compositions with the highest Z-scores are depicted. In **A**) HPV16+IR+ (n=11), **B**) HPV16+IR- (n=4), and **C**) HPV- (n=5) OPSCC. Clusters, frequency and Z-score are described below each composition. Interaction neighborhood is defined as 5µm (direct spatial cellular interaction). Threshold for the 360° compositions: ≥20 occurrences.



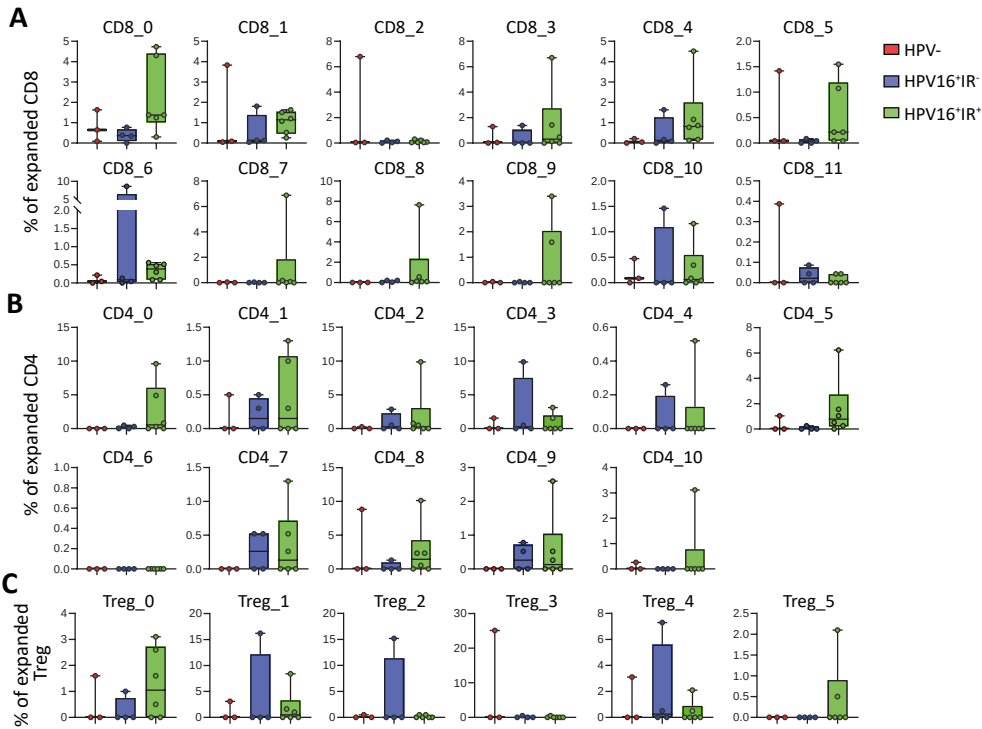
Supplemental Figure 9. Distribution of the identified clusters among HPV-, HPV16+IR- and HPV16+IR+ OPSCC tumors.

Magnetic-bead sorted CD3+ T cells and CD56+ NK cells from 13 OPSCC samples were analyzed by integrated single-cell transcriptome and TCR repertoire RNA sequencing analysis. Box plots depict the distribution of the Tother (A) and NK (B) cell clusters among HPV- (red, n=3), HPV16+IR- (blue, n=4) and HPV16+IR+ (green, n=6) OPSCC tumors. Data is represented as percentage of cluster. * p-value<0.05.



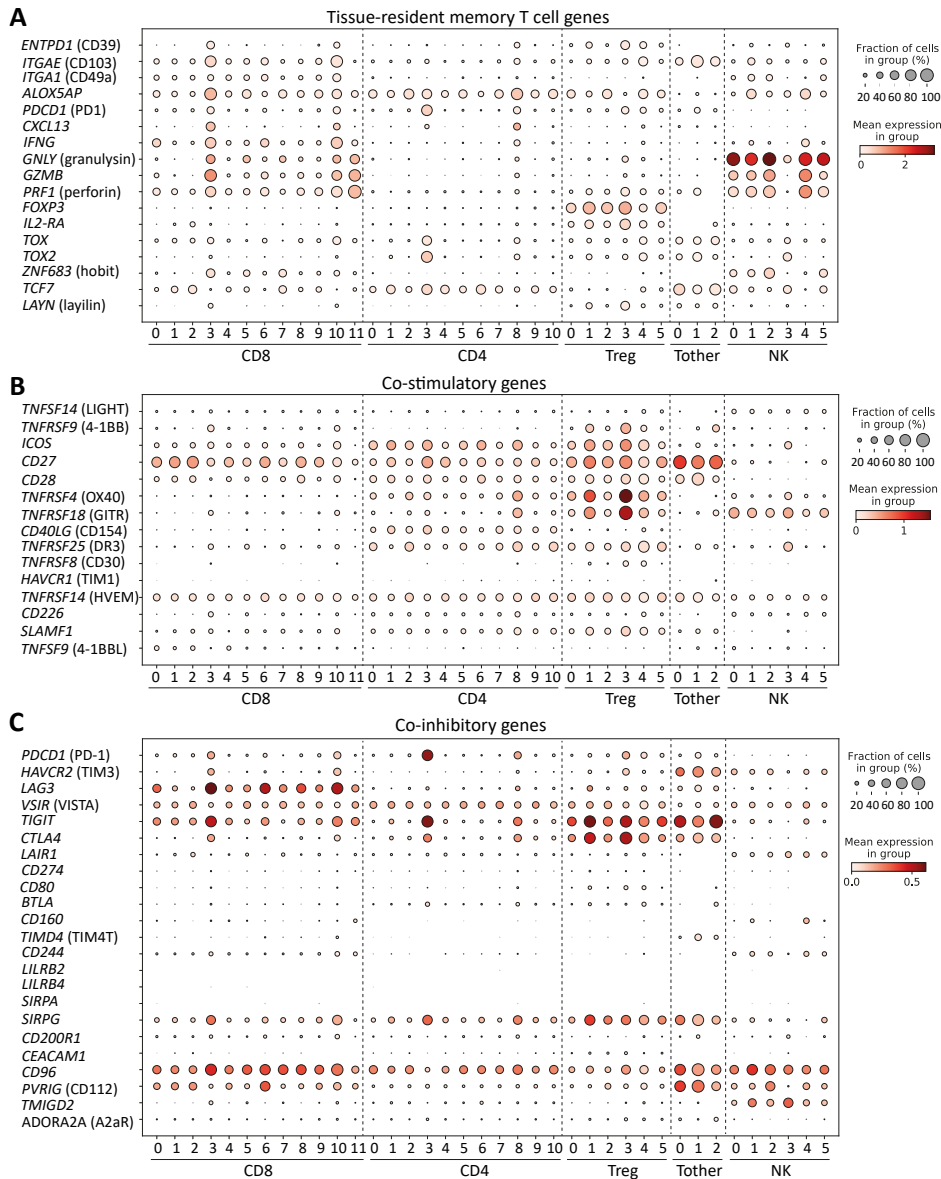
Supplemental Figure 10. Clonotype size among individual OPSCC and T cell clusters. Magnetic-bead sorted CD3+ T cells and CD56+ NK cells from 13 OPSCC samples were analyzed by integrated single-cell transcriptome and TCR repertoire RNA sequencing analysis.

A) Graphs depicting the expanded clonotype clusters, colored by patient (left) and cell type (right). B) Box plots displaying the number of expanded cells (top) and clonotypes (bottom) within Tregs (left) and Tother (right) cells detected in HPV- (red, n=3), HPV16+IR- (blue, n=4) and HPV16+IR+ (green, n=6) OPSCC patients. C, D) Graphs depicting the clonotype size of the expanded TCR in CD8+ T cells, CD4+ T cells, Treg and Tother cells per patient (C) and per cluster (D). Data is given as percentage of expanded CD8, CD4, Treg or Tother cells. Colored outline indicates the HPV- and immune response status (HPV- (red, n=3), HPV16+IR- (blue, n=4) and HPV16+IR+ (green, n=6) OPSCC patients).



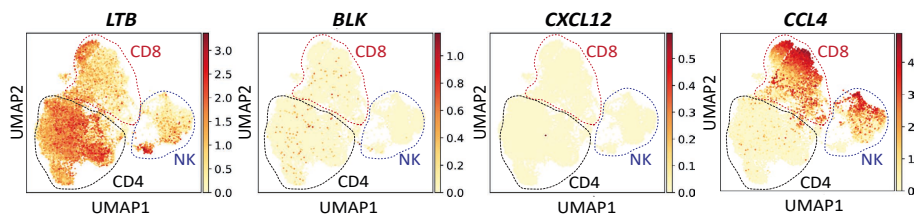
Supplemental Figure 11. T cell expansion among the different T cell clusters.

Integrated single-cell transcriptome and TCR repertoire RNA sequencing analysis was performed on magnetic-bead sorted CD3+ T cells and CD56+ NK cells from 13 OPSCC samples. A-C) Box plots displaying the percentage of expanded cells within the identified CD8 (A), CD4 (B) and Treg (C) clusters in HPV- (red, n=3), HPV16+IR- (blue, n=4) and HPV16+IR+ (green, n=6) OPSCC patients. Data are represented as percentage of total expanded CD8, CD4 and Treg cells.



Supplemental Figure 12. Expression of tissue-resident memory T cell, co-stimulatory and co-inhibitory markers within the intratumoral CD8, CD4, Treg, Tother and NK cell cluster in OPSCC.

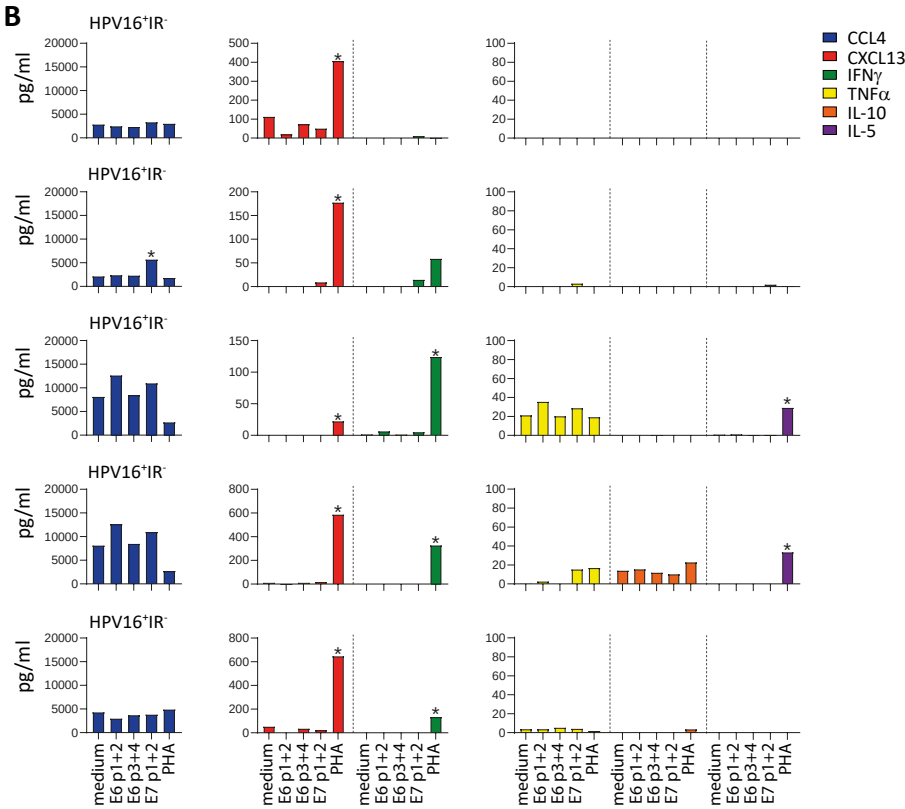
Single-cell transcriptome RNA sequencing analysis was performed on magnetic-bead sorted CD3⁺ T cells and CD56⁺ NK cells from 13 OPSCC samples. Dot plots depict the expression levels of **A)** tissue-resident memory T cell, **B)** co-stimulatory and **C)** co-inhibitory genes (Y-axis) for all identified CD8, CD4, Treg, Tother and NK clusters (X-axis, from left to right). The size of the dots represent the percentage of cells expressing the genes, and the color scale indicates the mean expression of the genes.



Supplemental Figure 13. Expression of LTB, BLK, CXCL12, CCL4, XCL1 and LMNA within T cells and NK cells.

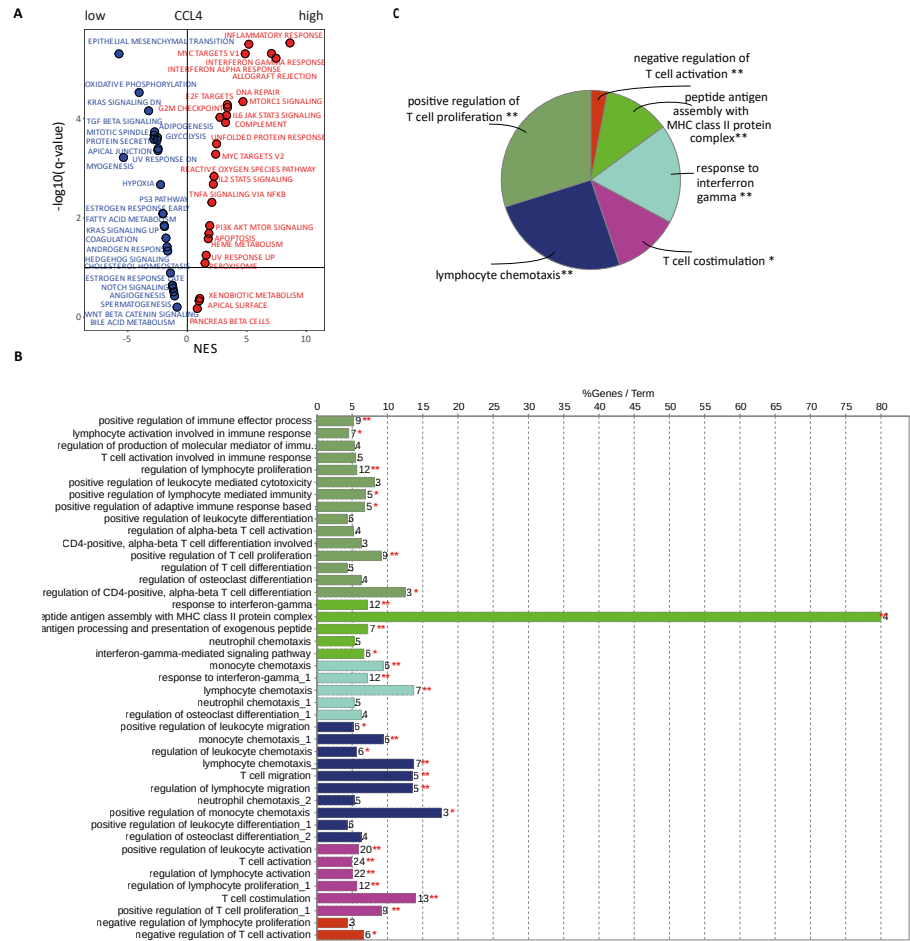
Two-dimensional UMAP plots displaying single cell transcriptomics of 14,242 T cells and 2,820 NK cells from 13 OPSCC patients. Each dot represents a single cell. Expression levels of LTB, BLK, CXCL12 and CCL4 are depicted in color code. Dotted borders denote where CD4, CD8 and NK cell clusters are located.





Supplemental Figure 14. HPV reactivity of cultured OPSCC TIL.

Graphs displaying CCL4 (blue), CXCL13 (red), IFN γ (green), TNF α (yellow), IL-10 (orange) and IL-5 (purple) production of cultured OPSCC TIL in response to medium, HPV16 E6 peptide (pool 1+2 and 3+4), HPV16 E7 peptide (pool 1+2)-loaded autologous monocytes for HPV16*IR⁺ (n=9; **A**) and HPV16*IR⁻ (n=5; **B**) OPSCC. Cytokine production in response to PHA served as positive control. Positive cytokine production, which is defined as at least twice above that of the cells stimulated with medium-loaded monocytes, is indicated by the asterisk. CCL4 and CXCL13 were determined by a multiplex cytokine assay and IFN γ , TNF α , IL-10 and IL-5 by cytometric bead array.



Supplemental Figure 15. CCL4 expression is associated with a productive tumor immune microenvironment.

A) High expression of CCL4 is positively associated with the hallmark gene sets of tumor rejection and negatively associated with the hallmarks of tumor promotion. The log2 fold change level of each gene between a high and low group was used as input for the GSEA-preranked. The GSEA was performed using the Molecular Signatures Database (MSigDB). The 50 hallmark gene sets were illustrated by their normalized enrichment score (NES). Red and blue dots show the enrichment and depletion of hallmark gene sets, respectively. **B)** ClueGO analysis of highly significant associated genes with high expression of CCL4 in the tumor microenvironment ($R > 0.7$ and $R > 0.4$, respectively, $\text{adj-value} < 0.05$). The bars represent the percentage of highly significantly correlated genes associated with the GO terms (%Genes/term). The number of expressed genes per term is shown as bar label. P-value is indicated by asterisks. **C)** Overview chart with functional groups including specific terms for highly significantly associated genes with high expression of CCL4. * mid-P values of two-sided (enrichment/depletion) tests based on the hyper geometric distribution (Rivals, 2007, PMID. 17182697).

Supplemental Tables

Supplemental Table 1. OPSCC patient characteristics.

Patient ID *	Sex	Age **	Tumor location	pTNM stage	HPV16 status	IR status ***	Received treatment	Analysis techniques
H62	M	61	Tongue base	4a-2c-0	+	-	RT	NanoString
H68	F	64	Tonsil	3-2b-0	+	+	RT	Hyperion, scRNAseq, NanoString, Luminex
H71	F	62	Tongue base	2-2c-0	+	+	RT	Hyperion, NanoString
H72	M	58	Tongue base	2-0-0	-	-	RT	Hyperion, NanoString
H74	F	65	Tonsil	4a-0-0	-	-	none	Hyperion, NanoString
H77	M	48	Tonsillar fossa	1-2a-0	+	+	S+RT	Hyperion, NanoString
H78	M	54	Tonsillar fossa	2-0-0	-	-	RT	NanoString
H93	M	57	Tongue base	1-2b-0	+	+	S+RT	Hyperion, NanoString
H95	M	59	Tongue base	2-1-0	+	+	RT	NanoString
H125	M	64	Posterior wall	3-0-0	-	-	CT+RT	NanoString
H136	V	47	Tonsil	2-2b-0	+	+	CT+RT	Hyperion, NanoString, Luminex
H138	M	74	Tongue base	2-0-0	+	+	RT	Hyperion, Luminex
H141	M	67	Tongue base	2-2b-0	+	+	CT+RT	Hyperion, scRNAseq, NanoString
H143	V	74	Tonsil	1-2a-0	-	-	RT	Hyperion, scRNAseq, NanoString
H148	V	64	Tonsil	2-2b-0	+	+	S+RT	Luminex
H149	V	47	Tonsil	2-2a-0	+	-	S+RT	Hyperion, scRNAseq, NanoString, Luminex
H150	M	59	Tonsil	2-2a-0	+	-	S+RT	Luminex
H159	V	67	Tonsil	4a-2c-0	+	+	CT+RT	Luminex
H160	M	69	Tongue base	2-2b-0	+	+	CT+RT	Hyperion, scRNAseq, NanoString, Luminex
H161	V	65	Tongue base	2-2b-0	+	+	S+RT	Luminex

Supplemental Table 1 Continued

Patient ID *	Sex	Age **	Tumor location	pTNM stage	HPV16 status	IR status ***	Received treatment	Analysis techniques
H176	M	52	Tongue base	3-0-0	+	+	CT+RT	Hyperion, scRNAseq, NanoString
H180	M	59	Tonsil	2-2a-0	+	+	RT	Luminex
H182	M	66	Tongue base	4-3-0	+	-	CT	Hyperion, scRNAseq, NanoString
H185	M	58	Tongue base	2-0-0	+	+	RT	Hyperion, scRNAseq, NanoString
H187	V	67	Pallatum mole	2-0-0	-	-	RT	NanoString
H188	V	56	Tonsil	3-0-0	+	+	CT+RT	Hyperion, scRNAseq, NanoString, Luminex
H191	M	39	Tonsil	2-2b-0	+	-	RT	Luminex
H197	V	63	Tonsil	4b-0-0	-	-	CT+RT+CX	Hyperion, scRNAseq, NanoString
H205	M	56	Tonsil	4a-2b-1	-	-	RT	Hyperion, scRNAseq, NanoString
H208	M	60	Tongue base	4-2a-0	+	-	CT+RT	Hyperion, scRNAseq, NanoString, Luminex
H211	M	62	Tongue base	3-2b-0	+	-	CT+RT	Hyperion, scRNAseq, NanoString, Luminex

* 'H' indicates OPSCC patients included in the P07-112 head and neck cancer study

** Age at diagnosis and sampling of tumor tissue pre-therapy

*** Immune response (IR) status was determined by analyzing cultured tumor infiltrating lymphocytes for the presence of HPV16-specific T cells using a [3H]-thymidine-based proliferation assay and antigen-specific cytokine production assay

CT: chemotherapy; CX: cetuximab; F: female; M: male; pTNM: pathological Tumor, lymph Nodes, Metastasis; RT: radiotherapy; S: surgical resection

Supplemental Table 2. Definition of NanoString nSolver cell types.

Cell type	Gene	Cell type	Gene
CD45	<i>PTPRC</i>		<i>CTSW</i>
	<i>CD3D</i>		<i>GNLY</i>
	<i>CD3E</i>		<i>GZMA</i>
T cells	<i>CD3G</i>		<i>GZMB</i>
	<i>CD6</i>	Cytotoxic cell	<i>GZMH</i>
	<i>SH2D1A</i>		<i>KLRB1</i>
	<i>TRAT1</i>		<i>KLRD1</i>
	<i>BLK</i>		<i>KLRK1</i>
	<i>CD19</i>		<i>NKG7</i>
	<i>FCRL2</i>		<i>PRF1</i>
B cells	<i>MS4A1</i>		<i>CD163</i>
	<i>PNOC</i>	Macrophage	<i>CD68</i>
	<i>SPIB</i>		<i>CD84</i>
	<i>TCL1A</i>		<i>MS4A4A</i>
	<i>TNFRSF17</i>		<i>CEACAM3</i>
	<i>CCL13</i>		<i>CSF3R</i>
DC	<i>CD209</i>	Neutrophils	<i>FCAR</i>
	<i>HSD11B1</i>		<i>FPR1</i>
			<i>S100A12</i>
CD8	<i>CD8A</i>		<i>SIGLEC5</i>
	<i>CD8B</i>		
Th1	<i>TBX21</i>	NK cells	<i>NCR1</i>
Treg	<i>FOXP3</i>		<i>IL21R</i>
	<i>CD244</i>	NK CD56dim	<i>KIR2DL3</i>
	<i>EOMES</i>		<i>KIR3DL1</i>
Exhausted CD8	<i>LAG3</i>		<i>KIR3DL2</i>
	<i>PTGER4</i>		<i>CPA3</i>
		Mast cells	<i>HDC</i>
			<i>MS4A2</i>

Cell types as automatically defined by the expression of the indicated genes by NanoString nSolver software

Supplemental Table 3. Imaging mass cytometry 33-marker panel.

Marker	Antibody clone	Conjugated heavy metal	Antibody dilution	Incubation conditions
HLA-DR	TAL1B5	¹⁴¹ Pr	1:100	5 hours at room temperature
CD11b	D6X1N	¹⁴⁴ Nd	1:100	5 hours at room temperature
CD4	EPR6855	¹⁴⁵ Nd	1:50	5 hours at room temperature
CD8	D8A8Y	¹⁴⁶ Nd	1:50	5 hours at room temperature
CD73	D7F9A	¹⁴⁸ Nd	1:100	5 hours at room temperature
TGFβ	TB21	¹⁴⁹ Sm	1:100	5 hours at room temperature
PD-L1	E1L3N(R)	¹⁵⁶ Gd	1:100	5 hours at room temperature
FoxP3	D608R	¹⁵⁹ Tb	1:100	5 hours at room temperature
IDO	D5J4E(TM)	¹⁶² Dy	1:50	5 hours at room temperature
CD204	J5HTR3	¹⁶⁴ Dy	1:100	5 hours at room temperature
CD45ro	UCHL1	¹⁶⁵ Ho	1:50	5 hours at room temperature
CD38	EPR4106	¹⁶⁹ Tm	1:50	5 hours at room temperature
CD163	EPR14643-36	¹⁷³ Yb	1:100	5 hours at room temperature
CD7	EPR4242	¹⁷⁴ Yb	1:50	5 hours at room temperature
P16	D3W8G	¹⁷⁵ Lu	1:100	5 hours at room temperature
Vimentin	D21H3	¹⁹⁴ Pt	1:100	5 hours at room temperature
β-catenin	D10A8	¹¹⁵ In	1:100	overnight at 4 degrees Celsius
CD20	H1	¹⁴² Nd	1:100	overnight at 4 degrees Celsius
CD68	D4B9C	¹⁴³ Nd	1:100	overnight at 4 degrees Celsius
CD31	89C2	¹⁴⁷ Sm	1:100	overnight at 4 degrees Celsius
CD57	HNK-1/Leu-7	¹⁵¹ Eu	1:100	overnight at 4 degrees Celsius
Ki67	8D5	¹⁵² Sm	1:100	overnight at 4 degrees Celsius
CD3	D7AGE(TM)	¹⁵³ Eu	1:50	overnight at 4 degrees Celsius
VISTA	D1L2G(TM)	¹⁵⁸ Gd	1:50	overnight at 4 degrees Celsius
ICOS	D1K2T(TM)	¹⁶¹ Dy	1:50	overnight at 4 degrees Celsius
CD14	D7A2T	¹⁶³ Dy	1:100	overnight at 4 degrees Celsius
D2-40	D2-40	¹⁶⁶ Er	1:100	overnight at 4 degrees Celsius
CD56	EPR2566	¹⁶⁷ Er	1:100	overnight at 4 degrees Celsius
CD103	EPR4166(2)	¹⁶⁸ Er	1:100	overnight at 4 degrees Celsius
CD15	BRA-4F1	¹⁷¹ Yb	1:100	overnight at 4 degrees Celsius
Cleaved-caspase	ASP175	¹⁷² Yb	1:100	overnight at 4 degrees Celsius
CD11c	EP1347Y	¹⁷⁶ Yb	1:100	overnight at 4 degrees Celsius
Keratin	C11 and AE1/ AE3	¹⁹⁸ Pt	1:50	overnight at 4 degrees Celsius

Supplemental Table 4A. Differentially expressed genes (DEGs) between HPV16+IR+ and HPV16+IR- OPSCC patients.

DEG	Log2 fold change	SD	P-value	Method	Corrected P-value
CCL20	-3.67	0.647	2.20E-05	Lm.nb	0.0124
BMP2	-2.68	0.512	5.60E-05	Lm.nb	0.0124
BLK	5.85	1.13	6.35E-05	Lm.nb	0.0124
HLA-DRB1	3.55	0.705	8.73E-05	Lm.nb	0.0124
CXCL3	-3.25	0.652	9.56E-05	Lm.nb	0.0124
LTB	5.22	1.06	0.00011	Lm.nb	0.0124
PFKFB3	-1.69	0.347	0.000123	Lm.nb	0.0124
CXCL12	4.59	0.98	0.000186	Lm.nb	0.0164
CCL19	4.1	0.943	0.000386	Lm.nb	0.028
CSF2	-4.26	0.984	4.00E-04	Lm.nb	0.028
TNFSF13	4.08	0.962	0.000489	Lm.nb	0.028
NLR5	1.84	0.439	0.000539	Lm.nb	0.028
HLA-DQA2	-6.12	1.45	0.000586	Wald	0.028
HSD11B1	3.21	0.771	0.000639	Wald	0.028
TNFRSF18	3.71	0.9	0.00064	Lm.nb	0.028
PIK3CD	3.03	0.745	0.000721	Lm.nb	0.028
HLA-A	2.87	0.706	0.000737	Lm.nb	0.028
TREM1	-2.56	0.633	0.000762	Lm.nb	0.028
INHBA	-3.93	0.98	0.000819	Lm.nb	0.028
IL1B	-3.16	0.791	0.000834	Lm.nb	0.028
LILRB4	3	0.751	0.000837	Lm.nb	0.028
HRA5	3.66	0.921	0.000891	Lm.nb	0.028
KLRD1	3.38	0.845	0.000913	Wald	0.028
CXCL8	-3.65	0.928	0.000968	Lm.nb	0.0285
IL11	-3.43	0.885	0.00112	Lm.nb	0.0308
IKBKB	2.79	0.724	0.00115	Lm.nb	0.0308
HLA-F	3.62	0.946	0.00123	Lm.nb	0.0308
CD79A	3.74	0.985	0.00132	Lm.nb	0.0308
TNFSF9	3.57	0.931	0.00132	Wald	0.0308
APOL6	2.87	0.755	0.00132	Lm.nb	0.0308
CD58	2.59	0.684	0.00135	Lm.nb	0.0308
PNOC	2.84	0.75	0.00148	Wald	0.0325
IRF5	1.55	0.417	0.00156	Lm.nb	0.0325
NEIL1	2.11	0.569	0.00159	Lm.nb	0.0325
IRF4	2.97	0.801	0.00161	Lm.nb	0.0325
IL6R	2.16	0.586	0.00167	Lm.nb	0.0326
LYZ	1.79	0.487	0.00171	Lm.nb	0.0326
CD6	2.59	0.709	0.0018	Lm.nb	0.0329
PGPEP1	2.61	0.715	0.00182	Lm.nb	0.0329
GZMM	2.41	0.656	0.0019	Wald	0.0336
PSMB10	2.03	0.577	0.00246	Lm.nb	0.0424
CTSW	2.79	0.798	0.00258	Lm.nb	0.0434
TGFB1	2.9	0.845	0.00295	Lm.nb	0.0474

Supplemental Table 4B. Differentially expressed genes (DEGs) between HPV16+IR- and HPV- OPSCC patients.

DEG	Log2 fold change	SD	P-value	Method	Corrected P-value
<i>CCL20</i>	3.25	0.628	6.43E-05	lm.nb	0.0454

Supplemental Table 4C. Differentially expressed genes (DEGs) between HPV16+IR+ and HPV- OPSCC patients.

DEG	Log2 fold change	SD	P-value	Method	Corrected P-value
<i>CXCL12</i>	5.74	0.912	6.21E-06	lm.nb	0.00259
<i>LTB</i>	6.04	0.986	8.60E-06	lm.nb	0.00259
<i>KLRD1</i>	4.19	0.743	2.92E-05	Wald	0.00259
<i>BBC3</i>	2.71	0.493	3.22E-05	lm.nb	0.00259
<i>BLK</i>	5.75	1.05	3.40E-05	lm.nb	0.00259
<i>PIK3CD</i>	3.73	0.693	4.06E-05	lm.nb	0.00259
<i>TNFSF13</i>	4.8	0.895	4.28E-05	lm.nb	0.00259
<i>IKBKB</i>	3.61	0.673	4.29E-05	lm.nb	0.00259
<i>UBA7</i>	4.44	0.831	4.42E-05	lm.nb	0.00259
<i>HRAS</i>	4.57	0.856	4.49E-05	lm.nb	0.00259
<i>APOL6</i>	3.73	0.702	4.77E-05	lm.nb	0.00259
<i>HLA-F</i>	4.65	0.88	5.06E-05	lm.nb	0.00259
<i>C5</i>	3.3	0.628	5.40E-05	lm.nb	0.00259
<i>DNMT1</i>	3.69	0.703	5.43E-05	lm.nb	0.00259
<i>DDB2</i>	3.49	0.666	5.50E-05	lm.nb	0.00259
<i>ICAM5</i>	5.15	0.988	5.89E-05	lm.nb	0.0026
<i>GNLY</i>	3.58	0.704	7.82E-05	lm.nb	0.00325
<i>GZMB</i>	3.65	0.712	8.35E-05	Wald	0.00327
<i>POLD1</i>	1.78	0.357	9.52E-05	lm.nb	0.00329
<i>LILRB4</i>	3.48	0.698	9.58E-05	lm.nb	0.00329
<i>VHL</i>	2.91	0.586	9.77E-05	lm.nb	0.00329
<i>CD58</i>	3.13	0.637	0.000113	lm.nb	0.00362
<i>GLS</i>	4.02	0.831	0.000134	lm.nb	0.00406
<i>CCL19</i>	4.22	0.877	0.000138	lm.nb	0.00406
<i>DLL4</i>	3.03	0.636	0.000157	lm.nb	0.00426
<i>TGFB1</i>	3.73	0.786	0.00016	lm.nb	0.00426
<i>PGPEP1</i>	3.16	0.666	0.000163	lm.nb	0.00426
<i>MSH6</i>	3.56	0.757	0.000177	lm.nb	0.00446
<i>CCND1</i>	-2.87	0.612	0.000186	lm.nb	0.00453
<i>PIK3R2</i>	3.52	0.758	0.000203	lm.nb	0.00477
<i>CTLA4</i>	3.04	0.659	0.000212	lm.nb	0.00477
<i>CSF1</i>	2.87	0.623	0.000216	lm.nb	0.00477
<i>NLRP3</i>	4.44	0.97	0.000235	lm.nb	0.00503
<i>CD7</i>	3.14	0.691	0.000252	lm.nb	0.00516
<i>CD80</i>	2.73	0.604	0.000261	lm.nb	0.00516
<i>IRF7</i>	1.43	0.316	0.000263	lm.nb	0.00516
<i>HSD11B1</i>	3.23	0.707	0.000277	Wald	0.00529
<i>FANCA</i>	2.29	0.513	0.000301	lm.nb	0.00559
<i>EZH2</i>	1.02	0.228	0.000309	lm.nb	0.0056

Tumor-specific T cells support immune microaggregates needed for survival

DEG	Log2 fold change	SD	P-value	Method	Corrected P-value
IRF1	2.44	0.549	0.000318	lm.nb	0.0056
CSF2RB	2.75	0.622	0.00033	lm.nb	0.00568
JAK2	2.41	0.55	0.000361	lm.nb	0.00585
HLA-DRB1	2.87	0.656	0.000364	lm.nb	0.00585
NLRCS	1.78	0.408	0.000377	lm.nb	0.00585
NOTCH1	2.32	0.531	0.000377	lm.nb	0.00585
TNFRSF25	2.29	0.526	0.000389	lm.nb	0.00585
CHUK	2.66	0.611	0.000393	lm.nb	0.00585
AXL	3.3	0.761	0.000397	lm.nb	0.00585
JAG2	3.41	0.782	0.000427	Wald	0.00615
MB21D1	0.921	0.215	0.000455	lm.nb	0.00632
CCL18	2.68	0.626	0.000456	lm.nb	0.00632
PPARGC1B	2.1	0.494	0.000472	lm.nb	0.00641
ULBP2	-2.19	0.518	0.000505	lm.nb	0.00654
TMEM140	2.53	0.599	0.000507	lm.nb	0.00654
CYBB	2.78	0.657	0.000509	lm.nb	0.00654
PSMB10	2.25	0.537	0.000555	lm.nb	0.00689
LGALS9	2.78	0.664	0.000557	lm.nb	0.00689
NCR1	2.86	0.682	0.000616	Wald	0.00729
CD68	2.7	0.653	0.000617	lm.nb	0.00729
TNFSF9	3.65	0.873	0.000622	Wald	0.00729
TNFRSF18	3.46	0.837	0.000629	lm.nb	0.00729
RAD51C	2.42	0.591	0.000687	lm.nb	0.00782
CD79A	3.74	0.916	0.000707	lm.nb	0.00792
SIGLEC1	2.65	0.651	0.00073	lm.nb	0.00805
CTSW	3.01	0.743	0.000747	lm.nb	0.00811
PVRIG	2.82	0.699	0.000776	lm.nb	0.0082
MLH1	1.72	0.427	0.000791	lm.nb	0.0082
TIE1	2.82	0.7	0.000798	lm.nb	0.0082
BRD3	2.56	0.638	0.000801	lm.nb	0.0082
MTOR	1.32	0.333	0.000892	lm.nb	0.00885
CD6	2.62	0.659	0.000898	lm.nb	0.00885
TYMS	2.51	0.632	0.000902	lm.nb	0.00885
MELK	1.17	0.295	0.000918	lm.nb	0.00887
IL6R	2.15	0.545	0.00093	lm.nb	0.00887
NFAM1	2.24	0.564	0.000978	Wald	0.00911
TAP2	1.62	0.413	0.00098	lm.nb	0.00911
CD74	2.57	0.656	0.00102	lm.nb	0.00936
JAK3	2.42	0.622	0.00106	lm.nb	0.00958
ZAP70	3.07	0.789	0.00107	lm.nb	0.00958
RELA	2.53	0.652	0.0011	lm.nb	0.0097
NFKB2	2.37	0.612	0.00112	lm.nb	0.00973
BAX	1.24	0.322	0.00115	lm.nb	0.00991
CDK2	1.5	0.393	0.00127	lm.nb	0.0107
TNFRSF1B	2.14	0.563	0.00132	lm.nb	0.0108
IRF3	1.18	0.312	0.00136	lm.nb	0.0108
ITGAL	2.82	0.745	0.00136	lm.nb	0.0108
STAT2	1.13	0.298	0.00137	lm.nb	0.0108
HLA-C	2.67	0.708	0.00138	lm.nb	0.0108

Supplemental Table 4C *Continued*

DEG	Log2 fold change	SD	P-value	Method	Corrected P-value
HLA-DMA	2.4	0.637	0.00139	lm.nb	0.0108
HELLS	1.45	0.385	0.0014	lm.nb	0.0108
SBNO2	1.25	0.331	0.0014	lm.nb	0.0108
IRF4	2.78	0.745	0.00153	lm.nb	0.0113
FUT4	2.68	0.72	0.00153	lm.nb	0.0113
CD3D	2.84	0.762	0.00156	lm.nb	0.0113
DUSP5	1.52	0.409	0.00156	lm.nb	0.0113
IKBK	2.1	0.563	0.00156	lm.nb	0.0113
TREM1	-2.18	0.59	0.00165	lm.nb	0.0118
CD8A	3.13	0.846	0.00166	lm.nb	0.0118
CCL4	1.85	0.503	0.00169	lm.nb	0.0119
TNFSF12	1.91	0.519	0.00175	lm.nb	0.0122
BAD	2.19	0.597	0.00176	lm.nb	0.0122
ITGB2	1.69	0.462	0.00178	lm.nb	0.0122
TRAF1	2.35	0.644	0.00181	lm.nb	0.0122
VSIR	1.87	0.511	0.00181	lm.nb	0.0122
NECTIN2	1.86	0.512	0.00187	lm.nb	0.0125
CX3CL1	1.66	0.462	0.00204	lm.nb	0.0135
TNFRSF14	1.68	0.466	0.00206	lm.nb	0.0135
CD47	1.6	0.445	0.00213	lm.nb	0.0138
CCL5	2.36	0.667	0.00232	lm.nb	0.0149
APOE	2.29	0.653	0.00249	lm.nb	0.0159
TAPBPL	1.38	0.394	0.00253	lm.nb	0.0159
PNOC	2.49	0.707	0.00266	Wald	0.0166
PDGFRB	2.6	0.748	0.00268	lm.nb	0.0166
CXCL9	2.55	0.735	0.00273	lm.nb	0.0168
TMEM173	1.74	0.502	0.00276	lm.nb	0.0168
CD45RO	1.92	0.555	0.0028	lm.nb	0.0169
PTGER4	1.66	0.482	0.00288	lm.nb	0.0172
C1QB	1.82	0.527	0.0029	lm.nb	0.0172
IFNGR2	1.55	0.453	0.00306	lm.nb	0.018
TREM2	2.02	0.592	0.00313	lm.nb	0.0183
LAG3	2.1	0.618	0.00324	lm.nb	0.0188
CEBPB	1.83	0.543	0.00347	lm.nb	0.0199
TGFB3	2.78	0.83	0.00356	lm.nb	0.0203
DLL1	2.02	0.604	0.00368	lm.nb	0.0208
CD3E	2.36	0.709	0.0037	lm.nb	0.0208
TLR7	2.25	0.671	0.00374	Wald	0.0208
IL2RA	1.78	0.535	0.00376	lm.nb	0.0208
HLA-E	1.25	0.376	0.00384	lm.nb	0.0209
GLUD1	1.63	0.491	0.00384	lm.nb	0.0209
EGFR	-1.67	0.505	0.00394	lm.nb	0.0211
CCND3	1.73	0.525	0.00408	lm.nb	0.0214
MMP9	2.72	0.828	0.00412	lm.nb	0.0214
BRD4	1.02	0.311	0.00416	lm.nb	0.0214
MAP3K7	1.55	0.473	0.00421	lm.nb	0.0214
BCAT1	-1.7	0.52	0.00422	lm.nb	0.0214

Tumor-specific T cells support immune microaggregates needed for survival

DEG	Log2 fold change	SD	P-value	Method	Corrected P-value
ITGAX	1.75	0.536	0.00422	lm.nb	0.0214
TAPBP	1.6	0.49	0.00426	lm.nb	0.0214
ITGA4	1.79	0.548	0.00428	lm.nb	0.0214
HLA-A	2.14	0.657	0.00432	lm.nb	0.0215
BCL2L1	1.26	0.388	0.00438	lm.nb	0.0216
RSAD2	2.04	0.632	0.00461	lm.nb	0.0225
IFIH1	1.11	0.343	0.00462	lm.nb	0.0225
BRIP1	1.3	0.404	0.00475	lm.nb	0.023
IFI35	1.69	0.529	0.00503	lm.nb	0.0241
HLA-B	2.02	0.632	0.00505	lm.nb	0.0241
MYD88	0.857	0.269	0.00514	lm.nb	0.0243
GBP2	1.61	0.507	0.00519	lm.nb	0.0244
HERC6	1.8	0.57	0.00542	lm.nb	0.0253
IRF5	1.22	0.387	0.00555	lm.nb	0.0257
MFNG	1.56	0.496	0.00557	lm.nb	0.0257
ICAM3	1.31	0.42	0.00588	lm.nb	0.027
OAS3	1.03	0.329	0.00592	lm.nb	0.027
TNF	1.78	0.571	0.00597	lm.nb	0.027
OAS1	1.03	0.332	0.00601	lm.nb	0.027
MAGEA1	-2.29	0.732	0.00617	Wald	0.0276
PSMB9	1.72	0.556	0.00627	lm.nb	0.0276
VEGFA	1.8	0.582	0.00627	lm.nb	0.0276
CXCL10	2.06	0.668	0.0063	lm.nb	0.0276
APC	1.45	0.472	0.00647	lm.nb	0.0282
BMP2	-1.47	0.477	0.00654	lm.nb	0.0283
DUSP2	1.59	0.52	0.0067	lm.nb	0.0288
IRF9	1.08	0.353	0.00682	lm.nb	0.0292
GBP1	1.41	0.463	0.00697	lm.nb	0.0296
CDKN2A	2.81	0.935	0.00756	lm.nb	0.032
NKG7	2.18	0.726	0.00765	lm.nb	0.0322
OAS2	1.26	0.422	0.00794	lm.nb	0.0329
MARCO	2.47	0.823	0.00797	Wald	0.0329
BRCA2	1.11	0.373	0.00798	lm.nb	0.0329
IFITM1	1.2	0.407	0.00874	lm.nb	0.0359
CXCL6	-2.82	0.964	0.00904	lm.nb	0.0368
CD69	1.37	0.471	0.00919	lm.nb	0.0368
ICAM1	1.02	0.351	0.0092	lm.nb	0.0368
GIMAP4	1.37	0.47	0.00921	lm.nb	0.0368
PARP4	1.14	0.391	0.00925	lm.nb	0.0368
SNAI1	1.74	0.597	0.00929	lm.nb	0.0368
CASP9	1.12	0.386	0.00941	lm.nb	0.0371
CD3G	2.14	0.738	0.00952	lm.nb	0.0371
GPSM3	1.38	0.476	0.0096	lm.nb	0.0371
P4HA2	-3.58	1.24	0.00963	loglinear	0.0371
SGK1	1.58	0.547	0.00974	lm.nb	0.0371
MXI1	1.81	0.626	0.00975	lm.nb	0.0371
FYN	1.16	0.404	0.00993	lm.nb	0.0375
CD4	1.44	0.503	0.0102	lm.nb	0.0383
FAP	-1.33	0.463	0.0104	lm.nb	0.0385

Supplemental Table 4C *Continued*

DEG	Log2 fold change	SD	P-value	Method	Corrected P-value
MYC	1.23	0.433	0.0105	lm.nb	0.0389
C1QA	1.3	0.458	0.0106	lm.nb	0.0389
ITGAM	1.61	0.566	0.0106	lm.nb	0.0389
PALMD	2.11	0.744	0.0109	lm.nb	0.0397
IL2RB	1.59	0.561	0.0111	lm.nb	0.0402
IL11	-2.33	0.825	0.0112	lm.nb	0.0402
SH2D1A	2.22	0.787	0.0113	lm.nb	0.0404
BCL2	1.81	0.643	0.0113	lm.nb	0.0404
STAT1	1.15	0.41	0.0116	lm.nb	0.0409
SOX2	2.38	0.848	0.0116	lm.nb	0.0409
MKI67	1.18	0.421	0.0117	lm.nb	0.0411
TIGIT	1.92	0.687	0.012	lm.nb	0.0418
HIF1A	-1.02	0.368	0.0122	lm.nb	0.0423
HES1	1.11	0.4	0.0122	lm.nb	0.0423
FCGRT	1.28	0.463	0.0128	lm.nb	0.0437
MMP7	-2.39	0.866	0.0129	lm.nb	0.0437
CXCL14	-1.76	0.636	0.0129	lm.nb	0.0437
CXCL5	-4.06	1.47	0.0129	loglinear	0.0437
CD2	1.14	0.412	0.0131	lm.nb	0.044
COL6A3	-1.03	0.377	0.0134	lm.nb	0.0449
FZD8	2.03	0.741	0.0139	Wald	0.0463
CD14	1.37	0.504	0.014	lm.nb	0.0463
CXCL3	-1.65	0.608	0.0141	lm.nb	0.0467
IL1R2	2.28	0.841	0.0143	lm.nb	0.0469
NDUFA4L2	2.02	0.748	0.0148	lm.nb	0.0483

Differentially expressed genes (DEGs) that are upregulated in (A) HPV16⁺IR⁺ compared to HPV16⁺IR⁻ OPSCC, (B) HPV16⁺IR⁺ compared to HPV⁻ OPSCC and (C) HPV16⁺IR⁻ compared to HPV⁻ OPSCC are given as a positive log2 fold change value, while downregulated DEGs are indicated as a negative log2 fold change value. The standard deviation (SD), P-value and corrected P-value (method lm.nb, linear model.negative binomial or Wald) is given for each DEG.

Supplemental Table 5. Imaging mass cytometry clusters and superclusters.

Supercluster number	Supercluster name	Cluster number	Cluster name
sc01	HLADR+ tumor cell	c11	11_HLADR+_Ki67+_D240+_P16+_Bcat+_Ker+_tumor_cell
		c23	23_HLADR+_Bcat+_Ker+_tumor_cell
		c26	26_Ki67+_D240+_TGFb+_Bcat+_Ker+_tumor_cell
sc02	HLADR-Ki67+ tumor cell	c28	28_Ki67+_Bcat+_Ker+_tumor_cell
		c30	30_Ki67+_D240+_P16+_Bcat+_Ker+_tumor_cell
		c31	31_Ki67+_TGFb+_P16+_Bcat+_Ker+_tumor_cell
sc03	HLADR-Ki67- tumor cell	c07	07_CD103+_Bcat+_Ker+_tumor_cell
		c24	24_P16+_Bcat+_Ker+_tumor_cell
		c25	25_D240+_Bcat+_Ker+_tumor_cell
		c27	27_TGFb+_Bcat+_Ker+_tumor_cell
		c40	40_VISTA+_CD103+_Bcat+_Ker+_tumor_cell
		c46	46_PDL1+_P16+_Bcat+_Ker+_tumor_cell
		c47	47_IDO+_PDL1+_P16+_Bcat+_Ker+_tumor_cell
		c50	50_Caspase+_Bcat+_Ker+_tumor_cell
sc04	CD3+CD4+ FOXP3- T cell	c01	01_CD3+_CD4+_CD7+_ICOS+_CD45ro+_T_cell
		c05	05_CD3+_CD4+_VISTA+_CD73+_CD38+_CD45ro+_T_cell
		c14	14_CD3+_CD4+_CD7+_CD45ro+_T_cell
		c34	34_CD3+_CD4+_CD7+_CD73+_CD38+_TGFb+_CD45ro+_T_cell
		c35	35_CD3+_CD4+_CD7+_CD73+_TGFb+_CD45ro+_T_cell
		c37	37_CD3+_CD4+_CD7+_ICOS+_CD103+_D240+_CD45ro+_T_cell
sc05	CD3+CD4+ FOXP3+ Treg	c42	42_CD3+_CD4+_CD7+_FOXP3+_CD45ro+_D240+_Treg
		c43	43_CD3+_CD4+_CD7+_FOXP3+_ICOS+_CD45ro+_Treg
sc06	CD3+CD8+ T cell	c03	03_CD3+_CD8+_CD4+_CD57+_CD7+_CD45ro+_T_cell
		c04	04_CD3+_CD8+_CD7+_CD103+_CD45ro+_T_cell
		c36	36_CD3+_CD8+_CD7+_TGFb+_CD45ro+_T_cell
sc07	CD20+HLADR+ B cell	c02	02_CD20+_HLADR+_CD57+_CD45ro+_B_cell
		c41	41_CD20+_HLADR+_Ki67+_CD45+_B_cell
sc08	CD56+ NK cell	c08	08_CD56+_CD73+_VISTA+_NK_cell
sc09	CD3-CD7+ ILC	c06	06_CD7+_CD73+_TGFb+_ILC
sc10	CD11b+CD15+ granulocyte	c48	48_CD11b+_CD15+_VISTA+_CD45ro+_granulocyte
		c49	49_CD11b+_CD15+_CD45ro+_granulocyte
sc11	CD11c+CD14+ HLADR+ DC	c12	12_CD11c+_CD14+_HLADR+_D240+_CD45ro+_DC
		c13	13_CD11c+_CD14+_HLADR+_CD45ro+_DC

Supplemental Table 5 *Continued*

Supercluster number	Supercluster name	Cluster number	Cluster name
sc12	CD68+CD163- M1 macrophage	c15	15_CD68+_CD14+_CD204+_HLADR+_TGFB+_macrophage
		c32	32_CD68+_VISTA+_CD38+_CD31+_macrophage
		c38	38_CD68+_CD14+_CD204+_CD11c+_HLADR+_macrophage
sc13	CD68+CD163+ M2 macrophage	c39	39_CD68+_CD163+_CD14+_CD204+_CD11c+_HLADR+_macrophage
		c44	44_CD68+_CD163+_CD14+_CD11c+_HLADR+_VISTA+_macrophage
		c45	45_CD68+_CD163+_CD14+_VISTA+_CD38+_CD31+_macrophage
		c51	51_CD68+_CD163+_macrophage
sc14	CD68- CD14+HLADR+ myeloid cell	c16	16_CD14+_CD204+_HLADR+_CD45ro+_myeloid_cell
		c17	17_CD14+_HLADR+_CD73+_TGFB+_myeloid_cell
sc15	CD45ro+lin- memory immune cell	c22	22_CD45ro+_memory_immune_cell
		c29	29_HLADR+_CD45ro+_memory_immune_cell
sc16	Vim+lin- fibroblast	c18	18_TGFB+_Vim+_fibroblast
		c19	19_Vim+_fibroblast
		c20	20_Vim+_D240+_fibroblast
sc17	CD38+Vim+lin- stromal cell	c21	21_CD38+_stromal_cell
		c33	33_CD38+_CD31+_CD73+_TGFB+_stromal_cell
sc18	CD31+ blood vessel endothelial cell	c09	09_CD31+_CD73+_blood_vessel
		c10	10_CD31+_CD73+_TGFB+_blood_vessel
sc19	D240+ tumor cell	c11	11_HLADR+_Ki67+_D240+_P16+_Bcat+_Ker+_tumor_cell
		c25	25_D240+_Bcat+_Ker+_tumor_cell
		c26	26_Ki67+_D240+_TGFB+_Bcat+_Ker+_tumor_cell
		c30	30_Ki67+_D240+_P16+_Bcat+_Ker+_tumor_cell
sc20	CD103+ tumor cell	c07	07_CD103+_Bcat+_Ker+_tumor_cell
		c40	40_VISTA+_CD103+_Bcat+_Ker+_tumor_cell
sc21	Ki67+ tumor cell	c11	11_HLADR+_Ki67+_D240+_P16+_Bcat+_Ker+_tumor_cell
		c26	26_Ki67+_D240+_TGFB+_Bcat+_Ker+_tumor_cell
		c28	28_Ki67+_Bcat+_Ker+_tumor_cell
		c30	30_Ki67+_D240+_P16+_Bcat+_Ker+_tumor_cell
		c31	31_Ki67+_TGFB+_P16+_Bcat+_Ker+_tumor_cell

Supercluster number	Supercluster name	Cluster number	Cluster name
sc22	CD3+FOXP3-TGFb+ T cell	c34	34_CD3+_CD4+_CD7+_CD73+_CD38+_TGFb+_CD45ro+_Th_cell
		c35	35_CD3+_CD4+_CD7+_CD73+_TGFb+_CD45ro+_T_cell
		c36	36_CD3+_CD8+_CD7+_TGFb+_CD45ro+_Tc_cell
sc23	CD11c+ cell	c12	12_CD11c+_CD14+_HLADR+_D240+_CD45ro+_DC
		c13	13_CD11c+_CD14+_HLADR+_CD45ro+_DC
		c38	38_CD68+_CD14+_CD204+_CD11c+_HLADR+_macrophage
sc24	CD38+CD31+ stromal cell	c32	32_CD68+_VISTA+_CD38+_CD31+_macrophage
		c33	33_CD38+_CD31+_CD73+_TGFb+_stromal_cell
		c45	45_CD68+_CD163+_CD14+_VISTA+_CD38+_CD31+_macrophage
sc25	CD103+ T cell	c37	37_CD3+_CD4+_CD7+_ICOS+_CD103+_D240+_CD45ro+_Th_cell
		c04	04_CD3+_CD8+_CD7+_CD103+_CD45ro+_Tc_cell
sc26	ICOS+ T cell	c01	01_CD3+_CD4+_CD7+_ICOS+_CD45ro+_Th_cell
sc27	TGFb+ myeloid cell	c15	15_CD68+_CD14+_CD204+_HLADR+_TGFb+_macrophage
		c17	17_CD14+_HLADR+_CD73+_TGFb+_myeloid_cell
		c12	12_CD11c+_CD14+_HLADR+_D240+_CD45ro+_DC
sc28	CD14+ myeloid cell	c13	13_CD11c+_CD14+_HLADR+_CD45ro+_DC
		c15	15_CD68+_CD14+_CD204+_HLADR+_TGFb+_macrophage
		c38	38_CD68+_CD14+_CD204+_CD11c+_HLADR+_macrophage
		c39	39_CD68+_CD163+_CD14+_CD204+_CD11c+_HLADR+_macrophage
		c44	44_CD68+_CD163+_CD14+_CD11c+_HLADR+_VISTA+_macrophage
		c45	45_CD68+_CD163+_CD14+_VISTA+_CD38+_CD31+_macrophage
		c16	16_CD14+_CD204+_HLADR+_CD45ro+_myeloid_cell
		c17	17_CD14+_HLADR+_CD73+_TGFb+_myeloid_cell

Cluster name is based on the markers that are expressed by that cluster, indicating that that cluster is negative for all other tested markers that are not mentioned in the cluster name.

Supplemental Table 6. Single cell sequencing counts per identified cluster.

Cluster	H143		H197		H205		H149		H182		H208		H211		H141		H160		H176		H185		H188		H68	
	HPV-	HPV+	HPV-	HPV+	HPV-	HPV+	HPV+ IR-	HPV+ IR-	HPV+ IR-	HPV+ IR-	HPV+ IR-	HPV+ IR-	HPV+ IR-	HPV+ IR-	HPV+ IR+	HPV+ IR+	HPV+ IR+	HPV+ IR+	HPV+ IR+	HPV+ IR+	HPV+ IR+	HPV+ IR+	HPV+ IR+	HPV+ IR+	HPV+ IR+	HPV+ IR+
CD8_0	31*	66	10	5	45	10	15	19	73	50	302	184	49													
CD8_1	276	7	10	10	90	8	23	63	81	70	54	18	64													
CD8_2	446	2	5	2	12	0	6	8	32	10	14	6	1													
CD8_3	0	71	6	0	7	41	2	0	10	83	65	18	212													
CD8_4	9	8	4	17	96	2	11	7	9	33	81	205	33													
CD8_5	7	87	9	10	15	15	10	21	11	30	90	132	15													
CD8_6	0	15	2	1	9	316	2	2	5	15	17	24	22													
CD8_7	1	1	1	9	11	3	4	12	1	12	7	40	305													
CD8_8	0	0	0	0	8	5	4	2	3	4	8	26	255													
CD8_9	2	2	1	0	0	5	3	0	1	101	0	0	151													
CD8_10	0	16	3	0	2	45	0	1	1	10	13	4	38													
CD8_11	1	19	1	0	3	5	1	0	6	1	0	2	0													
CD4_0	2	0	4	202	51	5	28	7	1	33	40	324	224													
CD4_1	103	12	53	87	82	7	78	48	251	23	136	8	11													
CD4_2	3	17	12	9	98	172	28	27	10	33	14	82	379													
CD4_3	203	0	43	74	52	1	175	96	104	2	16	5	3													
CD4_4	17	2	15	11	15	16	112	516	13	9	8	8	22													
CD4_5	16	61	19	47	81	7	32	34	24	45	113	239	42													
CD4_6	9	3	33	309	54	0	5	19	8	11	85	16	8													
CD4_7	19	46	16	15	40	41	44	22	87	113	53	21	10													
CD4_8	11	99	8	8	26	41	7	10	16	34	38	90	100													
CD4_9	38	6	14	3	54	19	74	39	93	95	7	3	14													

Cluster	H143	H197	H205	H149	H182	H208	H211	H141	H160	H176	H185	H188	H68
	HPV-	HPV-	HPV-	HPV+ IR-	HPV+IR-	HPV+IR-	HPV+IR-	HPV+IR+	HPV+IR+	HPV+IR+	HPV+IR+	HPV+IR+	HPV+IR+
CD4_10	2	3	0	0	0	2	0	2	6	29	0	4	107
Treg_0	4	67	8	17	46	23	31	71	31	58	61	101	116
Treg_1	3	20	6	6	41	94	7	5	7	42	15	51	102
Treg_2	0	7	0	0	4	181	4	10	0	7	0	7	13
Treg_3	0	141	1	0	4	3	0	0	0	2	2	1	3
Treg_4	0	16	3	3	18	38	8	5	10	20	5	8	21
Treg_5	0	0	0	0	0	5	0	0	0	25	1	1	36
Tother_0	18	0	4	0	23	2	13	10	8	2	9	11	3
Tother_1	2	0	7	0	11	1	1	4	0	0	0	3	5
Tother_2	1	0	1	3	5	1	1	2	1	0	0	3	0
NK_0	2	21	21	46	149	14	20	16	28	78	59	97	272
NK_1	1	222	8	6	46	34	14	11	42	55	288	35	22
NK_2	0	12	1	1	9	586	0	22	0	9	6	5	11
NK_3	2	5	13	7	54	22	40	19	37	16	13	8	8
NK_4	0	146	5	1	13	18	4	4	6	8	6	7	14
NK_5	0	0	1	0	0	2	0	0	1	36	2	0	35

* Number of cells identified per patient in each cluster.

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The background of the entire page is a deep space image showing a complex cosmic web. It features a dark blue to black background filled with numerous small, distant stars. Overlaid on this are intricate, glowing filaments of light in various colors, including green, orange, yellow, and purple. These filaments represent the large-scale structure of the universe, connecting galaxy clusters and individual galaxies. The overall effect is a sense of vastness and the interconnectedness of the cosmos.

Chapter 9

**Summary, discussion
and future perspectives**

In **chapter 1**, I provided an overview of the state of knowledge on the role of the immune TME in HPV-induced tumors at the onset of this PhD. I identified two key unanswered questions in HPV tumor immunology: whether the TME influences the response to immunotherapy in HPV-induced precancers (part I), and whether the TME impacts survival in patients with HPV-associated cancers (part II). We integrated the latest available spatial technologies to study the TME in relation with clinical outcome. Here, I summarize and discuss the main findings of this PhD thesis, as well as provide a future outlook.

The insights presented in this PhD thesis, derived from studies of HPV-induced tumors, a relatively homogeneous group of tumors, are increasingly generalizable to broader oncological contexts. These findings illustrate for example how antigen-rich tumors can evolve mechanisms of immune exclusion. Consequently, HPV-positive malignancies serve as a valuable model system for dissecting mechanisms of immune regulation in solid tumors. These strengths are particularly pertinent in the era of spatial and single-cell technologies. The relative uniformity of the antigenic landscape in HPV-positive tumors, driven by common oncogenic drivers, contrasts with the heterogeneity observed in many other cancer types. This uniformity enables more confident attribution of spatial and cellular features within the TME, such as immune exclusion, microaggregate formation and myeloid reprogramming, to genuine immunobiological processes rather than to variability in tumor genetics or etiology. Leveraging these advantages, this thesis investigated the spatial organization of anti-tumor immunity, mechanisms of therapeutic resistance and the development of biomarkers to inform future personalized immuno-oncological strategies.

Summary

Part I: immunotherapy and TME of HPV-induced precancer

In part I we comprehensively analyzed the TME with multiple high-dimensional *in situ* imaging methods, and studied the impact of the TME on the response of HPV-induced precancers to various types of immunotherapy. In **chapter 2**, we elucidated the different etiological pathways of VSCC, and outlined the immunosuppressive nature of the TME in vulvar (pre)cancer, marked by T cell exhaustion, infiltration by regulatory T cells and M2-like macrophages and downregulation of HLA class I. This immunosuppressive milieu poses significant challenges for the immune system to clear these lesions, but also highlights

the potential of immunotherapy. The key challenge is how to overcome the immunosuppressive TME that is observed.

In **chapter 3**, we demonstrated that a pre-existing coordinated type 1 inflammatory microenvironment, characterized by Tbet⁺ (IFN- γ producing) or PD1⁺ (activated) T cells and CD14⁺HLADR⁺ inflammatory myeloid cells, is associated with the clinical response of vHSIL to therapeutic HPV16 vaccination. In **chapter 4** we found similar findings for the response to imiquimod, however the pre-therapy presence of inflammatory myeloid cells were found to be more important for response to imiquimod therapy, fitting with the fact that this therapy mostly results in local activation of myeloid cells as they express the cognate TLR. These findings have recently been confirmed in an independent cohort.¹ Interestingly, the immune TME of vHSIL complete responders to immunotherapy was found to resemble healthy vulvar tissue in terms of coordinated type 1 immunity, however vHSIL displayed higher numbers of Tregs than healthy vulvar tissue. These findings suggest that "cold" tumors, lacking baseline inflammation, are unlikely to benefit from monotherapy. It has also been well documented across various other tumor types (particularly pancreatic cancer, prostate cancer, colorectal cancer and breast cancer) that immunologically cold tumors exhibit limited responsiveness to therapeutic vaccination.² Clinical trials have demonstrated that the immunogenicity and efficacy of therapeutic vaccines are limited in these contexts, largely due to the lack of pre-existing T cells.^{3,4} In addition to vaccine resistance, these cold tumors often exhibit poor clinical responses to other forms of immunotherapy, including adoptive cell transfer using expanded TILs and immune checkpoint blockade.⁵⁻⁷ Therefore, the success of these immunotherapies may be limited, unless if combined with interventions that first induce a pro-inflammatory TME. Current experimental co-treatments are for example intratumoral cytokine delivery⁸ or oncolytic viruses⁹, however their clinical applicability is still very limited and it is unknown whether they cause sustainable reversal of immune exclusion.

The application of single-cell spatial transcriptomics in **chapter 5** provided unprecedented in-depth insights into the cell states, of all cell types not merely immune cells, and spatial ecosystems dictating response of vHSIL to immunotherapeutic vaccination. We uncovered a higher ratio of immune-supportive to immune-suppressive cells (classified based on function described in literature) in responding patients, in contrast to non-responders. This pattern was also found in other solid tumor patient cohorts treated with ICB.

Notably, we discovered response specific spatial ecosystems. These findings challenge the prevailing view that solely infiltration by immune cells suffices for therapeutic response, highlighting instead the crucial role of their spatial organization in shaping their function. However, understanding the formation and maintenance of these immune ecosystems remains incompletely understood.

In **chapter 6**, we identified an immune-based biomarker that predicts response to imiquimod immunotherapy in cHSIL: the CHSIL Immune Biomarker for Imiquimod (CIBI). CIBI comprises the sum of intraepithelial CD4⁺ T helper cells, M1-like macrophages and dendritic cells, thereby grasping the most important components of coordinated anti-tumor immunity, offers a promising tool for patient stratification. Patients with baseline immune exclusion (low CIBI score) responded poorly to topical imiquimod, indicating the necessity of priming the immune system, similar to in vHSIL (as described in chapter 3 and 4). Interestingly, the cell types important for response in cHSIL are slightly different from the ones found in vHSIL, most likely due to differences in anatomical location.¹⁰ However, retrospective studies are inherently limited by potential biases and confounding factors. Therefore it is essential to confirm these findings through prospective validation. To this end, the CIBI is currently being evaluated in a prospective multi-center clinical trial (Predict-TOPIC).

Part II: survival and TME of HPV-induced cancer patients

In part II we investigated the impact of the TME on the survival of HPV-associated cancer patients. The intraepithelial infiltration of CD14⁺ monocytes emerges as a novel positive prognostic biomarker in VSCC in **chapter 7**. High intraepithelial monocyte infiltration correlates with significantly improved survival, likely through enhanced recruitment of T cells and modulation of the TME towards a pro-inflammatory state. In cervical cancer it was previously described that high infiltration by CD14⁺ monocytes was associated with improved survival.¹¹ Similarly, in vHSIL we found monocyte-rich lesions to be associated with improved response to immunotherapy (chapters 3 and 4). It remains unclear whether these monocytes directly clear dysplastic epithelial cells, or merely reflect broader immune cell recruitment to the TME, and that these monocytes might undergo differentiation into monocyte-derived dendritic cells or M1-like macrophages, both capable of promoting anti-tumor immunity, and that this might be the reason why they are positively associated with response to immunotherapy.^{12 13} This finding challenges previous assumptions that myeloid cells are primarily tumor-promoting. However, therapeutically activating

monocyte recruitment pathways could serve as a double-edged sword, necessitating careful modulation to reach an anti-tumor balance.¹⁴ It would be of interest to investigate whether specific chemokines can be targeted to selectively recruit or expand beneficial myeloid populations to achieve this¹⁵, or whether TLR agonists can be used to promote their polarization¹⁶.

Chapter 8 highlights the pivotal role of tumor-specific T cells in organizing chemokine-driven immune microaggregates, consisting of CD4⁺ T cells, CD8⁺ T cells and dendritic cells, that were shown to be essential for long-term survival in head and neck cancer patients. This is likely a causal relationship, since the T cells in these microaggregates secrete XCL1, a chemokine that attracts dendritic cells, thus they are likely orchestrating the formation of these microaggregates. Also in literature, T cells have been found to recruit antigen presenting cells that were critical for therapeutic efficacy via chemokines.¹⁷ This finding emphasizes that the coordinated spatial organization of immune cells may be more prognostically and therapeutically relevant than immune cell density alone, as also supported by the findings in chapter 5 and corroborated by recent studies across multiple tumor types.¹⁸ Notably, also in literature the formation of these immune cell triads has been described, which appear to be central to sustaining effective anti-tumor immunity.¹⁸⁻¹⁹ These triads may facilitate local T cell priming, but also likely serve to re-engage or reconfirm T cell effector function through interactions with antigen-presenting cells and helper T cells. Within such triads, CD8⁺ T cells are frequently observed to express Ki-67, indicating *in situ* proliferation and sustained activation, suggesting that T cells do not merely arrive "pre-armed" at the tumor site but are maintained and possibly reprogrammed locally to continue responding.

This phenomenon is not restricted to head and neck cancers; similar immune architectures have been described in melanoma²⁰, non-small cell lung cancer²¹ and renal cell carcinoma²², where they are often associated with improved prognosis and better response to immunotherapy. Importantly, these immune ecosystems may also underlie favorable responses to conventional therapies such as chemotherapy and radiotherapy²³, further reinforcing the concept that a permissive immune contexture (characterized by organized, functional immune cell interactions) plays a central role in cancer treatment efficacy across modalities. Despite these findings, it remains unclear how to therapeutically induce or stabilize such spatial structures. A deeper mechanistic understanding of the cellular interactions and molecular signals

that govern their formation will be essential for developing next-generation immunotherapies aimed at shaping the TME toward durable tumor control.

The findings of this PhD thesis strengthen the growing consensus that cold tumors represent a biologically and clinically distinct phenotype. For example, vaccination does not turn cold tumors hot (**chapter 3 and 4**). These tumors are marked by a lack of functional effector T cells; as a result, they frequently show resistance to single-agent immunotherapy. This observation aligns with a broader paradigm shift in immuno-oncology: treatment efficacy depends not merely on immune cell abundance, but also on their activation status, spatial localization and ability to engage with tumor cells directly.²⁴ Previous studies have looked into treating immunologically cold tumors first with TME-modulating strategies, such as TLR agonists (e.g. imiquimod), oncolytic viruses²⁵ and STING pathway activators²⁶, to initiate local inflammation^{27 29}, and subsequently with checkpoint blockade. However, so far results of these combination trials have been disappointing, therefore we need better understanding of the spatial component of these tumors and how to therapeutically employ this. These immunologically cold tumors should be recognized as a distinct pathological subclass. For certain cases, this distinction implies that such tumors may be intrinsically resistant to immunotherapeutic interventions and may instead benefit more from conventional treatment modalities, such as chemotherapy, surgery, radiation therapy, or completely different treatment approaches.

Discussion

Through a series of comprehensive TME analyses in HPV-induced tumors in this thesis, we provide a better understanding of how pre-existing immunity, spatial immune architecture and immunological biomarkers are related to responsiveness to immunotherapy and patient survival. Collectively, the chapters of this thesis advance several critical areas in translational immuno-oncology.

Spatial organization of the TME as orchestrator of anti-tumor immunity

Spatially resolved technologies have illuminated a new principle in immuno-oncology: the placement of immune cell subtypes, tumor cells and stromal elements in the TME dictates whether effective anti-tumor immunity is orchestrated or suppressed.³⁰ We demonstrate that features such as the

formation of structured immune cell aggregates in the vicinity of tumor cells are more informative than bulk immune cell density. This evolution in understanding has been propelled by spatially resolved technologies. Early multiplex immunohistochemistry enabled phenotypic mapping of immune cell subtypes and their localization, which fundamentally changed how immune cell distribution was interpreted.³¹ Imaging mass cytometry added dimensionality by allowing simultaneous detection of >40 markers at a subcellular resolution, thereby revealing unexpected cellular interactions and phenotypes within the TME.³² In this thesis, the application of single-cell spatial transcriptomics further expanded these insights by offering untargeted, wide profiling of a thousand genes *in situ* at single-cell resolution. This holistic approach (no predefined targeting of immune subsets) enabled the identification of novel or underappreciated cell populations, such as the CCL18⁺ macrophages that we identified and the triads it makes with CD8⁺ T cells and Th2 skewed CD4⁺ T cells in **chapter 5**.

Among the most striking discoveries made with these spatial technologies are tertiary lymphoid structures (TLS): ectopic lymphoid aggregates within tumors that recapitulate many features of secondary lymphoid organs. Two landmark studies in melanoma and renal cell carcinoma demonstrated that the presence of mature, B cell rich TLS strongly predicts responsiveness to PD-1 blockade and improved survival.^{19,33} TLS are now recognized as intratumoral “immune organs” that sustain antigen presentation, support CD8⁺ T cells and enable local B cell maturation. Importantly, TLS not only mark immune infiltration but also shape it, providing the infrastructure for ongoing priming and renewal inside the tumor rather than in distant lymph nodes. Within these structures, B cells and stromal organizers support ongoing priming and renewal of anti-tumor responses; functions traditionally attributed to distant lymph nodes. These insights repositioned B cells and stromal organizers from passive bystanders to central architects of effective anti-tumor immunity.³³

A second spatial breakthrough came from the concept of immune triads, where tumor control requires CD4⁺ T cells and CD8⁺ T cells to simultaneously engage the same intratumoral dendritic cell¹⁸, in line with what we found in HNC (**chapter 8**). This spatial interaction is necessary to license CD8 cytotoxicity, explaining why inflamed but disorganized tumors do not respond to immunotherapy. These findings suggest a hierarchical architecture: TLS for priming and renewal and licensing, and triads for execution.

These spatial insights force a critical re-examination of what it means for a tumor to be immunologically hot. A high estimation of T cell prevalence bulk sequencing does not guarantee effector function if those cells are sequestered by stromal barriers.³⁴ Conversely, tumors with lower overall infiltration but rich in TLS or spatial triads can mount effective anti-tumor responses. This reframing highlights organization over abundance as a determinant of outcome. It also raises unresolved questions: why do some tumors spontaneously generate TLS and triads, while others remain anarchic despite strong antigenicity? And to what extent can we therapeutically engineer such spatial architecture, without destabilizing systemic immune homeostasis and inducing auto-immunity related toxicity?

The translational implications are profound. Rather than simply unleashing T cells, emerging strategies should aim at re-designing spatial architecture: inducing TLS with chemokine scaffolds³⁵, recruiting and programming dendritic cells to seed triads³⁶, or dismantling stromal and myeloid barriers that cause T cell exclusion³⁴. Equally important is the recognition that intact lymphatic circuits are indispensable; preclinical work shows that ablation of tumor-draining lymphatics abrogates checkpoint inhibitor responses, highlighting the importance of preserving the tumor-lymph node axis.³⁷ The field is now beginning to integrate these spatial biomarkers into clinical trial design, using proximity scores, neighborhood analyses and TLS maturity indices as predictors of therapeutic response.

Advancing cancer immunotherapy requires not only understanding of the baseline tumor characteristics, but also how therapies actively reprogram the TME. Static biomarkers often struggle to reliably predict response, whereas longitudinal analyses of on-treatment samples provide a temporal dimension that captures immune dynamics. Currently we are applying single-cell spatial transcriptomic profiling on the on-treatment vHSIL samples from the cohort in **chapter 5** to elucidate this. In neoadjuvant trials, effective PD-1 blockade has been linked to the maturation of TLS^{19 33}, and the breakdown of fibroblast and myeloid barriers that exclude T cells³⁴. Multiplexed imaging further shows that therapy can induce new immune neighborhoods, such as T cell-macrophage hubs³⁸, while neoadjuvant PD-1 blockade in NSCLC demonstrated the induction of TLS-like aggregates and T cell redistribution as early correlates of regression³⁹. These findings highlight that responsiveness is less about overall immune abundance and more about whether therapy reshapes spatial ecosystems to enable effective interactions. On-treatment sampling thus offers

not just predictive biomarkers, but mechanistic insights into resistance when remodeling fails. However, challenges still remain: sampling is invasive, tumors are heterogeneous and standardized analytic frameworks are still evolving.

These recent advances in single-cell spatial technologies are revolutionizing our understanding of the TME, enabling *in situ* mapping of cell states, phenotypic heterogeneity and precise cellular neighborhoods. Nevertheless, each platform captures only a facet of tumor biology. The next frontier lies in integrative multi-omics: combining genomic, transcriptomic, proteomic and metabolomic data within spatial contexts, to holistically chart the complex multi-level tumor-immune interactions. Such a comprehensive "TME atlas" is pivotal for uncovering metabolic crosstalk, signaling networks and functional immune states and networks that underly therapeutic response and resistance. While transcriptomic studies have charted immune neighborhoods, it is proteins and metabolites that ultimately dictate function. Post-translational modifications, receptor signaling and metabolic fluxes determine whether a CD8⁺ T cell near a dendritic cell becomes cytotoxic, exhausted, or senescent.

Advances in ultrasensitive proteomics and spatial metabolomics by mass spectrometry imaging now make these layers visible *in situ*. Early studies already highlight their impact, for example imaging mass cytometry can be used for spatial cancer metabolomics analyses, which revealed that distinct metabolic profiles correlate with specific cellular phenotypes and spatial niches within the TME, highlighting metabolic heterogeneity and its role in tumor progression and immune interactions.⁴⁰ Single-cell metabolomics has identified mitochondrial dysfunction and lipid peroxidation as hallmarks of exhausted T cells⁴¹, while spatial metabolomics uncovered lactate and adenosine gradients enforcing immunosuppression.⁴² Similarly, proteomic studies of TLS revealed differential cytokine signaling and antigen presentation signatures that distinguish mature from *abortive* TLS^{19 33}, suggesting biochemical cues underlying their prognostic relevance. Together, these insights show that effective immunity is determined as much by biochemical context as by spatial arrangement.

A major breakthrough is that these technologies are now increasingly compatible with formalin-fixed paraffin-embedded (FFPE) samples, which has been the cornerstone of clinical pathology for over a century. This compatibility is transformative: FFPE tissue blocks represent an immense, largely untapped worldwide biobank of patient material, meticulously archived from countless

clinical trials and routine diagnostics. With FFPE compatible spatial technologies, researchers can now re-examine these specimens, opening an entire world of possibilities, e.g. the discovery of conserved mechanisms of resistance, acceleration of biomarker discovery and validation of therapeutic targets.

Looking ahead, spatial biology is going to be the next paradigm shift in immunotherapy. If immunity is governed by neighborhoods and hubs, then the next generation of interventions will not be judged merely by changes in cell counts or transcriptomic signatures, but by their ability to remodel the immune landscape: to build TLS where none exist, to increase triad frequency, to open stromal corridors and to sustain crosstalk between tumors and draining lymph nodes. The TME, in this view, is less a battlefield of isolated cells and more a city: its fate depends on infrastructure, circulation and neighborhood planning. The challenge, and the opportunity, is to learn how to re-engineer that city so that immunity is not only present but architecturally enabled. The key question is, what molecular cues govern the formation and maintenance of these essential spatial structures? Therapies may be designed to normalize nutrient competition, provide localized cytokine or metabolic support, or selectively inhibit suppressive pathways. Ultimately, spatially resolved single-cell proteomics and metabolomics will reveal not only where immune ecosystems are built, but also how they are powered and regulated, providing the foundation for interventions that rewire both structure and function to achieve durable tumor control.

AI-driven data analysis

As the data retrieved with the novel spatial technologies grows exponentially in scale and complexity, traditional analytical approaches are increasingly insufficient to extract biologically meaningful insights. AI-powered software tools are emerging as essential facilitators, transforming raw high-dimensional datasets into interpretable insights. Recent work has demonstrated that AI models trained on radiomic, pathologic, genomic and spatial-omic layers can accurately predict immune phenotypes, stratify patients by therapeutic response and identify novel targets for therapy.^{43 44} A striking emblem of this transformation is AlphaFold, which has shown remarkable accuracy in predicting protein-protein, protein-ligand and protein-nucleic acid interactions, thereby accelerating target discovery, antigen-antibody modeling and therapy design in immuno-oncology.⁴⁵ With the increasing application of AI in biomedical research, more of such breakthroughs are expected to happen. These developments underscore a paradigm shift: AI is not just an analytical

tool, it is reshaping how we understand biological structures and interactions at all scales. High-dimensional spatial technologies generate vast data on cell states, interactions and tissue architecture. AI has the ability to prioritize the most relevant features, highlighting key cellular players within ecosystems and identify targets for these mechanistic validations.⁴⁶

In pathology, AI has begun to demonstrate equally profound potential. Deep learning approaches have been applied to hematoxylin-eosin (HE) slides to predict prognosis directly from routine histopathology, including work in HNC.⁴⁷ Similarly, AI classifiers have successfully distinguished HPV-positive from HPV-negative HNC, providing clinically relevant stratification.⁴⁸ AI is beginning to provide explainability, identifying which features (e.g. cell types, gene signatures, or spatial configurations) drive predictions, thus facilitating biological interpretation and biomarker discovery.⁴⁹ In parallel, foundation models trained on tens of thousands of whole-slide images across multiple tumor types have demonstrated robust generalizability and superior performance compared to single-institution models, enabling more reliable patient stratification across diverse clinical settings.⁵⁰

One of the most exciting applications is perturbation simulation with AI, enabling *in silico* experiments that model the consequences of specific genetic, cellular, or environmental interventions. These approaches allow hypotheses to be prioritized before wet-lab testing, thereby reducing costs and the use of animal models, while also making it possible to explore perturbations that were previously infeasible *in vivo*. Studies have demonstrated that such simulations can predict the outcomes of genetic and metabolic perturbations, identifying transporters and pathways that are most informative for cellular states.⁵¹ Moreover, benchmarking efforts across diverse single-cell datasets have evaluated AI models for their ability to predict unseen perturbations, revealing both opportunities and challenges in generalization and transferability.⁵² These advances illustrate how computational perturbation can move the AI field from correlative association towards causal inference, bridging the gap between data-driven discovery and mechanistic understanding.

Taken together, AI is not merely an advanced analytical tool, it is reshaping how we conceptualize and interrogate biological systems. By re-using archived, clinically annotated tissue from past trials, integrating multimodal datasets and enabling *in silico* perturbation experiments, AI allows us to move from association-driven discoveries towards causal and mechanistic insights. That

said, AI-enabled discoveries carry important caveats. Many current tools function as “black boxes”, lacking transparency. Additionally, computationally intense multi-omic studies often feature limited sample sizes or small tissue regions, raising concerns about generalizability. As a result, validation in independent cohorts or via orthogonal, lower-dimensional assays remains essential. To ensure these advances translate into clinical benefit, it is imperative that analytical pipelines remain transparent, benchmarked and investigator-led, with universal standards that promote reproducibility and interpretability across laboratories and platforms.⁵³

Emerging immunotherapeutic strategies: beyond T cells

For the past years, the field of immuno-oncology has been dominated by T cell centered strategies, ranging from immune checkpoint blockade and vaccines to adoptive T cell therapies, bispecific T cell engagers (BiTEs) and antibody-drug conjugates (ADCs). These approaches have demonstrated the power of harnessing cytotoxic T lymphocytes but have also exposed the limitations of focusing narrowly on a single cellular axis. A revised view of the cancer-immunity cycle highlights that sustaining durable antitumor immunity requires continuous rounds of T cell priming and reactivation within the TME, supported by a complex interplay of innate and stromal components.⁵⁴

Recent work underscores that myeloid cells are not merely suppressive bystanders but can act as potent effectors of tumor rejection. Macrophages, neutrophils and eosinophils are increasingly recognized as direct antitumor agents: macrophages can phagocytose tumor cells and produce cytotoxic mediators⁵⁵, neutrophils have been shown to mediate antibody-dependent cytotoxicity and release reactive oxygen species that damage tumor tissue⁵⁶, and eosinophils can promote T cell recruitment and exert direct cytotoxic effects.⁵⁷ This broader appreciation of innate effectors expands the immunotherapeutic landscape substantially.

The discovery of spatial ecosystems has revealed that T cell efficacy is sustained by repeated, localized interactions with myeloid and stromal cells. Spatially resolved profiling studies consistently identify multicellular clusters that correlate with favorable immune activity and therapeutic response. Within these triads, macrophages and dendritic cells provide co-stimulatory signals that maintain T cell proliferation and prevent exhaustion, as indicated by persistent Ki-67 expression in tumor-resident T cells.⁵⁸ These spatial networks reflect a dynamic immunity cycle, where T cells are not activated once but

require re-engagement with dendritic cells and macrophages to sustain function.¹⁷ Similar mechanisms are well established in TLS, where recurring antigen presentation reinforces adaptive responses.¹⁹

Therapeutically, selective targeting immunosuppressive myeloid populations has shown striking results. Depletion of CD163⁺ macrophages in murine tumors induces inflammatory monocyte recruitment and T cell activation, restoring sensitivity even in checkpoint-refractory settings.⁵⁹ Reprogramming M2-like macrophages towards pro-inflammatory M1 phenotypes synergizes with T cell directed therapies, highlighting the dual role of myeloid cells as both effectors and facilitators of anti-tumor immunity.⁵⁵ Moreover, the recognition that fibroblasts and stromal components shape immune niches further extends the therapeutic scope, suggesting that truly effective immunotherapy must sculpt entire ecosystems rather than single cell types.

Taken together, these insights signal a paradigm shift in immuno-oncology: future immunotherapies will not rely solely on T cells, but instead will strategically target multicellular ecosystems, leveraging both lymphoid and myeloid arms of immunity and their spatial orchestration, to achieve durable tumor control.

Biomarkers for personalized immunotherapy: function over numbers

The identification of reliable predictive biomarkers for immunotherapy response remains a central challenge. Commonly used biomarkers such as PD-L1 expression and tumor mutational burden (TMB) have limited performance in clinical settings. PD-L1, assessed mostly by immunohistochemistry, suffers from variability in antibody clones, scoring algorithms and intratumoral heterogeneity, resulting in only modest predictive accuracy (AUC ~0.5-0.6).⁶⁰ TMB, although conceptually linked to neoantigen load, is hampered by inconsistent sequencing platforms and poorly defined thresholds.⁶¹ Moreover, they only reflect one characteristic of the tumor, which is only partly responsible for the clinical outcome.

While numerous studies, including our own, have demonstrated associations between immune cell infiltration and therapeutic response, these observations often fail to generalize across patient cohorts due to inter-patient heterogeneity, technical variability and the lack of standardized thresholds. For instance, although we observed an enrichment of immunostimulatory over immunosuppressive immune cells in vHSIL responders to immunotherapeutic

vaccination compared to non-responders (**chapter 5**), this ratio does not translate into a clinically actionable cut-off, limiting its predictive utility in real-world settings. One likely explanation for these inconsistencies is that traditional metrics based on immune cell abundance overlook the spatial organization and functional state of immune cells within the TME. Our spatial analysis of OPSCC (**chapter 8**) provides evidence in support of this paradigm. In patients with immune responsiveness (IR⁺), we identified structured immune microaggregates composed of CD4⁺ and CD8⁺ T cells together with dendritic cells, microaggregates that were absent in IR⁻ patients. These findings are in line with recent reports showing that the spatial proximity and interactions between T cells and dendritic cells is a critical determinant of effective anti-tumor immunity across cancer types.¹⁹

In contrast to quantitative cell type measurements, transcriptional signatures such as the IFN- γ signature^{62,63} and the T cell inflamed gene expression profile have shown more consistent predictive value in multiple tumor types.⁶⁴ Also in **chapter 8** we found that the expression of certain chemokines was associated with the immune response status of the tumor. These signatures integrate downstream signals from a wide range of tumor-immune interactions, maybe even ecosystems, capturing functional immune activity rather than isolated cell type presence. Our spatial transcriptomics data (**chapter 5**) reinforce this point: inflamed gene expression profiles frequently co-occur with organized immune structures, suggesting that transcriptional activity may act as a surrogate for functional spatial immunity. Future biomarker strategies may benefit from combining these transcriptional readouts with spatial profiling to better reflect the complex cellular ecosystems that underlie therapeutic success.

Our findings contribute to the growing body of evidence advocating for composite biomarkers: incorporating immune cell types (like we did in **chapter 6**), their spatial relationships, activation states and local cytokine milieus. However, we must also acknowledge the limitations of current technologies: advanced tools like spatial transcriptomics and high-dimensional imaging are powerful for discovery, but remain impractical for routine clinical use due to cost, complexity and incompatibility with standard pathology workflows. For example in our study, advanced profiling revealed previously unappreciated immune subsets and interactions (e.g. CCL18⁺ macrophages interacting with Th2-skewed CD4⁺ cells in spatially organized triads in **chapter 5**), highlighting how emerging technologies can uncover novel cellular players in anti-tumor immunity. Nevertheless, these insights currently serve

more as a foundation for hypothesis generation than as tools for clinical diagnostics. AI could help to reduce the number of features that need to be tested and to identify variables that are truly important.

In summary, while immune infiltration, activation status and spatial structure all contribute to immunotherapy responsiveness, no single biomarker today offers sufficient sensitivity and specificity for widespread clinical use. A multimodal biomarker approach, integrating (surrogates of) molecular, functional and spatial dimensions of the immune response, will be essential to advance precision immunotherapy from bench to bedside.

Future perspectives

The findings presented in this thesis highlight the complexity of tumor-immune dynamics in HPV-induced (pre)malignancies and demonstrate how integrating spatial, cellular and functional perspectives can inform immunotherapeutic strategies. Yet, their broader significance extends beyond any single cancer type. They exemplify a larger transformation in immuno-oncology: a shift from cell type-centric models to an ecological view of the TME.

This transition requires not only richer data but a deeper shift in mindset: from targeting immune checkpoints to engineering immune ecologies. As cancers evolve, they rewire their microenvironments not only to exclude immune attack, but to structurally suppress it. Therapeutic progress will depend on our ability to dissect and reprogram these local ecosystems, often in ways that are temporally and spatially dynamic, and shaped by tumor etiology, tissue context and therapeutic history. In this light, novel spatial immune profiling methods are not just a technical advance, they enable a conceptual evolution. It allows us to see the tumor not as a collection of mutations or cells, but as a living ecosystem with emergent properties that determine responsiveness or resistance to therapy. The translational path forward is to measure spatial signatures prospectively, intervene on topology (TLS induction, triad boosting, barrier remodeling), and preserve the lymphatic circuitry that couples tumor and secondary lymphoid organs. If the last decade was about cold versus hot tumors, the next will be about making the right neighborhoods in the right places. When paired with emerging *in situ* multi-omics tools and AI-powered analytical tools, the stage is set for a future where we can model, predict and rationally design immune interventions that are tailored for the individual patient.

However, translating these discoveries into clinical impact will require discipline-wide shifts: robust data standards, collaborative consortia for independent validation and clinically deployable versions of today's discovery platforms. Ultimately, the promise of immunotherapy is not merely tumor destruction, but the re-establishment of immune equilibrium within tumor ecosystems. By using HPV-driven cancers as a model, the work in this thesis contributes to elucidating and therapeutically exploiting the spatial architecture of anti-tumor immunity.

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Chapter 10

**Nederlandse samenvatting
(Dutch summary)**

Introductie

Het humaan papillomavirus (HPV) is een virus dat verschillende tumoren in het hoofd-hals en genito-anaal gebied kan veroorzaken. HPV-geïnduceerde tumoren vormen wereldwijd een belangrijk gezondheidsprobleem. Hoewel profylactische vaccins effectief zijn in het voorkomen van nieuwe infecties, blijven jaarlijks miljoenen vrouwen en mannen wereldwijd gediagnosticeerd worden met HPV-geïnduceerde tumoren. De huidige behandelopties zijn voornamelijk chirurgisch en leiden tot aanzienlijke morbiditeit en mortaliteit omdat er in gevoelig gebied wordt geopereerd (**hoofdstuk 1**).

Een beter begrip van de interactie tussen deze tumor cellen en hun omgeving is daarom essentieel voor de ontwikkeling van succesvolle, minder invasieve therapieën. Dit proefschrift richt zich op het ontrafelen van de micro-omgeving van HPV-geïnduceerde tumoren en het identificeren van (immunologische) biomarkers voor het voorspellen van respons op immuuntherapie en overleving. Door het integreren van cellulaire en spatiale analyses beoog ik met dit proefschrift bij te dragen aan de verdere ontwikkeling van therapie op maat voor patiënten met HPV-geïnduceerde tumoren, om zo hun overleving en kwaliteit van leven te kunnen verbeteren.

Deel I: immuuntherapie en de tumor micro-omgeving van HPV-geïnduceerde premaligniteiten

In het eerste deel van dit proefschrift wordt onderzocht hoe de compositie van de immuun micro-omgeving bijdraagt aan de effectiviteit van immuuntherapie bij HPV-geïnduceerde premaligne afwijkingen.

In **hoofdstuk 2** heb ik aan de hand van een systematische literatuuranalyse de bestaande kennis over de immuun micro-omgeving van vulvaire (pre) maligniteiten weergegeven. Deze studie toonde aan dat de micro-omgeving een dynamisch samenspel vormt tussen tumor cellen, stroma cellen en verschillende immuun cel populaties, en dat verstoringen hierin bijdragen aan de progressie van premaligniteit naar maligniteit. Dit hoofdstuk vormde de conceptuele basis voor de hypothese dat de toestand van de micro-omgeving vóór behandeling mede bepaalt of (immuun)therapie succesvol kan zijn.

Om deze hypothese te toetsen, heb ik in **hoofdstuk 3** de immuun micro-omgeving van vHSIL (vulvaire hooggradige squameuze intra-epitheliale laesie) onderzocht bij patiënten die behandeld werden met een therapeutisch HPV16 vaccin, door multispectrale immunofluorescentie te gebruiken. We konden aantonen dat een pre-existente pro-inflammatoire micro-omgeving, gekenmerkt door de aanwezigheid van geactiveerde T cellen en antigeen-presenterende cellen, significant samenhang met het compleet kunnen klaren van de premaligne afwijking. Dit was een van de eerste studies die liet zien dat pre-existente lokale immuniteit een belangrijke biomarker is voor respons op therapeutische vaccinatie in vHSIL.

In **hoofdstuk 4** heb ik deze observatie uitgebreid door twee bestaande vormen van immuuntherapie bij vHSIL te vergelijken: therapeutische vaccinatie en topicale imiquimod (een Toll-Like Receptor 7 agonist). We vonden dat een gecoördineerde, pro-inflammatoire micro-omgeving met een nauwe interactie tussen CD8⁺ T cellen, dendritische cellen en macrofagen geassocieerd was met complete respons op immuuntherapie. Verder zagen we dat het pre-existent hebben van pro-inflammatoire lymfoide cellen het belangrijkste was voor succesvolle behandeling met therapeutische vaccinatie, terwijl myeloïde cellen het belangrijkste waren voor respons op imiquimod. Dit bevestigt dat het onderliggende werkingsmechanisme van de immuuntherapie dicteert wat pre-existent belangrijk is om te kunnen responderen.

Om het belang van de spatiele architectuur van de tumor micro-omgeving beter te begrijpen, heb ik in **hoofdstuk 5** single-cell spatial transcriptomics toegepast. Deze innovatieve en geavanceerde benadering maakte het mogelijk om cel fenotypes en interacties met ruimtelijke dimensies in kaart te brengen. We identificeerden specifieke spatiele ecosystemen, bestaande uit CD8⁺ T cellen en antigeen-presenterende cellen, die vHSIL patiënten die responderden op therapeutische vaccinatie karakteriseerden.

In **hoofdstuk 6** heb ik onderzocht of bepaalde immunologische kenmerken van cHSIL (cervicale hooggradige squameuze intra-epitheliale laesie) de respons op imiquimod behandeling konden voorspellen. We hebben aangetoond dat een pro-inflammatoir immuun profiel samenhang met respons op imiquimod behandeling. Hiermee werden de conceptuele bevindingen uit de vulvaire studies bevestigd, wat wijst op gedeelde onderliggende immunologische principes tussen verschillende HPV-geïnduceerde afwijkingen.

Gezamenlijk tonen deze hoofdstukken in het eerste deel van dit proefschrift aan dat de effectiviteit van immuuntherapie in hoge mate bepaald wordt door de pre-existente immuun status van de tumor. Toekomstige strategieën zouden daarom gericht moeten zijn op het moduleren van de tumor micro-omgeving om de kans op succes van immuuntherapie te vergroten.

Deel II: overleving en de tumor micro-omgeving van HPV-geïnduceerde maligniteiten

Het tweede deel van dit proefschrift richt zich op HPV-geïnduceerde maligniteiten en onderzoekt welke immunologische kenmerken bijdragen aan de lange termijn overleving van deze patiënten.

In **hoofdstuk 7** heb ik aangetoond dat hoge infiltratie van monocytën in vulvacarcinoom een onafhankelijke prognostische factor is voor lange overleving. Deze observatie doorbrak het klassieke paradigma waarin monocytën en macrofagen primair als tumor bevorderend werden beschouwd. De data suggereren dat bepaalde monocytën populaties juist kunnen bijdragen aan een effectieve anti-tumor respons, mogelijk via versterking van T cel activatie en antigeenpresentatie.

Vervolgens onderzocht ik in **hoofdstuk 8** hoe tumor-specifieke T cellen bijdragen aan langdurige overleving bij hoofd-hals kanker patiënten. We vonden door het toepassen van high-dimensional imaging mass cytometry dat patiënten met een goede overleving een opvallende spatiele organisatie van immuun cellen vertoonden: T cellen en dendritische cellen vormden goed georganiseerde microaggregaten binnen deze tumoren. Deze microaggregaten bleken aangestuurd te worden door chemokines en bleken essentieel voor langdurige overleving. Dit inzicht benadrukt dat niet alleen de kwantiteit, maar vooral de spatiele organisatie van immuun cellen bepalend zijn voor adequate anti-tumor immuniteit.

De resultaten uit dit tweede deel van het proefschrift benadrukken dat spatiele interacties tussen immuun cellen en tumor cellen een sleutelrol spelen in de effectiviteit van anti-tumor immuniteit. Deze bevindingen bieden aanknopingspunten voor de ontwikkeling van therapieën die gericht de spatiele organisatie binnen tumoren kunnen beïnvloeden.

Conclusie en toekomstperspectief

Dit proefschrift beschrijft hoe de samenstelling en compositie van de immuun micro-omgeving bepalend zijn voor de uitkomst van immuuntherapie en de overleving bij HPV-geïnduceerde tumoren. Door toepassing van innovatieve imaging technieken zoals multispectrale immunofluorescentie, high-dimensional imaging mass cytometry en single-cell spatial transcriptomics zijn nieuw inzichten verkregen in de heterogeniteit van de micro-omgeving tussen patiënten en de klinische relevantie hiervan. De resultaten laten zien dat specifieke immunologische kenmerken voorspellend zijn voor respons op therapie en voor duurzame tumor controle.

Gezamenlijk leggen de bevindingen uit dit proefschrift de basis voor de ontwikkeling van biomarkers voor gepersonaliseerde behandeling van HPV-geïnduceerde (pre)maligniteiten; een paradigma verschuiving van een uniform behandelstrategie op basis van tumor type, naar een gepersonaliseerde behandelstrategie op basis van de micro-omgeving status van de individuele patiënt (**hoofdstuk 9**).

Toekomstige studies zouden zich moeten richten op de validatie van de geïdentificeerde immuun kenmerken en op de integratie van spatiele data met genomische en klinische informatie in een prospectieve setting. Door verdere toepassing van kunstmatige intelligentie en geautomatiseerde beeldanalyse kan de complexiteit van de tumor micro-omgeving nog beter gekarakteriseerd en begrepen worden.

Appendices

The background of the page is a deep space image showing a complex network of galaxies and intergalactic matter. Thin, glowing filaments of light in various colors (blue, green, orange, purple) crisscross the dark void, representing the cosmic web. Numerous bright, multi-pointed star-like objects are scattered throughout, some appearing as dense clusters of light.

List of publications

The immune microenvironment in vulvar (pre)cancer: review of literature and implications for immunotherapy

Z. Abdulrahman, K.E. Kortekaas, P.J. de Vos van Steenwijk, S.H. van der Burg, M.I.E. van Poelgeest

Expert Opinion on Biological Therapy, October 2018

doi: 10.1080/14712598.2018.1542426

Cancer immunophenotyping by 7 colour multispectral imaging without tyramide signal amplification

M. IJsselsteijn, T. Brouwer, Z. Abdulrahman, A. Ramalheiro, A. Vahrmeijer, K. Jordanova, N.F.C.C. de Miranda

Journal of Pathology: Clinical Research, January 2019

doi: 10.1002/cjp2.113

High numbers of activated helper T cells are associated with better clinical outcome in early-stage vulvar cancer, irrespective of HPV or p53 status

K.E. Kortekaas, S.J. Santegoets, Z. Abdulrahman, V. van Ham, M. van der Tol, I. Ehsan, H.C. van Doorn, T. Bosse, M.I.E. van Poelgeest, S.H. van der Burg

Journal for ImmunoTherapy of Cancer, September 2019

doi: 10.1186/s40425-019-0712-z

Pre-existing inflammatory immune microenvironment predicts the clinical response of vulvar high-grade squamous intraepithelial lesions to therapeutic HPV16 vaccination

Z. Abdulrahman, N.F.C.C. de Miranda, E.M.G. van Esch, P.J. de Vos van Steenwijk, H.W. Nijman, M.J.P. Welters, M.I.E. van Poelgeest, S.H. van der Burg

Journal for ImmunoTherapy of Cancer, March 2020

doi: 10.1136/jitc-2020-000563

Lack of myeloid cell infiltration as an acquired resistance strategy to immunotherapy

E. Beyranvand, Z. Abdulrahman, C. Labrie, M. van Elsas, E. Rademaker, J. Kleinovink, T. van der Sluis, S. Duikeren, A. Teunisse, A. Jochemsen, J. Oosting, N.F.C.C. de Miranda, T. van Hall, R. Arens, S.H. van der Burg

Journal for ImmunoTherapy of Cancer, September 2020

doi: 10.1136/jitc-2020-001326

A pre-existing coordinated inflammatory microenvironment is associated with complete response of vulvar high-grade squamous intraepithelial lesions to different forms of immunotherapy

Z. Abdulrahman, N.F.C.C. de Miranda, B.W.J. Hellebrekers, P.J. de Vos van Steenwijk, E.M.G. van Esch, S.H. van der Burg, M.I.E. van Poelgeest
International Journal of Cancer, November 2020
doi: 10.1002/ijc.33168

CD161 expression and regulation defines rapidly responding effector CD4+ T cells associated with improved survival in HPV16-associated tumors

C.L. Duurland, S.J. Santegoets, Z. Abdulrahman, N.M. Loof, G. Sturm, T.H. Wesselink, R. Arens, S. Boekestijn, I. Ehsan, M.I.E. van Poelgeest, F. Finotello, H. Hackl, Z. Trajanoski, P. ten Dijke, V.M. Braud, M.J.P. Welters, S.H. van der Burg
Journal for ImmunoTherapy of Cancer, October 2021
doi: 10.1136/jitc-2021-003995

Tumor-specific T cells support chemokine-driven spatial organization of intratumoral immune microaggregates needed for long survival

Z. Abdulrahman, S.J. Santegoets, G. Sturm, P. Charoentong, M.E. Ijsselsteijn, A. Somarakis, T. Höllt, F. Finotello, Z. Trajanoski, S.L. van Egmond, D. Mustafa, M.J.P. Welters, N.F.C.C. de Miranda, S.H. van der Burg
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Immune-based biomarker accurately predicts response to imiquimod immunotherapy in cervical high-grade squamous intraepithelial lesions

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doi: 10.1136/jitc-2022-005288

The ratio between monocytic myeloid derived suppressor cells/monocytes and dendritic cells is a prognostic intratumoral and blood biomarker for survival of epithelial ovarian cancer patients

A.F. de Groot, S.J. Santegoets, Z. Abdulrahman, E.M. Dijkgraaf, J.R. Kroep, S.H. van der Burg
Gynecology & Obstetrics Open Access, June 2024
doi: 10.29011/2577-2236.100202

Monocyte infiltration is an independent positive prognostic biomarker in vulvar squamous cell carcinoma

Z. [Abdulrahman](#), K.E. Kortekaas, M.J.P. Welters, M.I.E. van Poelgeest, S.H. van der Burg

Cancer Immunology, Immunotherapy, July 2024

doi: 10.1007/s00262-024-03755-w

Single-cell spatial transcriptomics unravels cell states and ecosystems associated with clinical response to immunotherapy

Z. [Abdulrahman](#), R.C. Slieker, D. McGuire, M.J.P. Welters, M.I.E. van Poelgeest, S.H. van der Burg

Journal for ImmunoTherapy of Cancer, March 2025

doi: 10.1136/jitc-2024-011308

Pre-existing infiltration with T cells and CD14+ myeloid cells is associated with treatment response to imiquimod in primary and recurrent vulvar high-grade squamous intraepithelial lesions

C.L. Muntinga, P.J. de Vos van Steenwijk, L.F.S. Kooreman, S. Regauer, Z. [Abdulrahman](#), R.L.M. Bekkers, S.H. van der Burg, G. Trutnovsky, E.M.G. van Esch

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doi: 10.1136/jitc-2025-012666

The local cellular response to Human Papillomavirus focuses on basal layer restoration as visualized in situ by specific cellular neighborhoods near infected cells

R.C. Slieker, Z. [Abdulrahman](#), S.J. Santegoets, M.I.E. van Poelgeest, M.J.P. Welters, S.H. van der Burg

Frontiers in Immunology, December 2025

doi: 10.3389/fimmu.2025.1728629

Frailty of older patients is not reflected in the melanoma tumor microenvironment and treatment response to immune checkpoint blockade therapy

A. Özkan, M.E. Ijsselsteijn, Z. [Abdulrahman](#), M. van der Ploeg, E. Kapiteijn, M. Slingerland, E.M.E. Verdegaal, M. J. P. Welters, S.H. van der Burg, J.E.A. Portielje, N.F.C.C. de Miranda, N. A. de Glas

In press, 2026

Curriculum vitae

Ziena Abdulrahman studied at Leiden University. Following her graduation, she conducted doctoral research at the departments of Gynaecology, Oncology and Pathology at Leiden University Medical Center. Her PhD research focused on the tumor immune microenvironment in HPV-induced tumors and its impact on immunotherapy response and patient survival. She applied various high-dimensional imaging techniques and uncovered novel insights about the clinical importance of spatial immune architectures.

Acknowledgements

الْعُلْمُ فِي الصِّغَرِ كَالنَّقْشِ عَلَى الْحَجَرِ

'Knowledge in childhood is like engraving on stone' (Arabic proverb)

A heartfelt thank you to all colleagues, teachers, patients, friends and family whose guidance, encouragement and support have made this PhD journey possible. Their contributions and belief in the pursuit of knowledge have shaped the path that led to this thesis. Every encounter, conversation and shared moment of wonder has shaped my development as a scientist. I am profoundly grateful to all who supported and inspired me along the way.

