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Perspectives of the myeloid APC – T-cell axis

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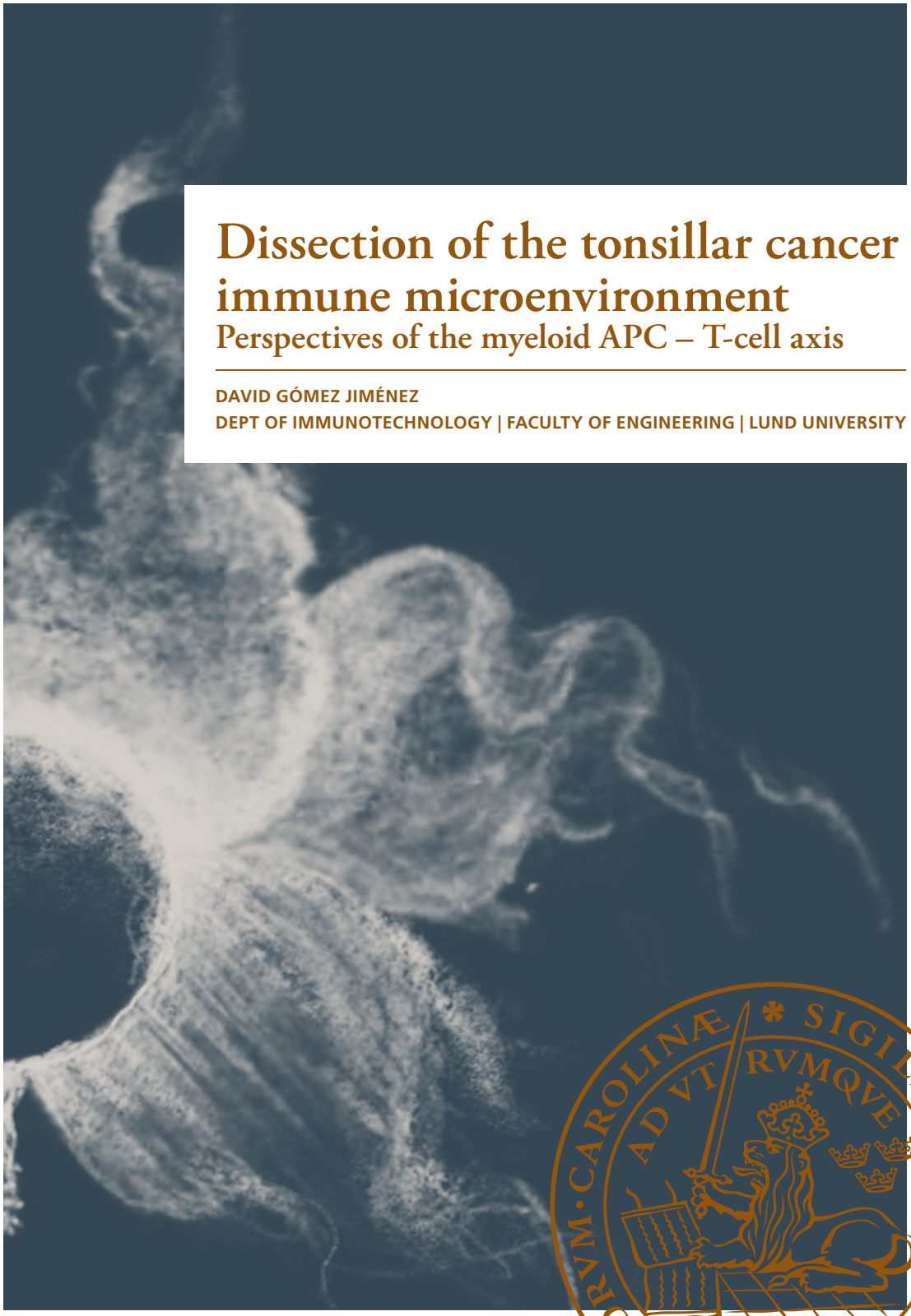

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LUND UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Tonsillar cancer (TC) is a subset of head and neck cancer (HNC). TC incidence is sharply rising due to an increased prevalence of human papillomavirus (HPV) infection in the western world. Currently HPV status is considered during clinical staging due to its positive association to patient prognosis. Presumably, the relation between HPV and patient survival is dependent on the generation of an effective anti-viral immune-response. However, there is a lack of knowledge of the immune-phenotypes of TC, and of the activity of the cancer-immunity cycle in these lessions. Such knowledge is essential to develop predictive biomarkers of prognosis and treatment response. In the papers included in this thesis we aim to characterize the immune-phenotypes of HPV$^+$ TC, with special attention to the myeloid antigen presenting cell (APC)-T-cell axis, to broaden our understanding of the tumor immune-microenvironment and its clinical implications.

In paper **I**, we described an increased infiltration of CD8$^+$ T-cells, dendritic cell (DC), and macrophages in HPV$^+$ TC compared to paired healthy tonsil. Intra-tumoral levels of CD8$^+$ T-cells and DC were correlated to one another in HPV$^+$ lesions, indicative of a cross-talk between these populations. In paper **II**, we investigated the distribution and targeted protein expression of CD8$^+$ cells, using spatial proteomics in a cohort of 105 TC patients. We observed that increased CD8 infiltration was associated with higher 5-year overall survival, independently of TNM7 classification, age at diagnosis and HPV status. HPV$^+$ TC patients with low degree of CD8$^+$ cell infiltration displayed a similar 5-year overall survival as HPV$^-$ patients, highlighting interpatient variations. Upon segmentation of CD8$^+$ regions according to their location in the tumor, we observed an upregulation of immune checkpoint and effector molecules in CD8$^+$ cells infiltrating cancer-cell nests, compared to CD8$^+$ cells in the stroma. In addition, CD8$^+$ T-cell density in cancer-cell nests, but not in stromal regions, was associated with a higher 5-year overall survival. Together, these results suggest an effective anti-tumoral function of CD8$^+$ T-cells in cancer-cell islets. In paper **III,** we explored myeloid APC diversity in HPV$^+$ TC and healthy tonsil using single-cell RNA-seq and flow cytometry. We identified four DC lineages, two macrophage polarization events, and described their maturation processess. Among the findings with clinical implications, we described that HPV$^+$ TC featured higher levels of cDC1, and that high score of a cDC1-specific 3-gene signature was associated with higher 5-year overall survival in TC as well as HNC in general. Finally, in paper **IV** we developed an *in vitro* 3D holographyc imaging protocol to study TC-derived organoid biology. Using this protocol, we described that CD44$^+$NGFR$^-$ cells displayed higher organoid forming capacity and higher motility than CD44$^-$NGFR$^-$ and CD44$^+$NGFR$^+$ cells.

Taken together, the research included in the present thesis highlights the interplay between myeloid APC and T-cells, and suggests several biomarkers that may be applied in clinical settings to assess prognosis. Our data also illustrate mecahnisms involved in immunosuppression as well as in succesful immunosurveillance related to HPV$^+$ TC biology.

Key words: Dendritic cell, macrophage, CD8$^+$ T-cell, human papillomavirus, tonsillar cancer, tumor microenvironment, immuno-oncology, flow cytometry, single-cell RNA sequencing, spatial proteomics.

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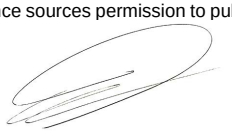
Signature  Date 2022-09-23

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"*When approaching a topic, we can adopt two basic attitudes, we either have a hypothesis about the matter, or we simply aim to learn from it. The first attitude can be called confirmation, while the second might be called research. There is no chance of understanding a matter without investigating it. We learn by allowing ourselves the benefit of surprise, replacing prejudice with judgement. We develop judgment through the compilation of successive facts, and sometimes it takes many quantitative measurements for a qualitative change to occur. However, if we analyze any idea with patience, unfeelingly, welcoming surprise, that idea will change*." – **Antonio Escohotado**.

Table of contents

Original research articles

This thesis is based on the following articles, referred to by their Roman numerals (I-IV):

I. **Jimenez D.G.**, Sobti A., Askmyr D., Sakellariou C., Carreira Santos S., Swoboda S., Forslund O., Greiff L., Lindstedt M. *Tonsillar cancer with high CD8$^+$ T-Cell infiltration features increased levels of dendritic cells and transcriptional regulation associated with an inflamed tumor microenvironment.* Cancers (Basel). 2021;13(21):5341.

II. Altunbulakli C., **Jimenez D.G.**, Askmyr D., Sobti A., Swoboda S., Greiff L., Lindstedt M. *Spatial proteomic characterization of the tumor microenvironment of tonsillar cancer*. 2022. Manuscript.

III. **Jimenez D.G.**, Altunbulakli C., Swoboda S., Sobti A., Askmyr D., Ali A., Greiff L., Lindstedt M. *Single-cell analysis of myeloid cells in HPV$^+$ tonsillar cancer.* 2022. Submitted manuscript with preprint available on bioRxiv.

IV. **Jimenez D.G.**, Carreira Santos S., Greiff L., Alm K., Lindstedt M. *Subpopulations of organoid-forming cells have different motility.* Appl. Sci. 2020, *10*, 4673.

Author's contributions to articles

I. Participated in experimental design. Performed sample processing, flow cytometric analysis, and RNA-seq and qPCR data analysis together with co-authors. Prepared figures and participated in manuscript writing and revision. Did not perform the HPV DNA and RNA quantification.

II. Participated in the conceptualization and design of the study, spatial biomarker analysis, conducted survival and recurrence data analysis, shared the biological interpretation of the data. Participated in manuscript writing. Did not perform the selection, normalization, and differential expression analysis of spatial proteomics data.

III. Conceived the study together with co-authors, participated in study design and performed the flow cytometric analysis and cell sorting. Performed the data analysis and interpreted the results in collaboration with other authors. Prepared the figures and was the main writer of the manuscript.

IV. Conceived the study and designed the analysis with co-authors, participated in method development, experiments, data analysis and visualization, and participated in manuscript writing.

Other original research articles

I. Askmyr D., Abolhalaj M., **Jimenez D.G.**, Greiff L., Lindstedt M., Lundberg K. *Pattern recognition receptor expression and maturation profile of dendritic cell subtypes in human tonsils and lymph nodes. Hum Immuno*l. 2021;82(12):976-981.

II. Hägerbrand K., Varas L., Deronic A., Nyesiga B., Sundstedt A., Ljung L., Sakellariou C., Werhau D., Thagesson M., **Jimenez D. G.**, Greiff L., Celander M., Smedenfors K., Rosén A., Bölükbas D., Carlsson F., Levin M., Säll A., von Schantz L., Lindstedt M., Ellmark P. *Bispecific antibodies targeting CD40 and tumor associated antigens promote cross-priming of T cells resulting in an anti-tumor response superior to monospecific antibodies.* Journal for ImmunoTherapy of Cancer. Accepted, 30th Sept 2022.

Popular scientific summary

Cancer is a group of more than 200 distinct illnesses, each characterized by its tissue of emergence as well as its cellular and molecular properties. The goal of cancer treatment is to maximize the results of the therapy while minimizing the side effects for the patient. However, due to the diversity of illnesses, and the intrinsic differences between patients, the effectiveness of the treatment varies. Precision medicine is an approach that accounts for this variability, in contrast to the traditional "one treatment suits all patients". Precision medicine can be used for instance to predict the probability of survival, tumor re-appearance after treatment, and to identify patients likely to respond to a certain treatment. This is accomplished by using clinical and/or biological markers, which are measurable traits that represent key events of the disease.

A solid tumor is composed of cancer cells, and their microenvironment including immune cells, blood and lymphatic vessels, among other cell types. Recent technological advancements enable us to study the components of a tumor at single-cell resolution, which makes it possible to study in detail the properties of individual cells and their location in the tumor tissue. We focus our research on tonsillar cancer (TC), a fast-growing disease caused by the infection of human papillomavirus, tobacco smoking, and alcohol abuse. The presence of the virus is used as a biological marker in the clinic, because patients with papillomavirus-driven tumors have a better prognosis compared to those patients with tumors caused by tobacco and alcohol. The viral particles in the tumor act as a flag marking infected cancer cells, and ultimately facilitate their detection and destruction by immune cells. Among immune cells, we investigate cells that are key in the generation and execution of immunological memory and tumor cell killing: dendritic cells, macrophages, and T-cells. Dendritic cells and macrophages can "eat" tumor cells, and use parts of these (the antigens) to instruct T-cells. Some instructed T-cells (known as $CD8^+$ T-cells) can then kill tumor cells when they recognize the antigens on the surface of the tumor cell.

We monitor the immune responses that take place in the tumor by quantifying the type and number of immune cells, by quantifying the genes they express, and by estimating how these parameters influence patient prognosis. Ultimately, we can use this information to estimate if an immune response happens and suggest biological markers and treatments.

In paper **I**, we quantified and compared the abundance of human papillomavirus, T-cell, macrophages, and dendritic cell subtypes in biopsies of TC lesions and healthy tonsil obtained from the same patient. We observed that tumors with viral gene expression had higher level of $CD8^+$ T-cells, macrophages, and subtypes of dendritic cells, compared to tumors that lacked viral gene expression and to the healthy tonsil from the same patient. This indicated that patients infected with papillomavirus had an on-going immune-response in the tumor. We also observed that the abundance of dendritic cells and $CD8^+$ T-cells were correlated to each other in tumors with viral gene expression. At this point, we hypothesized that dendritic cells and perhaps macrophages influenced the levels of $CD8^+$ T-cells.

The architecture of a solid tumor is as complex as the one of a healthy organ. We hypothesize that the location of the immune cells, either inside isles of tumor cells, or surrounding these isles, reflects their function. In paper **II**, we assessed if the abundance, position in the tumor, and expression of a selection of proteins by $CD8^+$ cells affected TC patient survival. We observed that the overall abundance of $CD8^+$ cells, and specifically of those located inside tumor cell isles, was associated with greater probability of survival. Next, we described that $CD8^+$ cells in the tumor isles expressed proteins related to dysfunctional activity, that might impair their ability to destroy tumor cells. These observations indicate that $CD8^+$ cells are beneficial for the patient, but their activity might be partly dampened when these infiltrate tumor cell isles.

The emergence of single-cell RNA sequencing technology has revolutionized biomedicine by allowing us to detect the expression of thousands of genes and cells. Using this novel tool, we can now classify cells based on their gene expression without any previous information. Dendritic cells and macrophages are key cells that tailor T-cell immune responses, but they are also rare, and often they represent 0.1% of all the cells in the tumor. Because of this, they are under-studied. In paper **III**, we characterized subtypes of dendritic cells and macrophages, isolated from biopsies of papillomavirus-driven TC patients, using this novel technology. We identified thirteen groups of dendritic cells and macrophages, and described their differentiation and activation in the tumor. We also observed that, among the different groups, a subtype of dendritic cell known as cDC1 was more abundant in tumors, and that its gene

expression was correlated to longer patient survival. We predicted that cDC1 had a higher ability to activate $CD8^+$ T-cells compared to other dendritic cells and macrophages. We proposed cDC1 as the initial spur in the development of a beneficial immune response in TC patients infected with papillomavirus.

In paper **IV**, we used a three-dimensional cell culture model that allowed us to grow tumor cells, derived from a patient with papillomavirus-induced cancer, in the laboratory. We developed a protocol to monitor the formation of this artificial tumor using holographic microscopy. We described that, among all tumor cells, those expressing the protein CD44 engaged other tumor cells creating three-dimensional artificial tumor spheres within fifteen hours. This paper represents a first step to study the interaction of immune cells with the tumor in detail. When studying the biology of a tumor, we rely on the expression of genes and proteins to predict the function of immune cells. However, the protocol described in paper IV may be used to prove some of the predicted functions of dendritic cells and macrophages.

To sum up, we have characterized the subtypes, activation status, and abundance of immune cells engaged in the development of antigen-specific immune responses in TC lesions driven by human papillomavirus infection. The research of this thesis contributes to the understanding of immunological responses in the disease and provides foundation for the use of cDC1 and $CD8^+$ T-cell biomarkers in clinical practice.

Abbreviations

APC: Antigen presenting cell.
CD: Complex of differentiation.
cDC: Conventional DC.
DC: Dendritic cell.
FC: Flow cytometry.
HPV: Human papillomavirus.
HT: Healthy tonsil.
ICI: Immune checkpoint inhibitor.
LC: Langerhans cell.
MDSC: Myeloid-derived suppressor cell.
MHC-I/II: Major histocompatibility complex class I or II.
moDC: Monocyte-derived DC.
PCR: polymerase chain reaction.
pDC: Plasmacytoid DC.
RNA-seq: RNA-sequencing.
scRNA-seq: Single-cell RNA-seq.
TAM: Tumor-associated macrophage.
TC: Tonsillar cancer.
TCR: T-cell receptor.
TF: Transcription factor.
TFH: T follicular helper cell.
Th: T-helper cell.
TME: Tumor microenvironment.
Treg: T-regulatory cell.

1.Introduction

The capacity of the immune system to wage war on cancer tissue has been recognized for over 100 years (1). However, it was not until recently that we effectively directed the immune system to treat neoplastic diseases. Immunotherapy holds the promise of generating a diverse yet specific and long-lasting effect on cancer patients. Recent advances in the field have now allowed us to treat patients with fatal malignancies of over 50 cancer types (2). As a result, immunotherapy is now included among the five pillars of cancer therapy, along with surgery, chemotherapy, radiotherapy, and targeted therapy (3). Despite its impressive clinical success, immunotherapy has proven effective only in a subset of patients (2). Currently there are active efforts in understanding the interplay between the immune system and cancer, ultimately aiming to address a higher number of patients.

The main objective of this thesis was to explore the immune landscape of human tonsillar cancer (TC). We have studied the interplay between myeloid and T-cells, which governs the antigen-specific cellular response, and compared subtypes of TC and healthy tonsils (HT), with a focus on human papillomavirus (HPV) driven TC. In the following chapters, I will briefly present our current understanding of immuno-oncology and how immune responses and cancer development shape each other. I will examine the cellular dynamics of the tonsil, and the origins and progression of TC. Further, I will detail the treatment modalities of TC, including currently approved treatments, as well as those under evaluation in clinical trials. Next, I will delve into the methodology used to scrutinize myeloid cell diversity, the subtypes of myeloid antigen presenting cells (APC), and their impact on patient survival. Finally, I will present *in vitro* tools that are used to study immune-cell function and to test novel therapeutic approaches.

1.1. Immuno-oncology

Cancer, derived from the Greek *karkinoma*, was first recognized as a medical entity in the western world by the Hippocratic school in the city state of Athens, as reviewed in (4). The term *karkinoma* is thought to derive from *karkinos*, crab, due to the hardness of the tissue and the blood vessels that grow into the tumor, reminiscent of the shell and the legs of the crab. According to the texts of Galen and Archigenes, long-standing cancers were then considered incurable and were surgically removed (5). On the contrary smaller tumors were treated with drugs, from the Greek *phármakon*, meaning medicine, poison, and magic (6). These texts illustrate that concepts such as staging, early detection, and treatment were already managed by ancient practitioners. However, the paradigm used to explain the origin and development of cancer has completely shifted over time.

In 2000, Hanahan and Weinberg proposed "the hallmarks of cancer" theory, which describes a series of traits that a cell acquires in its progression to a neoplastic state (7). Revisited in 2011 and 2022, it currently comprises a total of eight hallmarks: "sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing/accessing vasculature, activating invasion and metastasis, reprogramming cellular metabolism, and avoiding immune destruction" (8, 9). The field of immuno-oncology builds on the last hallmark, and it studies the interplay between immunity and cancer. The notion that the immune system is able to recognize and destroy neoplastic tissue arose from the observations of William Coley in 1893 (1). Coley treated sarcoma patients using inactivated streptococcal toxins and described a reduction of the tumor volume. The concept of immunosurveillance was finally proposed in 1950s by Burnet and Thomas who suggested that the immune system constantly monitors tissues, eliminating most of the early cancer cells, thus avoiding tumor formation (10).

After 50 years of experimental research in the field of immuno-oncology, the immunosurveillance concept crystallized into the cancer immunoediting theory, postulated by Schreiber in 2004 (11). Immunoediting harmonizes a body of evidence indicating that the immune system has a dual role regarding cancer progression, limiting or favoring it depending on the type and stage of the tumor. Immunoediting is a dynamic process encompassing three steps: elimination, equilibrium, and evasion, which occur in parallel to tumor progression, even though the steps may temporally co-exist (11). Firstly, during elimination the immune system detects and destroys highly immunogenic tumor cells. It does so, partly, by recognizing tumor

(neo)antigens generated due to genomic instability of tumor cells. The second step, equilibrium, involves a situation of clonal selection. The immune system recognizes and eliminates highly immunogenic tumor cell clones, while less immunogenic clones are selected and progress within the tumor. This situation leads into the third step, escape, in which the tumor cells co-opt immune cells to render them inactive or favor tumor growth (11).

In 2013, Chen and Mellman introduced the concept of the cancer-immunity cycle to explain how effective anti-cancer immune-responses are mounted, using the antigen-specific dendritic cell (DC) – T-cell axis as backbone (12). The cancer immunity cycle is a stepwise process consisting of subsequent events. Initially, dying neoplastic cells release a battery of molecular signals, i.e., damage associated molecular patterns, such as HMGB1, ATP, ANXA1, CALR, HSPs, and type 1 IFNs, which trigger chemotaxis and activation of innate immune cells via pattern recognition receptors (13). DC phagocyte particulate, or endocytose soluble, tumor (neo)antigens, and process these via sequential trafficking from endosomes to either lysosomes or cytosol where the antigens are enzymatically digested via cathepsins or the proteasome, respectively. Peptide epitopes are loaded onto nascent or recycled MHC-I or II complexes, and trafficked to the cell membrane ready to be presented to T-cells (14). This process is accompanied by profound changes in DC gene expression profile known as maturation, which involve upregulation of CCR7, MHC-II, and co-stimulatory molecules CD40/80/86 (15). Thereafter, $CCR7^+$ mature DC acquire the ability to migrate out of the tumor into the draining lymph nodes via CCL19/21 gradients generated by endothelial cells of lymphatic vessels (16). In the draining lymph node, $CCR7^+$ mature DC may then encounter and prime naïve and memory T-cells. Three signals are needed for T-cell priming to occur: specificity through the formation of the T-cell receptor (TCR)-epitope-MHC complex, co-stimulation via CD80/86-CD28, and differentiation signals via cytokine production by DC. These signals induce T-cell clonal expansion producing both effector and memory clones, which access high endothelial venules, traffic back into the tumor, and infiltrate the tumor bed. Once in the tumor microenvironment (TME), antigen-specific $CD8^+$ T-cell clones will recognize tumor (neo)antigens loaded onto MHC class I molecules on the surface of tumor cells. The formation of the TCR-epitope-MHC-I complex results in the release of cytotoxic granules containing PRF1 and GZMs by terminal effector $CD8^+$ T-cells, inducing tumor cell death in an antigen-specific fashion. Tumor cell death increases the availability of tumor (neo)antigens further boosting the cancer-immunity cell cycle (12). In the context of immunoediting, the cancer-immunity cycle performs at its best during the elimination phase.

1.2. Immunology of head and neck cancer

Tumors can be classified according to their immune-phenotypes (17). Desert and immune-excluded tumors feature insufficient infiltration of T-cells due to a lack of immunogenic antigens, antigen presentation, chemotaxis, or the deposition of dense extracellular matrix by stromal cells. In comparison, inflamed tumors display effective immune infiltration, yet with impaired anti-tumor activity (17). Taking head and neck cancer (HNC) as an example, tumor progression is accompanied by a loss of immunogenic neoantigens with branch mutations and acquisition of mechanisms to hijack different steps of the cancer immunity cycle (17, 18). For instance, Ferris et al. reported an increased abundance of TGF-β in the TME of HNC compared to healthy tissue (19). Mainly produced by tumor cells or T-regulatory cells (Treg), TGF-β inhibits CCR7 expression by DC *in vitro* (20). Intra-tumoral retention mediated by CCR7 downregulation could result in a decreased migration to draining lymph nodes in humans, as shown in mouse models (21). During T-cell priming, lack of IL-12 stimulation and/or production of IL-10 by DC renders $CD8^+$ T-cells anergic, characterized by absence of cytolytic and IFN-γ production abilities, and favors Treg development from naïve $CD4^+$ T-cells (22-24). Seemingly, over-expression of CTLA-4 upon T-cell activation limits clonal expansion, by outcompeting CD28 in its interaction with CD80/86 (25). Further, Meissner et al., and Leibowitz et al., demonstrated defects in the antigen processing pathway of HNC cells, at RNA and protein level *ex vivo* and *in vitro* (26, 27). These defects included the downregulation of STAT1, LMP2/7, and TAP1, which may ultimately allow $CD8^+$ T-cell evasion, and were associated with lower overall survival (27). Lastly, tumor cell death mediated by $CD8^+$ T-cells can be impaired by overexpression and engagement of PD-1, LAG-3, and TIM-3 immune checkpoints. Krishna et al. showed that these checkpoints are stably expressed by exhausted $CD8^+$ T-cells in HNC upon continued antigen re-stimulation, resembling T-cell exhaustion in chronic viral infections (28, 29). More importantly, $CD8^+$ T-cells from patients treated with anti-PD-1 inhibitors maintain exhaustion through other immune checkpoints, resulting in the loss of IL-2, TNF-α, and IFN-γ production, leading to apoptosis (30).

To sum up, a variety of immunological processes can influence cancer progression and ultimately patient prognosis. However, these mechanisms may vary according to the type of tumor, level of disease progression, and even between patients due to interpatient variation. Hence, it is of utmost importance to accurately describe these events as well as to suggests biomarkers, points of intervention, and treatment modalities that can be beneficial for a given patient.

2.Pathology of the tonsil

2.1. The tonsil, structure, and function

The palatine tonsils are paired lymphoid organs located in the oropharynx. While most lymphoid organs receive antigens via blood or lymphatic vessels, the tonsils continually sample inhaled and ingested material (31). The tonsil is divided in three functional areas consisting of reticular crypt epithelium, lymphoid follicles, and extrafollicular area (32).

The reticular crypt epithelium is a type of non-keratinized stratified squamous epithelium of endodermal origin. The epithelium is characterized by 10-30 branched crypts, which are macroscopically visible on the surface of the tonsil (32). The crypts maximize the surface of the tonsil and entrap cellular debris and foreign antigen material. In the crypts, antigen transport is thought to be performed by specialized epithelial M cells, which would constantly transport antigens across their cytoplasm via transcytotic vesicles (33). On their apical side, M cells display pockets where APC process antigens and transport them through the epithelium, making them available to the underlying lymphoid tissue (34).

The extrafollicular area is mainly composed of T-cells and conventional DC (cDC). This region is vascularized by high endothelial venules, which allow the entrance of circulating naïve and central memory T- and B-cells. Mature cDC2, carrying processed peptide antigens onto their MHC-II, activate $CD4^+$ T-cells through antigen presentation. Upon activation, $CD4^+$ T-cells are polarized into T-helper (Th) type 1, 2, and 17, Treg, or T follicular helper (TFH), the latter of which will migrate to the lymphoid follicles. In turn, mature cDC1 cross-present peptides to naïve $CD8^+$ T-cells via MHC-I, inducing proliferation and differentiation of clones with a peptide-matching TCR. Effector and memory $CD8^+$ and $CD4^+$ Th exit the tonsil via high endothelial venules, transiting into areas, for instance, with local inflammation.

The lymphoid follicles are mainly composed of B-cells, but also harbor small numbers of TFH, macrophages, and follicular DC. In the follicles, B-cells obtain antigens from follicular DC through B-cell receptor-mediated endocytosis. Then, activated B-cells proliferate and undergo somatic hypermutation increasing the diversity of the antibody variable region genes, and differentiate into memory B-cells and antibody secreting cells (35). TFH are key players of this process, providing signals for B-cell survival and differentiation via CD40LG and IL-21 (36). Durand et al. recently proposed that in the tonsil, maturing TFH access the B-cell zone interface where they are further primed by macrophages to instruct their differentiation process (37). This observation contrasts with the classical function of macrophages in the draining lymph nodes, where these cells are positioned in the subcapsular sinus, sampling exogenous material that flows through the afferent lymphatics (31, 38).

Overall, the structure and the localization of immune cells within the tonsil dictate the development of adaptive immune responses in the region of the oropharynx. However, due to the constant exposure to pathogens, the tonsil is also subjected to infection, which can impair human health either in the form of tonsilitis or through the development of TC triggered by HPV.

2.2. HPV biology and epithelial cell transformation

HPV are circular double stranded DNA viruses without envelop. The HPV genome is 8Kb long and it has 7-9 open reading frames including early genes *E1-2* and *E4-7*, and late genes *L1-L2*, which encode components of the viral capsid (39). The *Papillomaviridae* taxonomic family consists of over 170 HPV types, which are divided into low and high-risk groups. Low-risk HPV types may lead to the emergence of warts, while high-risk types can trigger carcinogenesis in non-keratinized stratified squamous epithelia (40). The oncogenic potential of HPV was first described in cervical cancer patients by zur Hausen's team in 1983 (41). Among high-risk HPV types, HPV16 and HPV18 are the most prevalent in tumors, and besides cervical cancer they are also associated with anal, vaginal, vulval, penile, and oropharyngeal cancer, notably TC (42).

The strict association between HPV and stratified squamous epithelia lies in the close relationship between the HPV life cycle and epithelial cell differentiation (39, 43). HPV has a marked tropism for the basal layer of the squamous epithelium, which harbors the epithelial progenitor cell layer. Upon injury to the epithelium, HPV accesses its basal layer, binds to proteoglycans in the surface of epithelial cells through the L1 protein (43), and is endocytosed via tetraspanin (44). Once in the endosomes, the viral capsid is degraded, and cleaved fragments of the L2 protein form a complex with the HPV genome. The genome-L2-retromer complex then accesses the retrograde transport pathway, sequentially moving from lysosome to Golgi network, endoplasmic reticulum, and finally accessing the cell nucleus during mitosis (43, 45).

In the nucleus, HPV DNA is either maintained episomally or it integrates into the host DNA via disruption of the *E2* open reading frame (39). The *E2* gene codes for the E2 repressor protein, which controls the expression of genes downstream from HPV's p97 promoter, including *E6* and *E7*. Hence, disruption of the *E2* gene upon integration of the viral DNA into the host genome allows active transcription of the *E6* and *E7* oncogenes (39). The major effect of the E6 and E7 oncoproteins is forcing basal epithelial cells to escape cell cycle control. Briefly, E6 induces ubiquitin mediated degradation of the tumor suppressor protein p53 via the ubiquitin ligase E3A (46). p53 degradation has diverse effects including decreased apoptosis through the Smac/DIABLO mitochondrial pathway, blockade of the cyclin regulated cell-cycle arrest mechanism, and impairment of the p53R2 mediated DNA repair process (47). In turn, E7 binds to the pRb-E2F complex, forcing the release of the transcription factor (TF). E2F controls the expression of a gene network involved in the transition from cell cycle phase G1 into S. E7 also promotes de-methylation of histone 3 (H3K27), resulting in the upregulation of p16 (48). In fact, p16 is used as a surrogate diagnostic marker of high-risk HPV infection in the clinic (49).

At the basal layer, HPV's genome is maintained in the progenitor cell layer and passed on to cellular progeny during cell division. As the epithelium differentiates, HPV DNA is amplified in the nucleus in a circular double stranded format, along with a high production of late proteins L1 and L2 in the cytoplasm (50). Finally, in the most outer epithelial layers, pentamers of L1 and L2 are recruited into the nucleus via Hsc70 where the virions are assembled. HPV virions are then passively released upon disruption of the nuclear envelope when terminally differentiated keratinocytes die (50).

2.3. Epidemiology and prognosis of TC

HNC comprises a group of solid tumors of epithelial origin in areas including the oral and nasal cavities, pharynx, and larynx. TC is an oropharyngeal subset of HNC strongly associated with HPV infection. In fact, HPV^+ TC accounts for over 50% of cases in the US and Europe and over 70% in Sweden (51-53). In the US, the incidence rate of HPV^+ TC has been growing over the last 40 years, while the number of HNC cases driven by carcinogens such as tobacco and alcohol have either declined or remained stable (54). However, these patterns vary globally, since national tobacco and alcohol control measures as well as changes in sexual behavior, have a great influence on the incidence of TC (55).

HPV^+ TC is not only distinct because of its epidemiological trends, but also due to its prognosis. Compared to HPV^- TC patients, HPV^+ TC patients have a better 5-year-overall survival (OS) (36% vs. 81%), and a lower relapse rate (26% vs. 69%) (56). The acknowledgement of these clinical traits has resulted in the inclusion of HPV status in clinical staging and decision making of TC and other oropharyngeal cancers (57), often assessed by p16 detection via immunohistochemistry (IHC). In paper **I**, we observed an 88% overlap between p16 detection via IHC, *E7* HPV16 DNA, and RNA detection using PCR methods. Interestingly, we described that while HPV16 DNA and RNA was detectable in TC, over 50% of contralateral paired HT also featured HPV16 DNA but lacked RNA expression. Gillison et al. reported that 1% of healthy individuals displayed HPV16 DNA in the oropharyngeal cavity in a cross-sectional study in the US (58). In this context, we argued that active transcription of HPV16 oncogenes is a surrogate marker of epithelial transformation, possibly resulting in the production of viral antigens that may elicit an immune response.

TC is a unique entity among HNC subtypes due to the underlying tonsillar lymphoid tissue with APC that can transport tumor antigens and trigger adaptative immune responses *in situ.* In this context, two hypotheses could explain the different prognosis of HPV^+ and HPV^- TC. These refer to either the development of *de novo* antigen-specific immune responses in previously unchallenged individuals, or the mobilization of preestablished immunological memory in individuals previously exposed to HPV.

First, the presence of viral antigens may increase the immunogenicity of HPV^+ TC via an anti-viral immune response. This hypothesis is based on studies where $CD8^+$ T-cell infiltration was assessed via IHC, demonstrating that HPV^+ TC featured higher $CD8^+$ T-cell infiltration compared to HPV^- TC (59, 60). Näsman et al. also highlighted a positive correlation between $CD8^+$ T-cell infiltration and disease-free survival, further supporting this hypothesis (59). In papers **I** and **II**, we explored the immune-phenotype of TC, with a focus on the antigen-specific cellular immunity exerted through the APC-T-cell axis. Consistent with the studies from Näsman et al. and Nordfors et al., we showed that HPV^+ TC displayed higher abundance of $CD8^+$ T-cells compared to HPV^- TC and HT, respectively. Of note, these results were validated in two independent cohorts using different techniques (paper **I**, flow cytometry (FC), n=40; paper **II**, immunofluorescence, n=105). We also indicated that the overall abundance of $CD8^+$ cells, and in particular their abundance in cancer-cell islets, was associated to a lower all-cause mortality, independently of TNM7 staging, HPV status, and age at diagnosis (paper **II**), suggesting that $CD8^+$ T-cell responses might be HPV/tumor antigen-specific. To test the additive prognostic value of combining HPV status and CD8 quantification via immunofluorescence we stratified TC patients according to both parameters. HPV^+ TC featuring CD8 expression levels above the cohort's median featured higher 5-year OS compared to HPV^+ TC with CD8 expression below the median and HPV^- TC patients.

Besides, abundance and location, clonal T-cell specificity is another relevant prognostic factor. Two independent studies have highlighted the presence of a T-cell response against viral antigens in over 60% of HPV^+ oropharyngeal cancer patients (61, 62). Firstly, Bhatt et al. highlighted that 65% of HPV^+ patients displayed circulating $CD8^+$ and $CD4^+$ T-cell clones against at least one of the eight HPV proteins, and 30% displayed T-cell clones against three or more HPV antigens (62). Similarly, Welters et al. reported HPV specific intra-tumoral T-cell clones against E6 and/or E7 oncoproteins in 64% of HPV^+ oropharyngeal cancer patients before treatment (61). They also described a positive association between detectable HPV specific T-cell responses and overall survival, associated with smaller tumor size and lower frequency of local metastases. The authors linked these clinical observations to a Th1-polarized TME as well as a higher frequency of memory and tissue resident T-cells compared to HPV^+ oropharyngeal cancer patients with no detectable anti-viral T-cell response (61). Together these studies highlight the complex network of events involved in the generation of an effective antigen specific cellular immune-response as well as the need to account for inter-patient variation.

The second hypothesis used to explain the different prognosis of HPV^+ and HPV^- TC reflects on a pre-existent anti-HPV immune response. Chesson et al. estimated that over 80% of Americans will acquire HPV during their life (63). In addition, Bhatt et al., described that some healthy individuals (3 out of 22) display $CD8^+$ and $CD4^+$ HPV16-specific T-cell clones (62). This suggests that some unvaccinated HPV^+ individuals have a pre-existent adaptative immune response against HPV antigens before they develop cancer. Currently, over 100 countries world-wide have introduced HPV vaccination according to WHO (64). A recent study by Falcaro et al. highlighted a drastic reduction in cervical cancer incidence when comparing vaccinated and unvaccinated cohorts in England (65). Even though no study has addressed this topic in TC, similar results might be expected from the HPV vaccination programs.

Yet another distinct factor of HPV^+ and HPV^- HNC, is their tumor mutational burden (TMB), i.e., the total number of coding mutations in the tumor genome. In HNC, TMB is close to the average of other solid tumors (> 180 somatic mutations/megabase) (66), with lower mutation rates in HPV^+ compared to HPV^- tumors (2.28 vs. 4.83 mutation/megabase) (30, 67). While HPV affects the activity of p53 and pRb via E6 and E7, and features unique mutations in *FGFR2/3* and *KRAS*, HPV^- HNC frequently feature loss-of-function mutations in the *TP53* and *CDKN2A* genes (68, 69). In theory, a higher mutational burden leads to an increase in tumor neo-antigen frequency, and greater tumor immunogenicity, which would positively impact patient survival. However, a study by Plath et al. suggested that the impact of TMB in HNC patient survival might be subset specific (70). In their study, HPV^- active smokers with homozygous *CDKN2A* deletion displayed a higher mutational burden associated with lower disease specific survival (70). Thus, the impact of TMB on the survival of HNC patients remains unclear.

In summary, the combination of an anti-viral immune response, proximity to underlying lymphoid tissue, and moderate TMB suggests HPV^+ TC as a potential candidate for early immunotherapeutic treatment.

2.4. Approved treatments

First line of treatment of TC traditionally involves radiotherapy. The most common approach is conventional fractioning where a total dose of 60-70 Gy is administered at 2 Gy per day, 5 days a week, over 6-7 weeks (71). The aim with fractioning is to minimize the effect on neighbouring healthy tissue, taking advantage of a higher repair ability of sublethal doses of healthy cells compared to cancer cells. Side effects of radiotherapy are induced by tissue damage/inflammation and subsequent repair processes, resulting in skin wounds and mucositis associated with, and followed by, symptoms including pain, dysphagia, weight loss, and fatigue (71). In locally advanced TC, T category 3-4, notably with HPV^- disease, concomitant radio- and chemotherapy can be administered. In this group, concomitant radio- and chemotherapy treatment increases 5-year OS compared to radiotherapy alone (34% vs. 27%) (72). Cisplatin is administered intravenously to a total of 200 mg/m^2, generally at weekly doses of 30–40 mg/m^2. Side effects of concomitant radio- and chemotherapy are usually severe compared with radiotherapy alone (71). Patients with locally advanced tumors may also be treated with simultaneous EGFR blockade and radiotherapy, to inhibit the proliferative signalling mediated by EGFR overexpression on cancer cells (71). Cetuximab, an EGFR-inhibitor, administered weekly along radiotherapy has shown increased 5-year OS compared to radiotherapy alone (45.6% vs. 36.4%) (73). However, EGFR blockade has a response rate below 20% in HNC, and patients may develop resistance through activation of HER2/3 and MET (74).

Patients with distant metastatic TC have a median survival of 6-9 months and often enter palliative chemotherapy regimens with no demonstrated benefit of survival (71). Currently, there are also two immunotherapeutic drugs approved for the treatment of HNC patients with recurrent or metastatic disease. Nivolumab and pembrolizumab are immune-checkpoint inhibitors (ICI) that block PD-1, aiming to extend T-cell survival, and avoid T-cell exhaustion by reactivating the signaling through ZAP70 and PI3K (75). Nonetheless, PD-1/PD-L1 blockade has proven limited effect in terms of response rate (13.3% for nivolumab, and 17-23% for pembrolizumab) and overall survival compared to the standard of care (7.5 vs. 5.1 months for nivolumab; 13.0 vs. 10.7 months for pembrolizumab) (76, 77). The adverse effects of ICI, even though less frequent, are also of a different nature compared to conventional therapy (76). ICI related adverse events include inflammation of diverse organs, reflected as, e.g., skin rash and hypothyroidism (76). Given the moderate response rates, there is an active effort to elucidate factors predictive of ICI response, but to

date only a few biomarkers have proven predictive efficacy. PD-L1 expression and an inflamed T-cell gene signature correlate to one another and to ICI response (78). In addition, *TP53* mutation was recently associated with a lower ICI response rate (79). TMB is only predictive of ICI response in HPV^- HNC, irrespective of PD-L1 expression (30), while HPV status is not predictive of ICI response (76).

Interestingly, Bhatt et al. reported a decrease in the number of HPV specific T-cell clones of patients following curative treatment, involving either surgery, radiotherapy, chemotherapy, cetuximab, or combinations thereof, compared to treatment naïve patients (62). This observation may reflect a reduction in HPV antigens necessary to maintain T-cell responses in patients after curative treatment. It also suggests that ICI-based therapy may produce significant therapeutic effect if applied at an earlier point of intervention.

2.5. Immunotherapeutic treatments under evaluation

Even though patient stratification strategies are improving, still more than 80% of HNC patients with refractory or metastatic disease are resistant to PD-1/PD-L1 blockade. Novel immunotherapeutic approaches are needed to treat these patients, and examples of strategies to meet this end are detailed below. For instance, the combination of ICI with radiotherapy aims at synergizing the release of antigens by dying tumor cells and inflammation triggered by radiotherapy, with a sustained immune response provided by ICI. Weiss et al. reported that the combination of PD-1/PD-L1 axis blockade and radiotherapy in locally advanced HNC was well tolerated, with a distinct one year overall survival of 94% vs. 75% of $p16^+$ and $p16^-$ tumors, respectively (NCT02609503) (80). Combinatorial ICI therapy approaches that target multiples immune checkpoints aim to bypass resistance to PD-1/PD-L1 axis (NCT04080804). The rationale behind this approach is supported by a study by Hanna et al. where HNC patients, refractory to ICI targeting PD-1/PD-L1, displayed an increase in LAG-3 and TIM-3 expression on $CD8^+$ T-cells (30). Another strategy consists of eliciting HPV-specific immune responses by administering HPV16 E6 and E7 derived peptides, alone or in combination with ICI (NCT03258008, NCT02865135). Sousa et al. reported a promising overall response rate of 33% in HPV^+ HNC patients with recurrent disease when using an HPV16-peptide vaccine in combination with nivolumab (81,

82). Compared with the results of the check-mate 141 study, where the overall response rate to nivolumab was 13.3% (76), a synergistic effect of the HPV16 vaccine and nivolumab seemed to increase the response rate more than 2-fold. Personalized medicine approaches using patient specific tumor cell lysates alone (NCT01998542), or combined with transgenic cells expressing GM-CSF (NCT02999646), aim to elicit immune responses against the patient's own mutanome.

Yet another strategy consists of harnessing anti-tumor immunity by targeting APC *in situ*. This therapeutic modality has the advantage of generating a diverse yet antigen-specific immune-response while conditioning the TME. In fact, targeting the CD40-CD40LG axis has historically been one of the most popular myeloid-directed immunotherapies, either in monoclonal antibody format or in combination with other agents. For instance, the combination of FLT3-LG and agonistic anti-CD40 antibody aims at increasing the production rate of bone-marrow derived cDC and monocytes via FLT3-LG, while simultaneously providing activation signaling via CD40 stimulation (83, 84) (NCT03329950). However, myeloid cells are diverse, and currently there is an active effort to delineate their role in different cancer types. In a seminal paper, Zhang et al. compared the effect of targeting murine myeloid APC using two different monoclonal antibodies directed towards M-CSF and CD40 in colorectal cancer models (85). M-CSF blockade resulted in a depletion of inflammatory macrophages, while macrophages expressing *VEGFA* and *MMPs* persisted. On the other hand, the CD40 agonist activated cDC1 resulting in an expansion of type-1 $CD4^+$ T-cells and $CD103^+$ $CD8^+$ tissue resident memory cells, and a reduction of exhausted T-cells and Tregs. This pre-clinical study highlights the importance of characterizing myeloid populations when designing targeted therapies, to achieve the desired effect.

In paper **I,** we described that DC frequency was correlated with intra-tumoral $CD8^+$ T-cell levels in HPV^+ TC biopsies. Our re-analysis of an RNA-seq dataset from TC patients indicated that $CD8^+$ T-cell scores were co-expressed along a battery of genes associated to antigen processing and presentation, as well as T-cell exhaustion (68). Following the cancer-immunity cycle theory, DC and T-cells could influence each other's frequency in the TME of TC patients in a positive feed-back loop fashion (12). In paper **I**, we discuss the application of different immunotherapies according to $CD8^+$ T-cell intra-tumoral levels. We hypothesize that TC patients with a higher degree of $CD8^+$ T-cell infiltration may benefit more from PD-1 blockade, alone or in combination with other ICI strategies, to boost their activity and bypass exhaustion. In comparison, patients in the lower spectrum of $CD8^+$ T-cell

infiltration may instead benefit from myeloid targeted therapies or CTLA-4 blockade, aimed at increasing the numbers of tumor infiltrating $CD8^+$ T-cells. Targeting myeloid cells to create appropriate T-cell polarization is an attractive approach to bypass the current limits of ICI therapy. However, a detailed understanding of the biology and function of myeloid cells in HPV-driven TC is a pre-requisite for that approach.

3.Myeloid cell identity

Three factors have been proposed to define cell identity: ontogeny, function, and cell state (86). The study of ontogeny involves a description of the developmental pathway, from a stem cell into a fully mature somatic cell type, which is controlled by specific TF. In turn, cell function encompasses physical measurable features that can be used to classify cells. Finally, cell state refers to the phenotypes that a given cell can acquire when it is exposed to intrinsic (e.g., cell cycle) or extrinsic stimuli (e.g., viral infection, response to cellular interactions). This chapter is focused on concepts and methodology used to evaluate myeloid heterogeneity and origin in lymphoid organs and solid tumors, with a particular focus on summarizing the current knowledge from HNC subtypes.

3.1. Single-cell transcriptomics as a tool to study cell identity

In humans, cellular ontogeny cannot be studied using lineage tracing or other genetic engineering methods. Instead, it has traditionally relied on the development of *in vitro* culture systems and isolation of distinct progenitor cells (87, 88). Organ transplantation has further allowed researchers to investigate the dynamics of cell replacement in grafted tissue, describing the turnover of cells from donors to receivers (89, 90). In addition, naturally occurring loss-of-function mutations in TF have provided information on specific TF-dependencies of different developmental pathways (91).

The emergence of single-cell transcriptomics has revolutionized our understanding of cellular identity and diversity. Currently, single-cell RNA sequencing (scRNA-seq) methods enable the detection of thousands of transcripts per cell, obtaining a snapshot of hundreds to thousands of cells from a given tissue at a given time (92). An unbiased analysis of cellular heterogeneity can be achieved through the implementation of community

detection algorithms, which group cells in clusters based on their transcriptomic similarity. However, it is important to differentiate between the concepts of cell-type, cell state, and cluster. A cell type is stable, and it encompasses a collection of reversible cell states. In contrast, a cluster is a group of entities that are similar to one another with regards to measurable variables. In a seminal paper, Villani et al. re-evaluated the diversity of blood DC and monocytes, reporting a total of ten subtypes of myeloid cells as well as circulating progenitor cells (93). Besides detecting rare populations, the authors described transitional states between some clusters. In this context, scRNA-seq analysis has highlighted the heterogeneity of cellular states in a given cell type. For instance, Regev's group stimulated human bone marrow-derived DC in parallel with Pam3Cys, poly(I:C), and LPS, which activate TLR1/2, TLR3, and TLR4, respectively. They observed the induction of different gene expression profiles, but most interestingly they also captured an asynchronous and time-dependent change in the transcriptomes between the unstimulated and stimulated cell states in the three conditions (94).

Yet another application of scRNA-seq has been the reconstruction of developmental trajectories and cell fate. Cellular development is driven by transcriptional changes throughout a continuum of cells states. By ordering different DC precursor groups throughout a pseudo-time, Schlitzer et al. described that commitment to cDC1 or cDC2 lineages happens at the stage of common cDC progenitor in the bone marrow (95). They also highlighted that cell differentiation, in this case cDC, is an asynchronous process. The transition between cell states can be also interrogated *in silico* using RNA velocity analysis, which measures the speed by which splicing of intronic regions take place to infer the future state of cells. By calculating the kinetics of immature (unspliced) vs. mature (spliced) RNA, these models re-capitulate the directionality across a trajectory. Using RNA velocity, Zhang et al. recapitulated DC population dynamics throughout body compartments in hepatocellular carcinoma (96). They predicted that circulating cDC accessed the tumor bed and either migrated to tumor draining lymph nodes with a sequential maturation of their transcriptomic profile, or they exited the tumor accessing the inflammatory fluid in the abdomen known as ascites. These studies provide evidence that scRNA-seq technologies complement traditional methods such as FC and *in vitro* cell culture in the study of human cellular identity.

3.2. Myeloid cell development in steady state

Myeloid cells are key players of innate immunity, and they are broadly divided into myeloid APC (also known as mononuclear phagocytes) and granulocytes. Since myeloid APC display partly overlapping phenotypes and functions there has been an active effort to delineate their ontogeny in humans (Figure 1).

In steady state hematopoiesis, $CD34^+$ common myeloid progenitors in the bone marrow proliferate and differentiate into a series of intermediate progenitors (97), eventually giving rise to monocyte-DC progenitors. Monocyte-DC progenitors differentiate into restricted monocyte and common cDC progenitors which progressively leave the bone marrow as they differentiate, entering blood circulation (87, 88). The differentiation of common cDC progenitors into pre-cDC1 or pre-cDC2s is dictated by expression of BATF3 and IRF8, or IRF4 and KLF4, respectively (15, 91, 98-100). At steady state, fully differentiated cDC, along with circulating pre-committed cDC, enter lymphoid and non-lymphoid organs giving rise to the two major cDC subsets, i.e., $CD141^+$ cDC1s and $CD1c^+$ cDC2s, thus maintaining tissue homeostasis (101). Plasmacytoid DC (pDC) were proposed to derive from common cDC progenitors, based on results from *in vitro* differentiation cultures (88), and also due to their dependency on FLT3 *in vivo*, similar to myeloid APC (102). However, the pre-pDC restricted progenitor has not been found in humans, and some authors hypothesize that pDC originate from lymphoid precursors, as shown in mouse models (103, 104).

In contrast to a parallel ontogeny model as for cDC, the monocyte lineage has been shown to differentiate sequentially (105). Patel et al. demonstrated that fully differentiated $CD14^+CD16^-$ classical monocytes exited the bone marrow into circulation and sequentially differentiated into intermediate $CD14^+CD16^+$ monocytes, and finally into non-conventional $CD14^-CD16^+$ monocytes (105). Two different theories are used to explain the origin of macrophages in steady-state peripheral tissues. Initially it was proposed that monocytes, originating in the bone-marrow, replenished tissue macrophages in steady state conditions. The most notable example refers to intestinal macrophages, which are completely replenished in duodenal transplant patients by the receiver's circulating monocytes, as shown by Bujko et al. (89). The second theory refers to the embryonic origin, in which yolk-sac and fetal liver monocytes seed different organs, acting as the unique source of self-renewing tissue resident macrophages (106). Microglia represent the best example of this hypothesis since they have been shown to originate from yolk-sac primitive macrophages (107), and they cycle and divide in human adults (108). Furthermore, Kanitakis

et al. showed that Langerhans cells (LC) in skin allografts self-renewed for over ten years post-transplantation. However, how well these dogmas translate to other human tissue resident macrophage populations is unclear. Most of the data on monocyte-macrophage lineage dynamics derives from mice studies (109-111), and only recently similar transcriptional profiles have been reported between mouse and human steady-state tissue macrophages (112).

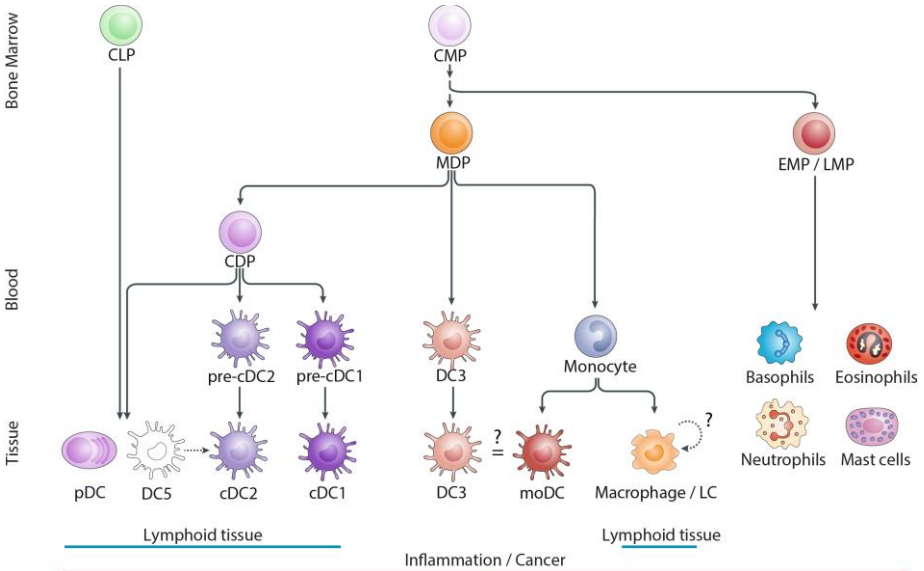

Figure 1. Development of distinct myeloid lineages in steady state and inflammation/cancer. CMP: Common myeloid progenitor; MDP: Monocyte dendritic cell progenitor; CDP: common dendritic cell progenitor; CLP: Common lymphoid progenitor; cDC: conventional dendritic cell; pDC: plasmacytoid dendritic cell; EMP: Erythro-myeloid progenitor, LMP: Lympho-myeloid progenitor. Adapted from (113).

3.3. Revision of myeloid heterogeneity

The emergence of single-cell omic techniques has broadened our understanding of myeloid cell heterogeneity in different body compartments (Figure 1). scRNA-seq studies have confirmed the identity of cDC1, cDC2, pDC, and monocyte lineages, while simultaneously providing evidence for a wide variety of novel myeloid cell types and states (85, 93, 94, 96, 112, 114-119). Motivated by these advances in the field, in paper **III** we explore the diversity of myeloid APC at single cell level, using scRNA-seq and multiplex-

FC in HPV$^+$ TC and HT. We and others have provided proof of cDC2 and pDC subpopulations, while highlighting the uniformity of cDC1. Surprisingly, and in agreement with the state of the art, we have shown a transcriptomic confluence of DC subtypes upon maturation, making it impossible to discern activated DC subtypes at the present sequencing depth.

Villani et al. described novel myeloid subtypes in blood, encompassing CD14$^+$ DC3 and CD123$^+$ DC5, besides the cDC and monocyte subsets described previously (93). Further characterization of DC3 in blood and bone marrow has confirmed that the novel CD14$^+$CD1c$^+$CD163$^+$ DC3 population shares transcriptional and phenotypical traits with both cDC2 and classical monocytes (93, 120, 121). Bourdely et al. described a GM-CSF based *in vitro* culture system that allowed differentiation of DC3 from monocyte-DC progenitors, independently from restricted common cDC progenitors and monocyte progenitors (120). Together, these studies suggest that DC3 represent a lineage of their own. Further research is required to assess if the same GM-CSF process drives DC3 development *in vivo*, and which are the TF responsible for the DC3 developmental program.

An Axl$^+$Siglec6$^+$CD5$^+$ DC5 (also referred to as 'AS'DC or transitional DC), which shares CD123 expression with pDC, is currently under extensive study. In steady state, this population has been described in blood and lymphoid organs such as tonsil and spleen (93, 114, 122). Villani et al. showed that DC5 have a capacity to differentiate into cDC2 *in vitro* (93), contrary to other DC and monocytic cells where the developmental potential is lost along lineage commitment. Of note, the authors reported that differentiation from DC5 into cDC2 did not involve up-regulation of cell cycle genes, and that the DC5 and pre-cDC2 did not share a common transcriptional profile, suggesting that DC5 do not represent pre-cDC2. Using ATAC-seq, Leylek et al. reported that KLF12 is a DC5 specific TF, as compared to other TF shared with pDC such as TCF4 and RUNX2 (116). In contrast to DC5, *bona-fide* proliferating cDC progenitors, pre-committed to either cDC1 or cDC2 lineage, have been identified in blood and spleen (93, 114). Consistent with these observations, in paper **III** we described the presence of DC5 in HPV$^+$ TC and HT using scRNA-seq and FC. Using a gating strategy based on the expression of CD45, CD13 and HLA-DR, we sorted myeloid APC along with DC5, while excluding pDC. Our transcriptomic data showed that DC5 did not cycle and represented a different cluster from cycling pre-cDC2. Compared to the *in vitro* studies conducted by Villani et al., we did not observe evidence of DC5 differentiating into cDC2 in the tumor tissue in our RNA-velocity analysis. This observation suggests that the TME lacked appropriate niche cues to induce DC5

differentiation in HPV^+ TC. Further phenotypic characterization using FC revealed that a subset of $CD123^+CD5^+$ DC5 expressed CD11c, while $CD123^+CD5^-$ pDC did not. Together, these studies suggest that DC5 represent a distinct DC lineage, perhaps of myeloid origin. In fact, a study of Rodrigues et al. highlights that $CD123^+$ DC may develop from both myeloid and lymphoid progenitors in mice (103). Moreover, the authors point out that $CD123^+$ DC of the myeloid branch present antigens, while $CD123^+$ DC of the lymphoid branch does not. If we assume that equivalent "pDC" differentiation processes occur in mice and humans, DC5 may represent the myeloid branch of $CD123^+$ DC, while *bone fide* pDC may develop from lymphoid progenitors.

3.4. Systemic perturbations of the myeloid compartment

Another layer of complexity is added during cancer and inflammation when myeloid cells and their progenitors/precursors are exposed to systemic and local stimuli that promote their expansion, recruitment, and differentiation. The most notable systemic effect is emergency myelopoiesis. In cancer and chronic inflammation, the affected tissues release a battery of cytokines into the blood stream, e.g., IFNs, CSFs, IL-1, IL-6, TNF-α, and TGF-β (123), triggering myelopoiesis through activation of bone marrow hematopoietic stem and progenitor cells. Even though the effect of these cytokines has been mostly described in mouse and *in vitro* models, increased levels of circulating (124-126) and intra-tumoral (126) $CD34^+$ myeloid progenitors have been described in a variety of human cancers including HNC. Notably, stimulation of circulating $CD34^+$ myeloid progenitors with SCF and GM-CSF produced cells of DC phenotype (126), indicating that circulating myeloid progenitors have the potential to differentiate into DC. In fact, in paper **III** we demonstrated an expansion of the myeloid compartment and observed the presence of cycling progenitor cDC in HPV^+ TC and HT, indicating that emergency myelopoiesis and myeloid cell recruitment were active. We hypothesize that HPV^+ TC feature an enrichment in pre-cDC1 in detriment of pre-cDC2, compared to HT. However, we lacked statistical power and further experiments are required to prove that hypothesis.

The combination of emergency myelopoiesis and systemic stimuli derived from the tumor results in the pathological activation of monocytes and neutrophils in circulation (127). Myeloid-derived suppressor cells (MDSC)

encompass cell states of monocytes (M-MDSC), neutrophils (or G-MDSC), and possibly progenitor cells (e-MDSC) with potent immunosuppressive effects (128, 129). Among others, the group of Gabrilovich has characterized MDSC in a variety of cancer types and described their accumulation along cancer progression (128). Using their proposed gating strategy (128), we have identified the presence of MDSC subtypes in peripheral blood and tumor lesion of TC patients (Figure 2A). We observed an increase of circulating MDSC subpopulations in TC patients when compared to healthy donors (Figure 2B). Interestingly, the same tendency was observed upon comparison of TC lesion and paired HT for G-MDSC and e-MDSC populations. However, M-MDSC were mostly absent in TC lesions (Figure 2C). In fact, HLA-DR$^{low/-}$ M-MDSCs have been reported to differentiate into tumor associated macrophages via HIF-1α upon extravasation (130, 131). Upon differentiation in the tumor bed, the resulting macrophages produce PGE_2 and TGF-β, impairing $CD8^+$ T-cell activation and expansion via IL-2 (132). In turn, $CLEC8A^+$ G-MDSC are present in both circulation and tumor tissue. Lang et al. demonstrated that G-MDSC overexpress Arginase I and iNOS in HNC, resulting in the depletion of arginine and impairing T-cell proliferation (133).

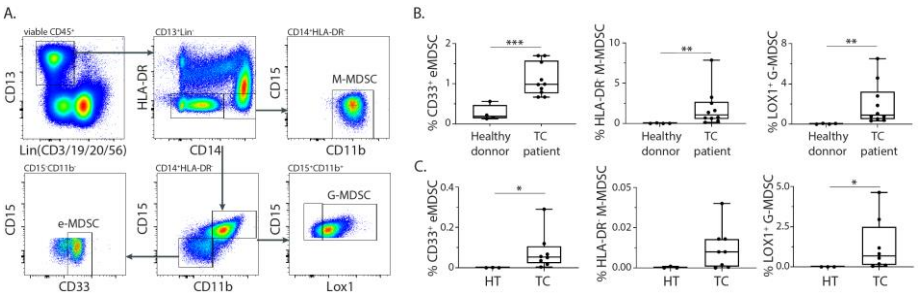

Figure 2. MDSC distribution in peripheral blood and tonsillar tissue in TC patients and healthy donors. Gating strategy used to identify MDSC in blood and tissue (A). Frequency of MDSC subtypes identified in (A) in peripheral blood (B) and TC and paired HT from TC patients (C). Frequency is indicated as % out of $CD45^+$ cells. TC: Tonsillar cancer; HT: Healthy tonsil; MDSC: Myeloid-derived suppressor cell.

In contrast to well characterized myeloid lineages such as cDC1, cDC2, and monocytic cells, MDSCs have not been extensively studied through scRNA-seq. In fact, only two papers have described the emergence of G-MDSC in the spleen of tumor bearing mice (134, 135). Further scRNA-seq studies are warranted to elucidate how monocytes are pathologically activated in blood and which macrophage phenotype they acquire upon differentiation in the tumor.

3.5. Local perturbations of the myeloid compartment

The potential of $CD14^+$ classical monocytes to differentiate upon exposure to inflammatory signals is a well characterized phenomenon. Driven by CCL2, $CCR2^+$ classical monocytes access the tumor/inflamed tissue where they are exposed to a variety of cytokines influencing their differentiation. Combinations of cytokines such as M-CSF, GM-CSF, IL-4, TNF-α, and IFN-γ have been shown to induce monocyte differentiation into monocyte-derived DC (moDC) (136, 137) and/or macrophages *in vitro*. The group of Segura has recently characterized *in vivo* moDC in inflammatory fluids from rheumatoid arthritis patients, tumor ascites (137, 138), and breast tumors (139). Similarly to LC and DC3, moDC expressed *CD1C*, *CLEC10A*, and *FCER1A*, as well as *CD14* and *S100A8/9*, revealing a hybrid transcriptional profile, half-way between cDC2 and monocytes (119). Another similarity between DC3 and moDC is their capacity to prime naïve $CD4^+$ T-cells into IL-17-producing Th17 cells in allogenic *in vitro* cultures (115, 138). However, differences have been reported between the three populations. First, moDC differentiate from monocytes under the control of the transcriptional factors BLIMP-1 and IRF4 (137), while LC differentiate from cycling primitive macrophages under the control of RUNX3, ID2, and MAFB. In contrast, DC3 differentiation from monocyte-DC progenitors has only been described *in vitro* (120), and no TF has yet been proposed to regulate this process. Secondly, moDC and LC were characterized by CD1A and CD207 expression, while DC3 uniquely expressed CD163. A closer comparison of the DC3 and moDC subpopulations is necessary to elucidate if these cells represent equivalent blood and tissue counterparts, or if they rather belong to distinct lineages. In paper **III**, we conduct a careful assessment of the identity of a $CD14^+CD1c^+$ myeloid cluster using a combination of these parameters. Based on the expression of *CD207*, *CD1A*, and *TGF-β*, its emergence from cycling cells, and a high LC gene-signature score, we categorized this cluster as mucosal LC. Careful examination of myeloid cells from HPV^+ TC by 26-plex FC showed that LC did not overlap with $CD163^+$ $CD1c^+$ DC3, demonstrating that these are two distinct entities. In our analysis, we did not observe evidence for monocyte differentiation into moDC, suggesting that either the process does not happen in HPV^+ TC, or that the sequencing depth of 10x scRNA-seq is limited to capture this event.

Macrophage heterogeneity in tissue is greatly influenced by the microenvironment in which these cells are embedded in. Traditionally, macrophages have been classified into two polarization states, namely M1 and M2, which represent canonical models of inflammatory and anti-inflammatory cells (140, 141). The M1/M2 paradigm has been extensively characterized by culturing monocytes *in vitro* either in IFN-γ + LPS/TNF-α or IL-4 + IL-13/IL-10 to obtain M1 and M2 macrophages, respectively (140). However, this theory has been re-challenged throughout the development of transcriptomic technologies. Xue et al. conducted a comparative transcriptomic study using 28 different stimuli on *in vitro* differentiated macrophages. They suggested a total of nine polarization states, and described common expression of the TF *NFKB1*, *JUNB*, and *CREB1* in all subtypes (142). In addition, recent scRNA-seq studies have suggested that the monocyte lineage is transcriptionally more plastic across tissue and cancer types than cDC lineages. This was shown in a scRNA-seq metadata-analysis by Mulder et al. where the identity of myeloid APC from 13 different tissues was assessed by canonical correlation analysis and unsupervised clustering. A total of 14 different monocytic cell states with strong dependence on the tissue of origin were detected, as compared to single cDC1 and cDC2 clusters (117). Currently, the diversity of the monocyte lineage is under extensive characterization.

Despite the disparity of profiles reported in scRNA-seq studies, some macrophage profiles have been recurrently reported in cancer. *FCN1*$^{+}$*S100A*$^{+}$ monocyte-like cells have been shown to differentiate into C1Q-expressing TAM (tumor-associated macrophage) in hepatocellular carcinoma and colon cancer (85, 96). Interestingly, C1Q macrophages expressed high levels of *TREM2*, a canonical marker used to define monocyte-derived TAM in breast cancer (118). However, the use of single transcripts to define cell identity has limitations, and when these studies are considered together contradictions arise. First, *C1QA*/*B*/*C* gene expression was detected in all macrophage clusters in breast cancer, including FOLR2^{+} tissue resident macrophages. Second, TREM2 TAM in breast cancer also expressed high levels of *SPP1*. In turn, SPP1^{+} TAM were reported as an independent cell state from C1Q TAM in colon cancer. Finally, while high C1Q macrophage scoring correlated with poor survival in hepatocellular carcinoma, it had the opposite effect in colon cancer (85, 96). Altogether, the understanding of macrophage diversity has drifted from a dual to a network paradigm. Further research is required to assess niche-specific cell states and to examine if macrophages can transit several of these states in cancer during a cellular life-span.

In paper **III** we characterized six monocytic lineage clusters in HPV^+ TC, along with two macrophage differentiation pathways triggered by different polarization states of monocytes. Similar to what Mulder et al. described in pancreatic cancer (117), we observed a monocyte cluster expressing an interferon-inducible gene signature and high levels of the TF *IRF7*, which sequentially differentiated into *C1QA*/*B*/*C* expressing macrophages and *SDC2*, *PD-L1*, and *CCR7* expressing activated macrophages. We validated the presence of the activated macrophage population using FC, and suggested CD163 as an additional surface marker. Their expression of CCR7 suggests that activated macrophages may respond to CCL19 and CCL21 gradients, like mature DC on their migration to draining lymph nodes. However, migratory capacity experiments must be conducted before claiming any migratory properties of this population. The second differentiation pathway involved the sequential differentiation of *FCN1* monocytes into *NLRP3* expressing macrophages followed by *CXCL1*/*3*/*5*/*8* expressing macrophages, similar to the description from Zhang et al. in colon cancer (85). Interestingly, we only detected CXCL macrophages in locally advanced patients, compared to earlier T-categories and HT, respectively. Even though we did not predict an impact of any monocyte-macrophage population on survival in TC or HNC using gene signature scoring, we hypothesize that the CXCL macrophage could be involved in the recruitment of pathologically activated neutrophils (G-MDSC) and angiogenesis through the CXCL1/3/5 - CXCR2 receptor-ligand axis (143).

4.Models applied in immuno-oncology to assess cell function

Cancer models aim to recapitulate key events of tumorigenesis and are useful to test scientific hypothesis and drugs. All cancer models have advantages and limitations, and thus careful consideration should be taken when selecting them. Conventional 2D monolayer cultures are easy and cheap to stablish, but lack most of the environmental traits of tumors, including matrix-cell interactions, immune-tumor cell interactions as well as nutrient and oxygen gradients, among others. In turn, *in vivo* models can recapitulate the complex features of tumorigenesis described above, but are limited by time and monetary cost, as well as by experimental limitations in terms of visualization, response monitoring, and inter-species differences. For instance, common *in vivo* rodent models, *Mus musculus* and *Rattus norvegicus*, do not have palatine tonsils (144), and are therefore not suitable for the study of myeloid cell trafficking and function in the context of HPV^+ TC.

3D *in vitro* models such as organoids recapitulate tissue (145), and even organ-(146), specific cellular interactions with the simplicity and capacity to control conditions of a 2D *in vitro* culture. Originally established by the group of Hans Clevers (147), organoids are self-organizing structures that recapitulate epithelial tissue architecture and are based on the use of Wnt-3a, R-spondin-1, and Noggin to control stem cell survival and differentiation (148). In the field of immuno-oncology, organoids have been used to reproduce patient-specific tumors *in vitro* (145), generate patient specific T-cell lines (149), and reproduce antigen-specific antibody generation in tonsillar germinal centers (146).

In addition, patient-derived organoids represent a useful tool to study myeloid cell functions such as chemotaxis-driven tumor-infiltration and phagocytic activity. The limitations of these 3D models include high costs and time needed to develop patient derived organoids.

One option to monitor these processes is to use label-free non-invasive digital holographic microscopy. Digital holography is based on the phase shift of light when it crosses an entity such a cell. The beam of a diode laser is split in two based on the wavelength, referred to as sample and reference beams. The sample beam hits the cell resulting in light scattered off and delayed light that passes by. A sensor then detects the phase shift of the light in the sample beam and overlaps it to the reference beam. Digital holography allows us to quantify morphological characteristics of cells at the resolution of one micron. The area is proportional to the number of pixels of the imaged cell, while cellular shape can be discerned by determining its edges. The cellular thickness is quantified as a measure of the phase shift of the sample beam, the wavelength, and the refractive index of the cell. Hence, by measuring the phase shift using a known refractive index and wavelength, we can estimate the thickness of the cell (150).

In paper **IV**, we used digital holographic microscopy to monitor organoid formation, as a proof of principle that the technology is suitable to study cell-cell interactions in a microscopic 3D model. Using the $HPV16^+$ TC cell line LU-HNSCC-26 (151), we observed that seeding of 100 to 1000 single cells in a hydrogel-embedded culture format generated 3D structures with stable optical thickness and volume in a time lapse of fifteen hours. Some authors have hypothesized that the ability of tumor cells to form 3D structures, such as organoids, reflects on their stemness potential (152, 153). We sorted TC cells based on their surface phenotype of CD44 and CD271, two reported cancer stem cell markers, and compared the motility and organoid forming capacity of these cells (154). Compared to the results from Elkashty et al. (154), we observed that the $CD44^+NGFR^-$ population had higher motility and organoid forming capacity than $CD44^+NGFR^+$ and $CD44^-NGFR^-$ cells. The disagreement between the two studies regarding the stemness phenotype, $CD44^+NGFR^-$ in our study and $CD44^+NGFR^+$ in Elkashty's, could be associated with the low number of cell lines used in our publication and theirs, i.e., one and two respectively. This would suggest that while CD44 is a pan-cell line HNC stem cell enriching marker, NGFR is not. Nonetheless, paper **IV** represents a first step towards the development of an assay to monitor tumor cell – DC interactions in a 3D format.

5.Outlook

The emergence of novel technologies, such as spatial omics and scRNA-seq, has enabled high resolution analysis of tissue and cellular heterogeneity. Improvement of scRNA-seq may provide an increased sequencing depth and median detected genes per cell. These parameters may be key to unbiasedly resolve questions arisen in the myeloid cell field. For instance, the development of a common DC maturation profile seems to eclipse lineage identity in scRNA-seq studies. However, there is extensive experimental research that indicates how the activation of different DC lineages dictates distinct antigen presentation and T-cell polarization abilities (93, 94, 155, 156). Hence, it would be expected that these properties were embedded in the DC transcriptome, and yet these have not been reported to my knowledge. Our median number of genes detected per cell was approximately 2.000 (paper **III**), while other myeloid cell studies have reported up to 5.000 detected genes per cell, which is far from the reported technical limit of 10.000 genes per cell detected in cell lines (157). Implementation of full-length scRNA-seq methods might also shed light on these questions, considering that 95% of protein coding genes undergo alternative splicing resulting in 70.000 transcripts (158). Another technique that holds the promise to deeply characterize single cells is proteomics. The advent of single cell proteomics may further increase the number of variables one order of magnitude given that each splicing variant can be subjected to different post-translational modifications which condition protein function. Thus, advances in single-cell omic technologies may provide a more mature picture of cellular identity and phenotype.

In this document, I have detailed some hypotheses that spin off the research projects I have been involved in. One hypothesis may hold clinical interest if proven certain. This hypothesis stems from observation in paper **III** that pre-cDC1 were more abundant than pre-cDC2 in HPV^+ TC. Even though this observation needs to be statistically confirmed, we did observe and statistically significant increase of cDC1 in HPV^+ TC compared HT, while cDC2 levels were similar. Given that cDC differentiation takes place in the bone marrow (88), and considering that tumor-derived factors can exert long range influence

over myeloid production in the bone marrow (123), I hypothesize that one or a combination of molecules derived from the tumor may be responsible for the increased production of cDC1 in the bone marrow of HPV^+ TC patients. If proven correct, the identification of soluble factors that allow cDC1 expansion and recruitment into the tumor may have clinical implications, since a high cDC1 gene-signature score has a positive impact in the 5-year OS of TC and HNC patients. A closer comparison of both tissue- and serum-derived proteins between HPV^+ TC and healthy donors, followed by screening of candidates using the *in vitro* culture system described by Lee et al. (88), may be a first step towards testing this hypothesis.

The cross-talk between myeloid populations in TC is another event that deserves attention. Specifically, the interactions involving the CXCL macrophage population (identified in **paper III**). We described the presence of this population in advanced tumor setting using scRNA-seq, but we could not validate it using 26-plex FC. Close examination of its gene expression profile suggests that CXCL macrophages are involved in granulocyte chemotaxis and angiogenesis. CXCL-macrophages may mediate G-MDSC recruitment into the tumor via CXCL1/3/5/8 - CXCR2 receptor-ligand axis. Interestingly, VEGF and CXCL1/3/5/8 also regulate angiogenesis upon exposure of $CXCR2^+$ endothelial cells (143). Since angiogenesis is triggered in hypoxic microenvironments, and as these are more common in advanced T category tumors, it would be interesting to study the distribution and frequency of CXCL macrophages, G-MDSC, and vasculature across T categories. Such experiments would shed light into the role of CXCL macrophages in the recruitment of G-MDSC and development of angiogenesis in hypoxic settings, and perhaps suggest novel targets or biomarkers.

Some studies relate myeloid cell location in lymphoid organs with function (37, 159). The experimental approaches that we have used exhibit limitations when it comes to decipher how myeloid lineages are compartmentalized within the tumor. Digital spatial protein profiling lacks single-cell resolution capacity, while FC and scRNA-seq methods lack cellular location information. Tertiary lymphoid structures are of increasing interest in the field of immuno-oncology due to their association with favorable prognosis in distinct cancer types including HNC (160-162). Tertiary lymphoid structures are self-organizing immune cell aggregates with varying degree of maturity, which form in sites of inflammation such as tumors. Recently, Ruffin et al. described the presence of tertiary lymphoid structures with mature germinal centers in HNC, with a higher positive impact in patient survival for HPV^+ compared to HPV^- individuals (162). It is possible that plasma cells producing antibodies against

tumor neoantigens are involved in eliciting antibody-dependent cellular cytotoxicity, for instance through NK cells, similar to monoclonal antibodies used in the clinical setting (163, 164). However, it is less probable that this mechanism is triggered by the antibody response against HPV viral antigens given that, as described by Wieland et al., the immunogenic B-cell receptor epitopes are located in the nucleus or cytoplasm of the infected cell (165). Nonetheless, antibodies directed against viral antigens may opsonize tumor cell debris favoring phagocytic uptake and antigen presentation, ultimately boosting the development of T-cell responses in the tertiary lymphoid structures of HPV^+ HNC. It would be interesting to assess if tertiary lymphoid structures are subjected to specific myeloid lineage segregation, and to examine the interplay of myeloid cells with B- and T-cells in these local lymphoid structures.

Deep transcriptomic characterization of the myeloid compartment within diseased and healthy tissue has provided insights on which myeloid subtypes influence cancer patient prognosis (85, 96). This knowledge opens the door to *in situ* targeting of specific myeloid populations using therapeutic antibodies. However, functional characterization of myeloid subpopulations is a pre-requisite to therapeutic targeting. FC-sorting followed by *in vitro* characterization of their phagocytic and antigen presentation activities as well as their cytokine profile will shed light on the function of these populations. Furthermore, assessment of standard and immunotherapeutic regimes on the composition of the myeloid compartment could provide mechanistic insights and perhaps help treatment selection.

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