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

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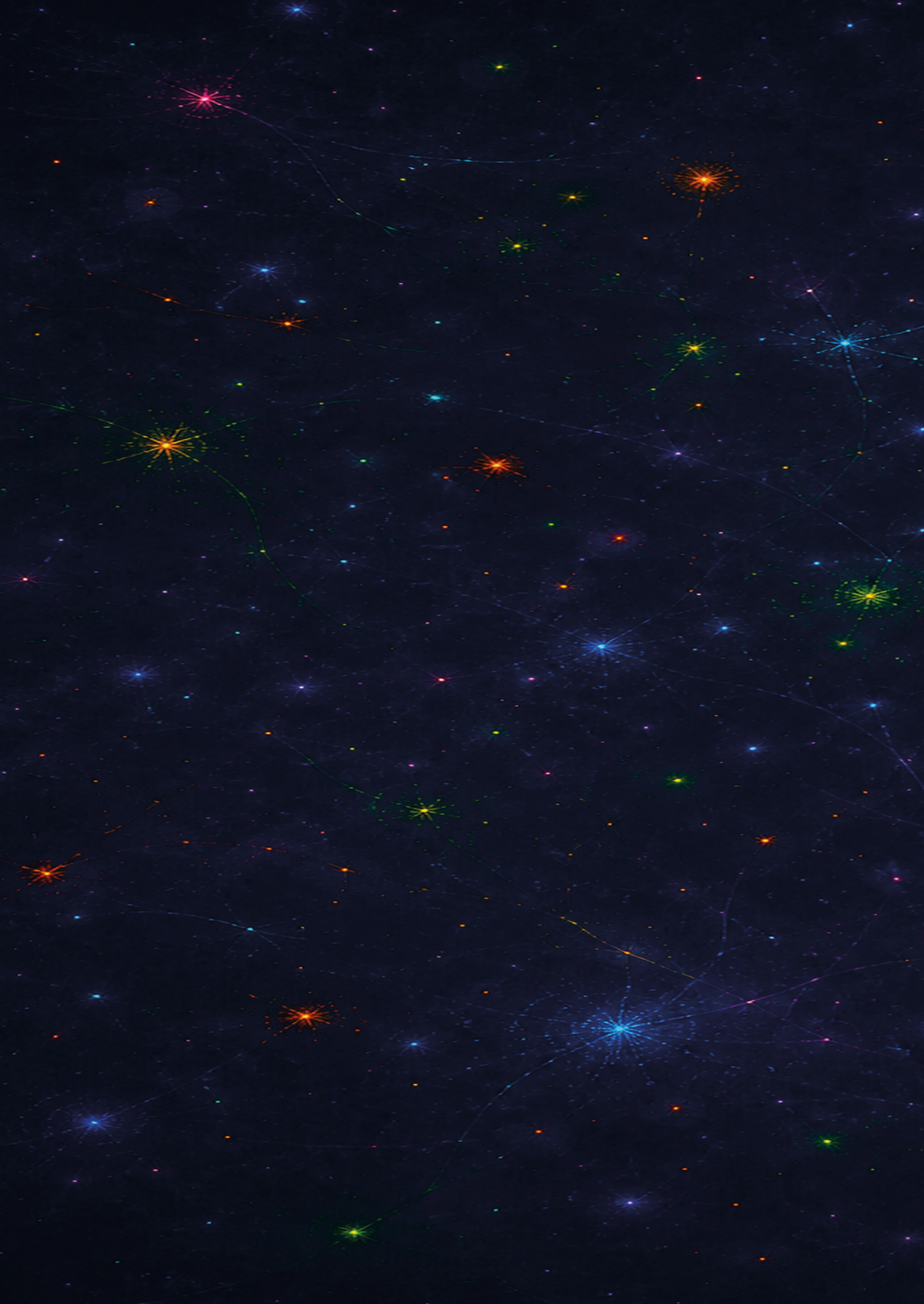

Chapter 9

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 delivery[8] or oncolytic viruses[9], 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 this[15], or whether TLR agonists can be used to promote their polarization[16].

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 melanoma[20], non-small cell lung cancer[21] and renal cell carcinoma[22], 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 radiotherapy[23], 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 viruses[25] and STING pathway activators[26], 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 cell[18], 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 scaffolds[35], recruiting and programming dendritic cells to seed triads[36], or dismantling stromal and myeloid barriers that cause T cell exclusion[34]. 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 cells[34]. Multiplexed imaging further shows that therapy can induce new immune neighborhoods, such as T cell-macrophage hubs[38], while neoadjuvant PD-1 blockade in NSCLC demonstrated the induction of TLS-like aggregates and T cell redistribution as early correlates of regression[39]. 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 cells[41], 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 mediators[55], neutrophils have been shown to mediate antibody-dependent cytotoxicity and release reactive oxygen species that damage tumor tissue[56], 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 abundancy 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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