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Novel mediators of anti-tumor immunity: dissecting intratumoral immune responses at the single-cell level

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1 General introduction

GENERAL INTRODUCTION

The complexities of cancer pathogenesis

In 1914, the German zoologist Theodor Boveri was the first to propose a genetic basis for cancer development and speculated that cancer is the result of chromosome alterations in cells.[1] Ever since, a cancer cell-centric vision has long dominated the field of cancer biology. Cancer was thought to form exclusively by multistep alterations in the genome of cells, leading to the progressive transformation of normal cells into cancer cells along with the acquisition of malignant features such as self-sufficiency in growth signals, evasion from apoptosis, and limitless replicative potential, all constituting important "hallmarks of cancer".[2] However, during the last decades it has become increasingly recognized that the study of cancer must also include other components of the cancer microenvironment, such as stromal cells, fibroblasts, and immune cells, to fully capture cancer biology.[3,4] Hence, for malignant transformation cells not only have to undergo alterations that result in their proliferation and migration, but also have to evade from control mechanisms, including those provided by cells surrounding the cancer cells such as immune cells.

The role of the immune system in cancer

Malignant transformation is accompanied by genetic alterations that eventually translate to modified proteins. The latter can be recognized during the process of immune surveillance[5], in which the immune system constantly surveys the body for transformed cells and eradicates such cells. The importance of the immune system in recognizing and eliminating cancer cells gained recognition by observations of an increased incidence of cancer among transplant patients using immunosuppressive drugs,[6] patients with immunodeficiencies,[7] and mutant mouse models lacking key components of the immune system[8-10]. Cancer immune surveillance incorporates contributions of both innate and adaptive immunity, which are each equipped with mechanisms to recognize and eliminate cancer cells (**Figure 1**). Innate lymphoid cells (ILCs), including natural killer (NK) cells, can be activated in response to cellular stress or DNA damage occurring in cancer cells, leading to increased expression of stress-induced self ligands on the surface of cancer cells that can be recognized by innate immune receptors.[11] In addition, ILCs and NK cells can be activated in response to the absence of self human leukocyte antigen (HLA) class I molecules,[12] the human counterpart of the major histocompatibility complex (MHC), on the surface of cancer cells. Myeloid cells, such as dendritic cells, macrophages, monocytes and granulocytes, also belong to the innate immune system and play important roles in the phagocytosis of cancer cells and the secretion of inflammatory cytokines.[13] By a highly orchestrated process dictated by multiple cues (e.g. inflammatory signals) that are provided by the innate immune system, cells of the adaptive immune system can become activated. The adaptive immune compartment comprises B and T lymphocytes. The latter can be separated into $CD8^+$ (cytotoxic) or $CD4^+$ (helper) T cells. In contrast to innate immune cells, B and T cells express diverse rearranged receptors which are specific for an antigen, and generate long-term immunological memory.

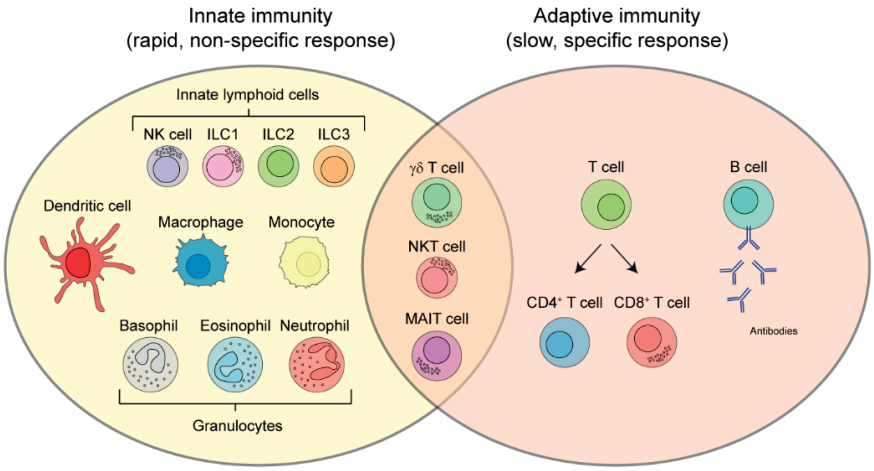

Figure 1. Cellular players of innate and adaptive immunity.
A simplified overview of the main cellular players of innate and adaptive immune responses. The innate immune system mounts a rapid and non-specific response, and comprises innate lymphoid cells, dendritic cells, macrophages, monocytes, and granulocytes. The adaptive immune response is slower to develop, but shows antigenic specificity and long-term immunological memory. It consists of T cells ($CD4^+$ and $CD8^+$ T cells) and B cells. γδ T cells, NKT cells, and MAIT cells are cytotoxic lymphocytes that span the interface of innate and adaptive immunity. Adapted from Dranoff *et al.* (2004)[14].

The genetic and epigenetic alterations that define cancer allow the immune system to generate anti-tumor T cell responses. Cancer cells express a range of aberrantly expressed proteins, including proteins originating from somatic mutations in cancer genomes (neoantigens[15]) and proteins expressed at elevated levels in tumor cells that can also be present on non-malignant cells (tumor-associated antigens, differentiation antigens[16]). During carcinogenesis, dying cancer cells release antigens that can initiate a stepwise process referred to as the cancer-immunity cycle[17] (**Figure 2**). For a successful anti-tumor T cell response, such cancer antigens need to be recognized and taken up during the process of immune surveillance via interactions with antigen-presenting cells such as dendritic cells accompanied by pro-inflammatory signals. Upon uptake of cancer antigens, dendritic cells migrate toward regional lymph nodes where they present captured antigens on HLA class I and II molecules to naïve $CD8^+$ and $CD4^+$ T cells, respectively. The process of uptake, processing, and presentation of antigens by dendritic cells to $CD8^+$ T cells is called cross-presentation. As a result, T cells against the cancer antigens are primed, activated, and start to clonally expand. Co-stimulatory signals provided by the interaction between co-stimulatory molecules expressed on dendritic cells (e.g. B7.1 and B7.2) and co-stimulatory receptors on T cells (e.g. CD28) further fuel anti-tumor T cell responses. Activated $CD8^+$ T cells migrate to and infiltrate the tumor, where they can recognize cancer cells via the interaction between their T cell receptor (TCR) and the cognate antigen bound to HLA class I molecules on the surface of cancer cells. $CD8^+$ T cells can eliminate cancer cells by two main pathways: i) the release of cytolytic granules such as perforin and granzymes, and ii) cell

death pathway-mediated apoptosis by the Fas-Fas ligand or TRAIL-TRAIL-R1/2 pathways.[18] In addition, production of the pro-inflammatory cytokine IFN-γ, by CD8+ T cells among others, can increase the expression of HLA class I antigens by cancer cells, thereby enhancing their targeting and killing. Activated CD4+ T cells provide help to promote CD8+ T cell responses via the secretion of effector cytokines (e.g. IL-2) and can, under specific circumstances, exert cytotoxicity against cancer cells (e.g. via IFN-γ, TNF-α). To oppose continued amplification of T cell responses, immune checkpoints and inhibitors are in place that negatively regulate T cells.[17] Last, in addition to the innate and adaptive immune arms, a variety of immune cell populations have been discovered that are in between innate and adaptive immunity, also called unconventional T cells. Unconventional T cells include γδ T cells, NKT cells, and mucosal-associated invariant T (MAIT) cells, and often reside at mucosal tissues where they can rapidly respond in an innate-like fashion. Unlike conventional CD8+ and CD4+ T cells, most unconventional T cells do not recognize peptide antigens associated with HLA molecules, but a broad spectrum of non-polymorphic ligands ranging from metabolite antigens such as phosphoantigens that can be expressed by cancer cells, tumor-derived lipid antigens, to stress-induced molecules on the surface of cancer cells, among others.[19]

The immune system and cancer immune evasion

The immune system plays a crucial role in the protection against cancer development and progression. However, the generation of diversity in tumors may enable the emergence of cancer cell clones that can escape the recognition and elimination by the immune system.[20,21] Cancer cells can escape immune surveillance through different mechanisms. One of the most studied cancer immune evasion mechanisms involves the loss of cancer antigen expression through downregulation or loss of HLA class I expression.[22] This would preclude HLA class I-mediated antigen presentation, thus rendering these tumors insensitive to CD8+ T cell-mediated immunity. Furthermore, cancer cells can escape immune cell killing by enhanced resistance to the cytotoxic effects of anti-tumor immunity via the induction of anti-apoptotic mechanisms or the loss of pro-apoptotic factors.[23] Third, cancer immune evasion can occur through the development of an immunosuppressive cancer microenvironment via the secretion of immunosuppressive cytokines (e.g. TGF-β, IL-10, VEGF) by cancer cells and/or the recruitment of immune cell types with immunosuppressive functions such as regulatory T cells and subsets of myeloid cells.[24] Hence, the immune system plays a dichotomous role in the development and progression of cancer, where different cells can antagonize or promote carcinogenesis. The type, density, and spatial localization of tumor-infiltrating lymphocytes (TILs) are well-known determinants for the prognosis of cancer patients.[25,26] High densities of CD8+ T cells within the center of different types of tumors correlated with improved patient prognosis, whereas high densities of CD8+ T cells at the tumor margin had no effect on survival.[26-28] Such intratumoral immune responses were found to have enhanced prognostic value for patients with colorectal cancer (CRC) than previous pathological criteria for tumor staging.[29] In addition to CD8+ T cells, high levels of intratumoral γδ T cells and subsets of CD4+ T cells represented favorable prognostic signatures in a collection of different cancer types, while high levels of intratumoral myeloid cell populations (polymorphonuclear cells, eosinophils, macrophages) primarily correlated with poor survival.[30]

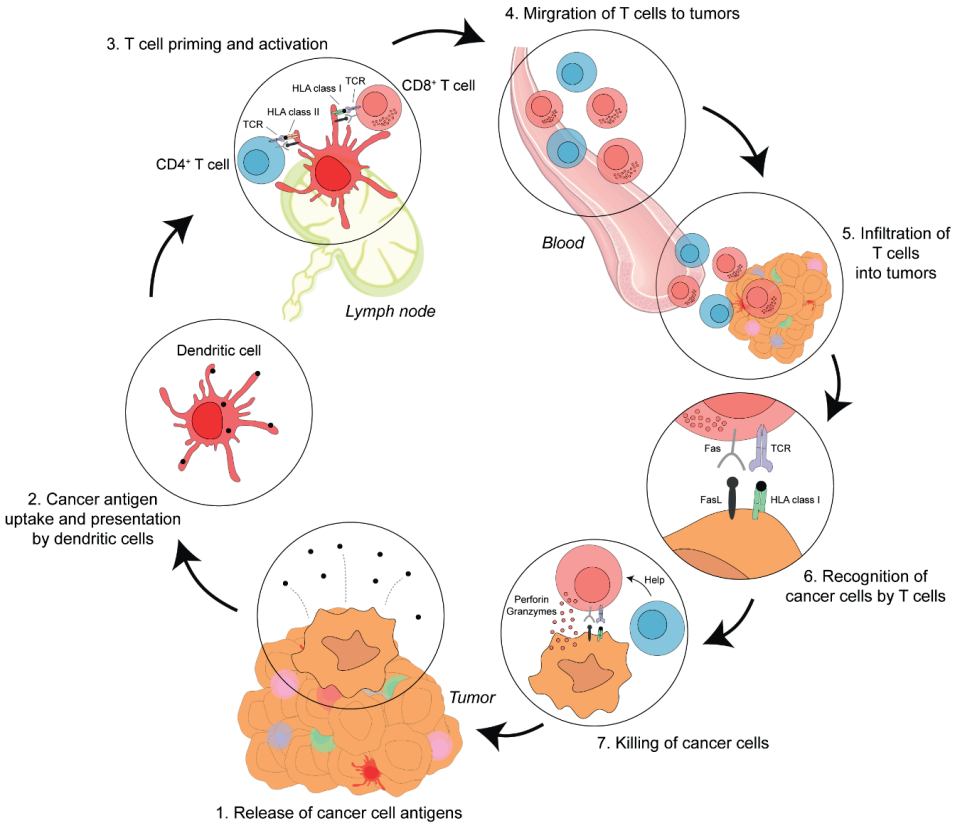

Figure 2. The generation of anti-tumor T cell responses.
A simplified overview of the main cellular players in the generation of anti-tumor T cell responses. Antigens released from dying cancer cells are taken up by antigen-presenting cells such as dendritic cells. Upon migration toward regional lymph nodes, dendritic cells activate naïve CD4⁺ (helper) and CD8⁺ (cytotoxic) T cells through interactions with antigen-HLA complexes on the surface of dendritic cells with the TCR on T cells (HLA class I-mediated antigen cross-presentation for CD8⁺ T cells and HLA class II-mediated antigen presentation for CD4⁺ T cells). The interaction of co-stimulatory molecules on the cell surface of dendritic cells with co-stimulatory receptors on T cells provides full activation. Upon priming and activation, CD8⁺ and CD4⁺ T cells migrate to and infiltrate the tumor. Here, CD8⁺ T cells can recognize cancer cells expressing the cognate antigen bound to HLA class I molecules on their surface, leading to the elimination of cancer cells via the release of perforin and granzymes or via the cell death pathway (e.g. Fas-FasL). CD4⁺ T cells provide help to promote CD8⁺ T cell responses via the secretion of effector cytokines (e.g. IL-2) and can, under specific circumstances, exert cytotoxicity against cancer cells (e.g. via IFN-γ, TNF-α). Adapted from Chen *et al.* (2013)[17].

The revolution of cancer immunotherapy

Remarkable progress in understanding cancer immunity has led to novel approaches for immune-based therapies. In contrast to chemotherapy, radiation, and targeted therapy that focus on eliminating the cancer cells themselves, cancer immunotherapy is aimed at stimulating the body's own immune system to recognize and eliminate the cancer cells or by engineering features of the immune system into therapeutic products. Cytotoxic T cells are viewed as central players in anti-tumor immunity, and T cell-mediated immunotherapies have emerged

as a successful treatment against cancer. The different steps involved in the generation of anti-tumor T cell responses provide a wide range of potential immunotherapeutic targets. Here we will discuss three main categories of cancer immunotherapeutic strategies, ranging from counteracting T cell inhibitory mechanisms to stimulating T cell effector mechanisms. First, cancer immunotherapies have been developed that intervene in the control of T cell activation by targeting negative regulators on T cells. T cells can express inhibitory immune checkpoints such as PD-1 and CTLA-4 that can bind to their ligands PD-L1/PD-L2 and B7.1/B7.2, respectively, on the surface of cancer cells (but also on other immune cell subsets).[31,32] Therapeutic blockade of these interactions with antibodies promotes the activation and expansion of T cells (**Figure 3**). This form of cancer immunotherapy is referred to as immune checkpoint blockade, and earned James P. Allison and Tasuku Honjo the Nobel Prize in Physiology or Medicine 2018. Immune checkpoint blockade therapies targeting the PD-1/PD-L1 and CTLA-4 axis are particularly effective in melanoma, non-small cell lung cancer, and DNA mismatch repair-deficient cancers.[33-37] These cancers are characterized by a high mutation burden, which increases the probability that neoantigens are presented in complex with HLA class I at the surface of cancer cells, allowing for enhanced immunogenicity.[38] As a consequence of their immunogenic character, these cancers generally show a dense infiltration by cytotoxic T cells, currently viewed as the main effector cell of immune checkpoint blockade-induced anti-tumor immunity.[39-41] Another immunotherapeutic strategy involves the modification of T cells to boost the recognition of cancer cells by adoptive cellular therapy with TILs, or genetically modified T cells expressing recombinant TCRs or chimeric antigen receptors (CARs). The most well-developed of such strategies is the use of CAR T cells. CAR T cell therapy involves the isolation of T cells from patients, which are genetically altered by transduction with a construct engineered to express CARs. CARs are composed of an extracellular antibody-binding domain, which can recognize cancer-specific antigens, fused to an intercellular TCR-signaling domain for T cell activation.[42] Further, CARs include co-stimulatory receptors to provide T cell co-stimulatory signals.[43] The modified T cells are subsequently infused back into the patient. CAR T cell therapy can yield substantial clinical benefit, mainly in B cell malignancies.[44] The last category, therapeutic cancer vaccines, primary promotes cancer antigen presentation. It is still a largely experimental therapy. Therapeutic cancer vaccines generally involve exogenous administration of selected cancer antigens, determined by sequencing of the expressed cancer genome, combined with adjuvants that activate dendritic cells.[45] The aim of therapeutic cancer vaccines is to activate or introduce cancer antigen-specific T cell responses.[46-48] Although successful responses can be achieved, therapeutic cancer vaccines generally fail to generate durable T cell responses.[45]

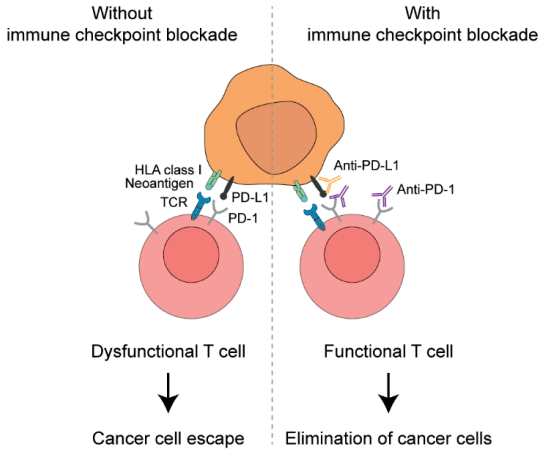

Figure 3. The effect of immune checkpoint blockade on anti-tumor T cell responses.
Inhibitory immune checkpoint protein PD-1 suppresses T cell response and function by engaging with its ligands (PD-L1, PD-L2) that can be expressed by cancer cells but also other immune cell subsets. Therapeutic antibodies directed against immune checkpoint molecule PD-1, or its ligands, block the interaction of PD-1 with PD-L1/PD-L2. As a result, the inhibitory signal is blocked and functional T cells can eliminate cancer cells upon the recognition of cancer antigens presented by HLA class I molecules on the surface of cancer cells with their TCR, accompanied by appropriate costimulatory signals.

The studies described in this thesis

This thesis has studied the tumor microenvironment of CRC and pancreatic ductal adenocarcinoma (PDAC). CRC is the third most frequently diagnosed cancer type worldwide.[49] Currently, more than 1.9 million new cases of CRC per year arise throughout the world, with an estimated 935.000 deaths annually.[49] The five-year survival rate is approximately 65%, but the prognosis of patients largely depends on the tumor stage at the time of diagnosis.[50] CRC is a heterogeneous disease with different pathways of carcinogenesis. One of the pathways of genomic instability involved in the pathogenesis of CRC is microsatellite instability-high (MSI-H). This phenotype is caused by a deficient DNA mismatch repair (MMR) system, involving MMR proteins MLH1, MSH2, MLH6, and PMS2.[51,52] MMR-deficiency occurs in approximately 15-20% of CRCs. As a consequence of the deficient MMR system in these tumors, somatic mutations (insertions, deletions) often accumulate at DNA microsatellite sequences. MMR-deficient cancers are characterized by the presence of numerous TILs.[53,54] The majority of CRCs are MMR-proficient (or microsatellite stable (MSS)), with chromosomal instability (CIN) as the most common form of genetic instability. The CIN pathway is characterized by structural chromosomal aberrations.[55] As compared to MMR-deficient tumors, MMR-proficient tumors generally show a lower mutation burden and less dense infiltration by immune cells.[56,57] The type of genomic instability observed in CRC is known to correlate with the clinical prognosis of patients, where MMR-deficient cancers show a reduced risk of recurrence and improved disease-free survival as compared to MMR-proficient cancers.[56] In addition, the type of genomic instability in CRC is a determining factor for the response to immunotherapy. Immune checkpoint blockade therapy, such as PD-1

blockade, has proven to be effective at reinvigorating T cell-mediated immune responses in patients with MMR-deficient CRC, while MMR-proficient CRCs are largely unresponsive to this therapy.[37,58-60] However, only a minority of MMR-deficient CRCs respond to immune checkpoint blockade. Immune evasion mechanisms counteracting T cell-mediated immune responses are common in MMR-deficient CRCs. For instance, loss of HLA class I expression is described to occur in the majority (78%) of MMR-deficient tumors.[61] In theory, HLA class I-mediated antigen presentation would be an essential component for the clinical activity of $CD8^+$ T cell-based immunotherapies. Surprisingly, the majority of HLA class I-negative MMR-deficient CRCs show durable clinical responses to PD-1 blockade,[62] suggesting that immune cell subsets other than $CD8^+$ T cells may contribute to these responses. Here, different types of innate and unconventional immune cells may come into play as they are capable of HLA class I-unrestricted tumor killing.

PDAC is one of the most lethal cancer types in the industrialized world, with a five-year survival rate of 10%.[63] The majority of PDAC patients present with locally advanced or metastatic disease, with only around 20% of patients eligible for surgical resection of their tumor.[64] Because of its poor prognosis, pancreatic cancer accounts for nearly as many deaths (466.000) as cases (496.000) per year worldwide.[49] The accumulation of genetic alterations in oncogenes and tumor suppressor genes drives the stepwise progression through non-invasive precursor lesions, including pancreatic intraepithelial neoplasia (i.e. PanIN), intraductal papillary mucinous neoplasia (i.e. IPMN), and mucinous cystic neoplasms (i.e. MCN), to invasive cancer.[64] PDAC is non-immunogenic and characterized by a lack of naturally occurring immune responses due to a generally low mutation burden as well as a large stromal compartment consisting of few and mostly immunosuppressive immune cells.[65] Defects in DNA repair systems such as MMR-deficiency are infrequent in PDAC, occurring in approximately 1% of cases.[66] Although patients with higher levels of infiltration by $CD8^+$ and/or $CD4^+$ T cells have significantly improved prognosis,[67] the numbers of tumor-infiltrating T cells are generally low and the cells mainly locate in the stromal compartment of the PDAC microenvironment.[68] Studies have reported minimal clinical benefit in response to immune checkpoint blockade in PDAC patients, with the exception of MMR-deficient PDAC tumors.[69,70] The low mutation burden, low numbers of cytotoxic T cells, large stromal compartment, and immunosuppressive immune cells may render pancreatic cancer insensitive to immunotherapy. In contrast to CRC, mutations in HLA, β2m, or other components of the antigen presentation machinery are not commonly found in PDAC.[71] Currently, combination therapies with immune checkpoint blockade are being examined that seek to enhance anti-tumor immunity.[72]

The need for high-dimensional analyses of the cancer microenvironment

The cancer immune microenvironment plays a critical role in the course of natural disease progression and the clinical prognosis of patients, but can also determine response to cancer immunotherapy. With the emergence of cancer immunotherapies, significant advances have been made that changed the clinical management of cancer treatment for a significant number of patients. However, durable clinical responses to cancer immunotherapy

treatment are observed in only a minority of patients, and the development of resistance to therapy remains an important complication for advanced cancer patients. To understand the mechanisms that are at play in the cancer immune microenvironment affecting responses to current immunotherapies, and for the development of novel, alternative immunotherapeutic strategies, it is crucial to characterize the cancer microenvironment with high-dimensional approaches (**Figure 4**). By simultaneously dissecting immune cell populations across multiple lineages, high-dimensional approaches allow the identification and characterization of intratumoral innate and adaptive immune landscapes and may discriminate immune cell subsets that can be exploited in an immunotherapeutic setting.

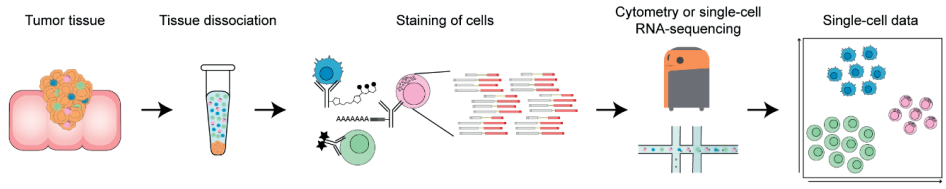

Figure 4. Immunophenotyping of tumor samples with high-dimensional approaches.
Tumor tissue samples are first dissociated into single-cell suspensions, and enriched for immune cells by density-gradient centrifugation. Single-cell suspensions are subsequently stained with a cocktail of antibodies, against different immune cell markers, that can be coupled to fluorochromes, heavy metal isotopes, or oligonucleotides. Stained cells are measured by flow cytometry, mass cytometry, or single-cell RNA-sequencing. Clustering of the obtained single-cell data based on their complete immune cell profile reveals the variety of immune cell types present in the cancer microenvironment.

OUTLINE OF THIS THESIS

In **Chapter 2**, we discuss the heterogeneity of cancer and the need for multidimensional analyses of the cancer microenvironment. We describe cutting-edge multidimensional single-cell technologies that have been paramount in studying the cancer microenvironment, and their strengths and weaknesses. We deliberate on potential avenues for the integration of multi-omics data, and how such integrated data will help to understand the complex role of the microenvironment in cancer.

In **Chapter 3**, we provide a blueprint of innate and adaptive immune cell populations in tumor and non-malignant tissues of patients with CRC analyzed by single-cell mass cytometry. We discover novel mediators of anti-tumor immunity in colorectal tumors, including PD-1$^+$ γδ T cells and a previously unappreciated ILC1-like population. We show that both immune subsets displayed cytotoxic activity and were particularly frequent in MMR-deficient CRCs. In addition, we find tumor-resident CD8$^+$ and CD4$^+$ T cell populations with highly similar activated (CD38$^+$PD-1$^+$) tissue-resident (CD103$^+$) phenotypes that are infrequent in non-malignant tissues. These observations formed the basis for new research questions that are studied in **Chapter 4**, **Chapter 5**, and **Chapter 6**.

In **Chapter 4**, we investigate the involvement of PD-1$^+$ γδ T cells in anti-tumor immune responses and as effectors of immune checkpoint blockade therapy in MMR-deficient colon cancers with loss of HLA class I-mediated antigen presentation. We apply a combination of transcriptomic and imaging approaches for an in-depth analysis of MMR-deficient tumor samples before and after immune checkpoint blockade, accompanied by *in vitro* functional studies, to provide evidence indicating that γδ T cells mediate responses to HLA class I-negative, MMR-deficient tumors during immune checkpoint blockade therapy.

In **Chapter 5**, we explore the full spectrum of ILC subsets and their functional differences in MMR-deficient CRCs by single-cell RNA-sequencing with mass- and flow cytometric characterization of immunophenotypic markers and transcription factors. We demonstrate that the majority of ILCs in MMR-deficient CRCs consist of CD127$^-$CD103$^+$ ILC1-like cells, and provide evidence for an active role for these cells in the recognition and cytotoxicity against MMR-deficient cancers.

In **Chapter 6**, we study TCR repertoires of distinct CD8$^+$ and CD4$^+$ T cell populations in colorectal tumors, defined by the expression of markers of activation (CD38, PD-1) and tissue-residency (CD103) along with their non-activated and non-tissue-resident counterparts. By comparing those TCR repertoires to T cells from adjacent healthy tissues, pericolic lymph nodes, and peripheral blood, we provide insights into the origin of tissue-resident activated (CD103$^+$CD38$^+$PD-1$^+$) CD8$^+$ and CD4$^+$ T cells and their clonal enrichment, a characteristic of antigenic responses.
In **Chapter 7**, we investigate the immune composition in tumors and non-malignant tissues of patients with PDAC by single-cell mass cytometry. We describe a suppressive immune landscape of PDAC, deprived of tissue-resident memory CD8$^+$ T cells with cytotoxic potential, and remarkable increased frequencies of B cells and regulatory T cells as compared to non-malignant pancreatic tissue. In addition, we find a tumor tissue-specific ILC1-like population resembling the one described in CRC (**Chapter 3** and **Chapter 5**). To some extent, the immune cell composition of PDAC could be reflected in portal vein blood, suggesting a regional enrichment of immune cells involved in the anti-tumor immune response.

In **Chapter 8**, we discuss the main findings of this thesis in a broader perspective and provide implications for future research and clinical practice.

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