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

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General discussion and future perspectives

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GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Cancer immunotherapy has established itself as a novel pillar of cancer care in recent years, despite its long history with the first experiments already performed in the late 19th century.[1] T cells, and their capacity for antigen recognition and cytotoxicity, have become a central focus in anti-tumor immunity. Remarkable progress in fundamental research elucidating the molecular and cellular biology of T cells have resulted in successful cancer immunotherapeutic strategies, such as immune checkpoint blockade therapy and chimeric antigen receptor (CAR) T cell therapy. In contrast to chemo- or radiotherapeutic approaches, these immunotherapies have shown curative potential in advanced cancers.[2] However, immune checkpoint blockade therapy and CAR T cell therapy are not yet applicable to a large proportion of cancer patients. Although T cells have long served as the cellular underpinnings of cancer immunotherapies, T cell-based immunotherapies might not be a solution for all cancer patients. Therefore, characterization of other immune cell subsets with anti-tumor potential in the cancer microenvironment is important and may pave the way toward exploiting previously unappreciated immune cells in an immunotherapeutic setting. Furthermore, it has recently been shown that cancer immunotherapies employed at earlier stages of cancer, in the neo-adjuvant setting, appear to be more successful than at advanced tumor stages.[3] This indicates that different mechanisms are at play in the primary tumor *versus* the metastatic immune microenvironment influencing the effect of immunotherapies. Moving forward to improving outcomes of patients with advanced cancer, it would be crucial to understand how to specifically target immune evasion mechanisms in the metastatic setting. Taken together, to progress cancer immunology research, we need to focus on: i) broadening the scope of anti-tumor immune mediators in primary tumors, before immune evasion can occur, and ii) improving our understanding of the metastatic immune microenvironment and how it can be targeted.

Broadening the scope of anti-tumor immune mediators in primary tumors

The work in this thesis focused on the characterization of the colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC) immune microenvironments for a comprehensive understanding of anti-cancer immune responses across the innate and adaptive immune compartments. Most cancer immunology research studied the role of cytotoxic T cells in both cancer types, while a comprehensive analysis of both innate and adaptive components of cancer immunity was largely lacking. With such an approach, we demonstrated an important involvement of understudied unconventional (γδ T cells) and innate (innate lymphoid cells (ILCs)) immune effector cells in anti-tumor immunity. Based on the findings of us and others, we foresee emerging roles for other immune effector cells in the coming decades such as Vδ1/Vδ3 T cells and ILC1-like cells in anti-tumor immunity and cancer immunotherapies. In addition, the recent findings that B cells, tertiary lymphoid structures, and the presence of antibodies to cancer antigens are associated with a favorable prognosis in several types of cancer[4-9] highlight further investigations into the potential of naturally-generated antibodies in the context of cancer. Research should prioritize the elucidation of the functions that these understudied immune effector cells perform and how they are

involved in anti-tumor immune responses. Exploitation of their anti-tumor reactivity may provide an alternative immunotherapeutic approach and/or may complement current T cell-mediated immunotherapies to enhance cancer immunotherapeutic success.

In this thesis, we observed that mismatch repair (MMR)-deficient colorectal tumors contained tumor tissue-specific PD-1$^+$ γδ T cells that were infrequent in colorectal healthy tissue, tumor-associated lymph nodes, and peripheral blood of the same patients. Furthermore, PD-1$^+$ γδ T cells were generally not observed in MMR-proficient colorectal tumors. γδ T cells are one of the least understood immune cell types at the interface of innate and adaptive immunity. The precise mechanisms of recognition by and activation of intratumoral γδ T cells remain largely unknown. Our findings of i) elevated frequencies of γδ T cells in MMR-deficient tumors with human leukocyte antigen (HLA) class I defects, ii) *in vitro* activation of intratumoral γδ T cells by CRC cell lines and tumor organoids, iii) killing of these tumor cells by γδ T cells, mainly by Vδ1 and Vδ3 subsets expressing PD-1, iv) increased presence of intratumoral γδ T cells upon immune checkpoint blockade therapy, and v) the localization of γδ T cells next to apoptotic cancer cells in these tumors indicate an active role for these cells in this context. Recent work has revealed an important involvement of butyrophilins binding to the T cell receptor (TCR) in Vδ2 T cell recognition and activation.[10,11] However, different subsets of γδ T cells have remarkably diverse functions. For Vδ1 and Vδ3 cells, it is not yet established whether tumor recognition is established through their γδ TCR, via innate immune receptors such as NKG2D or DNAM-1, or both. Like ILCs/NK cells, γδ T cells express important activating and inhibitory innate immune receptors that potentially are involved in tumor recognition including NKG2D and KIRs (**Figure 1**). For a precise understanding of γδ TCR – ligand interactions, it would be necessary to screen for (novel) γδ TCR ligands involved in the activation of γδ TCRs in combination with single-cell RNA/VDJ-sequencing to dissect antigenic specificities of intratumoral γδ T cells. This might help to elucidate the mechanism behind the specific enrichment and cytotoxicity of Vδ1 and Vδ3 T cells in CRC tissues, while Vδ2 cells are the main γδ T cell population in the circulation. Furthermore, such mechanistic studies might shed light on the observation that γδ T cells were virtually absent in PDAC tissues.

Furthermore, we detected tumor tissue-specific ILC1-like populations in both the CRC and PDAC microenvironment. In CRC, this population was particularly frequent in MMR-deficient tumors that show a relatively high number of somatic mutations and commonly lose HLA class I expression. In these tumors, the ILC1-like cells had a frequent intraepithelial localization and showed hallmarks of activation, cytotoxicity, and proliferation. In line, the ILC1-like population was most abundant in a PDAC tumor with a DNA repair defect, potentially underlying the increased mutational load and resulting inflammatory response observed in this tumor. However, the ILC1-like cells exhibited lower cytotoxicity in the PDAC microenvironment as compared to the CRC microenvironment. Possible reasons for this observation may be the largely immunosuppressive microenvironment in PDAC, the lack of immune evasion through loss of HLA class I-mediated antigen presentation in PDAC,[12] or the lack of expression of NKG2D ligands on PDAC cells. Interestingly, intratumoral NK cells are

scarce in both cancer types,[13,14] while these cells constitute a major immune cell population in the circulation. It would be of interest to study the possible conversion of peripheral NK cells to ILC1-like cells in the cancer microenvironment, as in murine cancer models ILC1-like phenotypes could emerge from NK cell differentiation driven by transforming growth factor-β (TGF-β) signaling.[15] TGF-β is a pleiotropic cytokine that, apart from its immunosuppressive activity, can drive the differentiation of immune cells.[16,17] In support of this, decidual stromal medium with TGF-β can convert peripheral NK cells into decidual NK cells exhibiting an ILC1-like phenotype.[18] Whether ILC1-like cells reflect a truly distinct lineage or acquired this phenotype upon encountering the cancer microenvironment remains elusive and requires further functional studies. In both cancer types, the presence of the ILC1-like population correlated with the presence of $CD8^+$ T cells with tumor-reactive phenotypes ($CD103^+CD39^+$[19-22]), raising questions about whether and how the functions of ILC1-like cells and $CD8^+$ T cells are related during anti-tumor immune responses. It is interesting that the ILC1-like and $CD8^+$ T cell populations shared such highly similar immunophenotypes, localization, and expression of cytotoxic molecules in different cancer types. Like cytotoxic T cells, intraepithelial ILC1-like cells have been shown to be cytotoxic against cancer cells.[23,24] However, they differ greatly in the way they sense cancer cells and in their kinetics of action. ILCs do not depend on the expression of antigen-specific receptors for their activation and respond rapidly, whereas T cell responses require clonal expansion and differentiation. Nevertheless the anti-tumor immune functions of these two diverse immune subsets appear related. From an evolutionary perspective, shared immunological features by subsets of ILCs and T cells[25] may account for robust immunity in the face of continuous cancer immune evasion.[25] The study of ILC1-like cells in CRC is currently being continued in which we examine i) the localization and interacting cells of ILC subsets in colorectal tumors with and without HLA class I expression by imaging mass cytometry, and ii) the cytotoxicity of ILC1-like cells toward CRC cell lines with and without HLA class I expression by *in vitro* functional assays. These follow-up studies may facilitate the exploitation of ILC1-like cells as targets for cancer immunotherapy. Of note, both the ILC1-like and Vδ1/Vδ3 T cell populations showed expression of HLA class II, suggesting that these cells might have antigen-presenting properties, another interesting direction for further research (**Figure 1**).

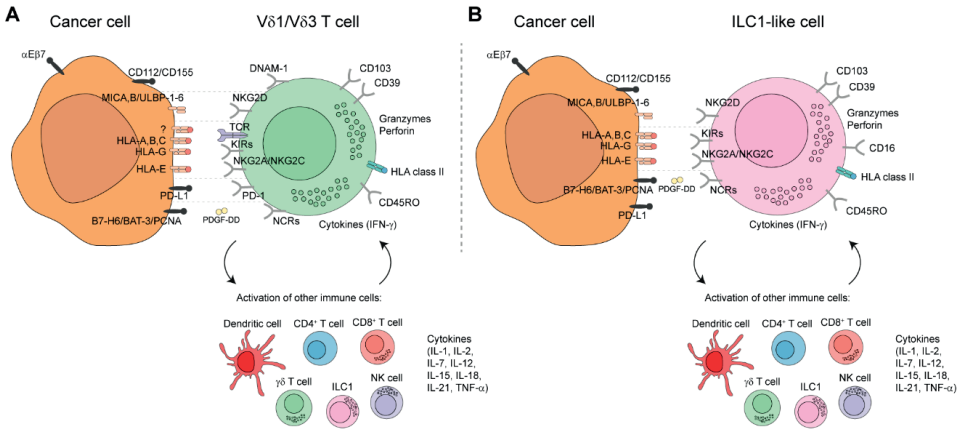

Figure 1. γδ T cells and ILCs are endowed with various recognition mechanisms to sense cancer cells and to initiate anti-tumor immune responses.

Schematic overview of possible receptor – ligand interactions for Vδ1/Vδ3 T cells (**A**) and for ILC1-like cells (**B**) in the context of cancer. NCRs; natural cytotoxicity receptors (NKp30, NKp44, NKp46), PDGF-DD; platelet-derived growth factor-DD.

Awaiting further insights into the function of these previously unappreciated unconventional and innate immune cells, we foresee multiple ways forward for the translation of Vδ1/Vδ3 T cells and ILC1-like cells into cancer immunotherapies. For γδ T cells, immunotherapeutic strategies could include: i) the induction of their activation by PD-1 blockade, ii) the upregulation of NKG2D (ligand) expression to enhance their anti-tumor functions (e.g. via cytokines), iii) the adoptive cell transfer of *ex vivo* expanded Vδ1/Vδ3 T cells, and iv) the transduction of tumor-reactive Vδ1/Vδ3 TCRs on donor αβ T cells. For ILC1-like cells, immunotherapeutic strategies could include i) the induction of their activation by NKG2A blockade, ii) the upregulation of NKG2D (ligand) expression to enhance their anti-tumor functions (e.g. via cytokines), iii) the adoptive cell transfer of *ex vivo* expanded ILC1-like cells, and iv) the engineering of ILC1-like cells to express CARs directed against surface molecules on cancer cells (CAR-ILC1-like cells) providing an "off-the-shelf" allogeneic product. Vδ1/Vδ3 and ILC1-like cancer immunotherapies could have broad implications for MMR-deficient cancers and other malignancies with common HLA class I defects. The first immunotherapies with γδ T cells (mainly Vδ2 subsets) and conventional NK cells are currently being developed for hematological malignancies.[26-28] These clinical trials have not yet reached the stage of conventional T cell-based therapies, but we foresee that we will see such products within the coming years. While it needs to be considered that the Vδ1/Vδ3 T cells and ILC1-like cells might also display alloreactivity, the application of these cells isolated and expanded from a universal donor rather than the autologous setting might be a reasonable approach for future application. Vδ1/Vδ3 T cells and/or ILC1-like cells from a universal donor with the highest cytotoxicity could, in theory, be distributed to many different patients. Harnessing such unconventional and innate immune cells may have the potential to lead to a cheaper, faster, and more universal form of cancer immunotherapy.

Improving our understanding of the metastatic immune microenvironment and how it can be targeted

The work in this thesis focused on the study of primary tumors, while the majority of cancer patients die from metastatic disease. Moving forward to improving outcomes of patients with cancer, it would be important to also study the role of innate and adaptive immune cells in metastatic lesions, how the metastatic immune microenvironment differs from the primary tumor, and how it could be targeted. The structure and composition of the cancer microenvironment vary between the primary tumor and metastasis, and between different metastatic niches in individual patients. During dissemination, cancer cells from primary tumors are exposed to different types of stromal cells, immune cells, platelets, and metabolic stress, and have to adapt to the new tissue microenvironment. In CRC, HLA class I expression can be a determining factor for metastatic behavior. Liver metastases originating from HLA class I-negative/β*2-microglobulin* (*B2M*)-mutated MMR-deficient CRCs are rare and HLA class I-positive.[29-31] This could be related to NK cell-mediated elimination of metastatic cancer cells that lack HLA class I expression in the circulation and/or the high abundance of NK cells (30-50% of intrahepatic lymphocytes) and, to a lower extent, γδ T cells (3-5% of intrahepatic lymphocytes) in the liver[32]. However, metastases could be derived through lymph nodes where NK cells and γδ T cells are virtually absent, or altered metastatic HLA phenotypes could be acquired during the process of dissemination. Analysis of such immune evasion mechanisms of metastases may provide important information required to determine to what type of cancer immunotherapy the metastatic lesions respond. It would be interesting to examine whether we can find similar tumor-resident immune cell populations in metastases as in the primary tumor. In PDAC, the majority of patients already present with advanced disease at the time of diagnosis. Hence, dissecting tumor-immune cell interactions in the metastatic lesions would be necessary to find out whether the approach to treating the primary tumor and metastases needs to vary.

Taken together, cancers are complex ecosystems deregulated at multiple levels. A "one-size fits all" approach will not suffice to achieve effective immunotherapy responses for the majority of cancer patients. We need to determine for individual tumors what immune evasion mechanisms are at play, and target these specifically to get an effective anti-tumor immune response. Multi-omics technologies that connect the immunophenotype, gene expression, and spatial landscape have the potential to accelerate personalized immunotherapy based on each individual's tumor and metastatic microenvironment (**Figure 2**). In parallel, genomic profiling of the primary tumor as well as metastatic lesions as standard diagnostics for patients is needed to gain insight into genomic differences between primary tumor and metastases, and to select (targeted) treatment based on their unique tumor DNA profile. Examining immune phenotypes in primary tumors and metastatic lesions of immunotherapy-responsive patients could provide new avenues on how we could introduce or adapt these tumor-reactive immune cells in non-responsive patients. These analyses could for instance indicate whether cancer immunotherapies with Vδ1/Vδ3 T cells and/or ILC1-like cells may also be effective in the metastatic setting. We can extract enormous amounts of relevant information from multi-omics data, but the challenge is to reconnect findings to their biological relevance.

Therefore, multi-omics technologies must always go hand in hand with laboratory research such as functional studies to properly interpret what we see. This requires an extensive collaboration between multi-disciplinary researchers including bioinformaticians, oncologists, and immunologists. The findings in this thesis will facilitate further mechanistical studies of γδ T cells and ILCs in anti-tumor immunity, and how such cells can be harnessed as novel cancer immunotherapies. We expect that these understudied immune effector cells hold promise for increasing the success rate of immunotherapy across a wide range of cancer types.

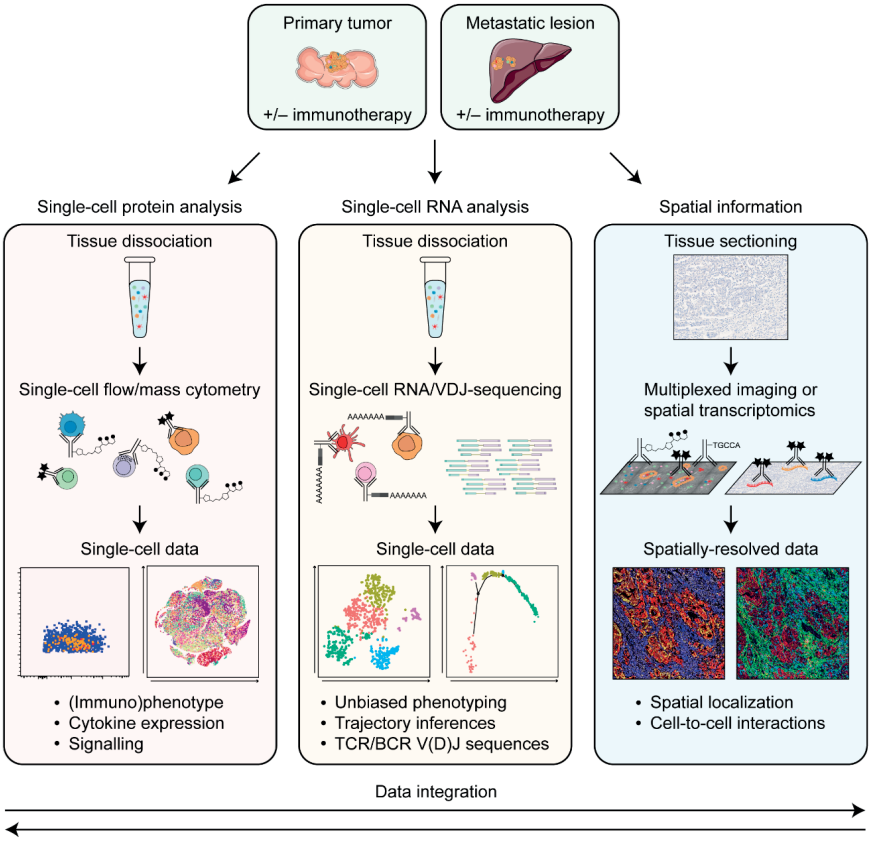

Figure 2. Toward understanding primary tumor and metastatic microenvironments by integrative single-cell technologies.
Schematic overview of the integration of single-cell data of dissociated cells and spatially-resolved data from primary tumors and metastatic lesions, which has the potential to reveal the full cellular landscape of the cancer microenvironment of the primary tumor and metastatic lesions. Adapted from de Vries *et al.* (2020)[33].

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