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

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SUMMARY IN ENGLISH

Overcoming challenges in cancer immunology with multidimensional technologies
Cancer immunotherapy treatment currently benefits only a minority of cancer patients, despite the successful application across a broad range of tumors. Cancer is a dynamic disease characterized by extensive heterogeneity, within a primary tumor, between lesions, and across patients (**Chapter 2**). Major challenges in the field of cancer immunology research are the identification of predictive biomarkers to select cancer patients that are likely to respond to specific immunotherapy treatments, the detection of resistance mechanisms to cancer immunotherapies, and the development of novel immunotherapeutic strategies to improve cancer survival. The advent of multidimensional single-cell technologies such as spectral flow cytometry, mass cytometry, single-cell RNA-sequencing, and imaging mass cytometry may play a crucial role to address the former challenges. As cancer is a complex biological system, we highlighted the need of integrating single-cell data derived from multidimensional technologies to i) improve our understanding of the variability in response to immunotherapy treatment and the resistance mechanisms that are at play, and ii) guide the development of alternative immunotherapeutic strategies (**Chapter 2**). As of now, only a handful of studies, including **Chapter 3** of this thesis, have used complementary forms of multidimensional single-cell analyses on the same tissue to study cancer.

Novel mediators of anti-tumor immunity in colorectal cancer
In **Chapter 3** of this thesis, we charted the complexity of colorectal cancer (CRC) immunity by single-cell mass cytometry complemented with single-cell RNA-sequencing. We identified tumor-resident immune cell populations across the innate (ILCs, γδ T cells) and adaptive (cytotoxic and helper T cells) immune compartments. The tumor tissue-specific T cell subsets showed a highly similar activated (HLA-DR$^+$CD38$^+$PD-1$^+$), tissue-resident (CD103$^+$CD69$^+$), memory (CD45RO$^+$) phenotype, and were infrequent in colorectal healthy tissues, tumor-associated lymph nodes, and peripheral blood. We found that two immune cell populations were particularly enriched in mismatch repair (MMR)-deficient CRCs, namely PD-1$^+$ γδ T cells and a previously unappreciated innate lymphoid cells (ILC) population (Lin$^-$CD7$^+$CD127$^-$ CD103$^+$CD45RO$^+$ ILC1-like).

With regard to the first immune cell population, the expression of PD-1 by the γδ T cells in conjunction with their cytotoxic potential suggested an active role for these cells in the anti-tumor immune response and potentially as effector cells of PD-1 immune checkpoint blockade therapy. These research questions were further addressed in **Chapter 4**. We found that intratumoral PD-1$^+$ γδ T cells, isolated from MMR-deficient colon cancers, were composed of Vδ1 and Vδ3 T cells, and showed potent cytotoxicity toward human leukocyte antigen (HLA) class I-negative/*β2-microglobulin* (*B2M*)-defect MMR-deficient CRC cell lines and patient-derived tumor organoids. This response was partly reduced upon blocking the interaction between NKG2D and DNAM-1, and their respective ligands on the cancer cells. As a next step, the relevance of γδ T cells in the clinical setting of patients treated with immune checkpoint blockade was investigated. We addressed the paradoxical observation

that MMR-deficient cancers show exceptional clinical responses to immune checkpoint blockade therapy, even when they lost the expression of HLA class I, an essential component for antigen presentation to $CD8^+$ T cells. This observation contradicts the current view that PD-1 blockade boosts anti-tumor immunity through $CD8^+$ T cells, but suggests the involvement of other immune effector cells than $CD8^+$ T cells. Our work on the in-depth analysis of MMR-deficient colon cancer samples before and after immune checkpoint blockade (**Chapter 4**) was the first to provide evidence that γδ T cells are cytotoxic effector cells of immune checkpoint blockade therapy in cancers with HLA class I defects. Overall, HLA class I deficiency was associated with an increased frequency of activated γδ T cells in treatment-naïve tumors. Furthermore, immune checkpoint blockade profoundly increased the intratumoral frequency of γδ T cells in HLA class I-negative/*B2M*-mutant cancers. In combination with the *in vitro* functional data, our observations illustrate the potential of γδ T cells, in particular Vδ1 and Vδ3 subsets, as novel targets for the development of cancer immunotherapies.

The second immune cell subset prominently enriched in MMR-deficient CRCs, Lin^- $CD7^+CD127^-CD103^+CD45RO^+$ ILC1-like cells, accounted for up to 80% of the innate lymphoid compartment in these tumors. The $CD127^-CD103^+CD45RO^+$ ILC1-like cells positioned, upon hierarchical clustering based on their immune cell profiles, in between conventional NK cells ($CD127^-CD56^+CD45RO^-$) and $CD127^+$ conventional ILCs. This positioning hints toward the possibility that the ILC1-like population can be an intermediate subset in between conventional NK cells and $CD127^+$ conventional ILCs. We attempted to isolate the ILC1-like cells from MMR-deficient CRC tissues for cell culture and functional studies to gain more insight into where the cells come from and whether they could further differentiate, however, this proved difficult. The ILC1-like population had a frequent intraepithelial localization and showed hallmarks of activation, cytotoxicity, and proliferation in MMR-deficient CRC tissues *ex vivo* (**Chapter 3 and Chapter 5**). We performed an unbiased characterization of sorted $CD7^+CD3^-$ ILCs, independently of CD127 expression, by a single-cell RNA-sequencing approach, and showed that high expression of genes encoding killer-cell immunoglobulin-like receptors (KIRs), NKG2A, immunomodulatory molecules, and HLA class II further distinguished the ILC1-like cells from conventional NK cells and conventional ILCs (**Chapter 5**). The ILC1-like cells are an attractive immune cell population to study in light of the expression of KIRs, implied in the recognition of HLA class I-negative cells, and the frequent loss of HLA class I expression observed in MMR-deficient cancers.

The origin of tissue-resident $CD8^+$ and $CD4^+$ T cell populations in colorectal cancer

We identified tumor-specific populations of tissue-resident ($CD103^+$), activated ($CD38^+$PD-1^+CD39^+) $CD8^+$ and $CD4^+$ T cells in CRC tissues (**Chapter 3**). The non-activated counterparts ($CD103^+CD38^-$PD-1^-) of the tumor tissue-specific $CD8^+$ and $CD4^+$ T cells were present in tumors and adjacent healthy tissue, but could not be found in pericolic lymph nodes (with the exception of tumor-positive lymph nodes). Co-expression of CD103 and CD39 has been reported to identify tumor-reactive $CD8^+$ T cells in different types of cancer, including CRC. Understanding the clonal relationships between such distinct $CD8^+$ and $CD4^+$ T cell

populations and their tissue of origin might shed light on the dynamics of anti-tumor T cell responses. In **Chapter 6**, we aimed to address the question where the tissue-resident, activated T cells in CRC come from and whether they are clonally expanded. Our TCRβ sequencing data of $CD8^+$ and $CD4^+$ T cell subsets in colorectal tumors and matched non-malignant tissue samples showed that the tissue-resident, activated ($CD103^+CD38^+PD\text{-}1^+$) $CD8^+$ and $CD4^+$ T cells largely originate from pericolic lymph nodes, while their non-activated counterparts ($CD103^+CD38^-PD\text{-}1^-$) showed frequent TCRβ clonal overlap with T cells from adjacent healthy tissue. Within colorectal tumors, highest clonality was observed for $CD103^+CD38^+PD\text{-}1^+$ $CD8^+$ and $CD4^+$ T cell populations, which co-expressed CD39, suggesting that tissue-residency and activation markers as CD103, CD38, PD-1, and CD39 may serve as surrogates for tumor-reactive $CD8^+$ T cells. With regard to $CD4^+$ T cells, phenotypic characterization of markers to identify tumor-reactive $CD4^+$ T cells are largely lacking, and it could be tested whether tumor reactivity is indeed associated with markers as CD103, CD38, PD-1, and CD39 in CRC. The observation that both activated ($CD38^+PD\text{-}1^+$) and non-activated ($CD38^-PD\text{-}1^-$) tissue-resident ($CD103^+$) T cells were infrequent in pericolic lymph node samples (**Chapter 6**) supports the current view that T cells are primed by antigen presenting cells in the lymph nodes, subsequently migrate to the tumor where they recognize their cognate antigen and clonally expand. Upon this TCR activation, the intratumoral T cells start upregulating tissue-residency and activation markers that retain the cells within the tumor.

Novel mediators of anti-tumor immunity in pancreatic ductal adenocarcinoma

In contrast to the immune cell-rich microenvironment of CRCs (**Chapter 3**), the immune microenvironment of pancreatic ductal adenocarcinoma (PDAC) was found to be immunosuppressive and deprived of infiltration by T cells with cytotoxic potential in the majority of pancreatic tumors (**Chapter 7**). The intratumoral $CD8^+$ T cells showed a lack of expression of activation markers as well as immune checkpoint protein PD-1, which might underly the lack of clinical responses to immune checkpoint blockade as of yet. As compared to non-malignant pancreatic tissues, B cells and regulatory T cells were remarkably increased in PDAC tissues. Interestingly, we identified a tumor tissue-specific ILC1-like population ($CD127^-CD103^+CD39^+CD45RO^+$) that resembled the ILC1-like cells found in CRCs, but showed lower expression of cytotoxic molecules. It would be of interest to study how the cytotoxicity of these ILC1-like cells in the PDAC microenvironment could be enhanced, and whether these cells exhibit a cytolytic response to PDAC cells. A unique feature of this study was the sample collection of portal vein blood, in addition to peripheral blood, enabling a characterization of immune cell profiles in portal vein blood in comparison to peripheral blood from PDAC patients. We showed that the immune composition in peripheral blood is extremely diverse, with large differences between patients, which troubles the discovery of disease-related features. Intriguingly, immune cells infiltrating the PDAC microenvironment could, to some extent, be detected in portal vein blood, but not in peripheral blood, suggesting a regional enrichment of immune cells involved in the anti-tumor immune response. For instance, the only patient that harbored $PD\text{-}1^+$ $CD8^+$ T cells in the pancreatic tumor, showed high frequencies of $PD\text{-}1^+$ $CD8^+$ T cells in the matched portal blood sample.

This observation may indicate that portal vein blood could be a novel source of T cells with tumor-reactive phenotypes that could be further exploited for PDAC patients.