The impact of one: single cell analysis of T cell states in human cancer

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Chapter 1

Scope of this thesis

The ability of T cells, and in particular $CD8^+$ T cells, to recognize and eliminate cancer cells forms the mechanistic basis for the immune checkpoint therapies that aim to reinvigorate the anti-tumor immune response. While ($CD8^+$) T cell infiltration is typically associated with a favorable outcome to checkpoint therapies, analysis of the phenotypes and functions of intratumoral T cells have demonstrated that T cells present at the tumor site are heterogeneous in their capacity to respond to surrounding tumor cells. In order to identify the cellular determinants that underlie an effective response to immune checkpoint blockade, it is therefore critical to gain insight into the diversity of T cell states at the tumor site and to dissect the roles of such distinct T cell states in anti-tumor immunity, and this is the scope of this thesis.

Direct evidence that T cells can actively influence tumor progression through eradication of cancer cells comes from adoptive T cell transfer studies that have demonstrated the capacity of *ex vivo* expanded T cells to eliminate tumors(*1*), a phenomenon that relies on the recognition of tumor-antigens derived from mutated or aberrantly expressed proteins(*2, 3*). The generation of such a T cell-mediated anti-tumor response is captured in the "cancer-immunity cycle" model, which starts with the uptake and presentation of tumor-antigens by antigen presenting cells, followed by the priming of tumor-specific T cells and their subsequent trafficking to and infiltration of the tumor, and – if all goes well – culminates in tumor recognition and elimination(*4*). Each step of this cycle can however be subject to immune-modulatory mechanisms that facilitate escape of tumor cells from immune pressure, including T cell-intrinsic factors such as the lack of tumor-specific T cell receptors (TCRs), or environmental factors that influence the efficacy of T cell priming, infiltration, and effector function. The latter environmental influences include – but are not limited to – production of soluble factors that modulate the tumor microenvironment, local expression of inhibitory molecules that reduce T cell function, and downregulation of components of the antigen-presenting machinery by tumor cells to evade T cell recognition. Together, these signals – or the lack thereof – shape the states of intratumoral T cells, leading to the diverse spectrum of phenotypes and functionalities that can be observed at the tumor site and that determines the effectivity of checkpoint therapies.

One of the T cell states that has received ample attention in the context of clinical response to immunotherapy, is the dysfunctional – or exhausted – $CD8^+$ T cell state. Dysfunctional $CD8^+$ T cells have first been described in the context of chronic viral infections, in which virus-specific $CD8^+$ T cells that are subject to continuous antigen exposure show a loss of classical T cell effector functions and reduced proliferation potential, increased expression of inhibitory receptors such as PD-1 and CTLA4, and a rewiring of their epigenetic landscape(*5-7*). While it is not entirely clear to what extent T cell dysfunction develops in an analogous manner in human tumors, dysfunctional $CD8^+$ T cells in human tumors do share many similarities with those described in chronic viral infections in mice, including loss of effector function and increased expression of inhibitory receptors(*8*). Notably, the enrichment of tumor-reactive cells in the dysfunctional $CD8^+$ T cell population(*9, 10*) underlines the relevance of this cell

pool in anti-tumor immunity, as the presence of tumor-specific T cells is deemed a prerequisite for a successful response to immune checkpoint therapy.
The observation that T cells are diverse in their capacity to recognize tumor antigens (*11*) and/or to be reinvigorated by immune checkpoint blockade (*12*) stresses the need to address the development of different intratumoral T cell subsets and their roles in tumor-immunity by addressing questions such as 1) which T cell states are present in human tumors and how do intratumoral T cell populations compare across cancer types? 2) how does the local microenvironment influence intratumoral T cell states? And 3) what is the relevance of different T cell states for clinical response to immunotherapy? The development of single cell analysis methods has opened a new chapter in the characterization of heterogeneous cell populations. The potential to perform unbiased transcriptome analysis on thousands of individual cells facilitates their in-depth characterization as well as the identification and analysis of rare cell subsets. For the dissection of T cell heterogeneity and T cell differentiation processes, the coupling of single cell transcriptomes to individual TCR sequences is of particular interest, as it allows one to compare the states of sister cells that share a given TCR and to assess tumor reactivity in a manner that is not influenced by cell state. While these technological advances have allowed tremendous progress in efforts to resolve the complexity of intratumoral T cell states, further innovations in single cell sequencing approaches remain of value, for instance to better understand the transcriptional dynamics of individual (intratumoral) T cells in space and time.

In this thesis, I aim to dissect the states of intratumoral T cells in human tumors and thereby extend our understanding of the role of diverse T cell states in response to immunotherapy, using a combination of single cell transcriptomics and novel experimental technologies for the analysis of patient tumor samples. In **Chapter 2**, we characterize the immune cell states that are present in human melanoma tumors**,** leading to the following conclusions: first, $CD8^+$ T cell dysfunction is not a single cell state, but should rather be viewed as a gradient of cell states, and $CD8^+$ T cell clones consist of cells with different levels of dysfunction. Second, in contrast to models that consider dysfunctional T cells as inert, $CD8^+$ T cells residing along this "dysfunctional gradient" show signs of active proliferation and express effector genes such as *CXCL13*. Based on these conclusions, we favor a model in which dysfunctional $CD8^+$ T cells acquire altered functional characteristics instead of solely losing canonical $CD8^+$ T cell effector functions. In **Chapter 3**, I discuss the T cell states that are identified across different tumor types in an extensive pan-cancer single cell RNA sequencing analysis, and how the characteristics of these different cell states are of value for predicting response to immune checkpoint blockade. Analysis of the T cell compartment of head and neck squamous cell carcinomas (HNSCCs) prior to and during immune checkpoint blockade treatment in patients that do or do not show a response to therapy, as presented in **Chapter 4**, suggests that the activation state of regulatory T cells (Tregs) is predictive for clinical response to dual anti-CTLA4 and anti-PD-1 therapy in this patient group. In addition, from these analyses we conclude that upon therapy, cells in the Treg and dysfunctional $CD8^+$ T cell compartments

adapt a state of reduced activation, while at the same time, a population of transitional $CD8^+$ T cells characterized by lower levels of dysfunction expands, presumably driven by recruitment of new T cell clones. Based on these observations, we propose that suppression of Treg activity plays a key role in the response of HNSCCs to dual CTLA4 - PD-1 blockade and that, in responding patients, the profound decrease in tumor load after two cycles of treatment results in reduced activation levels in independent T cell compartments.

Collectively, these studies demonstrate the diversity of intratumoral T cell states and reveal the cell state alterations that occur upon treatment with immunotherapy. In **Chapter 5**, the insights obtained through single cell analyses of T cells, in particular $CD8^+$ T cells, in human cancer by us and others are highlighted. In this chapter, we present a model for the relationship between $CD8^+$ T cell states in human tumors and the response of these cell states to immune checkpoint therapy. In this model, plausible drivers of an anti-tumor response following checkpoint blockade include reactivation and expansion of intratumoral T cells that reside along the dysfunctional gradient, and recruitment of novel T cells from the periphery. In **Chapter 6**, I further discuss the value of single cell genomics approaches to dissect the early response to treatment through analysis of patient material shortly after treatment initiation, and the importance of these types of analyses for our understanding of the anti-tumor response over time.

In **Chapters 7** and **8**, I outline the development and exploitation of 2 novel technologies to address the impact of localization of T cells in (tumor) tissue on their cell state and to unravel the early immunological response to immune checkpoint therapy. The observed diversity of intratumoral T cell states, as discussed in **Chapters 2** to **5**, has urged us to understand which factors drive this cell state heterogeneity. To assess the potential relationship between the spatial organization of T cells within tumors and their cell states, we have developed the novel spatial sequencing technology (termed SCARI) that is presented in **Chapter 7**. SCARI allows single cell analysis of cells from primary human tissues while preserving information on their location in the tissue. The feasibility of the method to specifically isolate and analyze live cells from small-scale regions of interest is demonstrated by the analysis of spatially defined $CD8^+$ T cells in *in vitro* systems and human tumor tissue. Our benchmarking experiments demonstrate the significant value of this technology for the dissection of the effects of local environmental factors on the phenotype and function of cells that reside in different tissue regions. In **Chapter 8**, we assess the early response to anti-PD-1 using single cell profiling of immune cells in an *ex vivo* patient-derived tumor fragment (PDTF) platform, in order to distinguish the primary effects of PD-1 blockade from downstream effects following response to therapy. The PDTF platform allows analysis of the cellular response that happens at the tumor site early after blocking PD-1, a time window that is not captured by the analysis of sequential tumor biopsies, and therefore complements our insights into response dynamics derived from pre- and on-treatment biopsies. We conclude that during the early response to PD-1 blockade, T cells at the tumor site maintain stable transcriptional profiles, while anti-CD3 induces profound gene expression alterations in the intratumoral T cell population. In spite of the lack of profound changes in the T cell compartment, anti-PD-1 does induce

expression of IFNγ-responsive genes in other intratumoral cell populations, and this response can be abrogated through blockade of the IFNγR1 receptor. Together, these data suggest a model in which PD-1 blockade induces a broad, IFNγ-mediated, downstream response in multiple immune cell types in the absence of profound transcriptional changes in the T cell compartment, and we propose that in patients, this downstream response may lead to activation and attraction of immune cells that has been observed in on-treatment biopsies. Finally, in **Chapter 9** I place the results presented in this thesis in context, based on our current knowledge on intratumoral T cells and their role in response to immune checkpoint blockade. In addition, in this chapter, I bring up a number of remaining questions that I feel will be important to address, and discuss how technological innovation can further increase our understanding of the mechanisms underlying response and resistance to therapy, in order to improve the prospects of cancer patients.

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