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## Therapeutic strategies to restore intratumoral immune activity in human cancer

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## **Chapter 1**

### Scope of this thesis

T cells are key players in cancer immunity, with the ability to selectively detect and eliminate cancer cells. However, it took time before their potential for cancer treatment was fully recognized. The concept of using the own body's immune defense to fight cancer dates back over a century when William B. Coley observed that bacterial infections could induce tumor regression<sup>1,2</sup>, but only in the past two decades the field of cancer immunotherapy took off. A pivotal breakthrough came with the clinical approval of high-dose interleukin-2 (IL-2), a cytokine that promotes T cell expansion and activation<sup>3</sup>. IL-2 was the first immunotherapeutic to provide clinical proof that stimulating the immune system alone could eliminate established tumors, particularly in metastatic melanoma and renal cell carcinoma<sup>4,5</sup>. This success paved the way for additional T cell-based therapies, such as adoptive T cell transfer<sup>6,7</sup>, which involves infusing T cell products derived from tumor-infiltrating lymphocytes expanded *ex vivo* with IL-2, as well as immune checkpoint blockade (ICB)<sup>8-10</sup>, which inhibits suppressive checkpoint signals on T cells. Different forms of immunotherapy are currently used for treatment of various cancer types<sup>10-13</sup>, and even more are undergoing clinical trials<sup>14-16</sup>. Yet, their efficacy is ultimately determined at the tumor site, where T cells directly interact with cancer cells and must overcome a range of immunosuppressive mechanisms. Many patients still fail to respond, underscoring the need to unravel how these therapies shape T cell responses within the tumor microenvironment (TME).

## T cell dysfunction in the tumor microenvironment

A robust and sustained immune response is essential for controlling infections and eliminating malignant cells. When antigen exposure becomes chronic, T cells however undergo profound functional changes that lead to the acquisition of a “dysfunctional” state<sup>17,18</sup>. This state, commonly referred to as T cell “exhaustion”, was first characterized in mouse models of chronic infections<sup>19,20</sup>, but later also found in other contexts of chronic antigen stimulation, including human cancers<sup>21-23</sup>. These dysfunctional T cells are characterized by surface expression of multiple inhibitory receptors such as PD-1, LAG-3, CTLA-4, and TIM-3<sup>22,24</sup>. Moreover, they are impaired in their proliferative capacity, the production of key effector cytokines like IL-2 and interferon-gamma (IFN- $\gamma$ ) as well as cytotoxic molecules such as granzyme B<sup>21,25</sup>.

Dysfunctional T cells do not form a uniform population but rather exist along a continuum of states with increasing characteristics of dysfunction<sup>23,26,27</sup>. Overlap in transcriptional profiles and T cell receptor (TCR)-sharing analyses revealed that these cells are related and follow a progressive differentiation trajectory, where progenitor-like dysfunctional T cells give rise to early dysfunctional cells, which then transition into a terminally dysfunctional state<sup>26,28-30</sup>. This differentiation process, described as the dysfunctional gradient<sup>31</sup>, is at least partially governed by epigenetic regulation. A key driver of this process is the transcription factor TOX, which drives chromatin remodeling and locks T cells into an epigenetically fixed dysfunctional state<sup>32-34</sup>. This state restricts the plasticity of late-dysfunctional T cells, preventing conversion into functional effector cells and limiting their ability to be

reinvigorated by immunotherapies<sup>35</sup>. In contrast, precursor-dysfunctional T cells residing in the lymph nodes retain proliferative capacity and express intermediate levels of TOX and inhibitory receptors<sup>36,37</sup>. Moreover, these cells were shown to provide a proliferative burst upon ICB and mediate long-term responses in mouse models<sup>38,39</sup>. As a result, less attention has been given to the intratumoral late-dysfunctional T cell compartment, which is often viewed as terminally exhausted and beyond therapeutic rescue. Late-dysfunctional T cells at the tumor site should not be dismissed as merely non-functional. Rather, they represent an adapted immune state that serves as a protective physiological mechanism to prevent excessive immune-mediated tissue damage<sup>40,41</sup>. As these cells progress along the dysfunctional gradient, they undergo more than just loss of effector function. Instead, they also gain functional properties, including the capacity to express and secrete CXCL13<sup>23,42</sup>, which plays a crucial role as a chemoattractant recruiting B and T cells into the TME as well as for the formation and maintenance of tertiary lymphoid structures<sup>43,44</sup>. Importantly, late-dysfunctional T cells in tumor tissues may hold significant therapeutic potential for multiple reasons: (1) These T cells are enriched for carrying tumor-reactive TCRs<sup>45,46</sup>, (2) they express high levels of effector RNA molecules<sup>42</sup>, (3) late-dysfunctional T cells express highest levels of inhibitory receptors<sup>25,42</sup>, which are the targets of ICB, and (4) their presence in tumors prior to treatment has been associated with responsiveness to ICB<sup>47,48</sup>. These observations raise the question of these cells primarily serving as biomarkers of an ineffective immune response, or whether they can still retain some function upon immunotherapeutic stimulation.

## Research questions and thesis outline

In this thesis, I aim to investigate the potential for reinvigorating dysfunctional T cells at the tumor site. By leveraging ex vivo tumor explant models<sup>49</sup>, I explore their responsiveness to ICB, in particular blockade of the PD-1 receptor, and investigate whether alternative strategies can further enhance their functional capacity.

In **Chapter 2**, we review relevant recent work about hallmarks of tumor-reactive T cells and their relationship with immunotherapy response. As these T cells are rare in the TME<sup>51,52</sup>, it is crucial to distinguish them from bystander cells in single-cell datasets. **Chapter 3** delves deeper into the mechanism of anti-PD-1 therapy in human tumor tissue. Herein, we disentangle local anti-PD-1 induced immunological responses driven by tumor-residing CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and show that both subsets can induce such responses upon ex vivo anti-PD-1 therapy. Transcriptome analyses revealed that anti-PD-1 primarily targets late-dysfunctional T cells of both subsets. In line with their fixed epigenetic state, PD-1 blockade induced limited transcriptional rewiring. Instead, responses were mediated by an increase in translational capacity of dysfunctional T cells at the tumor site.

Next, this thesis addresses novel strategies to tackle resistance to checkpoint blockade. **Chapter 4** examines the potential of IL-2 to overcome resistance to ICB. IL-2 was shown

to induce T cell activation and functional downstream immunological responses in tumor explants that were unresponsive to ICB, highlighting its potential for the treatment of ICB-resistant tumors. However, recognizing the toxicity and reduced efficacy associated with recombinant IL-2, **Chapter 5** explores a novel cis-targeted CD8 variant to specifically activate intratumoral CD8<sup>+</sup> T cells. CD8-IL2 treatment broadly armed the dysfunctional CD8<sup>+</sup> T cell pool at the tumor site with enhanced effector capacity, inducing widespread transcriptomic changes in this subset, possibly rewiring their fixed epigenetic state by decreasing expression of *TOX*. **Chapter 6** analyzes clinical samples from diffuse pleural mesothelioma patients treated with the tyrosine kinase inhibitor lenvatinib combined with ICB to identify response mechanisms in these patients. Interestingly, response was associated with increased pre-treatment presence of dysfunctional CD8<sup>+</sup> T cells. This finding is particularly noteworthy in a tumor type with significantly fewer mutations than typical immunotherapy-responsive tumors like melanoma and non-small cell lung cancer, underscoring the importance of these cells in cancer patients. **Chapter 7** reviews the challenges of predicting immunotherapy responses in human cancer. It discusses current biomarkers and their limitations in capturing the immune system's complexity, highlighting the need for improvement. The chapter examines how multimodal predictors for immunotherapy could be developed and incorporated into clinical practice in the future, aiming to better capture the reasons the immune system fails in certain patients. Finally, in **Chapter 8**, I explore how the findings from these studies are interconnected, particularly in relation to the current understanding of how T cells respond to immunotherapies, and discuss open ends.

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