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

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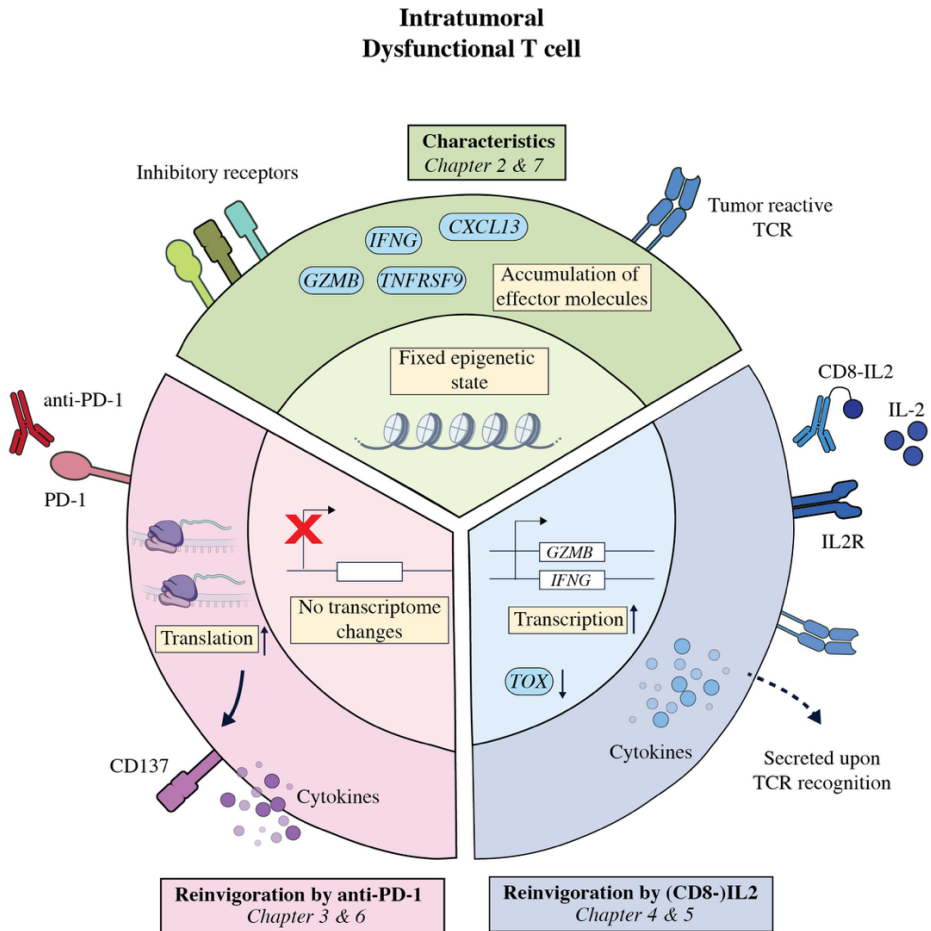
## **Chapter 8**

### Discussion and outlook

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T cells have the capacity to eradicate tumors and are therefore key targets for many immunotherapeutic strategies. However, despite the success of T cell-targeted therapies, many patients still do not benefit, and much remains to be understood about how T cells at the tumor site respond to these interventions. In the tumor microenvironment (TME), prolonged exposure to tumor-associated antigens drives T cells into a dysfunctional state<sup>1,2</sup>. As these cells progressively lose effector function, epigenetic reprogramming reinforces their dysfunctional identity<sup>3</sup>. Importantly, these epigenetic modifications limit their ability to fully respond to immunotherapies such as immune checkpoint blockade (ICB)<sup>4-6</sup>. As a result, this state is often referred to as “terminal exhaustion”<sup>7,8</sup>, suggesting that these cells have lost all functional potential. Yet, paradoxically, the presence of late-dysfunctional T cells in pre-treatment tumors has been shown to correlate with responses to ICB<sup>9-11</sup>, raising the question of whether these cells are entirely inert or can regain functional capacity in response to certain stimuli. Throughout this thesis, I have focused on how immunotherapeutic interventions influence T cells at the tumor site, revealing that these cells can regain function and may in fact represent key responders to immunotherapy.

This thesis begins by investigating the local immunological responses upon ICB, particularly examining anti-PD-1 therapy. The findings indicate that anti-PD-1 can reinvigorate intratumoral late-dysfunctional CD4<sup>+</sup> and CD8<sup>+</sup> T cells, likely by increasing their translational capacity. Despite a lack of transcriptional rewiring, protein-level activation of late-dysfunctional T cells resulted in increased secretion of cytokines and chemokines in response to anti-PD-1. Furthermore, decreased interleukin-2 (IL-2) signaling was identified as a potential factor contributing to non-responsiveness to neoadjuvant ICB in melanoma patients. This observation prompted an investigation into whether IL-2 could effectively overcome resistance to ICB. IL-2 was shown to induce T cell activation and immunological responses in tumors that are unresponsive to anti-PD-1+anti-CTLA4. However, the clinical application of IL-2 is complicated by toxicity due to its impact on natural killer cells and reduced efficacy resulting from the expansion of immunosuppressive regulatory T cells<sup>12-14</sup>. To address this, IL-2 was specifically targeted to CD8<sup>+</sup> T cells using a novel cis-targeting IL-2 molecule (CD8-IL2). The results showed that this approach effectively reinvigorated late-dysfunctional CD8<sup>+</sup> T cells at both the transcriptional and protein levels, leading to immunological responses where anti-PD-1 alone was ineffective. Notably, the combination of CD8-IL2 with anti-PD-1 further enhanced therapeutic responses, suggesting potential synergy between the two treatments. Lastly, clinical samples from patients with diffuse pleural mesothelioma were analyzed, revealing that dysfunctional CD8<sup>+</sup> T cells may also play a critical role in immunotherapy responses within this low-mutational burden cancer type. Collectively, these findings underscore the important –and previously underappreciated– role of dysfunctional T cells in anti-tumor responses to various immunotherapeutic strategies (summarized in **Figure 1**). In this chapter, I aim to discuss how these findings fit into the current understanding of anti-tumor T cell responses and highlight open ends for future investigation.



**Figure 1. Graphical summary of this thesis.** Chapters 2 and 7 review previously published research about the characteristics of intratumoral dysfunctional T cells and their relationship to immunotherapy response and tumor reactivity. Chapters 3 and 6 highlight the functional significance of these dysfunctional T cells for PD-1 blockade, emphasizing the role of translation in this process. Chapters 4 and 5 focus on the therapeutic potential of IL-2, demonstrating its ability to overcome resistance to PD-1 blockade by driving transcriptional rewiring of dysfunctional T cells and enabling a functional response when IL-2 signaling is combined with TCR triggering.

### ***Down but not out: dysfunction can turn into function***

T cell dysfunction represents a trajectory in which T cells, driven by continuous antigen recognition, progressively differentiate from progenitor-like dysfunctional T cells to a late-dysfunctional state characterized by reduced proliferative capacity, increased expression of inhibitory receptors, diminished effector function and decreased responsiveness to ICB<sup>1,15</sup>. This process likely evolved as a protective mechanism to prevent severe immunopathology caused by immune overstimulation<sup>7,16</sup>. Studies suggest that T cell dysfunction should be

“prevented”<sup>17-19</sup>, as these cells are presumed to not be reprogrammable into function effectors<sup>3,17,20</sup>. However, I propose reconsidering this perspective. T cell dysfunction allows these cells to persist in the tissue without succumbing to overstimulation<sup>6</sup>, and as demonstrated in this thesis, such persistence may be precisely what is required to initiate anti-tumor immune responses.

### Translational reinvigoration of late-dysfunctional T cells

**Chapter 3** demonstrates that late-dysfunctional T cells at the tumor site can regain partial functionality upon PD-1 blockade. Although it has been assumed that late-dysfunctional T cells merely reflect tumor immunogenicity and were not the direct responders to therapy, these findings reveal that late-dysfunctional T cells may actively contribute to ICB-induced anti-tumor responses. Consistent with their fixed epigenetic state<sup>17</sup>, this reinvigoration does not occur via transcriptional rewiring but appears to be mediated through the alleviation of a translational block, leading to the secretion of cytokines and chemokines in the TME. The protein-level mechanisms underlying this reinvigoration might explain why this phenomenon has been overlooked in single-cell RNA-sequencing studies.

The existence of a translational block raises the intriguing possibility that these cells use this “pause” mechanism to conserve energy—given that translation is highly resource-intensive—and to prolong survival. T cell metabolism is tightly regulated, with T cells needing to balance minimal energy expenditure in a resting state while rapidly meeting the demands of proliferation and effector molecule production upon activation<sup>21</sup>. Dysfunctional T cells are known to undergo metabolic reprogramming, likely as a response to the nutrient-poor and hypoxic conditions in tumors. Late-dysfunctional T cells show reduced mitochondrial mass, diminished oxygen consumption and impaired glucose intake<sup>22-24</sup>, which might lead to decreased ability to generate energy for protein translation. After anti-PD-1 therapy, it has been shown that T cells exhibit increased glucose uptake, enhanced glycolytic flux, and improved mitochondrial function<sup>25,26</sup>, which together provide the bioenergetic resources necessary for renewed protein synthesis.

Interestingly, translational repression is not unique to late-dysfunctional T cells and has also been observed in other T cell states in which effector function is restrained. For example, self-reactive anergic T cells<sup>27</sup> and tolerogenic T cells<sup>28</sup> also exhibit suppressed protein synthesis. Thus, this repression of translation seems to be a general mechanism to reduce effector function in T cells upon TCR-triggering. Additionally, Van der Byl *et al.*<sup>28</sup> demonstrated that inducing effector gene expression was not sufficient to overcome the tolerogenic T cell state, as protein translation remained suppressed. This reinforces the idea that T cell function is governed by two independent regulatory axes: one controlling transcriptional activation and another modulating effector function.

### Transcriptional reinvigoration of late dysfunctional T cells

In line with the previous notion, it was recently suggested that the loss of effector function and the acquisition of the exhaustion phenotype are driven by distinct regulatory modules<sup>29</sup>. The transcription factor TOX has been identified as a central coordinator of the exhaustion phenotype, enforcing the epigenetic remodeling that stabilizes dysfunction<sup>6,30,31</sup>. However, the regulation of effector function appears to be independent of TOX-mediated exhaustion pathways. For instance, functional effector and tissue-resident cells express TOX and retain effector capabilities. Furthermore, in murine models, TOX knockout T cells exhibit reduced expression of inhibitory receptors and fail to persist within the TME due to hyperactivation<sup>6</sup>. Thus, while TOX is critical for establishing and maintaining the exhausted transcriptional program, it does not directly control the loss of effector function.

**Chapter 5** demonstrates that late-dysfunctional T cells are also the primary intratumoral target of CD8-IL2 therapy, which—unlike anti-PD-1—induces transcriptional reprogramming in these cells. Both this thesis and a prior study suggest that IL-2 can reduce TOX expression via its downstream mediator STAT5<sup>32</sup>, potentially modifying the epigenetically fixed state of late-dysfunctional T cells. In murine models it was shown that sustained STAT5 activation extensively reshapes the epigenetic landscape of CD8<sup>+</sup> T cells, increasing accessibility of effector-related peaks and reducing this at dysfunctional-linked regions. This epigenetic reprogramming drives increased expression of cytokine, cytotoxicity, and activation genes and lowers expression of dysfunction genes. Additionally, a recent follow-up study showed that removal of TOX even allowed established late-dysfunctional T cells to differentiate into more functional effector-like T cells<sup>33</sup>. However, despite the IL-2 induced reprogramming, transcriptional changes alone do not immediately reinstate full effector function. In **Chapter 5**, IL-2-induced transcriptional rewiring occurred in the majority of T cells at the tumor site, yet only those recognizing antigen as well exhibited effector function. This further supports the theory that transcriptional and effector modules are distinct, with IL-2-mediated TOX downregulation enabling, but not singularly driving, effector molecule production.

These findings challenge the view of late-dysfunctional T cells as permanently fixed in an unresponsive state. Instead, they highlight a pathway through which IL-2 can transcriptionally rewire these cells, promoting functional recovery upon antigen recognition. Whether transcriptionally reprogrammed cells contribute to long-term immunity remains an open question. Nevertheless, results in this thesis demonstrate their ability to survive for extended periods under IL-2 treatment *in vitro*, and upon re-exposure to tumor cells these cells regained functional capacity, producing IFN- $\gamma$  and degranulating. In line with these observations, a recent adoptive T-cell transfer study revealed that a substantial fraction of clones in T cell products exclusively originated from late-dysfunctional T cells—particularly among treatment responders<sup>34</sup>. Following prolonged expansion with high-dose IL-2, these cells underwent significant epigenetic reprogramming, losing chromosome accessibility in the TOX-locus. Together, these observations hint at their potential to play a sustained role in anti-tumor responses upon IL-2.

### ***Anti-tumor T cell responses beyond the tissue***

This thesis highlights the potential of late-dysfunctional T cells as critical early responders to immunotherapeutic interventions such as anti-PD-1 and IL2. However, dysfunctional T cells are not isolated, but are connected to an upstream progenitor-dysfunctional population<sup>35-37</sup>. These progenitor-dysfunctional T cells have characteristics of both stem-like and dysfunctional T cells. On the one hand, they rely on transcription factor TCF1, retain substantial proliferative potential and predominantly reside within lymphoid tissues<sup>38</sup>. On the other hand, although lower than late-dysfunctional T cells, these cells express inhibitory receptor genes. Moreover, progenitor-dysfunctional T cells have the capacity to both self-renew and give rise to late-dysfunctional T cells, a differentiation step accompanied by reduced TCF1 expression, increased tissue residency, and elevated markers of dysfunction<sup>36,39</sup>.

Progenitor-dysfunctional T cells have emerged as essential responders to anti-PD-1 therapy. In murine models, PD-1 blockade was shown to effectively reinvigorate progenitor-dysfunctional T cells, which was required for driving durable responses<sup>40,41</sup>. In clinical settings, the proliferative expansion of these progenitor-like T cells correlates with responses to anti-PD-1 therapy<sup>42-45</sup>. Furthermore, these studies demonstrated that responses to PD-1 blockade involves the active recruitment and subsequent differentiation of these progenitor-like cells, replenishing the pool of terminally dysfunctional T cells within the TME. Consequently, the current paradigm is that anti-PD-1 therapy shifts the balance of intratumoral T cell populations, facilitating the differentiation rate from progenitor-like T cells toward more differentiated intermediate- and late-dysfunctional T cell states. Of note, this effect was also observed in clinical samples pre and post immunotherapy in **Chapter 6**.

Integrating these findings, a model emerges in which intratumoral late-dysfunctional T cells serve as initial responders to immunotherapeutic interventions. Upon activation by anti-PD-1 therapy, these cells release cytokines and chemokines, such as CXCL13, recruiting progenitor-dysfunctional T cells from peripheral lymphoid tissues, for example via interactions with their receptor CXCR5. Once recruited, progenitor-dysfunctional cells undergo rapid differentiation, replenishing the intratumoral T cell pool. This model explains the observed associations between both early and late-dysfunctional T cells and responses to anti-PD-1<sup>9,42</sup>, highlighting the complementary roles of these distinct T cell populations in anti-tumor immunity.

While this thesis emphasizes the effect of CD8-IL2 on the intratumoral T cell compartment, studies in mice highlight an additional critical role in reshaping progenitor-dysfunctional T cell differentiation. Specifically, CD8-IL2, as well as a cis-targeting PD1-IL2, were both shown to drive progenitor-like cells along an alternative differentiation trajectory distinct from the classical dysfunction pathway, resulting in the generation of a subset with enhanced effector functions called “better effectors”<sup>14,46</sup>. An interplay like that for anti-

PD-1 described above might also occur with CD8-IL2 therapy. Although late-dysfunctional T cells can regain effector function upon IL-2-mediated transcriptional rewiring, these cells lack substantial proliferative potential, necessitating continuous replenishment from progenitor-like subsets. Thus, progenitor-dysfunctional T cells, rewired into effector-competent subsets by IL-2, may critically support sustained anti-tumor responses.

### ***Intratumoral CD4<sup>+</sup> T cells respond to immunotherapy***

While CD8<sup>+</sup> T cells are often regarded as the primary effectors in immunotherapy due to their direct tumor-killing capabilities, CD4<sup>+</sup> T cells also play a crucial role in anti-tumor immunity<sup>47,48</sup>. Their infiltration has been shown to predict immunotherapy responses in various cancer types<sup>49-51</sup>, and they possess tumor-recognizing capabilities<sup>52</sup>. For instance, CD4<sup>+</sup> T cells have been shown to be important for the priming of naïve CD8<sup>+</sup> T cells<sup>48,53</sup>, recruitment of effector CD8<sup>+</sup> T cells<sup>54</sup> and the elimination of MHC-II expressing malignant cells<sup>55</sup>. In line with this, **Chapter 4** of this thesis described a low CD4/IL-2 gene signature associated with resistance to neoadjuvant ICB in melanoma patients, indicating that a lack of CD4<sup>+</sup> T cells and IL-2 could contribute to ICB resistance. As CD4<sup>+</sup> T cells are a major source of IL-2, addition of IL-2 could increase response rates in murine models and *ex vivo* tumor explants. However, the role of intratumoral CD4<sup>+</sup> T cells likely extends beyond merely supplying IL-2 to CD8<sup>+</sup> T cells.

**Chapter 3, 4 and 6** of this thesis show that intratumoral CD4<sup>+</sup> T cells are reinvigorated by different immunotherapeutic strategies. Additionally, **Chapter 3** highlights the capacity of CD4<sup>+</sup> T cells to independently initiate downstream immunological responses following PD-1 blockade, with evidence suggesting that, in certain tumors, CD4<sup>+</sup> T cells may even trigger a more robust cytokine and chemokine response than their CD8<sup>+</sup> counterparts. Although these findings do not contradict the established helper functions of CD4<sup>+</sup> T cells toward CD8<sup>+</sup> T cells, they emphasize the significant impact that CD4<sup>+</sup> T cells can independently have on the local cytokine and chemokine response. The CD4<sup>+</sup> T cells reinvigorated by anti-PD-1 predominantly belong to the dysfunctional and regulatory T cell pools, which aligns with Oliveira et al., who identified these subsets as harboring tumor-reactive TCRs<sup>52</sup>.

It has been shown that CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells and regulatory T cells acquire a shared transcriptional signature associated with dysfunction in the TME, indicating a parallel differentiation trajectory across these different intratumoral T cell subsets<sup>8,56</sup>. Functionally, intratumoral CD4<sup>+</sup> T cells, similar to their CD8<sup>+</sup> counterparts, have been shown to upregulate inhibitory receptor expression, exhibit reduced cytokine production, and possess limited proliferative capacity<sup>57-59</sup>. However, the dysfunctional trajectory of CD4<sup>+</sup> T cells is less well-established than that of CD8<sup>+</sup> T cells<sup>60</sup>. Interestingly, progenitor-dysfunctional CD8<sup>+</sup> T cells exhibit notable similarities to T follicular helper (Tfh) cells<sup>61</sup>, proposing that Tfh cells may represent a progenitor subset leading to dysfunctional CD4<sup>+</sup> T cells. Supporting this hypothesis, substantial TCR overlap is predominantly observed between Tfh and dysfunctional CD4<sup>+</sup> T cell subsets in **Chapter 3**.

### **Open ends**

While this thesis provides insights into the treatment-induced T cell responses in the TME, several important questions remain unanswered. Therefore, I outlined below the questions I would aim to answer if a PhD trajectory would extend over a decade.

First, although T cells are key initiators of anti-tumor immune responses, the magnitude and durability of these responses are substantially influenced by additional cellular players. Comparing cytokine and chemokine patterns induced by different treatments *ex vivo*, **Chapter 3, 4 and 5** showed that responsive tumors exhibited consistent cytokine and chemokine patterns regardless of treatment, whereas these patterns varied substantially across different tumors. Moreover, blockade of interferon-gamma receptor (IFN $\gamma$ R) signaling only partially reduced these cytokine responses, indicating that T cells, although critical initiators, rely on additional cells to fully establish and amplify immune responses, such as B cells producing additional mediators or dendritic cells (DCs) that enhance antigen presentation upon cytokine stimulation<sup>53,62</sup>. Additionally, the spatial localization of immune cells directly shapes their function. For instance, tertiary lymphoid structures (TLS) are spatially organized clusters of immune cells resembling secondary lymphoid organs strongly associated with favorable immunotherapy responses<sup>63-65</sup>. These structures were shown to maintain progenitor-dysfunctional T cell populations and enhance local antigen presentation by DCs and B cells. Moreover, it has been shown that the cytotoxic function of CD8<sup>+</sup> T cells is supported by the formation of “immune triads”, consisting of antigen-specific CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and antigen-presenting cells<sup>66,67</sup>. These cellular triads facilitate the differentiation of CD8<sup>+</sup> T cells into effector cells and their presence is associated with ICB response. Therefore, I would like to investigate how distinct cytokine and chemokine response patterns are mediated by specific downstream immune subsets, whether these subsets reside within defined immune niches in the TME, how treatment interventions influence the composition and structure of these niches, and whether such niche dynamics correlate with the strength and durability of clinical responses observed in patients.

Second, an unresolved question emerging from **Chapter 3** is the molecular mechanisms underlying the increased translational capacity observed in late-dysfunctional T cells following anti-PD-1 treatment. It is unclear whether PD-1 blockade induces a global enhancement of translation or selectively influences the translation of specific subsets of effector genes. Less complex systems that for instance generate late-dysfunctional T cells from peripheral blood mononuclear cells susceptible for reinvigoration by PD-1 blockade would enable the application of advanced techniques, such as ribosome profiling, whole proteome analysis, and transposase-accessible chromatin using sequencing (ATAC-seq)<sup>68</sup>—methods that have been challenging to implement in PDTFs due to limited cell numbers that can be retrieved. For example, ribosome profiling could further validate the translational response of PD-1 blockade in late-dysfunctional cells, whole proteome analysis could elucidate the array of mediators secreted by these cells, and ATAC-seq could shed light on how (and if) this influences the late-dysfunctional chromatin state. Additionally, the

signals within the TME mediating translational suppression of these cells have yet to be fully defined. Continuous antigen-driven TCR engagement likely contributes significantly to this translational block; however, it is plausible that other immunoregulatory mechanisms within the TME also play essential roles. For instance, regulatory T cells have been reported to inhibit translation in effector T cells via secretion of immunosuppressive cytokines such as IL-10 and TGF- $\beta$ <sup>69</sup>, suggesting that multiple, parallel pathways may maintain T-cell translational suppression.

Third, a critical yet understudied aspect arising from this thesis is the role of the CD4<sup>+</sup> T cell compartment in response to immunotherapeutic interventions. In **Chapters 3, 4, and 6**, CD4<sup>+</sup> T cells demonstrated substantial activation upon different immunotherapy treatments. Specifically, the late-dysfunctional CD4<sup>+</sup> T are the target of PD-1 blockade at the tumor site. However, the precise molecular mechanisms underlying their dysfunction remain incompletely understood, such as the epigenetic reprogramming and lineage relationships that give rise to this subset. Furthermore, while certain tumors in **Chapter 3** exhibited preferential activation of either the CD8<sup>+</sup> or CD4<sup>+</sup> T cell compartment upon immunotherapy, our observations predominantly indicated a coordinated response: tumors were typically either non-responsive—exhibiting a lack of activation in both T cell subsets—or responsive, with concurrent activation of both populations. This raises intriguing questions regarding antigen specificity and recognition: are CD4<sup>+</sup> and CD8<sup>+</sup> T cells within responding tumors targeting identical tumor antigens, or do they recognize distinct antigen repertoires? Moreover, both anti-PD-1 and IL-2 treatments mediated activation of intratumoral regulatory T cells, yet the functional consequences of these responses remain ambiguous. Regulatory T cells are known immunosuppressors, but it has also been indicated that these cells can adopt a fragile phenotype characterized by impaired immunosuppressive function, and potentially enhancing antitumor immunity<sup>70,71</sup>. Therefore, it would be of interest to selectively deplete these cells within the tumor fragments to clarify how their activation shapes the cytokine and chemokine secretion and influences the activation of other T cell subsets. However, achieving specific depletion remains challenging, as common markers such as CD25 are shared with activated effector T cells.

Lastly, the efficacy of the therapies explored in this thesis all depend on pre-existing anti-tumor immunity. However, throughout my PhD, I observed that many tumors our team collected lacked substantial immune cell infiltration, representing a major clinical challenge. Approaches such as TCR-based T cell therapy, which harness the potency of T cells independently of a patient's endogenous T-cell repertoire, could address this gap and potentially benefit patients lacking pre-existing immune responses.

## Concluding remarks

Dysfunctional T cells at the tumor site are far from inert. They represent a crucial subset of tumor-reactive T cells equipped with significant effector potential. However, in the face

of chronic antigen exposure in the tumor, they enter a paused state due to an adaptive physiological mechanism that prevents excessive immune-mediated tissue damage. This state, while often perceived as an endpoint, is in fact a dynamic equilibrium that can be therapeutically manipulated. With anti-PD-1 therapy, these cells can rapidly regain translational capacity, initiating local anti-tumor responses. Moreover, cytokine signaling, such as IL-2, has the potential to drive even deeper reprogramming by altering their transcriptional state. As our understanding of these cells evolves, so will our strategies to harness them.

As my PhD journey ends, it is heartening to see the field of immunotherapy rapidly advancing, with a growing consensus around the immense potential of the immune system to combat cancer. Unlike the more than a century that passed between Coley's early work and the emergence of immune checkpoint blockade, the progress made in recent years has been extraordinary. It is crucial that this momentum continues as we work to increase response rates and refine patient stratification. The unique value of immunotherapy lies in its ability to generate durable responses by empowering the immune system to recognize and fight cancer, transforming patients into their own best defense against a disease as complex and variable as cancer. I am optimistic that the strides made during this time are just the beginning of a future where immunotherapy fulfills its promise for countless patients.

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Chapter 8

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