# High-dimensional profiling of immunotherapy-responsive immune cells in cancer

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# SUMMARIZING DISCUSSION

# 7

## CANCER IMMUNOTHERAPY: WHAT IS THE RELEVANT TARGET?

The first clinical efforts to cure cancer were directed on stopping proliferation of tumor cells by blocking cell division of highly proliferating cells. The non-specificity of such approaches led to severe side effects. Alternative treatments options, such as immune checkpoint therapy, have been developed that aimed to target the immune system in order to unleash the cytotoxic power of CD4 and CD8 T cells, by blocking their inhibitory signals (immune checkpoint blockade, ICB) or by activating their stimulatory receptors.

The first ICB approved to treat metastatic melanoma was the anti-CTLA-4 inhibitor ipilimumab (1), followed by inhibitors of the PD-1/PD-L1 pathways. The clinical success of these ICBs led to their FDA approval for multiple types of cancer (2). New targets with the potential to strengthen the immune system have been discovered, leading to potential new types of cancer immunotherapy. This has conducted to a plethora of targets including CD27, CD40, CD47, CD73, 4-1BB (CD137), CSF1, GITR, ICOS, IDO, IL-2R, LAG3, OX40 (CD134), STAT3, STING, TLRs, TIGIT, TIM3. With the emerging development of new drugs targeting these molecules came the possibility of treatment tailoring to every patient. Therefore, there is an increasing need to anticipate the possible effect of immunotherapy at an early stage.

## DISSECTING COMPLEX IMMUNE RESPONSES: THE INESCAPABLE SINGLE-CELL TECHNOLOGIES

We investigated the complex patterns of immunotherapeutic responses with extensive mass cytometry panels (36-marker-murine, 46-marker-human) and analyzed the data with a self-made tailored bioinformatic pipeline *Cytofast* (**Chapter 3**). We developed *Cytofast* to compare different clustering methods, and validated this novel analysis platform with two previously published datasets. *Cytofast* offered a comprehensive view of proteomic data like flow and mass cytometry with detailed explanations to guide the user (**Chapters 3 and 4**). If traditional flow cytometry was largely used to analyze the tumor microenvironment (TME), such methods are generally limited to 15 markers and are sensitive to intrinsic fluorescence background generated by tumor and other cells. Alternative methods like mass cytometry avoided the fluorescence background to replace fluorophores by metal isotopes, which can be detected according to their mass. Such methods increased the number of detectable markers up to 51 (**Chapters 5 and 6**), resulting into a deep and unbiased phenotyping of the immune system. Whereas flow cytometry is restricted by the number of available fluorochromes spectra (around 30), mass cytometry is theoretically restricted to the number of isotopes available, slightly over a hundred. A recent technique named CITE-seq (Cellular Indexing of Transcriptomes and Epitopes by Sequencing), uses nucleotide tags to identify cellular subsets. The power of this method relies on the combination of cellular and genetical information by evaluating both gene expression and protein level simultaneously on cells (3). However, thereby the limit of detection level is increased, and thus the method might not be

able to capture rare cell subsets. Another drawback of single-cell mass/flow cytometry relies on the manual dissociation of the material before being processed thereby losing the organization of these cells in the tissues. The newly imaging systems coupled to mass cytometry keep spatial conformation of the tissue, by analyzing fixed paraffin embedded tissue slices, making this information clinically relevant as the spatial organization of T cells in the tumor micro-environment is linked to clinical outcome (4). Spatially resolved single-cell analysis can characterize phenotypic heterogeneity of TILs in a disease-relevant manner. It is also now possible to anchor sc-RNA-seq experiments with chromatin difference analysis together with protein expression and keep spatial gene expression patterns (5). Even if those methods provide deep characterization of the host, these studies investigated the immune system without considering the microbiota, assumed to be responsible to modulate the efficiency of immunotherapy (6).

An efficient investigating pipeline will embrace the diverse components interacting with the immune system and modulating the host response. Constant adaptations of visualization tools like *Cytofast* are required to filter and extract biological relevant information coming from single-cell technologies.

## THE PHENOTYPIC T CELL HETEROGENEITY IN THE TME REVEALS T CELL ACTIVATION

In the tumor microenvironment (TME), immunotherapy is shaping the immune system at an early stage. In a murine tumor setting, NK cells were shaped after three days of PD-L1 treatment (**Chapter 4**), which might contribute to immunotherapy efficacy. PD-L1 specifically upregulated a $CD54^{+}CD39^{+}NKG2A^{-}$ NK cell subset detected in all PD-L1 treated mice and absent in the untreated animals. The upregulation at the same time of activating markers like CD54 and inhibitory markers like CD39 triggered by PD-L1 therapy suggests that these cells might not be exhausted (7) but tumor reactive. The co-expression of activating and inhibitory molecules is not limited to NK cells.

Indeed, we further confirmed that PD-L1 treatment is upregulating a specific T cell subset co-expressing **a**ctivating and **i**nhibitory markers in the TME (**Chapter 5**). This subset, named $T_{AI}$ cells, co-expressed NKG2A, PD-1, LAG-3, CD43 and ICOS. This duality in their phenotype might be inherent to their cytotoxicity. Inhibitory molecules could be upregulated on those cytotoxic cells to limit their toxic effects to normal cells. A panel marker gathering activating and inhibitory molecules might be sufficient to delineate tumor-reactive T cells. Defining tumor-specific CTLs requires MHC class I tetramers, which need to be designed and might not integrate all tumor-reactive-lymphocytes (8). Surrogate markers replacing MHC class I tetramers to identify tumor-specific cells are of utility to understand the diversity of CTLs. For example, it has been recently reported that co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors (9). Deep phenotyping of tumor-infiltrating-lymphocytes (TILs) will allow personalized treatment by specific targeting.



## THE SYSTEMIC IMMUNE RESPONSE TO CANCER: A STEP TOWARDS BLOOD SCREENING FOR CANCER PATIENT IMMUNO-MONITORING

Our above described study was based on analysis of the TME (**Chapter 5**), requiring invasive surgery to access this compartment. An alternative to TME analysis would reside in the examination of immune cells in the blood. We discovered $T_{AI}$ subsets in the blood using scRNAseq and mass cytometry that correlated with efficient therapy in two different mouse cancer models (**Chapter 6**), suggesting the importance of an early blood-based test after administrating immunotherapy (**Chapter 2**). In both tumor models, efficient immunotherapy is accompanied by an upregulation of the same activating markers including the hyper-glycosylated form of CD43. These findings have been translated in human settings. In healthy donors, T cells appear to have a rested phenotype, limited to CD27 expression. In the responder group, and especially after therapy, patient CTLs in blood specifically show an upregulation of CD56 or CD161, demonstrating the systemic immunity response of immunotherapy. If CD161 is specific to responders only, not every single responding patient present a high level of CD161, making this molecule a reliable indicator of responsiveness of $CD8^{+}$ T cells in blood of cancer patients (**Figure 1**).

After profiling the immune response in blood with a 46-surface antibody panel we validate the results with a minimal set of markers identified by *Hypergate* (10). It will give

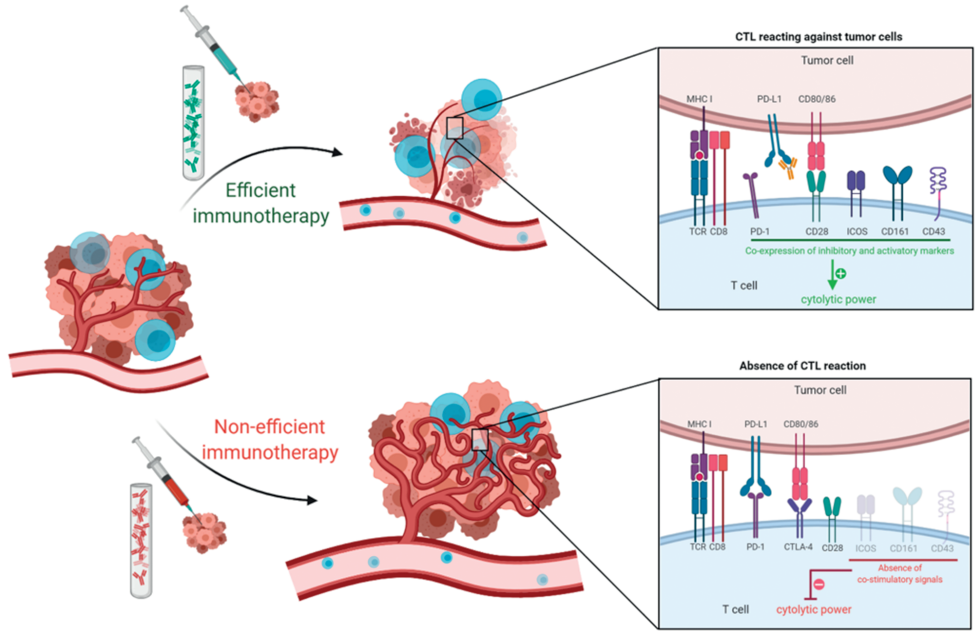


**Figure 1.** Summary of the differences found on T cells between different immunotherapy efficiency levels.

a faster way to discriminate healthy donor from responder and non-responder patients by flow cytometry, which is of faster use compared to mass cytometry and routinely used it in the clinic.

In the murine model we also showed the impact of our therapy in lymphoid organs like the bone marrow, spleen and lymph nodes. Our study suggests that T cells are systemically shaped by PD-L1 blockade in the bone marrow and the spleen, meaning that the T cell reservoir in the lymphoid organs plays a role in tumor immunity. A systemic impact of tumor cells and immunotherapy has also been observed by others (11, 12), suggesting the potential benefit of blood screening to follow tumor evolution. The underlined connections between the lymphoid organs demonstrated here reinforced the highlighted role of the draining lymph nodes and its potency to present tumor neoantigens through a cross-talk with the tumors already shown by others (13-15)

## COMBINATORIAL IMMUNOTHERAPY

The ultimate goal of single-cell analysis is to tailor cancer treatments on a patient-basis to reach a maximal effect. As illustrated in **Chapter 5**, identifying $T_{AI}$ cells by single-cell analysis could be a first step towards a tailored-treatment to reach tumor clearance. Indeed, we observe an concomitant increase in $T_{AI}$ cells in more effective combination ICB therapies in our mouse models. However, the timing component needs also to be taken in consideration. If the combination of successive OX40 and PD-L1 seems to be efficient in mice (**Chapter 6**) and in aggressive tumor models like pancreatic cancer (16), the simple concurrent addition of PD-1 to OX40 induces the opposite effect (17, 18) due to a significant induction of apoptosis on $CD8^+$ T cells. Such a pattern should be evaluated before engaging combinatorial treatment. Several clinical trials are ongoing with results being expected (NCT02221960, NCT02410512).

Combination therapy with CTLA-4 with PD-1 is clinically approved, and other combination approaches are under investigation regarding their efficacy in the clinic. As an example, LAG-3 and ICOS targeting, also studied in **Chapter 5**, is currently being tested in combination with PD-1 blockade (NCT02460224 and NCT03829501, respectively) after successful preclinical results being reported (19). A better use of such combinations might rely on the systematic deep phenotyping of patient material and avoid side effects, which can vary from colitis to diabetes (20, 21).

Combinatorial immunotherapy should not be limited in targeting extracellular markers expressed by T lymphocytes using antibodies but could also, for example, consist of enhancing antigen presenting cells by adjuvants such as CpG (**Chapter 6**, (22)) or create a pro-inflammatory environment in the tumor by injecting particular cytokines (23, 24) or oncolytic viruses (25).



## CONCLUDING REMARKS

The results presented here challenge the idea that inhibitory markers on T lymphocytes are delineating only exhausted T cells. The classical vision to restrict expression of markers like PD-1 or LAG-3 to an exhausted state can be questioned by high-dimensional phenotyping. These "exhausted" cells still present a cytotoxic power through the presence of activating markers, unleashed by blocking their inhibitory receptors (Figure 1).

Finally, transposing these findings in the clinic would suggest to investigate the presence of T cells co-expressing inhibitory and stimulatory molecules, being relevant immunotherapy targets. Current improvements in single-cell technologies like mass cytometry, single-RNA-sequencing or the combination of both together with better analyzing tools development will allow a more systematic phenotyping approach before treating patients with immunotherapeutic agents. The research presented in this thesis aimed for a closer connection between immunotherapy of cancer and bioinformatics (Figure 2) and integrate it as a whole, which may improve strategies for personalized therapy.

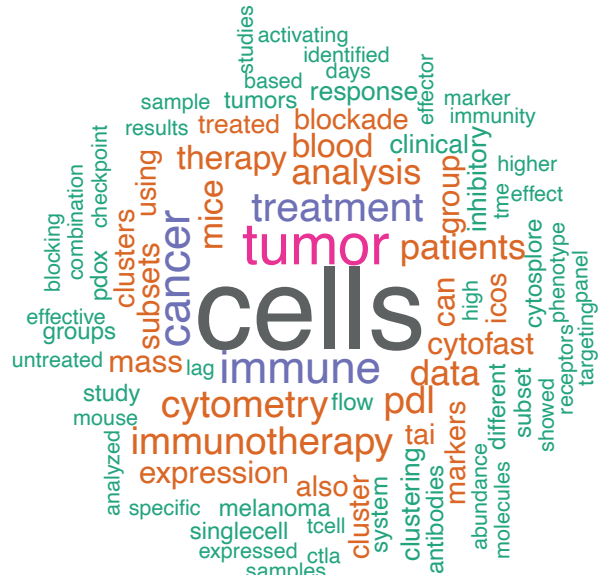


**Figure 2.** Word mapping of the presented thesis: an overview of the theme.

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