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

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INTRODUCTION

CONTEXT AND HISTORY

With an estimated 37.2 trillion cells (1), the human body is under constant massive cell division, which is prone to errors triggering therefore genetical mutations. Millions of potential cancerous cells are then accidentally created throughout the lifespan of an individual (2) leading to cancer in some cases. Among more than eight thousand different diseases classified by WHO in *International Classification of Diseases and Related Health Problems*, cancer jumped from the eighth to the second place between 1900 and 1940. Currently, cancer is still the second leading cause of mortality in the world (3). It is estimated that one person out of three will suffer from this disease during their lifetime, and about one in six deaths is attributed to cancer. The lethality of this disease is mainly due to the malignant behavior of the cancer cells, exemplified by uncontrolled proliferation and eventually spread throughout the body. Explanatory environmental or genetic factors are complex to ascertain: lung cancer is predominantly caused by smoking but five out of every six smokers will never get lung cancer (4). Therapeutic efforts have been concentrated to target tumor cells and stop their proliferation, thereby using generally non-specific treatments like chemotherapeutic agents or radiotherapy. However, as the primary intention implies, chemotherapy is associated with various side effects linked to the inhibition of any dividing cell, including hair loss, bowel issues or skin problems. To minimize side effects, an alternative way to cure cancer is to target the immune system with the recently developed immune checkpoint blockers by releasing the cytotoxic power of T cells.

The idea that the immune system might play a role against the proliferation of cancer cells is not new. In 1808, an attempt to immunize Louis XVIII against tumor cells with breast cancer tissue extract resulted in a local inflammation and lymph node enlargement (5). This first trial of a prophylactic vaccine was followed at the end of the 19th century by a therapeutic approach, consisting of the activation of the immune system against cancer. William B. Coley injected streptococcal organisms, known as the Coley's Mixed Bacterial Toxins, into a patient with inoperable cancer in 1891 (6). The infection surrounding the tumor triggered the immune system, which responded by attacking the tumor cells and resulting as one of the first immunotherapy examples. Two decades later, in 1909, one year after receiving the Nobel Prize for discovering treatment against syphilis, Paul Ehrlich proposed a first version of the "immune surveillance" hypothesis (7), stating that host defense may prevent neoplastic cells to develop into tumors. This concept of immune surveillance was decades later further developed, by Lewis Thomas, Gross and Macfarlane Burnett stating that the immune system can recognize and destroy transformed cells before they grow into tumors (8, 9).

One of the main actors of the tumor immunity are the T cells, which present the ability to kill tumor cells. Jacques Miller, born in Nice, France, discovered the T and B lymphocytes in 1961 (10). In 1983, the T cell antigen receptor (TCR) was discovered (11, 12), highlighting the specificity of T cells for a determined antigen. T cells recognize antigens

with their TCR, a complex of two protein chains. T cells are selected to not recognize self-antigens (by thymic negative selection), presented by major histocompatibility complex (MHC) molecules but to react strongly to non-self antigens. Both $CD4^+$ (helper T cells) and $CD8^+$ T cells (cytotoxic T cells, CTLs) can recognize tumor cells expressing mutated antigens, called neoantigens, which are not expressed by healthy tissues.

Steven Rosenberg and colleagues paved the path to use tumor infiltrating lymphocytes (TILs) as an adoptive cell transfer therapy to treat cancer in 1986 (13). Briefly, during this procedure, cancer patient's own lymphocytes, mainly CTLs, are expanded *in vitro* and reinfused into the patient. TILs are reinvigorated and can recognize tumor cells expressing neoantigens. However, specific inhibitory receptors can be expressed on the membrane of tumor-specific T cells. Two well-known inhibitory receptors are PD-1 (Program cell Death-1) and CTLA-4 (Cytotoxic T-Lymphocyte-Associated protein 4), which recognize their ligands, PD-L1 and CD80/86 respectively, expressed on either tumor cells or other immune cells. By blocking the interactions of PD-1 (14) or CTLA-4 (15) with their ligands using specific antibodies, cancer treatment was improved in experimental mouse models. Years later, this so-called immune checkpoint blockade (ICB) received FDA approval (CTLA-4 targeting in 2011, and PD-1 targeting in 2014) to be used as a cancer immunotherapeutic approach, and was shown to be especially efficacious against cancers with a high mutation load (16). This new treatment, which was originally based on the discovery of PD-1 and CTLA-4 function in mice, resulted in the award of the Nobel Prize in 2018, which underscores the importance of experimental *in vivo* mouse models to enhance the immunotherapy of cancer in the medical field.

HOW OUR IMMUNE CELLS PLAY A ROLE AGAINST CANCER

The application of new molecular and cellular technologies in the tumor immunology field is a powerful instrument to further understand the interactions between our immune system and cancer. Single-cell analysis that we and others have performed allowed us to have an overall view on the main lineages of the immune system in cancer: dendritic cells (DCs), macrophages, neutrophils, Natural Killer (NK) cells, T and B cells, and as well have an in-depth view regarding the phenotype and function of these cells. All of them are thought to play a unique role against cancer and all these immune cell subsets can be detected by mass cytometry.

Neutrophils, a lineage of the innate immune system, are the first recruited where danger signals are excreted into the tissue. The presence of neutrophils might be linked to pro- or anti-tumorigenic effect and often to increased metastatic potential of tumors (17). The chemokine products released by neutrophils could serve as enablers of tumor cell migration through the extracellular matrix, helping them to migrate to new metastatic sites (18).

After neutrophils have been recruited to the tissue, macrophages, from the innate lineage, reside in tissue after travelling as monocytes in the blood. Macrophages present such powerful phagocytic activity that they can clear approximately 200 billion erythrocytes each day (almost 3 kg of iron and hemoglobin per year) (19), making them strong candidates to engulf tumor cells and to present neoantigens. Pro-inflammatory macrophages, M1-type, play a relevant role in the elimination of malignant cells. The mechanisms by which macrophages can destroy tumor cells are similar to those that kill infectious agents, essentially through the production of nitric oxide. On the contrary, single-cell technologies frequently show the presence of M2-type macrophages in the tumor microenvironment (TME), promoting tumor growth (20) by different effects, such as stimulating angiogenesis via secretion of VEGF or inducing immunosuppression of anti-tumor effector immune cells. The liberation of IL-10 or TGF-β by macrophages is indeed impairing the activity of the T cells (21).

After the innate cell lineage components triggered an immune response, the adaptive immune system is undergoing drastic changes. T cells represent the principal way of cellular defense against cancer. Cytotoxic T lymphocytes (CTLs) perform surveillance by recognizing and killing tumor cells expressing antigens (such as neoantigens from mutated proteins) on their MHC-I receptors. The importance of CTLs in tumor immunity is such that a classification of tumors based on the number of tumor infiltrating lymphocytes (TILs) have been established and seemed to give reliable prognosis (22, 23). Clinically, TILs are relevant for several reasons. For example, TILs can be used for adoptive cell transfer therapy. In this treatment, lymphocytes are usually directly extracted from tumors, expanded ex vivo with different interleukins including IL-2 and infused back into the patient (24).

Major attention has also been given to the role of helper $CD4^+$ T cells. These cells are implicated in orchestrating the tumor response (25), and co-targeting of $CD4^+$ T cells might be required for efficient antitumor immunity. $CD4^+$ T cells can target tumor cells in multiple manners, either directly by eliminating tumor cells through cytolytic mechanisms or indirectly by modulating the TME and providing help to the $CD8^+$ T cells (26). Another type of $CD4^+$ T cells, the regulatory T cells (T_{regs}), are considered to have an opposite (negative) effect on tumor immunity. These cells, often characterized by the presence of FoxP3 and CD25 (27) can inhibit the cytotoxic power of CTLs thereby enhancing tumor growth.

Antigen presenting cells (APC) such as dendritic cells (DCs) are crucial to prime tumor-specific CTLs and helper $CD4^+$ T cells. During tumor cell death, the tumor cell debris containing tumor-antigens is ingested by DCs, which subsequently present the tumor antigens in both MHC-I and MHC-II molecules. The exogenous antigen uptake and presentation by MHC-I is called cross-presentation and considered as a decisive property of DCs to initiate tumor-specific $CD8^+$ T cell responses. DCs also need to express costimulatory signals to provide additional signals needed for differentiation of naïve T cells (28). However, a major hurdle to mount effective tumor-specific T cell responses is the lack of sufficient DC activation leading to insufficient display of neoantigens to naïve T cells (29), and the lack of upregulating co-stimulatory ligands such as CD80 or

CD86, the ligands of the primary T cell costimulatory receptor CD28, and members of the TNF superfamily ligating the costimulatory TNFR family members (e.g. CD27, OX40 and 4-1BB) expressed by $CD4^+$ and $CD8^+$ T cells.

NK cells are cells belonging to the innate lineage but like T cells, NK cells can also kill tumor cells. They can provide rapid cytolytic responses to infected or cancer cells. If tumor cells have down-modulated their MHC-I on the cell-surface, the NK cells are activated due to their receptors that are stimulated if MHC-I is deficient (30, 31). NK cells, as well as the recently discovered Innate Lymphoid Cells type 1 (ILC1), react especially to (MHC-I deficient) tumors (32), whereas ILC2 might enhance a pro-tumorigenic environment (33). Similarly, γδ T cells can play a role in cancer as their importance in cancer has recently been reported, like in colorectal cancer (34) where their activated phenotype was most exclusively found in mismatch-repaired-deficient cancer tissues. It was previously shown that human peripheral blood γδ T cells express PD-1 and exhibit natural killer-like activity, making these cells potential anti-PD-1-related-therapy targets (35).

Other immune cell subsets like B cells or eosinophils, basophils or myeloid derived suppressor cells are also implicated in the cancer immune response but their role is not further detailed in this thesis.

CANCER THERAPEUTIC STRATEGIES

Cells are often mutating and it is estimated that our body will carry respectively 8 million stem cells containing one mutation on a cancer-associated gene (2). Our immune system limits their development as described by the principles of immunoediting: Elimination, Equilibrium and Escape (36-38). Briefly, the elimination phase is the immunosurveillance where the immune system is killing efficiently tumor cells, followed by the equilibrium phase. During the latter, tumor cells are mutating, and tumor variants acquire resistance to elimination, entering the escape phase. During the escape phase, tumor cells grow and expand, leading to malignancies. The tumor microenvironment has then acquired a suppressive effect on the immune system. The immune system modulates this balance, acting either as a tumor growth promoter or inhibitor (39). Interventional therapies might be needed at this stage to reverse the pro-tumoral microenvironment. Oftentimes this cannot be achieved by only debulking of tumor mass by surgery and/or radio- or chemotherapy. These treatments are meant to target all dividing cells, not specific to tumor cells and are often accompanied by severe side effects. Therefore, more specific treatments, like immunotherapy, were developed to specifically modulate the tumor microenvironment with the advantage to limit the side effects. Immunotherapy treatments help the immune system to destroy cancer cells and stop cancer from spreading. There are several types of immunotherapy, including adoptive T-cell therapy, cancer vaccines, monoclonal antibodies and oncolytic virus therapy.

IMMUNOTHERAPY AND THE IMPORTANCE OF IMMUNE CHECKPOINT BLOCKADE

Activating and inhibitory immune checkpoint molecules have an important regulatory role in the immune system. They are expressed at the cell surface of immune cells and their role is to maintain self-tolerance or to regulate the magnitude of immune responses against microbes. Next to the natural role of these receptors and their ligands in regulating the normal T cells immune response, these molecules are often dysregulated in cancer. The expression is often higher or constitutive in the tumor microenvironment or in the tumor-draining lymphoid organs (40-42) thereby suppressing an anti-cancer immune response. As described above, the two best-defined inhibitory immune checkpoints are CTLA-4 and PD-1, and the targeting of these molecules have been FDA-approved for treatment of many cancers, for some tumor types even as first-line therapy. However, approximately 40% (43, 44) of melanoma patients responds partially or completely to therapy.

PD-1 is mostly expressed on activated cells of the lymphoid lineage, like T lymphocytes or NK cells ***(Figure 1)***. Its ligand, mainly PD-L1, is expressed on cancer cells but also on subsets of the myeloid lineage like macrophages or dendritic cells (45). When PD-1 on a T cell recognizes its ligand PD-L1, the T cell activation signal is inhibited, preventing cytolysis of the tumor (46). Blocking the PD-1/PD-L1 interaction with monoclonal antibodies could thus unleash the cytotoxic power of those T cells towards tumor cells. CTLA-4 is also mainly expressed on activated T cells. Initially, the ligands CD80/86 are expressed on APCs and bind to the T cell costimulatory receptor CD28. Upon activation of the T cell, CTLA-4 is upregulated, and then CD80/86 bind preferentially CTLA-4, thereby inhibiting further T cell activation. CTLA-4 is also a key molecule for T_{regs} to mediate suppression. Monoclonal antibodies blocking CTLA-4 (anti-CTLA4 antibodies) have been designed to specifically block the interaction between CTLA-4 and CD80/86, so that CD28 can still bind to CD80/86, leading to a stronger T cell activation (47). Both PD-1 (14) and CTLA-4 (15) have been historically first studied in animal models. To allow a better clinical success of immune checkpoint therapy, other targetable molecules have been tested such as lymphocyte activation gene-3 (LAG-3). This cell-surface molecule expressed on helper T cells and to a lower extent also on cytotoxic cells presents inhibitory functions, that are distinct from PD-1 and CTLA-4 (48). Since LAG-3 is over-expressed on tumor-infiltrating $CD8^+$ T cells in various tumor types, such as ovarian cancer, hepatocellular carcinoma, renal cell carcinomas and other solid tumors (49, 50),this molecule might be a potential target in cancer immunotherapy

Besides improving immune checkpoint therapy by targeting inhibitory molecules, currently many approaches are tested that are directed toward direct targeting stimulatory receptors. In this respect, the co-stimulatory TNF receptor (TNFR) superfamily members, and especially CD27, OX40 and 4-1BB, are promising new immunotherapeutic targets (51, 52). CD27 is constitutively expressed on the majority of CD4 and CD8 T cells, whereas OX40 and 4-1BB are upregulated on these cells upon activation. Whereas OX40

is most profoundly expressed by activated CD4+ T cells, 4-1BB is higher expressed on CD8+ T cells. The expression of the ligands, however, are all strictly regulated in their expression by APCs, and only transient expression has been observed after APC activation. All these costimulatory TNFR-like molecules play important roles in the proliferation, survival and differentiation of effector and memory T cells as exemplified by specific blockade or activation. Especially, the studies in which monoclonal antibodies to CD27, OX40 and 4-1BB with agonistic properties were used gained attention in the tumor immunotherapy field.

In most experimental tumor models, agonistic ligation of these TNFR-like molecules resulted T-cell activation but eradication of established tumors depended highly on the timing of administration and on combination with other therapies (53). Currently, the effectivity of targeting the costimulatory TNFR superfamily members is being evaluated in the clinic (54-56).

SINGLE-CELL TECHNOLOGIES: A HIGHLIGHT ON MASS CYTOMETRY AND SC-RNA-SEQUENCING

Major breakthroughs related to the function and heterogeneity of the immune system have been made possible through the apparition of novel single cell technologies.

On the proteomic level, flow cytometry has been existing for more than half a century (57), to sort cell populations based on their phenotypes (58). Briefly, cells are stained with

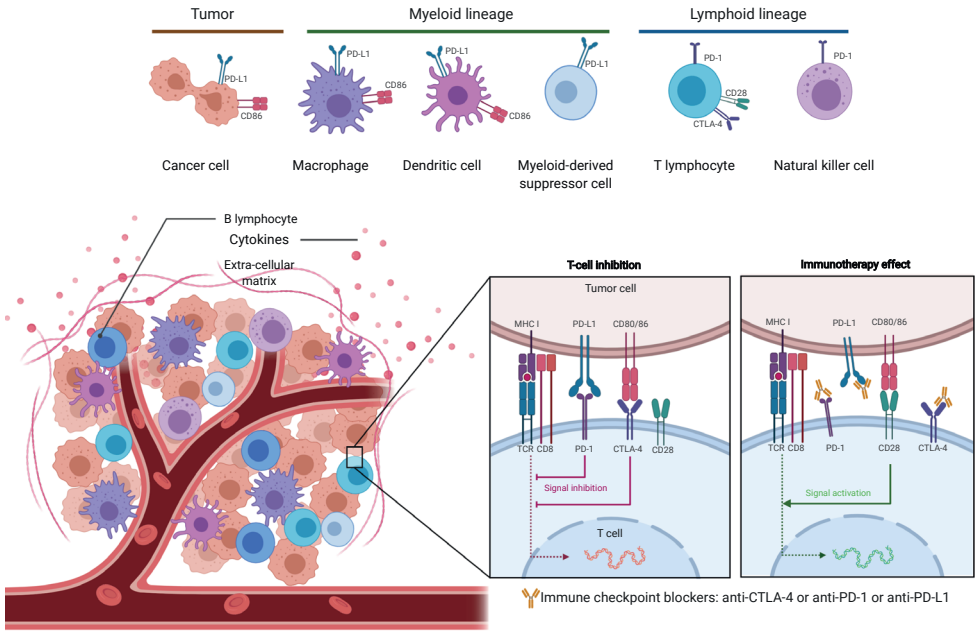

Figure 1. Presentation of immunotherapy effect in the tumor microenvironment.

fluorescent antibodies recognizing and binding specific extra or intra-cellular markers. Cells are then analyzed with a flow cytometer, passing through multiple lasers able to detect the fluorescent antibodies attached to each cell. Every single cell expressed different markers according to their phenotypes, meaning that the combination of the markers present at the cell surface defines a specific phenotype. The number of parameters or markers used can reach approximately 14 markers with conventional instruments, but the recent development of technologies based on full spectra increases this number up to 30, without decreasing the analysis flowrate. In contrast to most immune cells, tumor cells generally show a high auto-fluorescence background, which is difficult to discriminate with marker-related-fluorescence.

A new technology, named mass cytometry, is based on metal isotopes and not on fluorescence. This technique has revolutionized the way to analyze the immune system without compensation required or fluorescence background (59). The number of metals possibly extracted with a sufficient purity is currently set at 55, but the number of channels available to receive a specific signal is theoretically over a 100. The use of many more markers allows deeper surface phenotyping and also functional screening with intracellular signaling of the immune system (60). Although mass cytometry presents an unprecedented high resolution, there are still some challenges to overcome. The limit of detection of the instrument is not sufficient enough for the detection of subtle expression markers. The cell acquisition is more than 30 times slower (300 events/ sec in average) compared to flow cytometry analyzers (10 000 events/ sec). Cells can be sorted at the end of flow cytometry analysis for functional analysis, but this is impossible in mass cytometry, where cells are burnt.

On the genomic side, single-cell technology has improved the analysis of biological systems, via tumor genome sequencing (Navin et al., 2011; Vitak et al., 2017), tumor clonality dynamic (61), chromatin accessibility (62) and even spatial positioning (63). Whereas bulk RNA-sequencing measures the average expression analysis level for each gene in a large population of cells, single-cell-RNA sequencing (sc-RNA-seq) measures the expression analysis of each gene of every single cell of a sample, revealing heterogeneity of cell populations. Sc-RNA-seq research was first used in a four-cell-stage blastomere (64). At a larger scale, when used on e.g. the TME, it allows for example a deep genotyping of every single cell present in the TME, allowing to genetically characterize different subsets (65). The resolution of subset definition can be high enough to only focus on one lineage of the immune subset, previously sorted. For example, analysis of NK cells based on sc-RNA-seq have been performed (66) as well as on T cells (67).

ANALYSIS PIPELINE

Advanced flow and mass cytometers can generate highly complex data with up to 30 and 50 parameters, respectively. Conventional methods to analyze such data are implying biased, subjective and time-consuming gating strategies (68). They are usually based on

the interpretation of numerous two-dimensional plots by selecting different positive or negative populations with the help of markers historically defined by immunologists. This so-called gating strategy, if applicable in a known and limited panel, is unfeasible to analyze large data sets with many markers such as those we developed in our study; a 38-mouse-marker panel and a 46-human-marker panel. To analyze the data, we used automatic clustering techniques like Cytosplore (69), which are based on t-SNE (t-distributed Stochastic Neighbor Embedding) (70) and HSNE algorithm (Hierarchical Stochastic Neighbor Embedding) or FlowSOM (71). These clustering algorithms take all the markers into account and reduce the high-dimensional data into a two-dimensional plot. Such plots reflect the multidimensional relationship of the data and limit the intervention of the users by avoiding the definition of manual gates, known to be a significant source of variability (72). If these algorithms help the immunologists to define cell subsets in an unbiased manner and decrease variability, they do not automatically highlight treatment-related clusters and often do not offer a complete visualization of the data. An analyzing tool combining an efficient clustering method together with a visualization process was needed to automatically highlight immunological patterns between groups, leading to the creation of *Cytofast*, reported in this thesis.

OUTLINE

In this thesis, the latest single-cell technologies were used to advance insight in the complex immune responses to cancer raised by the variability of ICB immunotherapy.

The goal of this work is to improve current immunotherapy settings, by tailoring treatment and understanding the underlying factors preventing ICB to effectively lead to tumor regression. For this aim mass cytometry and software to properly analyze the complex data was used. A novel software method to analyze the data in an efficient manner by creating a tailored bioinformatic pipeline, named *Cytofast* was developed (**Chapter 2**). As a proof of concept, *Cytofast* analysis was run. Their results were confirmed and also new findings were discovered, reporting the usefulness of the new algorithm. The *Cytofast* method was further improved and explained in a visualized protocol (**Chapter 3**), where it was shown how PD-L1 treatment shaped the NK cell-tumor response at an early stage. Once the methodology was completed, the complexity of ICB immunotherapy was investigated (**Chapter 4**) and focused on the impact of PD-1/PD-L1 blockade. Extensive mass cytometry experiments were performed and processed by *Cytofast* to better understand the effect of PD-1 blockade on the immune response in the TME in order to improve this ICB. Next, the systemic immunity upon effective immune checkpoint therapy (PD-1 blockade combined with targeting direct T-cell costimulation) was studied in experimental models using the combination of genomic tools like sc-RNA-seq and proteomic technologies (**Chapter 5**). In **Chapter 6** the impact of immunotherapy on the blood immune cells as an important immunological compartment, as perceived in this thesis, was reviewed. Finally, the main findings of this thesis are discussed in **Chapter 7**.

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