The repercussions of recognition: imprints of T cells on the tumor microenvironment

Slagter, M.

CHAPTER 1

Scope of this thesis

This thesis is about immune cells of the adaptive immune system called T cells, how they interact with the microenvironment of tumors, which types of tumors are sufficiently visible to the T cell based immune system and how we can better boost T cell activity to treat patients with cancer. Put differently, the results in this thesis explore the effects of antigen-specific T cells on the tumor masses that they have homed to. Summarizing part of this work: a T cell's impact reaches beyond the directly adjacent antigen-expressing cell, but not detectably so in all manners we expected it to.

But first, what exactly are these immune cells called T cells? In the evolution of life, multicellular organisms have had to develop immune systems in order to fight of pathogens (such as bacteria and viruses) and to maintain homeostasis. The immune system of jawed vertebrates, the phylum that contains humans and mice as well 99% of all other modern vertebrates, consists of a plethora of different cell types, each with their own role to play in supporting immunity. The classical way to organize the ontology of immune cells is to group them into the *innate* and *adaptive* branches, but we now know of many cell types that exhibit characteristics of both branches and so the reality is not quite as binary. Sketching characteristics of the innate and adaptive banches in broad strokes one could say that the innate immune system offers a first line of defense, with rapid responses to a broad group of pathogens based on general molecular patterns. In contrast, the adaptive immune system

has the distinct potential for higher specificity (tailoring responses to individual pathogens), and without a requirement for recurring molecular patterns, albeit at a slower pace than the innate immune system. Importantly, the adaptive immune system has the capability to form immunological memory, such that it can respond with higher agility when a pathogen is encountered again. The adaptive system is also where we find the protagonist of this thesis: the T lymphocyte or T cell.

A distinguishing feature of T cells is that they can survey the entire proteome (including inner proteins) of an organism's own (i.e., host) cells, to try and discriminate between healthy cells and those that may have been compromised by a pathogen. To do this, T cells carry a protein complex called the T cell receptor (TCR) on their cell surface, which provides its molecular specificity. The TCR is capable of interacting with short peptides, derived from cellular proteins, that are presented on the cell surface by major histocompatibility complex (MHC) molecules. Together, they form the so called peptide MHC complexes (pMHCs). Essentially all host cells present pMHCs on their cell surface as a way of offering 'identification' to the T cell based immune system and can be instructed to further increase this activity by different kinds of molecular 'messengers' that put the immune system on alert. As highly specific sentinels, T cells migrate through the body and use their TCR to scan the pMHCs they encounter. Upon encountering a fitting 'match', the pMHC-bound TCR activates an intracellular signal transduction pathway within the T cell, consisting of a series of biochemical events mediated by associated co-receptors, adaptor molecules, and activated transcription factors, that will trigger various processes to neutralize the cell presenting the matching pMHC. Via this mechanism, T cells can track down virus (e.g. influenza, corona) infected cells. More specifically, infection of a host cell by a virus typically leads to expression of viral proteins, which can result in MHC-presentation of 'viral' peptides and T cell recognition of these infected cells.

However, the killing capacity of T cells reaches beyond virally infected cells. It has become abundantly clear that T cells can also detect and kill cancer cells, with large therapeutic potential for patients with cancer. Early evidence in favor of a potential role of T cells in the control of cancer came from the observation that, for a large variety of cancers, the intratumoral infiltration of CD8-$^{+}$ T cells correlates with positive prognosis[1,2]. Direct evidence for the anti-tumor potential of T cells came from data demonstrating that the administration of antibodies that target T cell inhibitory receptors, such as CTLA-4 and PD-1 (also known as T cell checkpoint blockade), shows clinical benefit in many different forms of cancer[3,4]. By the same token, the infusion of ex vivo–expanded autologous tumor-infiltrating lym-

phocytes (TILs) can induce clinically meaningful responses in melanoma patients[5], even when refractory to anti-PD-1 treatment[6]. Finally, early data on personalized (mRNA) vaccination, aiming to boost anti-tumor T cell reactivity and expand naturally occurring T cell clonotypes, points towards a potential clinical benefit for patients with melanoma[7–9] and pancreatic ductal adenocarcinoma[10].

As most human cancers are not associated with pathogen infection, how does the T cell-based immune system see cancer cells as foreign? Malignant transformation of cells depends on accumulation of DNA damage, which is a double-edged sword to an individual cancer cell. On the one hand, DNA damage may confer a fitness advantage over neighboring wild type (unmutated) cells, by enabling the acquisition of cancer hallmarks (e.g., increased proliferative capacity, resistance to cytostatic environmental cues). On the other hand, mutationally altered proteins can lead to the presentation of non-self *neoantigens*, which allow the T cell based immune system to identify the cancer cell as 'foreign'[11,12]. T cells can respond to the neoantigens that arise as a consequence of such genomic alterations[13,14] and this is likely to, at least in part explain, the clinical activity of both T cell checkpoint blockade[4] and adoptive TIL therapy[14].

Part I - The clinical utility of T cell therapies in cancer

The first part of this thesis is of a translational nature, with a relatively direct applicability to clinical care for patients with cancer.

My long PhD period spanned a pivotal time in the field of T cell targeting immunotherapies for cancer. Back in 2015 (the start of my PhD), highly encouraging data for the clinical utility of T cell checkpoint blockade started to emerge in melanoma and non-small cell lung cancer[15], tumor types that tend to carry a high mutational burden, and thereby neoantigen burden. If mutation derived neoantigens are indeed critical for T cell recognition of cancer, then one can wonder which other cancer types are sufficiently rich in this antigen class. There was a strong desire to get an early idea of the clinical utility of immunotherapies such as T cell checkpoint blockade beyond the aforementioned highly mutated tumor types. Leveraging large cancer sequencing projects like the Cancer Genome Atlas (TCGA) and the International Consortium for Cancer Genomes (ICGC), that have provided a molecular characterization of thousands of cancer samples, we addressed this need in **Chapter 2**. To learn what numbers of antigens may be sufficient for clinically relevant T cell activity, we first assessed the antigen load of 'benchmark' viruses, of which the controllability by

the T cell-based immune system is well documented. We then applied a custom neoantigen prediction pipeline, routinely used for the prioritization of neoantigens for experimental screening, to assess the different forms of DNA damage for their neoantigen generating potential and the cumulative predicted neoantigen load for each of the samples. Encouragingly, we found ~50% of the assessed cancer samples to be richer in predicted (neo)antigens than one of the benchmark viruses, suggesting potentially widespread applicability of T cell engaging therapies for cancer.

In **Chapter 2**, one subgroup of breast cancer patients, those of the basal subtype, appeared to have especially high predicted neoantigen loads. This basal subtype in the PAM50 subtyping system is largely similar to the triple negative breast cancer (TNBC) subtype of the molecular breast cancer subtyping system. TNBC typically affects younger women and is highly aggressive[16]. Until recently, the median survival for patients with metastatic disease was a meager 12-18 months[17,18]. Unlike other forms of breast cancer, TNBC is not driven by the hormones progesterone and estrogen, nor by amplification of the oncogene *HER2*. Instead, TNBC is typically defective in the apoptosis regulator P53, and less frequently in the DNA damage repair proteins BRCA1/2, contributing to TNBC's high mutational burden relative to other breast cancer subtypes. Based on data obtained in e.g., melanoma and non-small cell lung cancer, the high mutational burden and concomitant neoantigen load could offer a window of opportunity for T cell recognition. In **Chapter 3**, we describe the TONIC-study that evaluated the clinical utility of T cell checkpoint blockade, a treatment form that hinges on the availability of tumor specific antigens that can be recognized by T cells. In this phase II signal finding study, all 67 patients received nivolumab, a therapeutic antibody that antagonizes a regulatory protein on the cell surface of T cells (programmed death receptor 1, PD1). To try and elevate the intratumoral levels and/or activity of T cells prior to nivolumab administration, different induction treatments were evaluated: radiation therapy (3 times 8 Gray), oral cyclophosphamide, cisplatin, low-dose doxorubicin or a waiting period of two weeks. **Chapter 3** details an extensive molecular characterization of longitudinally acquired biopsies, aimed at comparing the induction treatments and gaining an understanding of their mechanism of action, as well as on identifying molecular markers of clinical benefit.

Part II - The effects of T cells on the tumor microenvironment

The second part of my thesis is of a more fundamental nature, primarily aimed at expanding our understanding of T cell biology in the context of cancer.

In **Chapter 5**, we investigate the way T cells communicate with other cells in the tumor microenvironment. Specifically, we report on the spreading behaviour of the T cell emitted cytokines IFN-γ and TNF-α. For this, we leverage the cytokine responsive behaviour of numerous endogenous genes and single cell RNA-sequencing of antigen-negative cells, which cannot directly be engaged by T cells. We found IFN-γ to spread beyond the antigen-presenting cell, consistent with earlier microscopy-based work[19,20], whereas TNF-α's activity is - surprisingly - more confined. We also acquired evidence for the notion that TGF-β-sensing is lowered in IFN-γ-experienced cells, potentially due to IFN-γ-instructed lowering of TGF-βsecretion in neighboring immune cells.

In **Chapter 4**, we revisit the neoantigen predictions of **Chapter 2** to study the ramifications of the selective pressure exerted by the T cell based immune system on developing cancers. It has long been hypothesized that, through their killing activity, T cells may exert evolutionary selective pressure on cancers[21], even before clinical manifestation and intervention. Such 'immune surveillance' is supported by murine models of cancer. Immunodeficiencies in mice increase tumor incidence and susceptibility to transplanted or chemical carcinogen-induced tumors[22]. For humans, (direct) evidence of immunoediting is more elusive. Some hints of its existence can be gleaned from the fact that immunosuppression, either due to AIDS[23] or purposefully induced to facilitate organ transplantation[24], is associated with a higher incidence of especially virus-induced cancers. However, these associations can also be attributed to decreased immunity against these (oncogenic) viruses, rather than a decreased ability to clean up nascent tumors.

Nevertheless, an important component of cancer development may be the evasion of immunity[25] and a multitude of mechanisms to this effect have been identified. For instance, tumors may lose components of the IFN-γ-signalling pathway[26], thereby conferring 'selective deafness' to IFN-γ, a central cytokine emitted by activated T cells. Tumors can also undergo genetic loss of the MHC locus[27], prohibiting T cell recognition via the T cell receptor. A third way to evade T cell immunity is to genetically lose DNA mutations associated with neoantigens[28] or transcriptionally silence expression of neoantigen coding genes[29,30], but the (bioinformatic) detectability of this type of immune editing in treatment-naïve cancer is still controversial[31]. In **Chapter 4** we leveraged the great statistical power that the

large availability of patient samples should offer, to try and robustly detect neoantigen depletion. We could not find a convincing signal of depletion, despite having corrected for a large series of potentially confounding processes. This result is likely to be in large part due to the high false positive rate that currently still plagues neoantigen prediction[32], a key tool in these analyses. As such, the statistical power of these analyses is limited and they should not be interpreted as fully ruling out some degree of sculpting of the neoantigen repertoire.

Finally, in **Chapter 6**, I first discuss how to build upon the methodology we developed for transcriptome-based cytokine exposure inference in **Chapter 5**. Due to the many cytokine-responsive genes and unique patterns with which different cytokines may up- or downregulate gene expression, the potential for this approach reaches far beyond 'just' the handful of cytokines we studied in **Chapter 5**, while its applicability also continues to rise with the developing revolution of *spatial* single cell sequencing. To enable the inference of exposure to stimuli (e.g., cytokines) from RNA-seq in a highly multiplexed fashion, algorithmic innovation will be of critical importance. In this chapter, I describe potential issues in applying transcriptome-based cytokine inference and how these can be surmounted with improved computational frameworks.

References

1. Galon, J. *et al.* Type, Density, and Location of Immune Cells within Human Colorectal Tumors Predict Clinical Outcome. *Science (New York, N.Y.)* **313,** 1960–1964. ISSN: 1095-9203 (Sept. 2006).
2. Fridman, W. H., Pagès, F., Sautès-Fridman, C. & Galon, J. The Immune Contexture in Human Tumours: Impact on Clinical Outcome. *Nature Reviews Cancer* **12,** 298–306. ISSN: 1474-175X, 1474-1768. (2024) (Apr. 2012).
3. Ribas, A. & Wolchok, J. D. Cancer Immunotherapy Using Checkpoint Blockade. *Science (New York, N.Y.)* **359,** 1350–1355. ISSN: 1095-9203 (Mar. 2018).
4. Morad, G., Helmink, B. A., Sharma, P. & Wargo, J. A. Hallmarks of Response, Resistance, and Toxicity to Immune Checkpoint Blockade. *Cell* **184,** 5309–5337. ISSN: 1097-4172 (Oct. 2021).
5. Hinrichs, C. S. & Rosenberg, S. A. Exploiting the Curative Potential of Adoptive T-cell Therapy for Cancer. *Immunological Reviews* **257,** 56–71. ISSN: 1600-065X. (2024) (2014).
6. Rohaan M. W. *et al.* Tumor-Infiltrating Lymphocyte Therapy or Ipilimumab in Advanced Melanoma. *New England Journal of Medicine* **387,** 2113–2125. (2024) (Dec. 2022).

7. Ott, P. A. *et al.* An Immunogenic Personal Neoantigen Vaccine for Patients with Melanoma. *Nature* **547,** 217–221. ISSN: 0028-0836. (2017) (July 2017).

8. Sahin, U. *et al.* Personalized RNA Mutanome Vaccines Mobilize Poly-Specific Therapeutic Immunity against Cancer. *Nature* **547,** 222–226. ISSN: 1476-4687 (Electronic) 0028-0836 (Linking). PMID: 28678784 (2017).

9. Weber, J. S. *et al.* Individualised Neoantigen Therapy mRNA-4157 (V940) plus Pembrolizumab versus Pembrolizumab Monotherapy in Resected Melanoma (KEYNOTE-942): A Randomised, Phase 2b Study. *Lancet (London, England)* **403,** 632–644. ISSN: 1474-547X (Feb. 2024).

10. Rojas, L. A. *et al.* Personalized RNA Neoantigen Vaccines Stimulate T Cells in Pancreatic Cancer. *Nature* **618,** 144–150. ISSN: 1476-4687. (2023) (June 2023).

11. Schumacher, T. N. & Schreiber, R. D. Neoantigens in Cancer Immunotherapy. *Science (New York, N.Y.)* **348,** 69–74. ISSN: 1095-9203 (Apr. 2015).

12. Schumacher, T. N., Scheper, W. & Kvistborg, P. Cancer Neoantigens. *Annual Review of Immunology* **37,** 173–200. ISSN: 0732-0582, 1545-3278. (2024) (Apr. 2019).

13. van Rooij, N. *et al.* Tumor Exome Analysis Reveals Neoantigen-Specific T-cell Reactivity in an Ipilimumab-Responsive Melanoma. *J Clin Oncol* **31,** e439–42. ISSN: 1527-7755 (Electronic) 0732-183X (Linking). PMID: 24043743 (2013).

14. Robbins, P. F. *et al.* Mining Exomic Sequencing Data to Identify Mutated Antigens Recognized by Adoptively Transferred Tumor-Reactive T Cells. *Nature Medicine* **19,** 747–752. ISSN: 1546-170X (June 2013).

15. Postow, M. A., Callahan, M. K. & Wolchok, J. D. Immune Checkpoint Blockade in Cancer Therapy. *Journal of Clinical Oncology* **33,** 1974–1982. ISSN: 0732-183X. (2024) (June 2015).

16. Bianchini, G., Balko, J. M., Mayer, I. A., Sanders, M. E. & Gianni, L. Triple-Negative Breast Cancer: Challenges and Opportunities of a Heterogeneous Disease. *Nature Reviews. Clinical Oncology* **13,** 674–690. ISSN: 1759-4782 (Nov. 2016).

17. Kassam, F. *et al.* Survival Outcomes for Patients with Metastatic Triple-Negative Breast Cancer: Implications for Clinical Practice and Trial Design. *Clinical Breast Cancer* **9,** 29–33. ISSN: 1526-8209 (Feb. 2009).

18. Skinner, K. E., Haiderali, A., Huang, M. & Schwartzberg, L. S. Real-World Effectiveness Outcomes in Patients Diagnosed with Metastatic Triple-Negative Breast Cancer. *Future Oncology (London, England)* **17,** 931–941. ISSN: 1744-8301 (Mar. 2021).

19. Thibaut, R. *et al.* Bystander IFN-γ Activity Promotes Widespread and Sustained Cytokine Signaling Altering the Tumor Microenvironment. *Nature Cancer* **1,** 302–314. ISSN: 2662-1347. (2024) (Mar. 2020).

20. Hoekstra, M. E. *et al.* Long-Distance Modulation of Bystander Tumor Cells by CD8+ T-cell-secreted IFN-γ. *Nature Cancer* **1,** 291–301. ISSN: 2662-1347 (Mar. 2020).

21. Ribatti, D. The Concept of Immune Surveillance against Tumors: The First Theories. *Oncotarget* **8,** 7175–7180. ISSN: 1949-2553. (2024) (Oct. 2016).

22. Schreiber, T. H. & Podack, E. R. A Critical Analysis of the Tumour Immunosurveillance Controversy for 3-MCA-induced Sarcomas. *British Journal of Cancer* **101,** 381–386. ISSN: 0007-0920, 1532-1827. (2024) (Aug. 2009).

23. Yarchoan, R. & Uldrick, T. S. HIV-Associated Cancers and Related Diseases. *The New England journal of medicine* **378,** 1029–1041. ISSN: 0028-4793. (2024) (Mar. 2018).

24. Engels, E. A. *et al.* Spectrum of Cancer Risk among US Solid Organ Transplant Recipients. *JAMA* **306,** 1891–1901. ISSN: 1538-3598 (Nov. 2011).

25. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* **144,** 646–674. ISSN: 0092-8674. (2024) (Mar. 2011).

26. Zaretsky Jesse M. *et al.* Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. *New England Journal of Medicine* **375,** 819–829. (2024) (2016).

27. Martínez-Jiménez, F. *et al.* Genetic Immune Escape Landscape in Primary and Metastatic Cancer. *Nature Genetics* **55,** 820–831. ISSN: 1546-1718. (2024) (May 2023).

28. Rooney, M. S., Shukla, S. A., Wu, C. J., Getz, G. & Hacohen, N. Molecular and Genetic Properties of Tumors Associated with Local Immune Cytolytic Activity. *Cell* **160,** 48–61. ISSN: 10974172 (2015).

29. Verdegaal, E. M. E. *et al.* Neoantigen Landscape Dynamics during Human Melanoma–T Cell Interactions. *Nature* **536,** 91–95. ISSN: 1476-4687. (2024) (Aug. 2016).

30. Rosenthal, R. *et al.* Neoantigen-Directed Immune Escape in Lung Cancer Evolution. *Nature* **567,** 479–485. ISSN: 1476-4687. (2024) (Mar. 2019).

31. Van den Eynden, J., Jiménez-Sánchez, A., Miller, M. L. & Larsson, E. Lack of Detectable Neoantigen Depletion Signals in the Untreated Cancer Genome. *Nature Genetics* **51,** 1741–1748. ISSN: 1061-4036, 1546-1718. (2023) (Dec. 2019).

32. Wells, D. K. *et al.* Key Parameters of Tumor Epitope Immunogenicity Revealed Through a Consortium Approach Improve Neoantigen Prediction. *Cell* **183,** 818–834.e13. ISSN: 00928674. (2023) (Oct. 2020).