Immunosuppression in breast cancer: a closer look at regulatory T cells

Kos, K.

1

General introduction & Scope of the thesis

The mechanisms of mutation and selection that have driven the breath-taking diversity of multi-cellular life present an inherent danger to an individual: the development of cancer. This risk is particularly inflated in our species, as we are able to live far beyond our reproductive age[1]. As we age past our reproductive years, evolutionary investments in tumor suppression wane, and when combined with the accumulation of oncogenic mutations throughout our lives, the stage is set for malignant cell growth to occur. These fast growing, resilient, expansionistic cells, when left uncontrolled, give rise to a cellular mass that ultimately spreads throughout the body, asphyxiates healthy tissue and perturbs vital physiological systems. The magnitude of this problem is best illustrated in numbers, with nearly 10 million reported cancer-related deaths annually worldwide[2].

These cellular masses, or tumors, are highly heterogeneous. Besides cancer cells, tumors consist of a wide variety of non-malignant host cells that are densely packed in a framework of extracellular matrix, collectively known as the tumor microenvironment (TME). Within the TME, cells continuously interact with each other through direct- or indirect cellular crosstalk governed by receptor-ligand interactions, and paracrine molecules like cytokines, chemokines and extracellular vesicles. A rich variety of cell types is found in the TME including stromal-, endothelial- and immune cells, which can be further classified in numerous subsets. While cancer cells have the malignant, viral-like nature of replicating *ad infinitum* at expense of their host, the function of non-cancerous cells in the TME is ambiguous and plastic. The behaviour of an intratumoral non-cancerous cell is simultaneously dictated by many factors like cancer stage[3], anatomical location of the tumor[3], composition and molecular cues of the environment[4], genetic make-up of the malignant cells[5] and treatment status[6]. With this level of complexity it becomes clear that each tumor is unique, and that the cell types therein are not fixed into a certain state, but are continuously adapting to their chaotic environment. This has important implications for tumor development as a whole, as many cells in the TME can have both tumor-limiting, and tumor-promoting effects, depending on the context[7].

The dogma that cancer is not solely a result of alterations in oncogenes and tumor suppressor genes has only begun to shift in the past decades, due to studies that showed the importance of components from the TME for tumor initiation, progression, metastasis and therapy response[8]. Particularly the observation that tumors are infiltrated by virtually any immune cell subset[9–11] has slowly sparked an interest to understand the role of immune cells in cancer progression. Today, we are aware of the paradoxical relationship that our immune system has with cancer. On one hand, chronic inflammation and the associated endless cycle of cell death and repair is a major driver of carcinogenesis[12,13]. On the other hand, our immune system can be employed as a means to combat cancer. This idea is stooled on the basis that the immune system has evolved to deal with external threats that compromise tissue function and homeostasis. To tell friend from foe, several detection mechanisms are in place that can recognise threats of foreign nature. This can, amongst

others, be mediated by recognition of pathogen-derived immunogenic antigens, pathogen- and damage-associated molecules, or loss of antigen presentation machinery on host cells. Detection of these "red flags" enables the elimination of threats of pathogenic nature like viruses, bacteria and parasites, but similarly licenses the eradication of cancer cells that have acquired these abnormal features, by cytotoxic cells like $CD8^+$ T cells and NK cells, that have been properly educated and activated to do so.

Mounting a tumor-specific immune responses is not trivial, and requires an intricate interaction between both innate and adaptive arms of the immune system. In general, the process starts with the uptake of tumor-associated antigens by dendritic cells in the TME, which upon maturation, carry their load to nearby lymph nodes. There, dendritic cells process and present the cognate antigen to naïve $CD4^+$ T cells which, upon successful priming, activation and differentiation, further orchestrate the induction of tumor-specific effector programs. Under ideal circumstances, this drives the influx of activated anti-tumoral immune cells into tumors, which either directly engage in tumor cell killing through release of cytotoxic molecules and engagement of death receptors, or create supportive pro-inflammatory conditions that facilitate anti-tumoral polarization of myeloid cells. Excitingly, the development of immunotherapeutics aimed at removing important brakes that limit anti-tumor immunity has led to never-before-seen response rates in cancer types that are traditionally difficult to treat like melanoma, lung, and hereditary colorectal cancers[14–16], thereby demonstrating the potential of engaging the immune system for cancer treatment.

In reality, an effective anti-tumor immune response can be impaired in any of its stages[17]. One obvious bottleneck relates to the fact that cancers are in essence not consisting of foreign cells, resulting in limited recognition of tumor antigens as "non-self", and thus poor priming and recruitment of T cells[18]. In fact, this is amplified by the removal of highly immunogenic cancer clones during early tumorigenesis in a process called immunoediting, which drives the propagation of sub-clones that are "invisible" to immune recognition[19]. Tumors that have high mutational burden due to external mutagenic factors, such as melanomas and non-small cell lung cancers[20], can have high levels of tumor-specific neo-antigens, which correlate to immunotherapy response[21,22]. However, having high mutational burden is not unequivocally related to T cell infiltration[23], or immunotherapy response[24], suggesting additional mechanisms exist that affect the capacity of the immune system to eradicate malignancies. One such crucial mechanism is tumor-associated immunosuppression. Immunosuppression is an essential component of an organism in homeostasis, and has evolved to tightly regulate immune activation[25]. This is essential to prevent deleterious (auto)immune reactions to harmless self- and environmental antigens, to restrain excessive immune activation during infection, and to resolve inflammatory conditions after pathogen clearance.

Intriguingly, tumors are able to hijack immunoregulatory mechanisms to their own benefit to undermine anti-tumor immunity, highlighting the duality of the immune system in cancer. Through cancer-cell induced co-option of anti-inflammatory stromal- and immune cells that abundantly accumulate in the TME, multiple overlapping mechanisms of suppression can be stacked. This typically occurs in the chronically inflamed context of the TME, as the immune system fails to resolve the cancer[7,13]. Chronically stimulated immune cells produce molecules like IL-10, TGF-β, adenosine, iNOS and IL-6 which, although pleiotropic, contribute to an immunosuppressive environment that is characterized by tissue repair, regeneration, and exclusion and inhibition of cytotoxic cells, instead of effector cell activation and cancer cell killing. Furthermore, co-inhibitory ligands like PD-L1 become widely expressed in the TME, which further stall T cell activation and function[26]. Finally, the TME can become even more hostile to infiltrated effector cells through changes in the availability of nutrients and growth factors[27], increased acidity[28], hypoxia[29], and release of radical oxygen species[30]. Combined, this heavily compromises the efficacy of anti-cancer immunity, leading the immune system to completely lose its grasp on tumor development.

The dual role of the immune system in cancer progression is not limited to the local tumor site, but is also intertwined with the final and most deadly stage of cancer; its spread to distant sites in a process known as metastasis. As invasive cancer cells enter surrounding tissue and circulation, those with high immunogenicity have been shown to be eradicated by extrinsic immune pressure in an effort to limit metastatic disease[31]. However, this pressure likely shapes the evolution of poorly immunogenic variants, which disseminate to distant sites[31]. In addition, it is becoming increasingly clear that cancer cells do not take the metastatic journey alone, but succeed in corrupting immunoregulatory mechanisms to increase their chances of successful colonisation of distant organs[32]. The chronically inflamed conditions within the primary tumor resonate throughout the host, leading to systemic mobilisation of immunosuppressive myeloid cells that confer protection against elimination by tumor-specific effector cells[33,34]. Furthermore, tumor-associated factors have been shown to instruct immune cells in distant organs to “prepare” a permissive niche prior to cancer cell arrival[32,35]. Even tumor-draining lymph nodes, the strongholds of effector cell activation, can become severely immunosuppressed during tumor progression[36,37].

The mechanisms underlying systemic immunosuppression and the formation of a (pre-) metastatic niche are only beginning to be understood, in particular how the adaptive immune system is involved in these processes. Furthermore, it is currently unclear how systemic immunosuppression impacts organ-specific metastasis. Ultimately, improved understanding of these mechanisms is critical to devise novel treatment strategies for patients with metastatic cancer. To acquire this fundamental knowledge, genetically engineered mouse models (GEMMs) are an important tool to dissect the complex immunoregulatory mechanisms at play during metastasis formation. Importantly, these models can reflect

crucial aspects of human primary tumor development and the metastatic cascade, including tissue-tropism of metastasis, heterogeneity within the primary tumor, and the chronic and systemic inflammation that underlies *de novo* tumor development and progression[38,39].

SCOPE OF THE THESIS

Although astonishingly complex and intriguing at a cellular level, the manifestation of cancer imposes an enormous burden on patients, their families, and society as a whole. This results in severe physical and emotional distress, reduced quality of life, premature death and large economic costs. With 2,261,419 cases diagnosed worldwide in 2020, breast cancer is the most common form of cancer across both sexes, despite over 99% of cases occurring in women[40]. These data illustrate the Herculean task laid out in the field of oncology to be tackled, and provides the context in which the work in this thesis was performed. However, by standing on the shoulders of giants, our current understanding on the pathogenesis of breast cancer has massively increased over time. In stark contrast to the Egyptian medical writer Imhotep (~2600 BC) who, while describing the first documented case of breast cancer, noted that no therapy could be offered[41], we are currently amidst a revolution in the development of immunotherapeutic approaches. To fully harness the potential of these novel immunomodulatory drugs, it is essential to increase our understanding of immunoregulatory mechanisms at play in breast cancer, in particular in the context of metastasis. This thesis aims to provide further mechanistic insight into the role of a key regulator of immunosuppression: the FOXP3$^+$CD4$^+$ regulatory T cell (T_{reg}). While essential for maintaining immune homeostasis and tolerance[42], this adaptive immune cell has emerged as a valuable asset for malignant cell growth, through suppression of anti-tumor immunity[43,44]. However, it is largely unclear exactly how, when, and where T_{regs} contribute to the formation of metastases. Using GEMMs for primary- and metastatic breast cancer[45,46], the work in this thesis describes how tumors succeed in corrupting T_{regs} for their own benefit, and provides more details into the intricate relationship that T_{regs} hold with the TME, metastasis, and immunotherapy response.

In **chapter 2**, I introduce the biology of T_{regs} in the context of breast cancer, and review exciting clinical advancements that have improved the prognostic and predictive value of T_{regs} in breast cancer, and the therapeutic potential of targeting these cells. I propose that the use of GEMMs that closely mimic the diversity and stepwise progression of human breast cancer subtypes is necessary to propel our understanding of T_{reg} biology to a higher level and to deepen our knowledge of underlying mechanisms, which is important to take full advantage of novel immunomodulatory drugs that may take the stage in breast cancer treatment.

CD4$^+$ T cells are plastic cells that respond to environmental cues by adapting their gene expression and function to local tissue environments. In **chapter 3** we describe how tumors

exploit this feature to increase the intratumoral accumulation of immunosuppressive T_{regs}, with the help of tumor-associated macrophages (TAMs). By characterizing the cellular crosstalk between TAMs and $CD4^+$ T cells in spontaneous mammary tumors, we show that TAMs can drive the conversion of conventional $CD4^+$ T cells into T_{regs}. Mechanistic studies reveal that this is mediated via two distinct mechanisms. TAMs can drive T_{reg} conversion directly via production of TGF-β, but can also prepare conventional $CD4^+$ T cells for conversion through regulation of PD-1 expression.

Ever since their discovery, T_{regs} have been in the crosshair of cancer immunology research, and a great effort has been made to characterize the function of T_{regs} inside primary tumors and metastases. However, recent studies implicate that in particular T_{regs} in blood and lymph nodes of breast cancer patients may have important clinical value. In line with this notion, in **chapter 4**, we move the scope from T_{regs} in primary tumors, to those in distant organs of tumor-bearing hosts. Through extensive characterization of these cells we discovered that T_{regs} expand systemically and undergo tissue-specific phenotypic and functional alterations during primary mammary tumorigenesis. This elicits a tissue-specific effect on metastasis formation, as neoadjuvant targeting of T_{regs} using an Fc-optimized anti-CD25 antibody reduces cancer spread to axillary LNs, but not to the lungs. Mechanistically, we found that T_{regs} suppress the anti-metastatic potential of NK cells specifically in the lymph node niche. **Chapter 4** provides evidence that tissue-specific mechanisms of immunosuppression are instrumental in shaping organotropism of metastases. **Chapter 5** highlights this concept from the angle of another key player in immunosuppression: neutrophils. I discuss a mechanism by which breast cancer cells co-opt neutrophils to enhance neutrophil extracellular trap formation specifically in the lungs, thereby increasing lung metastasis.

Immune checkpoint blockade (ICB) like anti-PD1 and anti-CTLA4 provide important tools to boost T cell activation, and are increasingly used in the context of cancer to overcome immunosuppression and to induce anti-tumor immune responses. However, T_{regs} themselves can express high levels of PD-1 and CTLA4, posing the central question of **chapter 6**: how does checkpoint inhibition affect T_{regs}? By performing intervention studies with combinations of ICB and T_{reg} depletion in preclinical mouse models, we demonstrate that ICB induces systemic activation and proliferation of T_{regs}, but not conventional T cells. Depletion of ICB-activated T_{regs} unleashes strong immune activation in blood, and improves the response to immunotherapy in a model for metastatic breast cancer, that is otherwise unresponsive to ICB. This chapter proposes that T_{regs} should be more prominently considered when using therapeutic approaches that modulate T cell function.

As is evident from work in this thesis, functional analyses are helpful to fully uncover the role of T_{regs} in cancer. In **chapter 7** we describe the details of a standardized method for assessing the immunosuppressive potential of freshly isolated T_{regs} from tumors and lymphoid tissue,

which has been used in **chapter 3**, **4** and **6**.

Finally, the theoretical and clinical implications of the findings in this thesis are discussed in in context of current literature in **chapter 8**.

REFERENCES

1. Casás-Selves, M. & Degregori, J. How cancer shapes evolution, and how evolution shapes cancer. *Evolution (N. Y).* **4**, 624 (2011).
2. Global Cancer Observatory. https://gco.iarc.fr/.
3. Thorsson, V. *et al.* The Immune Landscape of Cancer. *Immunity* **48**, 812 (2018).
4. Wu, S. Z. *et al.* A single-cell and spatially resolved atlas of human breast cancers. *Nat. Genet.* **53**, 1334–1347 (2021).
5. Wellenstein, M. D. & de Visser, K. E. Cancer-Cell-Intrinsic Mechanisms Shaping the Tumor Immune Landscape. *Immunity* **48**, 399–416 (2018).
6. Benavente, S., Sánchez-García, A., Naches, S., LLeonart, M. E. & Lorente, J. Therapy-Induced Modulation of the Tumor Microenvironment: New Opportunities for Cancer Therapies. *Front. Oncol.* **10**, 2169 (2020).
7. Garner, H. & de Visser, K. E. Immune crosstalk in cancer progression and metastatic spread: a complex conversation. *Nat. Rev. Immunol.* 1–15 (2020) doi:10.1038/s41577-019-0271-z.
8. Witz, I. P. The Tumor Microenvironment: The Making of a Paradigm. *Cancer Microenviron.* **2**, 9 (2009).
9. Hersh, E. M., Mavligit, G. M., Gutterman, J. U. & Barsales, P. B. Mononuclear cell content of human solid tumors. *Med. Pediatr. Oncol.* **2**, 1–9 (1976).
10. Vose, B. M., Vánky, F., Argov, S. & Klein, E. Natural cytotoxicity in man: activity of lymph node and tumor-infiltrating lymphocytes. *Eur. J. Immunol.* **7**, 753–757 (1977).
11. Brubaker, D. B. & Whiteside, T. L. Localization of human T lymphocytes in tissue sections by a rosetting technique. *Am. J. Pathol.* **88**, 323 (1977).
12. De Visser, K. E., Eichten, A. & Coussens, L. M. Paradoxical roles of the immune system during cancer development. *Nature Reviews Cancer* vol. 6 24–37 (2006).
13. Shalapour, S. & Karin, M. Pas de Deux: Control of Anti-tumor Immunity by Cancer-Associated Inflammation. *Immunity* **51**, 15–26 (2019).
14. Blank, C. U. *et al.* Neoadjuvant versus adjuvant ipilimumab plus nivolumab in macroscopic stage III melanoma. *Nature Medicine* vol. 24 1655–1661 (2018).
15. Forde, P. M. *et al.* Neoadjuvant PD-1 blockade in resectable lung cancer. *N. Engl. J. Med.* **378**, 1976–1986 (2018).
16. Chalabi, M. *et al.* Neoadjuvant immunotherapy leads to pathological responses in MMR-proficient and MMR-deficient early-stage colon cancers. *Nat. Med. 2020 264* **26**, 566–576 (2020).
17. Chen, D. S. & Mellman, I. Oncology Meets Immunology: The Cancer-Immunity Cycle. *Immunity* **39**, 1–10 (2013).
18. Apavaloaei, A., Hardy, M. P., Thibault, P. & Perreault, C. The Origin and Immune Recognition of Tumor-Specific Antigens. *Cancers (Basel).* **12**, 1–13 (2020).
19. Mittal, D., Gubin, M. M., Schreiber, R. D. & Smyth, M. J. New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. *Curr. Opin. Immunol.* **27**, 16–25 (2014).
20. Chalmers, Z. R. *et al.* Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Med.* **9**, 1–14 (2017).
21. Van Allen, E. M. *et al.* Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science (80-.).* **350**, 207–211 (2015).
22. Rizvi, N. A. *et al.* Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science (80-.).* **348**, 124–128 (2015).
23. McGrail, D. J. *et al.* Multi-omics analysis reveals neoantigen-independent immune cell infiltration in copy-number driven cancers. *Nat. Commun.* **9**, (2018).
24. McGrail, D. J. *et al.* High tumor mutation burden fails to predict immune checkpoint blockade response across all cancer types. *Ann. Oncol.* **32**, 661–672 (2021).
25. Huntington, N. D., Gray, D. H. & Nicholas Huntington, C. D. Immune homeostasis in health and disease. *Immunol. Cell Biol.* **96**, 451–452 (2018).
26. Binnewies, M. *et al.* Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat. Med.* **24**, 541–550 (2018).
27. Lim, A. R., Rathmell, W. K. & Rathmell, J. C. The tumor microenvironment as a metabolic barrier to effector T cells and immunotherapy. *Elife* **9**, 1–13 (2020).
28. Wu, H. *et al.* T-cells produce acidic niches in lymph nodes to suppress their own effector functions. *Nat. Commun. 2020 111* **11**, 1–13 (2020).
29. Barsoum, I. B., Smallwood, C. A., Siemens, D. R. & Graham, C. H. A mechanism of hypoxia-mediated escape from adaptive immunity in cancer cells. *Cancer Res.* **74**, 665–674 (2014).
30. Weinberg, F., Ramnath, N. & Nagrath, D. Reactive Oxygen Species in the Tumor Microenvironment: An Overview. *Cancers (Basel).* **11**, (2019).
31. Angelova, M. *et al.* Evolution of Metastases in Space and Time under Immune Selection. *Cell* **175**, 751-765.e16 (2018).

32. Kitamura, T., Qian, B. Z. & Pollard, J. W. Immune cell promotion of metastasis. *Nat. Rev. Immunol.* **15**, 73–86 (2015).
33. Coffelt, S. B. *et al.* IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* **522**, 345–348 (2015).
34. Wellenstein, M. D. *et al.* Loss of p53 triggers WNT-dependent systemic inflammation to drive breast cancer metastasis. *Nature* **572**, 538–542 (2019).
35. Doglioni, G., Parik, S. & Fendt, S. M. Interactions in the (pre)metastatic niche support metastasis formation. *Front. Oncol.* **9**, 219 (2019).
36. van Pul, K. M. *et al.* Selectively hampered activation of lymph node-resident dendritic cells precedes profound T cell suppression and metastatic spread in the breast cancer sentinel lymph node. *J. Immunother. Cancer* **7**, 133 (2019).
37. Rotman, J., Koster, B. D., Jordanova, E. S., Heeren, A. M. & de Gruijl, T. D. Unlocking the therapeutic potential of primary tumor-draining lymph nodes. *Cancer Immunol. Immunother.* **68**, 1681 (2019).
38. Gómez-Cuadrado, L., Tracey, N., Ma, R., Qian, B. & Brunton, V. G. Mouse models of metastasis: progress and prospects. *Dis. Model. Mech.* **10**, 1061–1074 (2017).
39. Kersten, K., de Visser, K. E., van Miltenburg, M. H. & Jonkers, J. Genetically engineered mouse models in oncology research and cancer medicine. *EMBO Mol. Med.* **9**, 137–153 (2017).
40. Ferlay, J. *et al.* Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int. J. Cancer* **144**, 1941–1953 (2019).
41. Mukherjee, S. *The Emperor of All Maladies: A Biography of Cancer*. (Schribner, 2010).
42. Kim, J. M., Rasmussen, J. P. & Rudensky, A. Y. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat. Immunol.* **8**, 191–7 (2007).
43. Plitas, G. & Rudensky, A. Y. Regulatory T Cells in Cancer. *Annu. Rev. Cancer Biol.* **4**, 459–477 (2020).
44. Kos, K. & de Visser, K. E. The Multifaceted Role of Regulatory T Cells in Breast Cancer. *Annu. Rev. Cancer Biol.* **5**, (2021).
45. Derksen, P. W. B. *et al.* Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell* **10**, 437–449 (2006).
46. Doornebal, C. W. *et al.* A Preclinical Mouse Model of Invasive Lobular Breast Cancer Metastasis. *Cancer Res.* **73**, 353 LP – 363 (2013).