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Remote control: the cancer cell-intrinsic mechanisms that dictate systemic inflammation and anti-tumor immunity

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General introduction

Immune co-option and corruption

The conundrum of cancer is one of tremendous magnitude. Cancer accounts for an estimated 9.6 million deaths annually, making it the second leading cause of death worldwide[1,2]. Although a hopeful note rings through the fact that advances in diagnosis and treatment have led to a steady decline of mortality rates of most tumor types over the last decades[3], cancer remains a major clinical challenge. The vast majority (±90%) of cancer-related mortality is due to metastasis[4], the spread of cancer throughout the body, showing an unmet clinical need for metastasis-limiting therapies for cancer patients. To improve existing treatments and develop new ones, it is therefore key to understand what mechanisms are employed by tumors to facilitate their spread throughout the body and how we can exploit these insights for the benefit of cancer patients.

Tumors are complex ecosystems that comprise a large variety of cell types, which can influence each other through cellular crosstalk. The complexity of cancer is tremendous: stemming from different cells of origin within a given tissue, acquiring mutations during its development, responding in a heterogeneous fashion to different treatments, and all the while constantly communicating with its surroundings and often manipulating that environment to its own advantage. Furthermore, tumors do not consist of one homogeneous entity of similar cells, but rather of many sub-clones of cells that can coexist in complex networks. These sub-clones can have differing properties, each with their own evolutionary benefit in terms of cellular proliferation, survival signaling and communication with their environment. Consequently, principles of evolution dictate that targeting one or several such clones will ultimately lead to survival advantage of another, resulting in treatment resistance. Additionally, cancer can arise in virtually all organs, with unique tissue characteristics for each anatomical location[5]. And to complicate matters further, even within one particular organ, such cancer ecosystems can differ between cancer subtypes and between patients with the same subtype. Ultimately, this vast degree of diversity and plasticity benefits tumors, as they find ways to grow, adapt to different metastatic sites and resist of anti-cancer therapeutics.

This thesis aims to shed some light on some of the constants within this complex ecosystem that we can potentially utilize for improvement of cancer treatment. It will focus on the interplay between tumors and their surrounding tissue, in primary sites as well as metastatic organs. This crosstalk between cancer cells and their environment, or among non-cancerous cells in a tumor-bearing host, is a key element influencing cancer progression[6,7] and treatment response[8]. These non-cancerous cells consist of a large variety of cell types, ranging from stromal cells such as fibroblasts, to endothelial cells and perhaps most importantly, immune cells. This collection of non-neoplastic cells is referred to as the tumor microenvironment. Over the last couple of decades, insights have emerged that cells in the tumor microenvironment, particularly immune cells, can be potently employed as anti-cancer therapeutics. The immune system consists of cell types that are endowed with cytotoxic capacities aimed to eradicate pathogenic infections. Cancer immunotherapy aims to boost these cytotoxic capabilities to generate and sustain an anti-tumor immune response. Key players in this type of treatment are cells of the adaptive immune system, such as cytotoxic T cells. These cells are able to recognize tumor-derived antigens that are presented by antigen-presenting cells (APCs) and consequently become activated[9]. Under optimal conditions, T cells can thus recognize and kill tumors and generate immunological memory against cancer[10].

The flipside of cancer-associated immune responses comes from the fact that some

immune cells are also capable of facilitating tissue regenerative processes, as is for example observed in wound healing responses. Mainly cells of the innate immune system, such as neutrophils and macrophages, are elemental in these types of processes. Under acute inflammatory conditions, these cell types can exhibit a wide range of anti-pathogenic and anti-tumor functions. However, under chronic inflammatory conditions, such as in unresolved infections or indeed cancer, these myeloid cells function in suppression of immune responses and facilitate tissue regeneration. Tumors profit from wound-healing-related mechanisms such as promotion of survival and proliferation signaling, blood vessel formation and suppression of immune attack[10]. In cancer, this duality in function of the immune system as a whole – cytotoxicity versus immunosuppression and tissue regeneration – is a balance that is often skewed towards the latter.

This cancer-immune crosstalk is not limited to primary tumor micro-environments. Importantly, cancer is also a systemic disease. Its systemic nature is apparent when cancer cells spread from their primary site to distant organs, but also holds true when primary tumors engage host cells to become alternatively activated at sites where no tumor is present (yet), in the pre-metastatic niche[11]. Tumors can generate chronic inflammatory conditions in its local microenvironment, but also throughout the body, so-called systemic inflammation[12]. An inflamed tissue normally produces proteins to recruit immune cells and/or promote immune cell generation in the bone marrow to resolve pathogenic infection. Mirroring these conditions, tumors engage the immune system systemically by secreting factors that lead to generation, activation and systemic accumulation of (mainly myeloid) immune cells. These tumor-derived signals can thereby also activate immune cells to their own benefit, for example by creating conditions in pre-metastatic organs that suppress the anti-tumor immune attack. This cancer-induced systemic inflammatory condition is dominated by neutrophils, the most abundant circulating leukocyte. These cells are present in vast numbers throughout the body, where they exert a variety of functions that can aid tumors and help their spread, one of which is immunosuppression. This is perhaps best evidenced when correlating systemic neutrophilia in cancer patients with disease outcome. In almost all solid tumor types, high systemic neutrophil-to-lymphocyte ratios correlate with poor disease outcome[13]. By manipulating systemic immune responses and creating conditions of chronic inflammation, tumors can have a long-distance reach and transform distant tissues that are intrinsically hostile to metastatic colonization into niches that are more hospitable for metastatic lesions to grow out[14]. Thus, the process of cancer metastasis is beset by an antagonizing host – in the form of an anti-tumor immune response – that is to be manipulated both in primary lesions and systemically for successful dissemination, colonization of distant organs and secondary outgrowth. In this thesis, we will examine the duality of anti-cancer immunity and chronic tumor-promoting inflammation in the tumor microenvironment and beyond. Understanding the mechanisms that tumors employ to generate systemic neutrophilia and suppress immune responses against primary and metastatic lesions will help us identify mediators that can be targeted therapeutically to limit metastatic progression.

The appreciation of cancer as a complex ecosystem greatly complicates our means of understanding it. While one can study processes that occur in the tumor microenvironment and systemically in a reductionistic manner – assessing specific aspects in isolation – it is inherent to complex systems that all elements influence each other, and therefore need to be examined as such. One could therefore argue that the complexity of cancer warrants study in equally complex, yet controllable, model systems. Such models would require spontaneous

development of cancer that is derived from one or several cells gone awry, as well as (chronic) inflammatory conditions both in the microenvironment as well as systemically. Moreover, such models should show spontaneous development of metastasis with organotropism similar to that of cancer patients. For several disease entities, mouse models have been developed that closely mimic human cancer in terms of subtype, mutated genes, cell- and tissue-of-origin, and composition and quality of the tumor microenvironment. These models are based on tissue-specific engineering of cancer-related genetic aberrations that induce spontaneous tumorigenesis, and include genetically engineered mouse models (GEMMs) and somatic genome editing models[15]. It is proposed that one of the reasons why many anti-cancer drugs that initially show promise in pre-clinical models fail in the majority of clinical trials is due to the use of inadequate or inappropriate model systems[15]. Indeed, the vast majority of studies of cancer development and progression use models that are based on inoculation of cultured (human or murine) cell lines[16]. These cancer cell line inoculation models fail to adequately recapitulate tumorigenesis, cancer development, metastasis, immune status and co-evolution with host cells as seen in patients. While these xeno- or allograft models are not uninformative, one needs to consider the shortcomings of conclusions drawn based on these. On the other hand, while GEMMs are superior in modelling human cancer, they still have major shortcomings, as their tumors harbor relatively few mutations compared to (some) human cancers, still have limited intratumoral heterogeneity and sometimes show a different metastatic spectrum than their human counterparts. Moreover, generation and usage of GEMMs is time-consuming and costly, rendering it difficult to use in a high-throughput fashion. In this regard, it is important to consider the balance between speed and adaptability of the model versus accuracy and complexity. Taking the above notions into consideration, the studies contained in this thesis will most heavily rely on GEMMs to allow a holistic view of the multidimensional systems involved in cancer development, evolution and most importantly, treatment efficacy.

Scope of the thesis

The four cancer types with the highest incidence worldwide occur in the lung, colon, prostate and breast[2]. This thesis focuses on the latter, breast cancer and its metastatic progression. Metastatic breast cancer accounts for over 600,000 deaths per year worldwide[2,17]. This work details investigations into the mechanisms of metastatic spread and how tumors employ a systemic inflammatory chain reaction of events to promote this process. Dissecting the crosstalk between breast cancer cells and their immune environment, both locally and systemically, may lead to the understanding of the means of their communication and thereby reveal novel vulnerabilities for therapeutic intervention.

The main hypothesis I pose here is that while cancer is highly heterogeneous and the host response to cancer is perhaps equally diverse, there are intricate relationships between cancer cell-intrinsic processes and inflammatory responses, which can potentially be exploited therapeutically. With cancer-intrinsic processes I specifically refer to oncogenic signaling pathways, the oncogenes and tumor suppressor genes that are mutated or dysregulated in cancer cells. This work broadly entails two aspects of the interplay between breast cancer and the immune system. One is a cancer-centered viewpoint, in which we try to find cancer cell characteristics that link to immune phenotypes. I describe how the genetic makeup of breast cancer dictates intratumoral and systemic immune recruitment and activation and how this can potentially be used for patient-tailored, personalized immune

intervention strategies. The second part focuses more specifically on the key players of cancer-induced systemic inflammation, neutrophils. It specifically examines the diversity of neutrophil phenotypes in cancer and how (systemic) neutrophilia in turn affects tumors.

In **chapter 2**, I introduce the conceptual framework that the perception of the biology of cancer evolved from viewing it as a genetic disease of mutated genes to a disease of cancer cells residing in complex microenvironments, and examine how tumor genetics and immunity are intricately connected. It discusses how cancer cell-intrinsic mechanisms, regulated by specific oncogenes or tumor suppressor genes that are aberrantly expressed in the tumor, can mold the immune system in ways that affect disease onset, progression and therapy response. It also discusses early pre-clinical and clinical studies on how this can be exploited to improve response to targeted or immune-based therapies.

The influence of the genetic makeup of tumors on the immune system reaches beyond the local microenvironment. **Chapter 3** describes how an oncogenic driver that is aberrantly expressed in breast tumors dictates systemic immune composition and function, thereby promoting metastatic progression. In this study, we have performed extensive examination of the systemic immune milieu of a large panel of GEMMs that model different subtypes of breast cancer with unique oncogenic mutations driving tumorigenesis. By comparing the genetic makeup underlying tumorigenesis in these GEMMs, we discovered that systemic expansion and activation of neutrophils is induced by cancer-intrinsic loss of the tumor suppressor gene p53, one of the most frequently mutated genes in human breast cancer. Mechanistic studies uncovered how loss of p53 activates the WNT signaling pathway to trigger a systemic cascade of inflammatory cells to drive metastasis. Using WNT-targeting agents in pre-clinical mouse models demonstrated that targeting these molecules reduced systemic inflammation and consequently metastasis in a p53-dependent manner.

Chapter 4 delves further into the effect of p53 aberrations in mammary tumors on the immune system and immunotherapy response. It describes the modelling of the most frequently occurring p53 mutations of human breast cancer in mouse tumors. Comparing these distinct p53-mutant tumors demonstrated that there are some mutations in p53 that create a tumor microenvironment generally devoid of cytotoxic T cells, while others induce an immune-enriched tumor. We show that p53 mutations that induce an immune-depleted tumor also impair the response to immunotherapy. This chapter describes the alterations in signaling pathways induced by these specific p53 mutations that stimulate immune cell recruitment in the microenvironment and demonstrates that interfering with these pathways influences the efficacy of immunotherapy.

Together, **chapters 3** and **4** provide pre-clinical evidence that studying the relationship between cancer genotypes and immune phenotypes could uncover potential novel anti-metastatic (immune-based) therapies for patients with specific mutations (in this case p53 aberrations), as well as show that the genetic makeup of tumors can be linked to immunotherapy response. These chapters are a testament to the utility of genotype/immune phenotype studies for tailoring cancer treatment.

The dissection of the tumor ecosystem benefits from examining some processes in isolation and connecting these to observations in complex models. As shown in **chapters 3** and **4**, the crosstalk between cancer cells and their environment and the mechanisms it entails was in part uncovered by *in vitro* modeling of this process. **Chapter 5** details a method to study the communication between cancer cells and macrophages, a type of immune cell that is present in high abundance in both murine and human primary breast tumors, and is a

key orchestrator of local and systemic inflammation.

Next, we focus specifically on the immune cell type that has over recent years been uncovered and acknowledged as one of the key players in breast cancer metastasis: the neutrophil. As described in **chapter 3**, systemic neutrophilia can promote metastatic progression. **Chapter 6** discusses the diverse roles these cells can play in cancer, how they develop, and how they can be used therapeutically in cancer. **Chapter 7** further zooms in on a key emerging concept in cancer-induced neutrophil biology: its metabolic reprogramming.

As a testimony to neutrophil diversity in breast cancer metastasis, **chapter 8** examines the molecular composition of a subset of neutrophils that emerges in mammary tumor-bearing hosts that is characterized by expression of the stem cell marker cKIT. Using proteomic profiling, we revealed that under homeostatic conditions, neutrophils have specific phenotypes according to the anatomical location in which they reside. While breast tumors induce a specific neutrophil phenotype, this organ-specificity is largely annulled by the presence of a tumor. Additionally, it shows preliminary evidence that targeting the cKIT receptor may limit metastatic progression. This chapter, along with **chapters 3** and **6**, demonstrates that systemic neutrophilia is a major hallmark of many cancers that can have dire consequences for tumor development. Normalization of systemic neutrophil activation may prove an important addition to the range of immune-based therapies currently in clinical development. The concepts contained in this thesis and their implications for cancer patients are extensively discussed in a larger framework in **chapter 9**.

This work aims to shed light on the complexity of systemic crosstalk between cancer cells and immune cells in breast cancer metastasis and how the genetic makeup of cancer is a key orchestrator of such responses. Moreover, it shows the key role neutrophils play in this process, and how this can potentially be exploited for therapeutic opportunities for cancer patients.

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