Immune cell complexity in the tumor microenvironment of breast cancer

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CHAPTER 1

Scope of the thesis

Breast cancer is the most prevalent tumor type in women, accounting for 28% of all cancers in female patients[1]. It is estimated that 1 in 8 women will receive a diagnosis of breast cancer during her lifetime[2]. Breast cancer is a heterogeneous disease that includes several subtypes defined by histological and molecular features. Histological type, hormone receptor status (estrogen, progesterone receptor), HER2 receptor status, tumor grade and size are all aspects of the disease that affect the prognosis, the selection of treatment options and the response to therapy. Chemotherapy represents one of the main treatments that breast cancer patients receive, however, response rates differ among patients. For example, patients with invasive lobular carcinoma (ILC), a histological subtype accounting for 10% of breast cancers, respond poorer to chemotherapy compared to patients with invasive ductal carcinoma (IDC), when administered before tumor resection (neoadjuvant setting)[3]. It was proposed that this was due to the enrichment of estrogen receptor positive/HER2 negative tumors in ILC patients, which are less sensitive to chemotherapy[4]. Because pathologic response to neoadjuvant chemotherapy of the primary breast tumor is associated with long-term survival[5,6], it is essential to discover new therapeutic strategies that improve the success rate of chemotherapy.

Since Theodor Boveri hypothesized the existence of a causal link between chromosomal abnormalities and tumor growth in 1914[7], the idea that tumors arise solely as a consequence of mutations in healthy cells and that their genetic aberrations are the determinants of therapy response dominated the oncology field for decades. However, this concept of cancer as a "disease of the genome", does not fully explain tumor progression and therapy outcome. In the 1970s, researchers started to acknowledge the tumor microenvironment (TME) as a key component able to influence tumor behavior[8]. Alongside cancer cells, tumors include different cell populations, such as immune cells, fibroblasts and endothelial cells. In addition, their secreted inflammatory mediators like cytokines, chemokines and metabolites, and elements of the extracellular matrix play a central role in the TME. It is now well established that the components of the TME are active players in tumor development and progression[9]. Of particular interest are immune cells because of their opposing abilities to interfere or promote tumor growth[10,11]. Indeed, in the past years, great emphasis was placed on understanding the impact of immune cells in tumor initiation, progression and metastasis formation that led to the development of different types of immunotherapies[12].

Tumor-associated inflammation

The immune system includes cells from the innate and adaptive arm which, by communicating with each other, protect the human body against foreign pathogens in a fine-tuned and regulated fashion. The immune system has developed in such a way that, upon perturbation of tissue homeostasis, it sets in motion a chain of

events that leads to the elimination of the assaulting agents and that culminates in wound healing and resolution of inflammation. Cells from the innate immune compartment, also called myeloid cells, like monocytes/macrophages, neutrophils, eosinophils, mast cells, dendritic cells (DCs) and NK cells, represent the first line of defense against foreign pathogens. They are also pivotal in bridging the innate and adaptive immune system by helping the activation of CD8+ and CD4+ T cells and B cells. Differently from the unspecific responses of myeloid cells, adaptive immune cells provide antigen-specific responses against pathogen-infected cells.

When the immune system fails to clear the initiating stimulus, chronically activated immune cells persist and induce tissue damage that may, in some cases, increase the risk of cancer development[10]. The first awareness of this concept was by Rudolph Virchow in 1863, when he proposed a causal link between inflammation and cancer after the observation that solid tumors are infiltrated by leukocytes[13]. Indeed, in some instances, inflammation can trigger tumorigenesis. For example, infections with the bacteria Helicobacter Pylori or the Hepatitis B and Hepatitis C viruses may lead to chronic gastritis and hepatitis, respectively, that may result in gastric or liver cancers[14,15]. Besides infectious pathogens, toxic agents and irritants that cause inflammation are also linked to tumor development, e.g. asbestos and malignant mesothelioma[16]. In addition to inflammation-induced cancers, many tumors induce inflammation, which can further promote tumor progression. Similar to a chronic infection, cancer is often described to be chronically inflamed or as a "wound that does not heal" due to the failed resolution of the original damage[17]. For decades, researchers have acknowledged that the majority of neoplastic lesions are highly infiltrated by cells of both the innate and adaptive immune system, resembling a chronic infection-induced inflammatory state. Tumor-associated chronic inflammation is characterized by accumulation of pro-inflammatory and pro-tumorigenic innate immune cells that produce cytokines, free radicals, growth and angiogenic factors, that sustain tumor growth and progression[10]. In addition, chronic inflammation supports immunosuppression in the TME, able to negate the anti-tumor cytotoxic activity of adaptive immune cells, such as CD8+ T cells[10]. Notably, the tumor-promoting capacity of inflammation was added to the list of hallmarks of cancer[18].

Macrophages, whose infiltration into tumors was already described in the late 70s – early 80s[19-21], represent one of the main immune cell types studied in the immune-oncology field. In a healthy organism, macrophages are found in every tissue with unique properties critical for the physiological function of a specific organ. Tissue-resident macrophages derive from the yolk sac and fetal liver during embryogenesis, but in certain conditions like infection or injury, damaged tissues recruit inflammatory monocytes from the circulation, blood and bone marrow which differentiate into macrophages[22]. Likewise, in mouse models for breast

cancer, tumor-associated macrophages (TAMs) have been proposed to derive from CCR2+ inflammatory monocytes[23], although local proliferation of differentiated macrophages has also been suggested[24]. Although early in vitro studies considered macrophages as tumoricidal cells[25], it is now appreciated that TAMs frequently function as orchestrators of tumor progression and metastatic disease via several mechanisms including support of angiogenesis, stimulation of tumor cell proliferation and migration, and suppression of anti-tumor immune responses[26]. Indeed, the majority of tumor types are densely populated by these cells, and for most of them, like breast, ovarian and oral cancers, the presence of macrophages is a negative prognostic factor[27-29]. Initially, TAMs were simplistically described as being either anti-tumoral (M1) or pro-tumoral (M2) by the expression of few markers. However, this dual definition has left room for a more complicated, but realistic view of macrophage phenotype. Indeed, using single cell RNA sequencing analysis, a recent study reported both M1- and M2-associated genes in the same macrophage isolated from the tumor of breast cancer patients[30]. TAMs, like tissue resident macrophages in general, are characterized by a remarkable plastic behavior; they adapt their phenotype depending on the environment they reside. Different stimuli, such as cyokines, metabolites and growth factors, derived from cancer cells or from stromal/immune cells can affect the macrophage polarization status and function in a dynamic and continuous fashion. This implies that macrophages are a heterogeneous population that differ between and within tumor types.

Apart from the role of inflammation in promoting tumor development, recent studies have shown that cancer cells are also able to dictate the intratumoral immune landscape and to induce systemic inflammation[31]. As the first cells that arrive in an inflamed site, neutrophils are well known to be mediators of inflammation. They are constantly produced in the bone marrow, in a process termed granulopoiesis, under control of the granulocytes-colony stimulating factor (G-CSF), the key regulator of neutrophil formation and differentiation[32,33]. Under critical circumstances, like cancer, malignant cell- and stromal cell-derived G-CSF stimulates the bone marrow and other organs (emergency granulopoiesis) to increase neutrophil generation[34,35]. Interestingly, this action also induces the release of immature neutrophils that have a less hypersegmented nucleus and that retain cKIT expression, which is usually found on progenitor cells[34]. Once in the circulation, the half-life of neutrophils is only about 7 hours, but it is increased to 17 hours in cancer patients[36]. Despite being short-lived cells, neutrophils can influence tumor progression[35]. Although a few preclinical studies showed an anti-tumorigenic role for neutrophils, the vast majority of preclinical studies reported a tumor-promoting function for these cells[35]. This effect is also mirrored in the clinic where a high neutrophil-to-lymphocyte ratio in the circulation of patients with several types of cancer is associated with worse disease outcome[37,38]. Like macrophages, also neutrophil phenotype is believed to

be constantly influenced by the environment, although our insights into neutrophil plasticity are still limited. Therefore, it is important to understand the function of neutrophils in each tumor settings in order to design new therapeutic strategies.

Not all cells of the immune system have pro-tumorigenic properties. For example, cytotoxic T cells are capable of killing cancer cells and controlling tumor progression. To obtain a successful anti-tumor response a process termed cancer-immunity cycle should be fulfilled[39]. Upon release of antigens from cancer cells and subsequent antigen uptake by dendritic cells (DCs), T cells are primed and activated. Effector T cells then infiltrate the tumor and kill the target cancer cells after recognizing the complex formed by a peptide and a major histocompatibility class I (MHC-I) molecule on the surface of malignant cells through their T cell receptor (TCR). The release of new antigens upon T cell-mediated killing of tumor cells reinforces the progression of the cycle. However, the generation of effective anti-tumor immunity is hampered by a series of bottlenecks, including paucity of released antigens, improper DC and T cell activation, and immunosuppressive mechanisms like the presence of myeloid cells and regulatory T cells (Tregs) that hamper the activity of cytotoxic T cells. Finding new ways to overcome these obstacles is essential in order to mount an effective immune response. Thus, the TME represents a complex dynamic environment in which immune cells paradoxically influence tumor growth in opposite ways. The pro- and anti-tumoral effects of the innate and adaptive immune system, respectively, often result in a tilted balance in favor of cancer progression.

The interplay between chemotherapy and immune system

The first chemotherapeutic drug, nitrogen mustard, a DNA alkylating agent, was discovered in the early 1940s and from that time on new chemotherapeutic agents and new combinations of cytotoxic drugs were used as standard of care in the clinic[40]. Apart from DNA damaging agents, other chemotherapies interfere with the DNA replication machinery or block cell mitosis. Although for a very long time chemotherapy was considered to solely target highly proliferating cancer cells, studies have shown that chemotherapeutic agents themselves have the capacity to modify the composition of the tumor microenvironment and the phenotype and function of intratumoral immune cells[41]. Because the immunomodulatory effects of some conventional cytotoxic therapies include inhibition of immunosuppressive cells, they could be strategically used in combination with immunotherapeutic agents to enhance anti-tumor immunity[41].

Although chemotherapeutic agents are frequently used for the treatment of the majority of tumor types, their anti-cancer effects are, most of the time, temporary. Cancer cells can escape the treatment regime because of the emergence of drug-resistant cells that, for instance, carry mutations in the target enzyme or enhance

DNA damage repair pathways, or overexpress drug efflux pumps[42]. Besides these cancer cell-intrinsic mechanisms, it is now clear that the tumor microenvironment also influences chemotherapy sensitivity[43]. Among the cells of the TME, macrophages have been the focus of a large number of studies addressing their role in response to chemotherapy. In breast cancer patients, a high intratumoral macrophage-to-CD8+ T cell ratio predicts poor response to chemotherapy[44,45]. In support of a detrimental function of macrophages in chemotherapy efficacy, preclinical mouse studies have shown that the elimination of these cells increased the efficacy of various chemotherapeutic drugs[45-52]. Several mechanisms have been accounted for the role of macrophages in limiting chemotherapy efficacy, including secretion of cathepsins that protect tumor cells from chemotherapy-induced cell death[51], production of lysophospholipids that affect the DNA damage response[46], and secretion of IL-10 that indirectly suppresses the cytotoxic activity of CD8+ T cells by inhibiting dendritic cell functions[50].

One clinically relevant strategy to deplete macrophages is to interfere with the CSF-1/CSF-1R signaling pathway, which is crucial for macrophage homeostasis, proliferation and survival[53]. Therapeutic agents include antibodies against CSF-1 or against the receptor, or small molecules inhibitors of the tyrosine kinase domain of CSF-1R[54]. Thus far, monotherapy with these drugs in clinical trials have shown limited efficacy in solid tumors, except for the high response rate of one particular cancer type, the diffuse-type tenosynovial giant cell tumor, characterized by CSF-1 overexpression and recruitment of CSF-1R+ cells[54]. In order to improve the therapeutic success of these drugs, it is crucial to understand the effects of these molecules on the TME and to identify their optimal therapeutic partner for combination treatment.

Similar to macrophages, neutrophils are also able to affect the response to chemotherapy. Although it was reported that depletion of neutrophils using the neutrophil-specific anti-Ly6G antibody modestly reduced the efficacy of doxorubicin in mice bearing inoculated cancer cells[55], other studies have shown that preventing their recruitment via inhibitors of the chemokine receptor CXCR2 in combination with chemotherapy decreased tumor growth in several mouse tumor models[56-58]. In line with these experimental data, clinical studies showed a better prognosis and a decreased recurrence risk in breast cancer patients that underwent chemotherapy-induced neutropenia[59,60]. It is thus important to understand in which settings neutrophils affect chemotherapy response and the underling mechanisms.

It is now clear that tumors hijack myeloid cells to sustain their growth and to hinder the anti-cancer efficacy of chemotherapy. Thus, targeting these cells represent a compelling strategy in combination with cytotoxic agents. However, understanding the mechanisms behind the effects of myeloid cells on therapy response is critical to design novel and more specific therapeutic strategies.

Description of the chapters in this thesis

Work described in this thesis aims to understand the role of myeloid cells in tumor progression and therapy response of breast cancer. Utilizing transgenic mouse models for breast cancer, I explored methodologies to study the role and phenotype of intratumoral immune cells, with a focus on how the tumor milieu affects macrophage phenotype. Moreover, I mechanistically addressed how immune cells impact the chemotherapy response of breast cancer.

The immune system is not a static component of the tumor microenvironment, but it constantly changes and adapts, co-evolving with the tumor during its initiation and progression. To study how cancer cells shape the phenotype of tumor-infiltrating immune cells, it is important to directly isolate the immune cells from freshly isolated tumors. In this regard, **chapter 2** describes a methodology to isolate immune cells from murine mammary tumors and other organs. This method is based on antibody-mediated enrichment of immune cells by magnetic cell sorting followed by purification of selected immune cell populations with multi-color flow cytometry-based sorting. By using this methodology, we studied in **chapter 3** whether and how distinct breast cancer models differently affect the phenotype of intratumoral macrophages. TAMs from two distinct genetically engineered mouse models (GEMMs) for *de novo* mammary tumorigenesis, *i.e. K14cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* and *MMTV-NeuT* mouse models which resemble human ILC and HER2-overexpressing tumors, respectively[61,62], were compared at the transcriptomic level. Computational analysis of RNA sequencing data showed that although clustering together with macrophages from the mammary gland, these TAM populations showed a common signature as well as a breast cancer type-specific core signature. Interestingly, a selection of the genes derived from the model-specific core gene signature from the *K14cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* model correlated with worse clinical outcome in two independent cohorts of ILC patients, while the core signature of TAMs derived from the *MMTV-NeuT* mammary tumors failed to predict prognosis in the same ILC patient cohort. These data highlight that the tumor of two independent GEMMs have such a strong impact on the transcriptome profile of intratumoral macrophages.

The second part of this thesis focuses on the impact of chemotherapy on the immune system and vice versa. There is growing evidence that chemotherapeutic drugs do not only target cancer cells, but also have immunomodulatory effects. **Chapter 4** reviews the preclinical and clinical studies investigating the impact of chemotherapeutic agents on various immune cell populations. For example, some chemotherapeutic agents target the lymphoid compartment, like low doses of cyclophosphamide that reduce regulatory T cells (Treg)[63], while other

chemotherapies influence the myeloid compartment like gemcitabine that reduces myeloid-derived suppressor cells (MDSC)[64] or trabectedin that selectively ablates monocytes and macrophages[65]. Because of the influence that chemotherapeutic agents have on these immunosuppressive immune cells, in this chapter we propose to rationally combine certain chemotherapies with immunotherapy in order to boost anti-tumor immunity.

As macrophage-targeting agents are under clinical investigation, **chapter 5** aims to maximize the success of these compounds and to uncover the mechanisms by which these agents can increase the sensitivity of breast cancer to chemotherapeutic drugs. By using a spontaneous mouse model for breast cancer, the *K14cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mouse model, we reveal that anti-CSF-1R synergizes with platinum-containing drugs by inducing an intratumoral type I interferon response. In particular, we discovered that anti-CSF-1R on one hand depletes the majority of TAMs and on the other hand changes the activation status of the remaining intratumoral macrophages into a type I interferon-producing phenotype. We further uncovered that the establishment of a type I interferon-enriched milieu and the elimination of immunosuppressive neutrophils were both critical to support anti-tumor immunity upon cisplatin treatment.

The most relevant method to study the role of immune cells in tumorigenesis or in chemotherapy response is to deplete these cells by either genetic manipulation of key factors involved in their recruitment to the tumor site or in their development (like chemokine receptors or transcription factors) or via antibody-mediated depletion. While for most immune cells this can be successfully achieved, the long-term depletion of neutrophils still remains challenging.
The most commonly used methods to target neutrophils *in vivo* are antibody-based techniques, which, however, lack specificity or fail to provide long-lasting depletion. Few genetic models have been generated of which the most satisfactory rely on the expression of the diphtheria toxin (DT) receptor in neutrophils and administration of DT to induce neutrophil apoptosis[66]. Nevertheless, durable depletion with this system is not achieved because of the generation of a neutralizing immune response against DT[67,68]. In order to overcome these limitations, in **chapter 6** we generated a novel mouse model for the conditional and reversible depletion of neutrophils using the Apoptosis Through Targeted Activation of Caspase 8 (ATTAC) approach[69]. This mouse model relies on the activation of apoptosis specifically in neutrophils upon injection of a compound. This chapter describes the model and characterizes the efficiency of neutrophil depletion, both in a homeostatic and in a cancer setting.

Finally, in **chapter 7** I contextualize the findings of this thesis in regards to the current literature. I also discuss the implications of my data concerning the current and future clinical strategies with immunomodulatory compounds.

In conclusion, in this thesis I focus on the non-cancer cell autonomous mechanisms that govern tumor progression and response to anti-cancer therapies, with a major emphasis on macrophages and neutrophils. I highlight the complex relationship between myeloid cells and tumor cells that might lead to the discovery of new therapeutic strategies.

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