Dissecting the immune microenvironment of breast cancer

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Chapter 1

Introduction

Toward understanding the role of the immune system in cancer progression and chemotherapy response

Scope of Thesis

Abstract

Over the last decades, it has become apparent that the immune system influences most of the hallmarks of cancer. Immune cells interact with cancer cells and other tumor-associated cells via direct cell-cell interactions and secretion of a variety of growth factors, cytokines, chemokines, and proteases. Historically it was thought that the immune system protects against tumor development. However, more recent clinical and experimental studies have reported pro-tumorigenic roles as well as anti-tumorigenic roles for various immune cell types during tumor progression and chemotherapy response. To date, it remains largely unclear why certain tumors elicit anti-tumor immune responses whereas other tumors elicit pro-tumor immune responses or are not regulated by the immune system at all. Here, we review current insights into how adaptive and innate immune cells participate in tumorigenesis and chemotherapy response. In addition, we highlight that understanding the inherent complexity of the immune system in cancer is paramount for the identification of novel prognostic and predictive biomarkers, and for the design of novel immunomodulatory treatment strategies to fight cancer.

1.1 Introducing the paradoxical role of the immune system in cancer

Currently, it is known that the inter-tumoral and intra-tumoral heterogeneous nature of cancer is not only a consequence of aberrant mutations but also of the composition and activation state of the tumor microenvironment (TME)[1]. The TME contains fibroblasts, endothelial cells and immune cells of which their secreted inflammatory mediators such as metabolites, cytokines, chemokines, growth factors and proteases play a vital part in the cancer cell's ability to grow and to metastasize [2]. The immune system is an important player in tumorigenesis. Over the last century, compelling evidence has indicated that the immune system sometimes protects against cancer [3-5]. Already in 1909, Ehrlich, and later Thomas and Burnet, proposed that the immune system has the capacity to spontaneously recognize and kill cancer cells, and therefore protects against tumor development [6,7]. Immunotherapy, a cancer treatment that is based on boosting the ability of the adaptive immune system to destroy cancer cells, has evolved from a promising therapy to a clinical reality [8]. Clinical trials with immune checkpoint inhibitors, anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) and anti-programmed death ligand 1 (PD-L1), anti-PD-1 or a combination of these

agents have shown remarkable success in patients with advanced metastatic melanoma, renal cancer, non-small cell lung cancer, Hodgkin's lymphoma, bladder cancer and microsatellite instability (MSI) high colorectal tumors, and are now FDA approved for various cancer types [9-11]. Current efforts to enhance the therapeutic benefit of immunotherapy are focused on targeting evolving immunomodulatory pathways, for example T-cell metabolism [12].

At the same time, however, an increasing body of evidence has shown that the immune system can also promote tumorigenesis [1,13-16]. The first link between cancer and inflammation was made by Virchow in 1863 when he hypothesized that cancer finds its origin at sites of chronic inflammation [17]. Indeed, as will be discussed below, epidemiological studies and molecular studies in genetically modified mouse models provide evidence for a causal link between chronic inflammation and cancer. Consequently, the tumor-promoting ability of inflammation was added to the hallmarks of cancer [18].

To date, it is largely unclear why different tumors are differentially influenced by the immune system. Hence, for the development of novel immunomodulatory strategies, it is important to understand how cancer-promoting and cancer-inhibiting immune responses are regulated. Here we discuss the current understanding of the inherent complexity of the inflammatory TME, with a focus on lymphocytes and macrophages, during tumorigenesis and chemotherapy response. Moreover, we review recent therapeutic strategies that target pro-tumorigenic immune cells.

1.2 Clinical observations supporting a link between cancer and the immune system

Cells of both the innate and adaptive immune system infiltrate the majority of solid tumors, often resembling a chronic inflammatory state. Various clinical observations support the hypothesis that inflammation predisposes to cancer. For example, chronic inflammation caused by pathogens such as the bacterium *Helicobacter pylori* is associated with gastric cancer [19]. In addition, chronic hepatitis B or C increases the risk of hepatocellular carcinoma and parasitic infections with schistosomes and trematodes can cause cancers of the urinary bladder, the intrahepatic and extrahepatic biliary tract [20,21]. Besides infectious pathogens, exposure to environmental chemicals and irritants, such as tobacco smoke and asbestos or silica particles, can lead to chronic inflammation and is linked to lung cancer [22-24].

Lastly, Crohn´s disease, a type of inflammatory bowel disease (IBD), increases the risk of colorectal cancer [25], and gallstones and chronic cholecystitis can increase the risk of gallbladder cancer [26,27].

Cancer formation in the context of chronic inflammation is possibly the result of the incapacity of the host to resolve the persistence of initiating factors leading to a prolonged inflammatory response. The chronically activated innate immune cells produce high levels of reactive metabolites of oxygen, nitrogen, growth factors, pro-angiogenic and inflammatory mediators and proteases [13,28], which can cause DNA damage and genomic instability, and lead to tumor development [28]. In addition, to sustain tumor growth and progression, tumors themselves can induce chronic inflammation [13]. Hence, chronically inflamed tumors are often described as “wounds that do not heal” [29]. Furthermore, chronic inflammation frequently leads to an immunosuppressive state, characterized by the exclusion or suppression of adaptive immune cells in the TME [30]. One of the most abundant immune cell types in tumors are tumor-associated macrophages [31]. Macrophage infiltration in many human cancers, such as breast cancer [32] and oesophageal cancer [33] is linked with poor prognosis. Also other immune cell types with immunosuppressive capacity, including neutrophils and regulatory T cells, are frequently observed in cancers and are linked with poor prognosis [34,35]. Importantly, long-term usage of non-steroidal anti-inflammatory drugs, such as aspirin, has shown to reduce cancer incidence and metastasis [36,37], illustrating that it is possible to prevent cancer by suppressing chronic inflammation.

On the other hand, anti-tumor roles of the immune system were suggested by studies correlating increased intratumoral T cell numbers, activated $CD8^+$ T cells and $CD4^+$ Th1 cells with better survival across various cancer types, including colorectal cancer, melanoma, multiple myeloma and pancreatic cancer [38-43]. In addition, congenital and viral-induced acquired immunodeficiency’s, such as AIDS, have been associated with increased incidence of certain types of malignancies such as leukemia and various viral-associated cancers, such as Kaposi sarcoma, skin cancer, cervical cancer and Merkel cell carcinoma [27,44-48]. However, the association between a suppressed immune system and cancer- outcome differs per tumor type [49,50]. For example, whereas breast cancer incidence is decreased in female immunosuppressed patients with organ transplants [51], immunosuppressed organ transplantation patients are at increased risk for viral associated cancers such as lung, skin, non-Hodgkin lymphoma and endometrial

cancer[52]. This is because the adaptive immune system is rather capable of fighting viruses as there are viral-antigens that can be easily recognized. Another example is that memory $CD4^+$ T cells correlated with favorable outcome in lung adenocarcinoma patients but were associated with adverse outcome in bladder cancer patients [53]. These clinical observations suggest that distinct cancer types are differentially regulated by the immune system. Indeed, a body of accumulating clinical data indicates that different molecular subtypes of tumors are characterized by distinct immune landscapes [54]. Different patient-specific or tumor-specific characteristics may underlie the inter-patient heterogeneity in immune landscape. The activation of oncogenes or loss of tumor suppressor genes (TSGs) in cancer cells, epigenetics but also the patient characteristics such as microbiome, age, gender and therapy history dictate the immune composition, activation states and therefore different immune responses [55].

Together these clinical observations illustrate a potential versatile impact of the immune system on tumorigenesis. The magnitude and phenotype of the immune response are shaped by various patient and tumor characteristics, including cancer location, cancer (sub)type and genetic make-up of the tumor. Identifying the exact mechanisms underlying the interactions between genetic aberrations in tumors and the immune landscape will be crucial for the design of personalized immunomodulatory treatment strategies. *In vivo* mechanistic studies will be key to understand the crosstalk between the immune system and cancer per cancer subtype. The various mouse models that can be used to dissect the immune composition and function in primary tumors as well as in metastatic lesions and therapy will be discussed in the next section.

1.3 Preclinical mouse models as tools to study the function of the immune system in tumorigenesis and cancer treatment

Though clinical observations suggest an involvement of the immune system in tumorigenesis, these correlative data do not provide insights into mechanisms underlying the interplay between the immune system and cancer. Hence, different preclinical mouse cancer models have been used to mechanistically investigate the role of the immune system in cancer biology and therapy response, *i.e.,* human and mouse tumor cell line inoculation models, tumor transplantation models, including patient-derived xenograft (PDX) models, carcinogen-induced cancer models and genetically engineered mouse models (GEMMs)[56]. These different models have both

advantages and disadvantages for studying the interplay between cancer and the immune system.

Tumor allograft models, which rely on the ectopic or orthotopic injection of cancer cells grown in culture, are frequently used to study the role of immune cells in cancer. One major disadvantage of tumor cell line allograft models is that cancer cell lines are adapted to grow under *in vitro* culture conditions and have thereby acquired mutations over time [57], which may not occur under *in vivo* conditions. Other disadvantages of cancer cell line allograft models include the diminished genetic heterogeneity, the derangement of the normal tumor architecture, the disparate tissue of origin location compared to spontaneous tumors and the fact that they are generally poor predictors of clinical response [56]. Furthermore, while human tumors develop via a multi-step process in which normal tissues progress through a pre-malignant phase into invasive cancers with co-evolving cancer cell-host interactions and an immunosuppressive microenvironment [58], tumors formed after inoculation of cancer cells skip the premalignant phase. As such, spontaneous experimental tumors have different chemotherapy response profiles compared to inoculated tumor cells isolated from these spontaneous tumors [59]. In addition, immunotherapy efficacy exhibited enhanced sensitivity in mice with subcutaneously implanted tumors compared to mice bearing orthotopic tumors [60], indicating that the endogenous T cell responses are niche-dependent or that the injection of a large number of cancer cells already primes the immune system or that immunosuppressive mechanism differ per location. Since several studies have demonstrated that cancer cells can disseminate from very early neoplastic lesions [61,62], this process will not be recapitulated in cancer cell line inoculation models. Consequently, the impact of the immune system on early neoplastic events and early metastasis formation cannot be investigated in tumor cell line inoculation models.

Many different types of patient-derived xenograft (PDX) models have been developed in which human tumor pieces or patient-derived circulating tumor cells (CTCs) are (orthotopically) transplanted into immunocompromised mice. The transplantation of small tumor fragments or CTCs into mice has some advantages over human cell lines because the resulting tumors frequently recapitulate the morphology, heterogeneity, vasculature, and molecular and genetic alterations of the original donor tumor [56,63,64]. However, given the necessity to use immunodeficient recipient mice, human tumor cell line inoculation models and PDX models, are not suited for

studies focusing on the interplay between the immune system and cancer cells [63]. This gap is currently being addressed by the generation of humanized mouse xenograft models where components of the human immune system, such as human CD34+ hematopoietic stem cells or precursor cells, are engrafted into immunodeficient mice [65]. The newer generations of humanized mouse models show a promising progress in mimicking human tumor heterogeneity, the TME and crosstalk between the tumor and immune cells [66].

In contrast to cancer cell inoculation and tumor transplantation models, chemical- or viral-induced tumor models and GEMMs develop *de novo* tumors in a natural immune-proficient microenvironment. Genomic and microenvironmental heterogeneity that defines human cancer is well represented in the spontaneous tumors arising in conventional, conditional or somatic GEMMs [67-70]. Chemical- or viral-induced tumor and GEMMs have proven to be tremendously important in the inflammation and cancer field as they allow in-depth mechanistic characterization of the complex interactions between cancer cells and components of the immune system at all the different steps of tumorigenesis, drug response, and resistance [70]. Most work underlying the differential crosstalk of cancer cells with the immune system has been done in mice by utilizing tissue-specific promoters that induce somatic inactivation of TSGs or activation of oncogenes [70]. Mouse model engineering has taken a new direction with the discovery of the clustered regularly interspaced short palindromic repeats (CRISPR)-based genome editing approach [71]. CRISPR/Cas9-has proven to be an efficient gene targeting strategy with the potential for multiplexed genome editing for a wide spectrum of mutations found in human cancers [72-76]. An important factor to keep in mind when investigating the immune system with this approach is to circumvent somatic Cas9-specific immune responses [77-79]. Experiments should be performed in mice that have immunological tolerance to Cas9 or methods should be used such as the CRISPR-Cas9 bone marrow delivery system CHimeric IMmune Editing (CHIME), which allows rapid evaluation of gene function in immune cells lineages *in vivo* while keeping normal immune development and function [80]. In the next paragraphs, insights are provided into the role of the immune system during carcinogenesis and chemotherapy response, that have been obtained with these different experimental mouse tumor models.

1.4 Immunosurveillance and Immunoediting

The adaptive immune system is capable of recognizing and killing cancer cells, , and thereby has the ability to protect against tumor development [81]. This process, when functioning optimal, is referred to as cancer immunosurveillance [82]. The effective recognition and elimination of cancer cells by the immune system in a stepwise process is nowadays also referred to as the cancer-immunity cycle [55]. The cancer-immunity cycle begins with DCs that take up and present tumor (neo)antigens on major histocompatibility class I (MHC-I) and MHCII molecules to T cells. Neoantigens are expressed by tumor cells and are generally tumor-specific antigens generated as a consequence of DNA mutations in cancer cells [83]. Subsequent, $CD8^+$ T cells and NK(T) cells (effector T cells) are primed in secondary lymphoid organs such as lymph nodes (LN) and spleen and then activated to travel to the tumor. After recognizing and attaching to the tumor cell through their T cell receptor (TCR) and the analogous neoantigen on the tumors MHC-I molecule, the tumor cell will be destroyed. The release of additional antigens upon the elimination of cancer cells improves the T cell response. In addition, type I interferons (IFNs) induced by stimulator of interferon genes (STING) in cancer cells can further augment the cancer–immunity cell cycle. The discovery and characterization of the cGAS–STING pathway in 2013 has provided a new understanding of the immune-stimulatory capacity of double-stranded DNA (dsDNA) [84]. Detection of tumor-derived DNA by cGAS in dendritic cells fuels the cGAMP-dependent activation of STING and subsequent secretion of type I IFNs [85,86]. In several tumor transplantation models these innate immune signals enhanced tumor antigen presentation and thereby augment the antigen-specific CD8+ T cell response, which linked to tumor regression [87-89].

The experimental basis for the cancer immunosurveillance hypothesis was established using mice that lack the recombinase activating gene (RAG)-2 [90]. *Rag2*-deficient mice [91] lack mature lymphocytes and developed MCA-induced sarcomas more rapidly and with greater frequency than wild-type controls [90]. Various experimental studies have subsequently addressed the mechanisms underlying immunosurveillance. For example, enhanced development of spontaneous tumors has been reported in mice lacking components of the immune system, such as perforin, granzyme, cytotoxic cytokines, lymphocytes, or in mice defective for IFN signaling [81]. Despite these reported mechanisms of immunosurveillance, cancer is a very prevalent disease. This raises the question why the adaptive immune

system frequently fails to protect us from cancer. In fact, in order to generate effective anti-tumor immunity, several bottlenecks need to be overcome, such as failure of T cell priming against tumor antigens which can occur due to the lack of immunogenic tumor antigens or defects and deficiencies in antigen presentation, for example loss of MHC expression or dysregulation of the antigen processing apparatus. Other bottlenecks are defective DC and T cell activation, impaired trafficking or infiltration of the anti-tumor T cells into the tumor, the activation of immunosuppressive myeloid cells and Tregs, and induction of immune checkpoint molecules that suppress the priming or activation of effector T cells [92].

The immune-mediated tumor-sculpting process is also referred to as immunoediting [93]. Developing tumors influence the anti-tumor immune response, while the anti-tumor immune response shapes the immunogenicity -the capacity of provoking an adaptive immune response- of the tumor. The immune-editing process is demonstrated by studies revealing that carcinogen-induced sarcomas and *de novo* epithelial carcinomas were more immunogenic when induced in mice lacking lymphocytes as compared to being induced in immunocompetent mice [90]. Furthermore, the so-called tumor 'equilibrium' phase can result in tumor dormancy, which can last for years [94]. During the equilibrium phase, cancer cells can become resistant against immune attack, escape immune control, and develop into full-blown tumors. Established tumors may subsequently benefit from immune cells and their soluble mediators present in the TME, favoring tumor outgrowth. This co-evolution of a tumor and the immune system explains why many established tumors are characterized by low immunogenicity and a high immunosuppressive state, which in most cases resembles chronic inflammation [95]. The goal of immunotherapy and immunomodulation is to unleash immunosurveillance and to change the odds in favor of elimination or at least equilibrium. It is therefore important to understand in which tumors immune cells are tumor-promoting or tumor-preventing.

1.5 The adaptive immune system in promoting tumorigenesis

The impact of the adaptive immune system during the development and progression of pathogen- and chemical-unrelated solid cancers is less well defined. Studies using GEM tumor models have shown opposite functions of various adaptive immune cell populations during *de novo* epithelial tumorigenesis [96-101]. For example, in experimental pancreatic islet tumors,

tumorigenesis in *RIP1-Tag2, Rag1*$^{-/-}$ mice was similar to *RIP1-Tag2, Rag*$^{+/+}$ mice [101]. While in a transgenic mouse model for skin tumorigenesis, *i.e., K14-HPV16* mice, it was demonstrated that B lymphocytes activate inflammatory responses through antibody-mediated activation of Fc receptors (FcRs) on macrophages and mast cells, which stimulated their proangiogenic abilities and led to cancer progression [96,99]. In addition, the development of hepatocellular carcinoma in a chronic hepatitis mouse model was dependent on both T and B-lymphocytes [97]. Importantly, T and B-lymphocytes do not only play distinct roles depending on tumor type, but tumor subtypes are also differentially influenced by the adaptive immune system. In several transgenic mouse models of breast cancer, different components of the adaptive immune system were reported to promote metastasis formation [98,102-104]. For instance, metastasis formation in a mouse model for spontaneous breast adenocarcinomas, *i.e.,* MMTV-PyMT mice, revealed to be dependent on interleukin 4 (IL-4)-expressing CD4^{+} T cells which promoted EGF secretion from tumor-associated macrophages (TAMs) [98]. However, regulatory T cells promoted the metastatic spread of orthotopically transplanted mammary tumors derived from the MMTV-*ErbB2* transgenic mouse model in a RANKL dependent manner [102]. And in a genetically engineered mouse model for invasive lobular carcinoma (ILC), i.e. *K14cre; Cdh1*$^{F/F}$*; Trp53*$^{F/F}$ mice, it was demonstrated that tumor-derived CCL2-mediated induction of IL1b in TAMs stimulated IL-17 expression from γδ T cells, which resulted in the systemic granulocyte colony-stimulating factor (G-CSF)-dependent expansion and polarization of neutrophils, which in their turn suppressed effector CD8^{+} T cells that were limiting the development of metastasis [103,105]. This systemic pro-metastatic inflammatory pathway was triggered upon loss of P53 in breast cancer cells [104], illustrating that the genetic makeup of breast tumors shapes the crosstalk with the immune system. Hence, because different breast cancer subtypes are characterized by distinct (epi)genetic features that trigger unique gene expression patterns, combined with patient specific features that impact the immune landscape, distinct breast cancers hijack the adaptive immune system in different ways to contribute to metastasis [104,106,107]. Thus, different tumor types as well as tumor subtypes employ different mechanisms to circumvent or exploit components of the adaptive immune system for their own benefit. Numerous studies have elucidated that adaptive immune cells interact with many different components of the innate immune system. As such, for the development of therapeutics, future studies should gain insights into the interplay between adaptive and innate immune cells per individual cancer (sub)type or per genetic driver mutation.

1.6 Tumor-associated macrophages

Macrophage plasticity

Macrophages were originally identified based on their phagocytic nature by Metchnikoff in 1882 [108]. He suggested that macrophages fight infection by phagocytosis and play an important role in injury repair [108]. After decades of study, we now know that macrophages do more than defending the host from external invaders. Many preclinical studies have established that macrophages contribute to various cancer hallmarks including cancer proliferation, suppression of anti-tumor immune responses, angiogenesis and migration [109-111]. The current concept is that macrophages arise from two different lineages. Most tissue-resident macrophages arise from yolk sac progenitors and fetal liver during embryogenesis and are maintained through local proliferation. On the other hand, macrophages that fight pathogens in damaged tissues originate from bone-marrow derived macrophages (BMDMs) which get into the circulation as monocyte and then differentiate into macrophages once they enter the tissue [112-115]. Both tissue-resident as well as bone marrow-derived macrophages have shown to be important in tumor development in several mouse models [116-119]. Whether TAMs derive from circulating monocytes [120,121] will be discussed in the next paragraph.

Originally, macrophages were classified as 'classically activated' M1 and 'alternatively activated' M2- macrophages, based on a limited set of produced cytokines and expressed surface markers [122,123]. It is now generally accepted that the standardization and nomenclature of macrophages originating from cell culture studies, even though practical, does not fit TAM complexity *in vivo* [124-126]. Macrophages are inherently plastic, and therefore they can adapt their phenotype and function to the evolving changes in the TME during tumor progression. Over the years many studies have found a wide spectrum of macrophages with different polarization states and with specific tumor regulatory features that can include both inflammatory and immunosuppressive characteristics [127]. For instance, in a study where immune cells were isolated from human breast tumors, single-cell RNA-sequence (scRNA-seq) demonstrated that both M1 and M2 signatures were present in the same macrophage [128]. Similar results were found in gliomas [129]. Macrophage polarization in tumors has shown to be dependent on several different factors including tumor and organ type, intratumoral location, tumor stage and origin. For example, by transplanting differentiated peritoneal macrophages into the alveolar cavity, a study found that fully differentiated macrophages switched to resemble the

transcriptomic profile of lung macrophages [130], demonstrating that the tissue environment in which macrophages reside dictates macrophage polarization as well as regulation. In addition, a recent story discovered that TAM heterogeneity is driven by tissue territories in human and mouse breast cancer by combining scRNA-seq with spatial localization, indicating that tumor regions, rather than defined activation states, are the key drivers of TAM plasticity and heterogeneity [131]. Furthermore, by performing lineage tracing and scRNA-seq a recent study showed that distinct populations of macrophages were enriched in mouse and human non-small cell lung cancer (NSCLC); *i.e*. tissue-resident macrophages provided a pro-tumorigenic niche to early tumors while during tumor growth monocyte-derived macrophages became dominant and tissue-resident macrophages were redistributed at the periphery of the TME [119]. Given the spatiotemporal and environmental context, gene-expression profiles and transcriptional regulatory pathways are crucial for the understanding of human and mouse tissue- and tumor macrophage regulation and discovery of novel marker genes as well as biomarkers [132-135]. For example, a study showed that a specific TAM gene signature derived from *K14cre; Cdh1$^{F/F}$; Trp53$^{F/F}$* mouse tumors could be used to predict poor survival in two separate cohorts of ILC patients when compared to the transcriptome profile of bulk tumor samples, indicating that matched mouse TAM transcriptome signatures can be used for outcome prediction [136].

Targeting tumor-associated macrophages via the CSF-1/CSF-1R pathway

Since TAMs represent orchestrators of various tumor-promoting processes, TAMs have become interesting putative targets for therapeutic intervention. Various approaches aimed at targeting survival, recruitment or polarization of TAMs have shown potential in preclinical studies [137].

Macrophage recruitment to tissues in mice can be initiated by a chemotactic factor, identified as colony stimulating factor-1 (CSF-1) [138]. The receptor for CSF-1, CSF-1R, a class III transmembrane tyrosine kinase receptor encoded by the *cfms* proto-oncogene [139], is largely restricted to and expressed on almost all macrophages[138]. Initial positive support for blocking TAM recruitment or function via targeting CSF-1/CSF-1R signaling was found in M-CSF-deficient (Csf1^{op}/Csf1^{op}) mice *i.e*., histopathological progression and metastasis of mammary tumors in Csf1^{op}/Csf1^{op} PyMT mice was delayed [140]. Later, others have found reduced tumor outgrowth of gastric cancer in M-CSF-deficient mice [141] and reduced tumor outgrowths of neuroblastoma xenotransplants and human MCF-7 mammary carcinoma

cell xenografts using antisense oligonucleotides and small interfering RNAs directed against mouse CSF-1 [142,143]. Pharmaceutical targeting of the CSF-1/CSF-1R pathway, including antibodies against the receptor (anti-CSF-1R), the ligand (anti-CSF-1), and inhibitors of the tyrosine kinase domain of CSF-1R (such as BLZ945), have predominantly demonstrated anti-tumor effects in several preclinical models [144-156]. Interestingly, in some studies, such as in a mouse model of glioma, treatment with inhibitors of CSF-1R did not deplete TAMs but instead altered macrophage polarization, which resulted in blocked glioma progression [145]. Different findings upon CSF-1/CSF-1R pathway targeting are likely caused by different cancer (sub)types and cancer mouse models with their different TME and the use of a different type of inhibitor, doses, and timing of treatment initiation.

These preclinical studies have laid the foundation for the development and clinical testing of CSF-1R signaling pathway inhibitors [137,157-161]. Ries *et al* were the first to demonstrate the clinical benefit of a macrophage-targeting agent: the humanized anti-CSF-1R IgG1 monoclonal antibody (RG7155) reduced macrophages in tumor tissues, which resulted in clinical objective responses in 83% of patients with diffuse-type giant cell tumor [157]. In light of recent clinical trials for CSF-1R blockade therapy in cancer treatment, there are still several questions that need to be addressed. For example, how do we predict sensitivity to CSF-1R inhibition? Immuno-phenotyping of TAMs in patients may be vital to find biomarkers that can predict sensitivity to CSF-1R blockade and facilitate personalized immunotherapeutic treatments. Furthermore, though immune cells are not under mutational pressure like cancer cells, bidirectional feedback between cancer cells and their microenvironment could induce resistance of the tumor microenvironment to immuno-modulation of CSF-1R targeting. In fact, a study showed that treatment with anti-CSF-1R or the CSF-1R kinase inhibitor GW2580 increased breast cancer lung metastasis in a breast cancer allograft model [162]. Neutrophil blockade using G-CSF-1R decreased anti-CSF1R-induced neutrophil influx in blood, tumor and metastasis-associated lung tissue and reduced metastasis in these mice [162]. Another study in a mouse model for glioma discovered resistance to CSF-1R inhibition in more than 50% of the mice that initially responded to CSF-1R inhibition [163]. The resistance was initiated through IGF-1R/PI3K signaling, driven by macrophage- derived IGF-1 [163]. These findings warrant that resistance to CSF-1R targeting needs to be taken into consideration. Furthermore, several studies have shown that TAMs presence was essential during therapies to elicit an anti-tumor

response [164-166]. As such, it will be important to understand whether macrophage depletion or repolarization is favored.

While preclinical experiments suggest that targeting TAMs, either by inhibiting pro-tumor macrophage function via depletion or by repolarization of macrophages, is an attractive anti-cancer approach, CSF-1R blockade alone has shown only marginal therapeutic benefit [137]. Therefore, current clinical and experimental- efforts are focused on finding the right combination partners for TAM targeting [137]. These optimally matched partners may vary from immune checkpoint blockades inhibitors [167], adoptive transfer [168 169], radiotherapy [170] to chemotherapy. The latter will be discussed in more detail in the next section [171].

1.7 The role of the immune system in chemotherapy response

Chemotherapy is frequently used to treat cancer patients. Although most tumors initially respond to chemotherapeutic drugs, tumors develop mechanisms of resistance to the treatment. Thus, it is urgently needed to investigate effective strategies to increase chemo-responsiveness and/or to prevent or eliminate chemoresistance. Cancer cell-intrinsic factors like resistance to apoptosis or overexpression of drug transporter proteins have been identified as causes of therapy resistance [172]. However, also cancer cell-extrinsic processes underlying poor chemotherapy response have been recognized [173-176]. In fact, an increasing amount of data reveals that both the adaptive and innate immune system play an important role in modulating the anti-cancer efficacy of chemotherapy [173,177]. There are many different types of chemotherapeutic drugs with different mechanisms of action, such as alkylating agents (*i.e*. cisplatin), anti-microtubule agents (*i.e.* paclitaxel), topoisomerase inhibitors (*i.e.* topotecan), anthracyclines (*i.e.* doxorubicin) and deoxynucleoside (*i.e.* gemcatibine). Besides differentially influencing cancer cells, these distinct cytotoxic drugs differentially affect immune cells, as has been observed in *in vitro* studies [95,178]. The influence of the immune system on chemo-responsiveness and/or chemo-resistance depends on the type of chemotherapeutic drug and dosing [179]. As such, immune cell depletion typically occurs with high-dose chemotherapy, while low-dose chemotherapy (also called metronomic) has immunomodulatory and anti-angiogenic effects [180]. Importantly, the interplay between chemotherapy and immune cells is bidirectional; *i.e*. chemotherapy can affect immune cells and the other way around, immune cells can affect chemotherapy efficacy**.** Various studies have elucidated that macrophages [181] and neutrophils [182-184]

counteract chemotherapy efficacy in certain cancer models. Similarly, combining chemotherapy with targeting treatments against MDSC's, B cells, Tregs or Th17, could as well be effective in certain cancer types [179]. On the other hand, $CD8^+$ T cell and DC functionality are necessary for a good chemotherapy response in several tumor cell line and tumor transplantation models [177]. Overall, numerous studies have illustrated the complexity of immunomodulation by conventional chemotherapeutics, which is highly context dependent. Hence, insights into the exact role of specific immune cell subsets in affecting the efficacy of chemotherapeutic drugs may contribute to the rational design of combinatorial therapies.

Chemotherapy response of tumor transplantation models is dependent on the adaptive immune system

The influx of high T cell numbers in multiple human cancers, including breast cancer, before chemotherapy treatment, has shown to correlate with improved chemotherapy response [185-190]**.** In line with these data, experimental studies in highly immunogenic tumor models, *e.g.,* cancer cell line allograft models and chemically- induced sarcomas, have indicated that T cells can contribute to the anti-cancer efficacy of certain chemotherapeutics [185-189]. As such, cytotoxic drugs, such as doxorubicin, oxaliplatin, cyclophosphamide, epothilone B, mitoxantrone, and melphalan lose their therapeutic efficacy on tumor cell line outgrowths in mice with a defective adaptive immune cell function, including *Rag*$^{-/-}$ mice [177,185-187]. The success of these chemotherapy treatments is dependent on the stimulation of an anti-cancer immune response through the induction of immunogenic tumor cell death (ICD) [191]. Chemotherapy-induced ICD starts with endoplasmic reticulum stress in dying cancer cells. This leads to phosphorylation of the signaling axis extracellular-signal-regulated kinase (PERK)-eukaryotic initiation factor 2α (eIF2α), which is required for the translocation of calreticulin to the plasma membranes of cancer cells that serves as 'eat-me' signals for DCs [185]. Next, nuclear protein high mobility group box 1 (HMGB1) from cancer cells in the extracellular space binds to TLR4 on DCs and triggers their functional maturation and facilitates antigen presentation [185]. Next, the active secretion of adenosine tri-phosphate (ATP) from dying neoplastic cells promotes the proteolytic maturation and release of pro-inflammatory cytokines such as IL-1β from DCs and stimulates the NLRP3 inflammasome [187]. In addition, anthracyclines also require the production of type I IFNs by malignant cells after activation of a TLR3-elicited signal transduction cascade [192]. At last, effective antigen cross-presentation by DCs results in the activation of $CD8^+$ T-dependent tumor-killing

responses[185-187]. Proof for the immunogenic cell death cascade is largely based on cancer cell line transplantation models and the immunogenic MCA fibrosarcoma model, in which tumor initiation on itself is already suppressed by host immunity [177,193]. As described previously, cancer cell line inoculation models do not accurately mimic *de novo* tumors [56]. *De novo* tumors are characterized by extensive local and systemic immunosuppression which may facilitate escape from immune control during chemotherapy. Indeed, a study in *PyMT* mice indicated that TAM-derived IL-10 indirectly prevented $CD8^+$ T cell-dependent tumor-killing responses to chemotherapy by suppressing IL12 expression in intra-tumoral DCs [194]. There is a need for more studies that use *de novo* tumor models to study the impact of components of the adaptive immune system on the therapeutic efficacy of different chemotherapeutic drugs. We hypothesize that poorly immunogenic tumors might benefit from chemotherapy in combination with immunosuppression inhibitors, for example macrophage-targeting therapies, to unleash cytotoxic T lymphocytes with anti-tumor reactivity.

Macrophages counteract chemotherapy response

Many preclinical studies have shown by direct targeting of macrophages that macrophages counteract the anti-cancer efficacy of chemotherapy [181,195,196]. Currently, clinical trials with CSF-1R signaling pathway inhibitors in combination with chemotherapies are ongoing in cancer patients [137]. To maximize the clinical success of such macrophage-targeting compounds various questions still need to be addressed. For instance, it is unclear whether the influence of macrophages on chemotherapy efficacy depends on the type of chemotherapeutic drug used. Understanding this will help to determine the optimally matched combination therapy. Also, resistance to CSF-1R targeting in a chemotherapy context needs to be considered. Furthermore, observations in human breast cancer patients indicate that intratumoral presence of high numbers of macrophages and low numbers of $CD8^+$ T cells is associated with poor neoadjuvant chemotherapy response [151,197]. Thus, to develop similar and more specific predictive markers for immune-modulation-based therapies, we need to know what the mechanisms are by which macrophages counteract chemotherapy.

It has been reported that macrophages counteract chemotherapy response through a variety of mechanisms, such as matrix deposition and/or remodeling, activation of angiogenesis or revascularization, reduction of chemotherapy delivery to tumors through modulating vessel leakiness, providing survival signals to tumor-initiating cells and perhaps most

importantly: suppression of cytotoxic T cell immunity [147,151,194,198-209]. Although the exact mechanisms of how macrophages counteract chemotherapy efficacy of certain tumor (sub)types remain to be evaluated, these mechanisms could reside in macrophage polarization. Evidence for this hypothesis comes from both *in vitro* and *in vivo* studies that have indicated that chemotherapy can modify macrophage polarization; either skewing macrophages to gain pro-tumor M2-like functions or anti-tumor M1-like functions. For example, treatment with cisplatin or carboplatin increased the potency of 10 different cervical and ovarian cancer cell lines to skew monocytes to M2-like macrophages *in vitro* [210] while docetaxel skewed macrophages to an M1-like phenotype in 4T1-Neu transplants [211]. Furthermore, in a recent *in vitro* study, it was found that reactive oxygen species (ROS) induced by paclitaxel upregulated PD-L1 expression in macrophages [212]. *In vivo* evidence comes from reports that have indicated that chemotherapy efficacy is linked with macrophage polarization [200,208,213,214]. For instance, a study in *K14-HPV16* mice showed that B cell-depletion changed the chemokine expression of macrophages, which resulted in an improved chemotherapy response due to activated $CD8^+$ T lymphocytes via CCR5-dependent mechanisms [213]. Furthermore, paclitaxel repolarized TAMs through TLR4 signaling toward an M1-like pro-inflammatory profile, which contributed to the antitumor effect of paclitaxel [214]. Thus, macrophage polarization might dictate chemotherapy efficacy and investigating the polarization status of TAMs during treatment with different chemotherapeutic drugs in different cancer (sub)types could contribute to the development of combinational therapies and the identification of predictive markers. What the mechanisms are by which TAMs integrate external signals and translate them into a transcriptional program following chemotherapy are unclear and should be under active investigation. In conclusion, TAMs are promising pharmacological targets, but we need to gain a better understanding of the interactions of anti-cancer therapies with the innate and adaptive immune system. TAM-targeting compounds could pave the way for a better precision medicine approach and innovative combinations of conventional therapies. However, it will be critical to consider individual tumor profiles (tumor type, mutation status and the immune profiles of the tumor) to match with the appropriate immunomodulatory intervention.

Scope of Thesis

Breast cancer is the most common type of cancer in women, representing 28% of all cancer cases (www.cijfersoverkanker.nl). 1 in 8 women is estimated to receive a breast cancer diagnosis during her lifetime (https://seer.cancer.gov/data/). Breast cancer is a heterogeneous disease, which consists of five molecular subtypes: luminal-A, luminal-B, basal, HER2 positive and normal breast-like. Chemotherapy is one of the main therapeutic modalities for breast cancer patients, however, response rates vary, and resistance occurs among patients. In the past few decades, it has become clear that the tumor microenvironment plays an important role in cancer development, progression and therapy response. To improve the success rate of current therapies and to develop novel (immune)therapies we need to have a better understanding of the mechanisms underlying the crosstalk between cancer and the immune system. The overall goal of the research described in this thesis is to investigate the role of the adaptive and the innate immune system in breast cancer progression, metastasis formation and chemotherapy response. To study this, we use two independent spontaneous mouse models of mammary tumorigenesis representing two different subtypes of breast cancer:

1.The MMTV-*NeuT* mouse model: Oncogenic signaling of the human epidermal growth factor receptor-2 (*HER2/neu* or *ErbB2*), a proto-oncogene that belongs to a family of transmembrane receptor tyrosine kinases, has been shown to play a major role in 15%–20% of breast cancer patients [215,216]. Overexpression of HER2, due to amplification of the *HER2* gene, is an adverse prognostic factor associated with poorly differentiated, high-grade tumors, metastasis formation, relative resistance to certain chemotherapy regimens and greater risk of recurrence [106,215]. Anti-HER2 therapies have dramatically improved survival [217]. MMTV-*NeuT* transgenic mice express a mutated form of the rat *c-erbB-2* (*neuT*) oncogene under control of the mouse mammary tumor virus (MMTV) promoter. These mice develop metastatic mammary carcinomas within 4 months of age, which resemble human $HER2^{+}$ breast cancer [218].

2. The *K14cre; Cdh1$^{F/F}$; Trp53$^{F/F}$* mouse model: Invasive lobular carcinoma (ILC), a histotype within luminal A breast cancer, is the second most common histotype of breast cancer after invasive ductal carcinoma and accounts for 5%–15% of all breast cancer cases [219-221]. ILC is often difficult to diagnose and less responsive to conventional chemotherapy [221-223]. Conditional

K14cre; Cdh1$^{F/F}$; Trp53$^{F/F}$ mice have combined stochastic loss of E-cadherin and p53 in mammary- and skin epithelial cells, resulting around 6-8 months of age in the development of skin tumors and metastatic, invasive mammary carcinomas which resemble human ILCs [222].

Chapter 1 summarizes the current understanding of the paradoxical roles of adaptive and innate immune cells, with a focus on macrophages, in tumorigenesis and chemotherapy response. Here the limitations of our knowledge of current strategies targeting macrophages are being discussed. In addition, we propose that a better mechanistic understanding of the interactions between cancer cells and the immune landscape per cancer subtype, and upon therapy response is needed. In particular, more knowledge is needed about how cancer cell-intrinsic features shape the crosstalk with the immune system. These insights will provide a basis for the design of personalized immune intervention strategies for patients with cancer.

The role of the adaptive immune system in mammary tumorigenesis is only beginning to be understood. Different cancer types and subtypes have been shown to be regulated differently by the adaptive immune system [98,102-104]. In **Chapter 2** we elucidate the functional significance of the adaptive immune system during (pre-) malignant progression and pulmonary metastasis formation in MMTV-*NeuT* transgenic mice. By genetically eliminating the adaptive immune system from the transgenic MMTV-*NeuT* mouse model via intercrossing with *Rag2$^{-/-}$* mice, lacking B and T lymphocytes [91], we demonstrate that spontaneous HER2-driven mammary tumorigenesis and metastasis formation are neither suppressed nor promoted by the adaptive immune system. As outlined in detail in **Chapter 2**, the outcome of the interplay between the adaptive immune system and tumors is not only dependent on the tissue context, but also on the genetic pathways underlying tumor initiation and tumor maintenance.

Based on studies using tumor cell line transplantation models it has been reported that the adaptive immune system contributes to the therapeutic efficacy of certain chemotherapeutics via a process referred to as immunogenic cell death [177]. A major limitation of tumor models based on inoculation of cancer cells is that they do not resemble *de novo* tumors with co-evolving tumor-host interactions and an immunosuppressive microenvironment [56]. In **Chapter 3** we explore whether the adaptive immune system influences chemotherapy response of established spontaneous

mammary tumors in MMTV-*NeuT* and *K14cre; Cdh1$^{F/F}$; Trp53$^{F/F}$* mice. We intercrossed both mouse tumor models with T and B cell–deficient *Rag$^{-/-}$* mice and treated tumor-bearing mice with various conventional chemotherapeutic drugs. In both mammary tumor models, the lack of T and B cells did not affect chemotherapy response. These data highlight that the role of the endogenous adaptive immune system in chemotherapy response might not be as crucial as proposed previously when using tumor cell line transplantation models [177].

Currently, clinical trials testing various compounds targeting macrophages are ongoing in cancer patients [137]. However, essential questions still need to be addressed to maximize the clinical success of compounds that inhibit macrophage function. For example, it is unclear whether the influence of macrophages on the anti-cancer efficacy of chemotherapy depends on the type of chemotherapeutic drug used and what the exact mechanisms are by which these agents can increase the sensitivity of breast cancer to chemotherapeutic drugs. In **Chapter 4** we demonstrate that macrophage targeting through CSF1R blockade acts synergistically with platinum-containing drugs, but not with docetaxel, by inducing an intratumoral type 1 interferon response. The elimination of neutrophils further enhanced the beneficial effect of cisplatin and CSF1R blockade due to the activation of anti-tumor immunity.

Finally, in **Chapter 5,** the findings of this thesis are summarized and put into context of the current literature. I also discuss how the field may move forward to use immunomodulatory compounds in the clinical setting.

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