Immune cell complexity in the tumor microenvironment of breast cancer

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CHAPTER 7

Discussion

Myeloid cells represent an important component of the tumor microenvironment (TME) as they have been shown to contribute to tumor progression and impact therapy response. As a result, immunomodulatory agents targeting myeloid cells are currently being evaluated in clinical trials. To maximize the success of these compounds and to develop new therapeutic approaches, understanding the biology of the targeted cell and the effects of these drugs is of utmost importance. Here, I will discuss important aspects of immune cell complexity, focusing on the phenotype of tumor-associated macrophages (TAMs), the impact of macrophage-targeting drugs on chemotherapy response, approaches to achieve anti-tumor immunity in poorly immunogenic tumors and the challenges to study neutrophils.

The challenging definition of macrophage functional identity

Initially, macrophages were described to have different polarization states: they could either be classically activated or alternatively activated. These terms originated from studies in the early 1990s which showed that macrophages cultured in the presence of IL-4 displayed a different phenotype as compared to ones cultured in the presence of IFNγ, which showed an "alternative" phenotype[1]. Years later, in 2000, the observation that macrophages isolated from different mouse strains had a different arginine metabolism which influenced lymphocytes to produce either Th1 or Th2 cytokines, led to the M1/M2 dichotomy[2]. Although the authors, who described these macrophage phenotypes in the original report, discussed the possibility of a "continuum of phenotypes between M1 and M2 macrophages", the M1/M2 dichotomy dominated the macrophage field for almost two decades. However, this binary generalization is rather limiting and unlikely to occur in a complex *in vivo* system. In fact, macrophages are one of the most plastic cells of the immune system. Based on *in vitro* experiments we now know that macrophages can change their phenotype based on various stimuli they receive in the environment[3]. This dynamic behavior of macrophages is critical for the protection against several insults for which a quick adaptation and response is necessary. To solve the complexity of macrophage definition, it was proposed that, besides an accurate description of the methodology used, studies should define these cells by the stimulus they receive[4]. For example, macrophages stimulated *in vitro* with IL-4 would be termed M(IL-4). Although this terminology would be useful for *in vitro* experiments, this nomenclature based on the type of activator would be challenging for *in vivo* studies, as multiple and unforeseeable mediators might be responsible for shaping the macrophage phenotype. Because of the effects that different stimuli have on the phenotype and function of macrophages, the local environment where they reside plays an important role in shaping their phenotype. An elegant study by Lavin *et al*. showed that transplantation of differentiated peritoneal macrophages into the alveolar cavity resulted in a transcriptomic switch that closely resembled the profile of lung macrophages[5]. These data reiterate the plasticity of fully differentiated macrophages that can reshape and acquire a different transcriptomic identity based on the tissue environment.

The M1/M2 terminology also became popular in the cancer field where it was used to define TAMs as pro-tumoral (M2) or anti-tumoral TAMs (M1)[6]. Interestingly, single-cell RNA-sequencing of immune cells isolated from human breast tumors revealed

the co-existence of M1 and M2 signatures in the same macrophage[7], strengthening the concept of a continuum of activation states as opposed to the static M1/M2 paradigm. This concept of plasticity implies that distinct TMEs differently affect TAM phenotype. To test this hypothesis, in **chapter 3** of this thesis, we isolated TAMs from the mammary tumors of two genetically engineered mouse models (GEMMs), in which macrophages and cancer cells co-evolve mimicking the human situation. While *K14cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* (KEP) mammary tumors[8] resemble an invasive lobular carcinoma (ILC) histotype of breast cancer, the *MMTV-NeuT* (NeuT) tumors, which overexpress an activated form of *neu*, model HER2^{+} breast cancer[9]. Interestingly, the transcriptomic profile of TAMs from the KEP and the NeuT model were found to be more similar to macrophages of the mammary gland compared to macrophages that reside in other tissues, like the spleen and bone marrow, indicating that the tissue of origin dictates macrophage identity. Indeed, we observed that these TAMs have a common signature probably related to the mammary tissue where they arose. Nevertheless, we also identified a unique signature for both TAMs, strengthening the concept that distinct tumor milieus differently influence the macrophage transcriptomic profile. Several reasons may explain the different macrophage reprograming, including the genetic make-up of the tumor, the histological subtype of the tumor, and/or the presence of different stromal characteristics like fibroblasts and collagen fibers. Expanding this analysis to additional GEMMs for mammary tumorigenesis could identify tumor characteristics that associate with similar TAM transcriptomic profiles. In addition, it would be interesting to perform the same computational analysis on macrophages isolated from different tumor stages to obtain a dynamic overview on how particular features are gained, lost or changed during tumor growth. Importantly, with our analysis we only focused on the *inter*-tumoral heterogeneity of macrophages between tumor models. It may also be interesting to address the *intra*-tumoral diversity of macrophages by single cell RNA-sequencing and spatial transcriptomic[10], in order to assess how the macrophage profile changes based on location (*e.g.*: centre, rim, vicinity to necrotic areas) or contact with neighbouring cells.

Although RNA sequencing is a powerful tool it is important to consider that these results on TAM phenotype provide only descriptive data and not functional information. Nevertheless, the potential functionality of these cells could be extrapolated from transcriptomic analysis. For example, TAMs from the NeuT model, and to a lesser extent also from the KEP model, upregulate genes involved in angiogenesis-related processes, suggesting that these macrophages may support tumor growth by secreting growth factors and promoting angiogenesis. In addition, gene expression data may also provide some insights on the biology of TAMs. In preclinical models, TAMs were demonstrated to originate from circulating CCR2^{+} monocytes in the *MMTV-PyMT* mouse model for breast cancer[11], while in the *MMTVneu* model, which bears the unactivated form of HER2, the increased accumulation of TAMs in the tumors was the result of major local proliferation[12]. In **chapter 3** of this thesis, we observed that TAMs isolated from the KEP model are highly proliferative compared to TAMs from the NeuT model, suggesting that in KEP tumors TAMs may originate from local proliferation rather than differentiation of monocytes. These data highlight that different, and probably multiple, mechanisms can give rise to TAMs and future studies should assess whether the transcriptomic

profile of macrophages can determine their ontogeny.
In **chapter 3** of this thesis we show that the specific gene signature of TAMs from the KEP tumors associates with poor survival in two separate cohorts of ILC patients, while the specific gene signature of TAMs from the NeuT tumors fails to do so in the same groups of patients. These findings were quite surprising as our signature, derived from a pure TAM population, was applied to the transcriptome profile of bulk tumor samples containing multiple different cell types. This association may suggest that this novel set of genes derived from murine macrophages could have prognostic value. However, it is important to note that more studies should confirm the value of the KEP TAM signature in predicting outcome and response to specific type of therapies. For example, it would be interesting to assess whether patients with tumors enriched in this signature would benefit from the treatment with macrophage-targeting agents. In addition, it would be interesting to apply the core signature of TAMs from the NeuT model to gene expression data from $HER2^+$ breast cancer patients, and perform the same analysis. It should be noted that in other breast cancer models, two populations of intratumoral macrophages are found and distinguished based on CD11b expression[11,12]. Differently from the KEP tumors which contains only $CD11b^{high}$ macrophages (**chapter 5** of this thesis), NeuT tumors display both macrophage populations, although with some variability. To fairly compare these two models, only $CD11b^{high}$ macrophages were analyzed in our study. However, both macrophage signatures of TAMs from the NeuT model should be tested for their ability to predict survival in $HER2^+$ patients.
In conclusion our data, together with other studies, indicate that one unique TAM phenotype does not exist; rather the plasticity of macrophages to different stimuli determines a distinct phenotype in certain environments and not in others. This diversity in TAM phenotype may have consequences for the efficacy of macrophage-targeting drugs, as targeting particular TAM phenotypes might not be therapeutically beneficial.

Targeting inflammation as an anti-cancer strategy

The importance of gaining knowledge on the therapeutic effects of CSF-1R blockade
The acknowledgment of the TME as a crucial component for tumor establishment and growth[13] and the realization that myeloid cells often facilitate tumor development formed a rationale for developing approaches to clinically target these cells. TAMs represent an important target because 1) their presence within the TME strongly associates with poor prognosis across tumor types[14], 2) they are abundant in the majority of tumor types[15], and 3) preclinical data show that they contribute to tumor growth and metastasis by supporting angiogenesis, stimulating cancer cell proliferation and migration and suppressing anti-tumor immune responses[16-21]. Approaches to therapeutically target TAMs aim at depleting the pool of macrophages, blocking their recruitment or skewing their polarization towards an anti-tumor phenotype.
Thanks to the knowledge gained over the last 40 years on macrophage biology, blocking CSF-1/CSF-1R signaling pathway, which is essential for macrophage survival, denotes an attractive strategy to eliminate macrophages and suppress tumor growth. Several therapeutic approaches aiming at interfering with this

pathway have been developed, including antibodies against the receptor (anti-CSF-1R), the ligand (anti-CSF-1), and inhibitors of the tyrosine kinase domain of CSF-1R. Monotherapy treatment with CSF-1R inhibitors exert anti-tumor effects in several preclinical models[22-30], but not in others[31], including the KEP mouse model (**chapter 5** of this thesis). These differences might reflect the different function of TAMs elicited by the distinct TME, the biology of the tumor model, or may reflect the different experimental designs used, such as the type of inhibitor, tumor stage at the start of the treatment and/or doses. Because cancer patients are often treated with chemotherapy, many preclinical studies also evaluated the effect of CSF-1R inhibition in the context of chemotherapy. Targeting the CSF-1R pathway in various experimental tumor models enhances the cytotoxic efficacy of chemotherapy[24,25,31-34], including in studies where anti-CSF-1R monotherapy does not have any effect on tumor control[31]. Indeed, in **chapter 5** of this thesis we observed that anti-CSF-1R monotherapy does not affect tumor growth in the KEP mouse model of breast cancer. However, anti-CSF-1R synergized with platinum-containing drugs, *i.e.* cisplatin and oxaliplatin, resulting in prolonged tumor-specific survival. Besides blocking the main survival signaling pathway in macrophages which results in decreased TAM numbers in some tumor settings, the secondary effects of anti-CSF-1R treatment on the TME are not fully elucidated. A better understanding of the impact of anti-CSF-1R *in vivo* may explain the therapeutic benefits achieved in combination with chemotherapeutic drugs. In this regard, **chapter 5** of this thesis describes a novel mechanism-of-action of the antibody against CSF-1R in improving cisplatin response. Anti-CSF-1R treatment induces an upregulation of type I interferon (IFN) in the KEP mammary tumor which significantly enhanced the efficacy of cisplatin. However, the increase in type I IFN does not enhance the efficacy of a different type of chemotherapeutic agent, the taxane docetaxel. A possible explanation could be that the anti-CSF-1R-induced expression of type I IFNs sensitizes tumor cells to the mechanism-of-action of platinum-containing drugs, which damage the DNA, and not of taxanes, which inhibit mitosis. In addition, it is now widely acknowledged that cytotoxic drugs exert also immunomodulatory effects, as described in **chapter 4** of this thesis. Therefore, it might be plausible that chemotherapy-induced effects on immune cells might underline the therapeutic activity of CSF-1R inhibition and cisplatin. Although promising, the number of chemotherapeutic agents tested in this study is limited, therefore correlating the synergistic effects of anti-CSF-1R with certain classes of drugs is currently not possible. Future studies should expand the number of chemotherapeutic agents to be examined, including other taxane-based chemotherapies such as paclitaxel and DNA-damaging agents that do not belong to the platinum-containing drug family, including doxorubicin and cyclophosphamide which are commonly used in the clinic. In addition, it would be interesting to assess the effect of anti-CSF-1R in combination with cisplatin on metastasis. Our study showed that anti-CSF-1R alone does not affect the metastasis-specific survival in the KEP-based model of spontaneous breast metastasis. However, as with the primary tumor growth, the combination could prolong distant metastasis free survival.

Currently, immunomodulatory drugs blocking the CSF-1/CSF-1R axis are under clinical evaluation as a monotherapy or in combination with other therapies, including chemotherapy, for several types of solid tumors[35]. Although preliminary results of phase I and II clinical trials display good tolerability to the drugs, early

results on their anti-cancer efficacy describe low and variable clinical benefits in several types of tumors[35]. Currently there are clinical trials ongoing with anti-CSF-1R in combination with paclitaxel[36], another taxane-based drug like docetaxel used in our study. However, our data suggest that this class of drug might not be suitable to achieve a synergistic therapeutic advantage. Particularly, in light of our data showing that anti-CSF-1R treatment induces type I IFN response in cancer patients, choosing the optimal cytotoxic drug is of utmost importance to maximize the effects of CSF-1R targeting agents. Interestingly, of all the tumor types tested for response to CSF-1/CSF-1R-targeting drugs, only diffuse-type tenosynovial giant cell tumor showed very encouraging results with a response rate higher than 80%[35,37]. These tumors are characterized by overexpression of CSF-1 and high numbers of CSF-1R$^+$ macrophages[38]. Although it is thought that the clinical activity of CSF-1R-targeting drugs mainly rely on blocking this signaling pathway in recruited myeloid cells, cancer cells can also express the receptor[38], raising the question on the importance of targeting this pathway in malignant cells for the outcome of the therapy.

Although our data showed that CSF-1R blockade depletes intratumoral macrophages, we also describe that a small population of intratumoral F4/80$^+$ cells expressing high levels of IFNα resists anti-CSF-1R treatment. In an attempt to identify the origin of these remaining TAMs, we showed that circulating monocytes have the potential to infiltrate the tumor of anti-CSF-1R-treated mice. However, we cannot exclude that the IFNα-producing F4/80$^+$ cells are anti-CSF-1R therapy-resistant TAMs or repolarized TAMs. Notably, in this study we used a dosing schedule of CSF-1R blocking antibody that depletes 80% of TAMs; it would be interesting to investigate whether reducing the compound dose would avoid macrophage depletion without affecting the capacity of the agent to convert the TME into a type I IFN-enriched milieu. Furthermore, of all the strategies developed to inhibit the CSF-1/CSF-1R signaling pathway, we only tested the use of the anti-CSF-1R blocking antibody, raising the question whether other compounds would also induce a type I IFN response. Another study has shown that targeting CSF-1 with a neutralizing antibody in a preclinical model for pancreatic cancer reduced macrophage numbers while concomitantly skewing their polarization status into an anti-tumor activated state[30]. Interestingly, the remaining macrophages expressed IFNα, suggesting that targeting CSF-1 or inhibiting its binding to the receptor by anti-CSF-1R treatment may trigger type I IFN signaling. In addition, in a preclinical model for glioblastoma, treatment with a small molecule inhibitor of CSF-1R did not deplete TAMs, due to tumor-derived factors that sustained their survival, but repolarized them towards a tumor-inhibiting state that resulted in improved survival[27]. Unfortunately, IFNα expression was not assessed in these macrophages. The molecular mechanisms that induce type I IFN expression in cisplatin/anti-CSF-1R-treated mice are still unknown. However, because type I IFNs are stimulated by pattern recognition receptors (PRR), I hypothesize that the newly recruited monocytes or the remaining TAMs scavenge debris, RNA or DNA released from anti-CSF-1R-mediated macrophage cell death or dying cancer cells that triggers IFNα expression.

Another aspect that was not addressed in **chapter 5** of this thesis is whether newly recruited or remaining macrophages in peripheral organs also start expressing type I IFNs and whether their overall phenotype is altered affecting their function. Beside reducing intratumoral macrophages[28], CSF-1R-targeting drugs also decreases the

number of skin macrophages in patients[39] and resident macrophages of other organs, like liver and colon, in cynomologus monkeys[28]. Data from CSF-1R-null mice revealed that tissue-resident macrophages of organs like skin and liver are depleted, while macrophages from organs such as spleen and lung are not, indicating that in these tissues other growth factors are involved in macrophage survival[40,41]. These data suggest that, if IFNα-producing macrophages derive from newly recruited monocytes, anti-CSF-1R treatment may result in monocyte influx in organs in which tissue-resident macrophages rely on CSF-1R signaling. On the other hand, *in vitro* treatment of bone marrow-derived macrophages with anti-CSF-1R showed an increase in IFNα expression, suggesting that macrophages in those organs where CSF-1R inhibition does not decrease macrophage number, may also be affected by anti-CSF-1R treatment. However, whether the recruited monocytes or resident-macrophages in peripheral organs produce type I IFN and whether IFNα prevents, in combination with cisplatin, metastasis formation still needs to be elucidated.

A fine balance between type I IFN-mediated immune-stimulation and immune-suppression

Our study demonstrates that it is pivotal to induce the adequate type of inflamed TME, *i.e.* type I IFN-enriched, in order to boost the efficacy of platinum-containing drugs in the KEP model. The discovery of type I interferon (IFN) dates back to 1957 when Isaacs and Lindenmann identified factors released upon exposure of cells to heat-inactivated influenza virus that protected or "interfered" with the replication of live viruses[42]. In humans, the type I IFN family includes 13 different IFNα proteins (14 in mice), one single IFNβ protein and other, less studied IFNs, such as IFNε and IFNω[43]. Every cell can potentially produce type I IFNs upon activation of PRRs, like toll-like receptors (TLRs) and the cyclic GMP-AMP synthase (cGAS)/stimulator of IFN genes (STING) pathway, that recognize bacterial or viral components, including DNA and RNA. Subsequently, type I IFN molecules bind to their receptor that is composed of IFNAR1 and IFNAR2 subunits, either in a heterodimer or in an IFNAR1 homodimer[43]. Upon binding, IFNs activate the kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) which phosphorylate STAT1 and STAT2 to promote the expression of type I IFN-stimulated genes (ISGs). Type I IFN signaling is well known for its role in host defense against viruses and most of our knowledge to date derives from infection studies[43]. More recently several studies have addressed the role of type I IFN in cancer settings. Blocking type I IFN signaling by knocking-out IFNAR1 or by antibody-mediated blockade of IFNAR1 results in increased tumor development in several tumor models, including tumor cell inoculation models, methylcholanthrene (MCA)-induced sarcoma model and dextran sodium sulfate/azoxymethane (DSS/AOM)-induced colitis-associated tumorigenesis[44-48]. Type I IFNs can have direct effects on tumor progression, by inducing apoptosis or blocking proliferation in cancer cells, by inhibiting angiogenesis, or indirectly by stimulating immune cells. Like during viral infections, also in a cancer setting type I IFNs exert their main immunomodulatory effects by activating DCs and stimulating antigen cross-presentation resulting in T cell activation and consequently tumor rejection[49,50]. Type I IFN signaling is also essential for the function and survival of cytotoxic T cells[51] and NK cells[47]. Indeed, experiments with IFNAR1 KO bone marrow chimeras and tumor transplantation in IFNAR1 deficient hosts revealed that IFNAR1

signaling in hematopoietic cells is critical for engaging anti-tumor immunity[44,50]. In addition, downregulation of the transcription factor interferon regulatory factor 7 (IRF7), which induces the transcription of type I IFNs and other ISGs, and its targets in 4T1 breast cancer cells fosters metastasis to the bone through immune escape[52]. Finally, sustained activation of type I IFN signaling in chemotherapy-treated breast cancer cells induced dormancy in a T cell-dependent manner[53]. Interestingly, the dose of type I IFN seems to play a role in dictating the mechanisms by which tumors are rejected; low doses induce a T cell-mediated anti-tumor responses[54], while high doses exert anti-angiogenic effects[55]. The role of type I IFNs in hindering cancer progression raises the question on their role in therapy response. Studies have described that radiotherapy and some chemotherapeutic drugs, like anthracyclines and cyclophosphamide stimulate type I IFN production in preclinical cancer models and patients[56-59]. Importantly, the secretion of type I IFNs are critical for the therapeutic efficacy of radiotherapy and anthracyclines as their anti-cancer efficacy is lost upon blockade of type I IFN signaling[56,57,59]. Differently from these studies, **chapter 5** of this thesis shows that neither cisplatin nor docetaxel alone stimulate type I IFN expression in the KEP model. In line with this, blockade of type I IFN signaling does not affect cisplatin response. Only the synergistic effect of the cisplatin/anti-CSF-1R combination is dependent on anti-CSF-1R-induced type I IFNs. The difference in the effect of chemotherapy in inducing type I IFNs may be due to the type of chemotherapy used, the tumor model or the different composition of the TMEs.

Our work still leaves a question open: how does type I IFN exert its anti-cancer efficacy in cisplatin/anti-CSF-1R-treated mice? Our data only partially answer this question. We observed that the activity of type I IFN in combination with cisplatin is independent of $CD8^+$ T cells, as the depletion of these cells did not influence the survival of cisplatin/anti-CSF-1R-treated mice. Additional studies are required to assess the role of other cytotoxic cells, such as NK cells. Our *in vitro* results showed that IFNα might have a direct inhibitory impact on KEP cancer cells, however, the *in vivo* relevance of this is still unknown. Notably, we only tested one IFNα subtype, IFNα1, which exert inhibitory effects on KEP cell line only at high concentrations. Despite the high sequence identity, the 14 IFNα molecules qualitatively differ in their potency against viral infections[60] and in their anti-proliferative effects on cancer cells[61]. It is possible that the combinatorial activity of multiple IFNα subtypes and also IFNβ might play a role in enhancing the cisplatin effect. In this regard, MDA MB231 breast cancer cells treated with cisplatin and IFNβ displayed cell growth inhibition and apoptosis[62]. Although we did not observe an increase in apoptotic cells number in cisplatin/anti-CSF-1R-treated KEP tumors, other cell death modalities like necrosis or necroptosis may occur. In addition, because both cisplatin[63] and type I IFNs[64] have been shown to induce senescence in cancer cells, it might be interesting to assess the number of senescent cells in cisplatin/anti-CSF-1R-treated tumors, as it may explain the absence of apoptosis and the reduced proliferation observed in these tumors.

Systemic administration of recombinant type I IFN is approved for haematological diseases and for melanoma at high-risk of recurrence after surgical resection, for which modest benefits were associated with intratumoral influx of DCs and T cells[65,66]. In breast cancer patients, type I IFN gene signatures have been shown

to correlate with increased bone metastasis-free survival or with metastasis-free survival in general[52,67,68]. However, administration of recombinant type I IFN in breast cancer and ovarian patients did not give the desired results because of the severe side effects and limited survival benefits[69-71]. These data suggest that a more physiological way to induce type I IFN production might promote better anti-cancer responses. In this regard, our study suggests that CSF-1R blockade may be used as a strategy to induce intratumoral type I IFNs. Importantly, we observed an increase in ISGs in the tumor of cancer patients treated with anti-CSF-1R compared to their baseline levels. Our data also implicate the use of agents that trigger type I IFN, like STING agonists[72], in order to enhance the anti-cancer efficacy of chemotherapy, bypassing the side effects of anti-CSF-1R on tissue-resident macrophages. Notably, STING agonists are now being tested in clinical trials[73-75].

Although type I IFNs are generally thought to be beneficial for anti-tumor immunity, prolonged activation of the pathway might lead to opposite effects. Indeed, like during infections, it is crucial that, after the initial inflammatory response against the pathogens, a resolution phase takes place to restore tissue homeostasis. In this regard, type I IFNs, which are important for the initial inflammatory response, also induce immunosuppression. For example in tumor-bearing and in non-tumor-bearing mice, type I IFNs can induce the expression of programmed death-ligand 1 (PD-L1), an inhibitory molecule that prevents T cell activation, IL-10, and indoleamine 2,3 deoxygenase (IDO), an enzyme involved in tryptophan metabolism that suppresses effector T cells[76-80]. In line with this, the therapeutic blockade of PD-L1 in combination with type I IFN treatment in cell line inoculation models resulted in better tumor control compared to mice that did not received the checkpoint inhibitor[80,81]. These data raise the question of whether the prolonged type I IFN signaling achieved in the KEP mice after CSF-1R blockade might induce immunosuppressive circuits. Indeed, we found that cisplatin/anti-CSF-1R treatment in KEP mice did not unleash an anti-tumor response. In order to engage anti-tumor immunity, we had to breach through the immunosuppressive layer by further targeting immunosuppressive neutrophils in this poorly immunogenic tumor model, resulting in a T cell dependent better tumor control and extended survival. RNA-sequencing analysis on intratumoral neutrophils isolated from cisplatin/anti-CSF-1R-treated mice displayed a pronounced type I IFN signaling compared to neutrophils in cisplatin/control antibody-treated mice. These data indicate that neutrophils signal through type I IFN receptor in the TME, however whether this signaling promotes immunosuppressive abilities in these cells has not been elucidated. While some studies have suggested that type I IFNs can induce anti-tumor properties in neutrophils[82], studies in chronic infections report that a type I IFN transcriptional signature in neutrophils in malaria-infected hosts and in patients with active tuberculosis correlated with tissue damage and disease pathogenesis[83,84]. It is possible that the type of IFN molecules, the set of ISGs expressed, the abundance of the receptor on cell surface and the duration of type I IFN signaling may all play a role in determining neutrophil function in different settings. Indeed, we observed that type I IFN-producing macrophages express higher levels of PD-L1 upon anti-CSF-1R treatment, raising the question of whether it is induced by IFNα in an autocrine mechanism occurring to resolve the inflammatory responses. In conclusion, we showed that although type I IFN is important to enhance the anti-cancer efficacy of cisplatin, future studies should

address whether sustained type I IFN signaling can be detrimental in cisplatin/anti-CSF-1R-treated mice.

Targeting neutrophil-derived immunosuppression

Because of the immunosuppressive function of neutrophils in the KEP model, **chapter 5** of this thesis suggests that targeting neutrophils may be an attractive therapeutic option for cancer therapy. Indeed, a high neutrophil-to-lymphocyte ratio in the circulation of several cancer types is linked to poor prognosis in patients[85]. However, total depletion of neutrophils is not desirable as it might increase the risk of serious opportunistic infections. Current approaches under clinical evaluation aim at blocking neutrophil migration by targeting CXCR1 and CXCR2, two chemokine receptors important for neutrophil recruitment in the tumor bed[86,87]. Other attractive approaches for inhibiting neutrophils include targeting the IL1β-IL-17-G-CSF inflammatory axis that leads to neutrophil expansion in several models, including the KEP model[88]. Importantly, drugs targeting this pathway are already clinically approved for the treatment of other inflammatory conditions like psoriasis, suggesting the possibility of a quick and easy transfer of these drugs to the treatment of cancer[89]. Future clinical studies should assess the efficacy of these neutrophil-targeting agents in patients with neutrophil expansion in combination with anti-CSF-1R treatment or type I IFN-stimulating drugs and chemotherapy.

Neutrophils can exert immunosuppressive functions by several mechanisms[90]. For example, neutrophils express enzymes, such as arginase I (ARG1) and IDO, that consume amino acids important for T cell function and survival like L-arginine and tryptophan, respectively[91,92]. Also, the release of reactive oxygen species and nitric oxide by the activity of ARG1 and inducible nitric oxide synthase (iNOS) inhibits the ability of T cells to recognize the specific peptide by affecting the T cell receptor (TCR) conformational flexibility[93]. Although our group has previously reported that in the KEP-based model of spontaneous breast cancer metastasis immunosuppressive and pro-metastatic neutrophils express high levels of iNOS[88,94], additional studies are required to elucidate the mechanisms employed by neutrophils to prevent an anti-tumor immune response in cisplatin/anti-CSF-1R-treated mice.

One proposed advantage of therapies targeting the immune compartment of the TME is that, differently from cancer cells, these cells are genetically stable cells, thus less prone to acquire therapy resistance. However, considering the plasticity and versatility of the immune system, resistance mechanisms to immunomodulatory drugs may derive from compensatory inflammatory cues. In several types of tumor models, macrophage inhibition triggered an influx of neutrophils with tumor-supporting functions in the TME that reinstates tumor progression[95-98]. Some of these studies showed that neutrophils compensate for the depletion of TAMs by employing the same pro-tumoral mechanisms of TAMs, such as production of metalloproteinase-9 and activation of the PI3K signaling pathway to induce angiogenesis and immunosuppression[96,97]. Differently from these studies, our work suggests that upon CSF-1R inhibition, neutrophils do not take over macrophage activities, but, unlike macrophages, exert immunosuppressive functions. The therapeutic efficacy of targeting macrophages and neutrophils in cisplatin-treated KEP is mediated by inducing type I IFNs by intratumoral macrophages and by unleashing anti-tumor responses.

The challenge to study neutrophils in cancer

Similar to macrophages, neutrophils are highly plastic cells which dynamically adapt to changes in the environment. Reflecting the M1/M2 binary definition, it was proposed that also tumor-associated neutrophils could be described as N1 or N2 with anti- and pro-tumoral properties, respectively[99]. TGFβ was found to be the key molecule to skew neutrophils towards a N2 phenotype[99]. G-CSF is another mediator known to induce pro-metastatic neutrophils with immunosuppressive or pro-angiogenic functions[88,100-103]. Conversely, IFNβ has been reported to shift neutrophil profile towards an N1 phenotype *in vivo*[104]. *In vitro* treatment of neutrophils with a cocktail of cytokines including GM-CSF and IFNγ has been shown instead to induce the generation of neutrophils with an antigen-presenting ability[105]. Interestingly, a subset of neutrophils with antigen-presenting abilities was also found in lung cancer patients[106]. These data suggest that different stimuli may sculpt neutrophils in unique ways. Like for macrophages, a static binary state of neutrophil activation is unlikely to exist; rather, their functional identity is dynamically and continuously determined by stimuli in the environment. As a consequence, the function of neutrophils in distinct TME may differ. Although the majority of the studies demonstrate the pro-tumorigenic and pro-metastatic role of neutrophils, others report tumor-inhibiting properties[100]. Neutrophil depletion is the main strategy used by these studies to identify the function of these cells. However, current approaches are either non-specific (*e.g.*: anti-GR1 antibodies or CXCR2 antagonists) or with non-durable effects (*e.g.*: anti-Ly6G antibody or genetic model based on administration of diphtheria toxin[107]). To circumvent these issues, **chapter 6** of this thesis describes a novel mouse model for the conditional and reversible depletion of neutrophils. This model, called hMRP8-ATTAC, expresses caspase 8 fused to the FKBP domain under the control of the human MRP8 promotor, which is mainly active in neutrophils. The model relies on the administration of a chemical dimerizer which binds with high affinity to two FKBP domains, leading to the dimerization and activation of caspase 8 and resulting in apoptosis[108]. Despite the high expression of the transgene mainly in neutrophils and their reduction after dimerizer injection, we did not observe neutrophil depletion in tumor-bearing hMRP8-ATTAC mice. The challenge of depleting neutrophils is that they are relatively short-lived, they are continuously produced in the bone marrow and ablating neutrophils activates feedback mechanisms to increase granulopoiesis[100]. In tumor-bearing hosts, this phenomenon is amplified, as tumors induce high levels of G-CSF pressing the bone marrow to release new neutrophils[88]. The continuous neutrophil production and the fact that the dimerizer has a half-life of approximately 5 hours, might explain why we did not observe neutrophil ablation in tumor-bearing hMRP8-ATTAC mice. Future studies should assess whether a more constant dimerizer treatment would be more successful in reducing neutrophil numbers. In this regard, it would be interesting to investigate a dimerizer formulation to be supplied in the animal chow or in the drinking water.

Besides total ablation of neutrophils, future studies should also focus on specific gene deletion in neutrophils. For example, it was discovered that the MET proto-oncogene in neutrophils is important for their chemotaxis and anti-tumoral and anti-metastatic effects[109]. The hMRP8;Cre mouse model, in which the cre recombinase is

expressed under the control of the neutrophil-specific promotor, could be crossed with a mouse model bearing LoxP-flanked genes of interest, resulting in neutrophil-specific gene knockouts. However, the generation of these transgenic mice is time and cost-consuming. A strategy to accelerate genetic studies in neutrophils could be the *in vitro* manipulation of bone marrow progenitors in order to obtain knocked-out or knocked-down genes specifically in neutrophils followed by bone marrow reconstitution with these cells, allowing for testing in parallel the function of multiple genes of interest. These types of experiments may be used to answer some of the questions described above. For example, by knocking-out IFNAR1 specifically in neutrophils, it would be interesting to assess the effect of type I IFN signaling in the immunosuppressive abilities of neutrophils in cisplatin/anti-CSF-1R-treated mice.
In conclusion, future studies should aim at generating new *in vivo* tools for the study of neutrophils, in order to obtain a better understanding of the role played by neutrophils in different aspects of tumor biology.

Concluding remarks

The TME is a complex network in which several cell populations exert a variety of functions that can favor or limit tumor growth and progression. This thesis focuses on the complexity of myeloid cells in the TME, highlights the need to better understand the effects of immunomodulatory agents and proposes the design of combinatorial strategies targeting several aspects of the TME for the treatment of breast cancer. In particular, our data suggest that creating a favorable TME and targeting immunosuppressive cells are two steps necessary to engage anti-tumor immunity in breast cancer. In addition to targeting pro-tumoral immune cells or creating an advantageous TME for immune-stimulation, directly stimulating the anti-tumor cytotoxic activity of T cells represents an attractive strategy against cancer. One of the most studied approaches is the use of immune checkpoint inhibitors, which are blocking antibodies directed against molecules that inhibit T cell function, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) on T cells, or against one of its ligands, PD-L1, expressed by a variety of cells including myeloid cells and tumor cells[110]. The use of checkpoint inhibitors in clinical trials showed remarkable results in several types of cancer including melanoma, non-small cell lung carcinoma, Hodgkin's lymphoma and bladder cancer[111-115]. The successes of this therapeutic approach resulted in the selection of cancer immunotherapy as the breakthrough of 2013 by the journal *Science*[116] and the award of the Nobel Prize in Physiology or Medicine 2018 to James P. Allison and Tasuku Honjo for their discovery of CTLA-4 and PD-1, respectively[117]. However, not all tumor types respond equally to checkpoint inhibitors. In triple negative breast cancer (TNBC) patients, checkpoint inhibitors as monotherapies show a modest response rate that varies between 5 and 30%[118], raising the possibility that a more immunosuppressed TME and/or a lower mutational burden, as compared to other tumors types, contribute to the poor response. Further evaluation of the efficacy of checkpoint inhibitors in combination with conventional chemotherapy in breast cancer patients is currently ongoing. A phase III clinical trial in TNBC patients, showed promising results of anti-PD-L1 therapy in combination

with paclitaxel, especially in the subgroup of patients that expresses PD-L1[119]. In addition, the immunomodulatory properties of low dose chemotherapy are also appealing to be exploited in combination with immunotherapy. In this regard, a clinical trial at the NKI led by Dr. Marleen Kok in metastatic TNBC patients is evaluating the effects of five different conventional therapies, including low dose chemotherapy and radiotherapy, in conditioning the tumor microenvironment to the following treatment with checkpoint inhibitors[120,121]. In addition to conventional therapies, the efficacy of checkpoint inhibitors is currently under evaluation in combination with CSF-1R-targeting agents[35]. Results from these clinical trials will provide further information on the importance of modulating the TME in order to achieve anti-tumor immunity.

Overall in my thesis I have explored the role of myeloid cells in conventional therapy response in breast cancer. My results suggest that suppressive cells are an important roadblock for successful anti-cancer therapy. Therefore, future therapeutic approaches, including immunotherapies, should include combinational therapies that counteract myeloid cells. Additionally, this thesis highlights the importance of considering the effects of conventional therapies on intratumoral cells other than cancer cells for treatment decisions.

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