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

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Discussion

Despite major advances in the treatment of breast cancer, metastatic disease remains largely incurable and is accountable for the vast majority of cancer-related mortality. The role of the immune system in regulating breast cancer progression is undisputed[1], but a duality in immune function exists: where one part of the immune system, when properly activated, can counteract cancer development and growth, the other part can be hijacked by tumors to aid progression. The balance between these two functions determines whether the immune system promotes or impairs cancer progression. With this thesis, I have attempted to shed light on some of the determinants of inflammatory processes in cancer progression, metastasis and immune-based treatment response. The main questions posed in this work are: 1) What is the role of the genetic makeup of tumors in cancer-associated inflammation and how can this be used to improve treatments?; 2) How can systemic inflammation, most importantly neutrophilia, be exploited therapeutically?

With the work described in **chapters 2 – 4**, we propose that the tumor genetic makeup can dictate the composition of immune landscapes, the activation of metastasis-promoting inflammation and can be used to tailor immunotherapies. We argue that linking these two fields, immunology and genetics, in the context of cancer may prove valuable for clinical utilization. Understanding how the tumor immune microenvironment is shaped by common drivers of tumorigenesis can lead to the identification of targetable molecules in patients bearing tumors with specific genetic aberrations. Moreover, these genetic aberrations can serve as biomarkers to guide existing (immunotherapeutic) treatments. **Chapter 5** details an experimental method that can be used to dissect tumor-immune crosstalk in genotype-immunophenotype studies, which was extensively applied in **chapters 3** and **4**. In **chapters 6 – 8**, we provide insights into a potential new recruit to the immunotherapeutic arsenal: neutrophils. These chapters outline the impact of these cells on cancer progression and provide a glimpse of their tremendous diversity and plasticity.

In this final chapter, I will discuss the implications of the work presented in this thesis. I will review how inflammatory processes in the tumor microenvironment and systemically can promote tumor progression and metastasis. Focusing on neutrophils, I will discuss the diversity of this cell type in cancer and what should be considered when attempting to utilize a cell type of such plasticity for therapeutic purposes. Furthermore, I will outline how cancer cell-intrinsic properties can shape the immune microenvironment, I will discuss potential therapeutic targets emerging from these findings, and I will share my views on how these types of genotype-immunophenotype studies may be improved moving forward. Lastly, I will examine how these findings can potentially be applied clinically, so that the insights from this thesis may set the stage for personalized immune-based therapies for cancer.

The balance between immunosuppression and immune attack in the tumor microenvironment and systemic immune milieu

When examining the immune response against cancer, it is important to consider the many elements determining such a response, as well as the counteracting mechanisms at play. Several checks and balances are in place in the immune system to ensure not only defense against a theoretically unlimited plethora of pathogens, but importantly also proper resolution after fending of such pathogenic attack. Resolution involves suppressing the cytotoxic effector cells and restoring perturbed tissue. This duality in immune function also exists in the tumor context. Whereas some immune cell subsets provide anti-tumor immune responses, others actively counteract these processes[2]. For breast cancer, this is reflected

by the observation that while presence of cytotoxic cells such as $CD8^+$ T lymphocytes or Natural Killer (NK) cells in tumors correlate with a favorable patient outcome, high levels of other immune cell types, such as neutrophils and macrophages, generally associate with poor prognosis[3-7]. This notion of tumor immune composition and quality, *i.e.* activation state, is a point of consideration when studying tumor immunity. Besides looking at immune influx, one must on the one hand examine the production of cytotoxic molecules and activation of cytotoxicity pathways, and on the other hand assess the immunosuppressive mechanisms at play.

Immunosuppression in the tumor microenvironment can consist of several layers. Firstly, immunosuppression is mediated by cellular components of the tumor-induced immune environment. Several cell types can be at play here, including regulatory T cells[8], cancer-associated fibroblasts[9], macrophages[10] and neutrophils (**chapter 6**). Secondly, soluble factors are also able to suppress cytotoxic immune cell function. These can be derived from immunosuppressive immune cells, but can also be directly produced by cancer cells. Proteins like IL-10 and TGF-β have profound effects on anti-tumor immunity and blockade of such molecules has been shown to improve immunotherapy responses in preclinical mouse models of cancer[11-13]. These and other soluble factors influence the tumor microenvironment, but also reach further than the primary site and influence the systemic immune milieu to regulate cells in the pre-metastatic niche (**chapter 3**). Thirdly, immunosuppression also occurs by the physiological properties of the tumor, such as oxygen levels, acidity (pH) and nutrient availability. Tumors often have an acidic, hypoxic and nutrient-poor microenvironment, which negatively affects anti-tumor immunity[14,15]. Normalization of these physiological properties, either by vessel normalization or altering cancer metabolism[16,17], may improve the efficacy of immunotherapeutics. The expression of immune checkpoint molecules, such as PD-L1[18], is also a key aspect impairing anti-tumor immune responses. Moreover, the host tissue in which the tumor is present is an important determinant of immunity in cancer[19]. Certain tissues, such as the brain, may be inherently devoid of certain immune cell types that are abundantly present in other anatomical locations, such as the lung. Lastly, the extracellular matrix in which cancer and immune cells reside must be considered, as this network of collagen, fibronectin and other macromolecules can render a tumor impenetrable for recruited immune cells.

Altogether, there is a diverse set of regulatory elements that can counteract anti-tumor immunity. In order to understand how to tackle immunosuppression, a holistic approach is necessary. As in any complex system, interfering with one aspect will have consequences for all elements. Compensatory mechanisms that are in place may interfere with therapies that aim to target immunosuppression. Depleting one myeloid cell type may induce recruitment of other cell types that take over the function of the depleted population[20-23]. Furthermore, cells that remain after incomplete depletion of a given cell population are often altered in phenotype, as was shown for neutrophils and macrophages[20,24]. Therefore, one cannot simply target one cell type that is counteracting anti-cancer therapies and expect every other cell to fall in line. Combination treatments, simultaneously targeting the immunosuppressive cells such as neutrophils and stimulating T or NK cell responses, would theoretically induce the most potent anti-tumor immune response. Understanding immune activation, immunosuppression and their complicated interplay in a holistic manner will ultimately help and improve immune-based therapies for cancer patients.

Hitting a moving target: neutrophil heterogeneity and plasticity

We have shown in **chapters 3, 6** and **8** that neutrophils play a key role in promoting cancer development and metastasis. To target neutrophils in cancer patients, it is important to consider their phenotypic plasticity. As we have discussed in **chapters 6** and **7** and expanded upon in **chapter 8**, neutrophils have the ability to constantly adapt to environmental changes, rendering one definition of 'neutrophil' challenging. There is a growing notion that neutrophils, despite their limited lifespan, can have remarkable phenotypic and functional heterogeneity in cancer and other disease entities. The nature of cancer-induced neutrophil diversity can be determined by their maturation state[25-32], their anatomical location[33,34], the tumor type in which they reside[35], the microbiome[36] and even circadian rhythms[37,38]. Even within one tumor type, several subsets of neutrophils have been shown to coexist[39,40]. Moreover, there is marked variability in the ability of neutrophils to form neutrophil extracellular traps (NETs), networks of extracellular DNA and cytolytic proteins expelled by these cells. NETs have an important role in promoting metastasis via mechanisms that range from trapping of metastasizing cancer cells[41], to protection of cancer cells from T cell attack[42], awakening of dormant cells[43] or increasing cancer cell motility[44]. Understanding the heterogeneity in the regulation of NETs will also be important for therapeutic targeting of neutrophils. Altogether, neutrophils wear many hats, but whether these different 'flavors' represent neutrophil subpopulations, with unique developmental trajectories and cellular programming, or different activation states, remains a matter of debate. This diversity can have an assortment of functional consequences, as immunosuppressive capacities, metabolic rewiring, effector molecule production and cell surface protein expression ultimately determine the impact of neutrophils on disease progression, as well provide targets for potential therapeutic targeting.

Neutrophil-redirecting therapies

When targeting neutrophils, it is important that such strategies do not deplete neutrophils, as the resulting neutropenia can render patients highly susceptible to infection and thus be very toxic. Indeed, such a phenomenon is observed in chemotherapy-induced neutropenia, which is potentially life-threatening. Rather than depletion, limiting tumor-induced neutrophil recruitment by targeting chemokine receptors expressed by neutrophils is presently under investigation. For instance, targeting of CXCR2, widely examined in preclinical and clinical studies to inhibit cancer-induced neutrophilia in cancer, has been shown to potently limit neutrophil recruitment and thus improve anti-tumor T cell responses[45-47]. Problematic in neutrophil recruitment-based therapies is that targeting an ever-changing entity such as neutrophils will be like hitting a moving target. CXCR2 inhibitors may not target immature neutrophil subsets, as immature neutrophils have much lower CXCR2 expression than fully differentiated neutrophils[30,32,48]. And these immature neutrophils are abundantly present in mouse tumor models and cancer patients[25-31]. It may therefore be important to target only the tumor-promoting group of neutrophils, rather than targeting recruitment of neutrophils. In **chapter 8**, we reasoned that antibody-mediated targeting of cKIT, the tyrosine kinase receptor expressed on immature neutrophils, may impair only this immature subset. We showed that anti-cKIT treatment in mice bearing *K14-cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* tumors does not deplete neutrophils from the circulation, but it limits development of metastatic disease. Although we could not yet discern whether the effect of anti-cKIT treatment was due to targeting of cKIT-expressing neutrophils alone, it may be of interest to carefully examine targetable cell surface proteins that tumor-promoting neutrophils may uniquely express to target and/or

deplete these cells specifically. Conversely, besides removing tumor-promoting neutrophil subsets, patients may benefit from activation of tumor-killing neutrophils by therapeutic redirection of these cells towards an anti-tumor phenotype. This can be done by promoting their ability to kill cancer cell through antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis, for example using CD47-targeting agents[49,50]. However, phenotypic plasticity may still be an issue in this case. This notion argues that, rather than finding targetable cell surface molecules on neutrophils themselves, one could target the effector molecules that neutrophils use in tumor-promoting contexts, such as reactive oxygen or nitrogen species (ROS/RNS). This still may prove problematic, since for example nitric oxide has been shown to both inhibit $CD8^+$ T cells[25], but also kill tumor cells[51].

Alternatively, it may be more useful to target upstream regulators of neutrophil phenotypes to redirect them to negate their tumor-promoting characteristics. Targeting (tumor-derived) soluble factors has been shown to reprogram neutrophils in mouse cancer models. For example, TGF-β was reported to be instructive for tumor-promoting neutrophil phenotypes[26,52], whereas interferons could induce antitumoral functions in neutrophils[53], both of which can be targeted *in vivo*. Another key neutrophil-stimulating protein, G-CSF, has been shown to induce an immunosuppressive, tumor-promoting phenotype in mice[25,54]. Even in non-tumor-bearing mice, injection of recombinant G-CSF can induce expression of enzymes responsible for nitric oxide production, which is key in their immunosuppressive function[25]. GM-CSF, another important neutrophil growth factor, can activate immunosuppression in neutrophils via metabolic reprogramming, as discussed in **chapter 7**, and could therefore also serve as a potential therapeutic target. Caution needs to be taken when aiming to target these two neutrophil colony stimulating factors, for inhibition may also lead to severe neutropenia. One could speculate that proper dosing may result in phenotypic changes but not depletion. However, this concept requires further study.

Other promising examples of targetable neutrophil-activating proteins are IL-17, for which inhibitors are used in patients with psoriasis[55] and IL-1, inhibition of which has been used as therapy for inflammatory diseases such as rheumatoid arthritis[56]. These interleukins have been shown to promote systemic neutrophilia in mouse breast cancer models (described in **chapter 3** and ref. [25]) and correlate with enhanced neutrophil levels in cancer patients[57,58]. Interestingly, a large study using IL-1 neutralizing antibodies to reduce the risk of atherosclerosis found that besides reducing cardiovascular disease, anti-IL-1 treatment also significantly reduced lung cancer incidence and mortality in this patient population[59]. As another interesting example, IL-8 has recently been shown to associate with increased neutrophil levels in tumors and low efficacy of immune checkpoint blockade in patients of different cancer types[60]. These examples hint that targeting cytokines upstream of neutrophils may have a beneficial role in cancer patients, by redirecting rather than depleting the entire neutrophil population. Furthermore, it shows that drawing parallels with inflammatory diseases such as psoriasis, atherosclerosis and rheumatoid arthritis yields important insights into the chronic inflammatory conditions that arise in cancer and the potential therapeutic targets they provide. When and for how long patients must be treated to obtain cancer-limiting responses however, remain as yet unknown, especially when considering metastatic disease, which can occur many years after the occurrence of a primary tumor. Nonetheless, examination of neutrophilia and serum levels of the abovementioned cytokines may inform on that and help therapeutic targeting to normalize neutrophils.

Using emerging insights in neutrophil heterogeneity to employ these cells for cancer therapy

Moving forward, what could be other important elements of neutrophil biology that may be examined for therapeutic targeting? One underexplored aspect of these highly plastic cells is the regulatory mechanisms underlying neutrophil heterogeneity. Once thought transcriptionally silent after terminal differentiation, examination of circulating neutrophils from healthy individuals demonstrated hundreds of genes that are dynamically and epigenetically regulated, including effector gene programs such as those related to inflammasome activation[61,62]. Single-cell RNA sequencing (scRNAseq) has further revealed that neutrophils have quite a diverse transcriptomic profile in cancer[39,40]. And as suggested in **chapter 8** and elsewhere[34,63], the translation dynamics of neutrophils may be an important aspect of their biology in peripheral tissues and perhaps also tumors. Moreover, DNA and histone modification have been described to underlie key transcriptional changes during differentiation[64,65], and it would be of interest to examine these epigenetic gene regulatory networks of neutrophils in cancer. One could imagine that diversity in the chromatin regulation of genes that respond to environmental stimuli, such as cancer-derived signals, would lead to diversity in transcriptional output and thus neutrophil phenotype[66]. Single cell-based techniques examining the transcriptome and epigenome of neutrophils will surely shed light on the heterogeneous nature of these cells in different contexts and disease settings. The relevance of this wealth of information is to be examined through functional intervention studies, to assess these insights for potential therapeutic value.

Another potential telling aspect of neutrophil biology that needs to be understood in order to successfully utilize these cells as immunotherapeutic agents, is their interactome. Such studies can be performed *ex vivo*, as we show for macrophages in **chapter 5**, but *in situ* interactions will be far more important to discern. For this, spatial information may be instrumental, provided by techniques such as multiplex immunohistochemistry or tissue mass cytometry[67-69]. These techniques provide a wealth of information regarding not just presence of cells in tumors, but also their relative position to other cells and activation states based on expression of cell surface proteins. Other interesting emerging techniques to examine cellular crosstalk are based on expression of ligand-receptor pairs distilled from scRNAseq data, such as CellPhoneDB or related methods[70,71]. The advantage of these types of techniques is that it can infer the transcriptional states in response to an interaction in the tumor microenvironment, thus directly providing insight into the consequence of the interaction. Whether these interactions then in fact occur in the intact tissue must subsequently be verified. Another interesting cellular interaction technique is based on a transferrable fluorescent dye expressed by cancer cells that labels nearby cells, which was used to show that neutrophils in close proximity to 4T1 breast cancer cells *in vivo* were markedly changed in metabolic pathways compared to more distant neutrophils[72]. Cellular crosstalk can also be probed by mildly dissociating tissue and examining cell aggregates by scRNAseq, as was done for example by looking at neutrophil interactions in bone marrow or in blood with circulating tumor cells[73,74]. These techniques are however inherently descriptive and follow-up functional assays need to be performed on the basis of these analyses. Interestingly, Szczerba *et al.* identified cell surface proteins by which pro-metastatic neutrophils interact with circulating tumor cells and selectively disrupted these, thus limiting neutrophil interaction with circulating tumor cells and impairing metastatic spread in mouse breast cancer models[74]. This revealed a proof-of-principle that probing the neutrophil interactome using single cell-

based techniques may indeed be informative for therapeutic targeting of neutrophils to limit metastasis.

Clinical targeting of neutrophils is still in its early steps, and will benefit from good models to study these cells and proper monitoring of fresh clinical samples. Neutrophils are notoriously short-lived in culture and poorly survive freeze-thawing cycles that are common in the processing of archived clinical material. Moreover, the highly plastic nature of neutrophils renders *in vitro* culture of these cell almost always inadequate, as subtle tissue- or tumor-specific phenotypes may change rapidly upon culture in a dish. Therefore, these cells are to be examined preferably *in vivo* or directly *ex vivo*. Fortunately, interesting models for tracking and manipulating neutrophils have emerged over the years[75], which will surely provide the field with a wealth of knowledge in the coming years.

Tumor genetics as orchestrator of immune phenotypes

Gene mutations, deletions or amplifications that occur in cancer cells are important regulatory aspects in shaping the tumor immune landscape. We have known for decades that cancer cells harbor mutations that give them growth advantage over non-transformed cells through dysregulated cellular signaling. Targeting aberrantly expressed, mutated or amplified genes as cancer therapy stems from the notion that, while these mutated genes provide evolutionary advantages to cancer cells, they also yield unique targetable molecular vulnerabilities. Because of the high degree of heterogeneity and swift adaptability of tumors, survival- and proliferation-signaling pathways can be rerouted, rendering the tumor resistant to molecular targeted agents[76]. For therapies that evoke or enhance the anti-tumor immune response, the high degree of plasticity and diversity of the immune system potentially equals that of the tumor, which in theory should lead to less therapeutic resistance. However, we know from clinical analysis that for most tumor types immunotherapy only works in a subset of patients. To employ the immune system as an anti-cancer therapeutic, it is therefore important to understand what dictates immune cell recruitment to tumors and activation therein. We and others have sought to understand inter-patient heterogeneity in tumor immune landscapes and immunotherapy response by linking immunology with cancer genetics. As we argue in **chapters 2 – 4**, this connection between oncogenic signaling and immunity is one mechanism underlying immune heterogeneity in tumors.

How the guardian of the genome (p53) controls the immune system

It is perhaps not surprising that dysregulated intracellular pathways in cancer cells have profound effects on the tumor environment. The interconnectedness of signal transduction routes can on the one hand regulate survival and proliferation while on the other regulate secretion of molecules. Indeed, we describe altered cellular crosstalk between two cell types abundantly present in breast tumors, cancer cells and macrophages, as a result of cancer-intrinsic p53 loss (**chapter 3**). As a consequence of this altered communication, a cascade of inflammatory signals activates systemic immune responses, thus linking oncogenic signaling with systemic inflammation. While a plethora of oncogenes and tumor suppressor genes have been identified to modulate cancer cell-extrinsic signaling (see **chapter 2**), we directed our focus mainly on p53, because its phenotype in modulating systemic inflammation was dominant over other oncogenic drivers assessed in **chapter 3**. In addition, p53 deletion or mutation is highly prevalent in breast cancer and other cancer types alike, with highest frequencies observed in small cell lung cancer, colorectal cancer and ovarian cancer (**Fig.**

9.1). This is mainly due to the elemental role of intracellular p53 signaling in many aspects of cell biology[77], but may also hint towards potential common p53-regulated cell-extrinsic signaling in different cancer types.

Reports of p53-mediated modulation of the immune system in other cancer types have emerged over recent years. In line with our findings in breast cancer, lung cancer mouse models with a deletion in p53 also show increased neutrophilia compared to p53-proficient tumors[78]. In prostate cancer mouse models, loss of p53 does increase intratumoral neutrophil levels, but has strongest effects on monocyte influx into tumors[79]. In mouse models for pancreatic cancer, the loss of p53 induces immunosuppression in the tumor microenvironment, but this is mediated by macrophages and regulatory T cells (Tregs), rather than neutrophils[80]. Also in colorectal cancer mouse models, macrophage levels are enhanced by p53 deletion[81]. Furthermore, using human genomic and transcriptomic datasets, reports have also shown an association between loss or mutation of p53 and immune activation in breast cancer[82], lung cancer[83] and myeloid leukemia[84], while showing lower immune gene activation signatures in gastric[85], head and neck[83] and colon cancer[83]. Furthermore, even in a non-cancer context, p53 deficiency induces enhanced neutrophilia, as for example demonstrated for pulmonary *Klebsiella pneumoniae* and *Streptococcus pneumoniae* infection in $p53^{-/-}$ mice, in which p53-loss-induced neutrophilia enhanced pathogen clearance[86]. These findings demonstrate that p53 has a marked influence on the immune system in general that holds true for cancer and pathogenic infections. However, even though there is a strong enrichment for this tumor suppressor gene to be mutated across different cancer types (**Fig. 9.1**), the immunological consequences of this aberration may differ between tumor types. Therefore, it will be important to assess these tumor type-specific effects for the cancer types in which p53 mutations are strongly enriched.

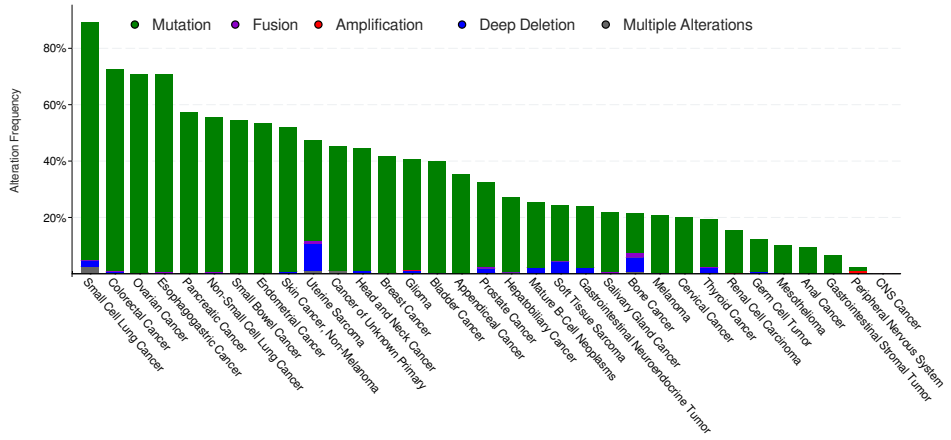

Figure 9.1. *TP53* aberrations in human cancer. Frequency of *TP53* (p53) aberrations across cancer types (MSK-IMPACT Clinical Sequencing Cohort, n=10336 patients). Only cancer types shown with more than 30 samples in the dataset. Bar plots generated using cBioPortal (http://www.cbioportal.org).

The biology of p53 further complicates generalizable conclusions on its role in immune modulation, as deletion and mutation of p53 can be functionally different[87]. Furthermore, as we demonstrate in **chapter 4**, even “mutant p53” is not a generalizable term; certain mutations induce changes that are distinct from other mutations, with (immuno)therapeutic

consequences. Because of the strong effect on immunotherapy outcome in p53 mutant mouse models (**chapter 4**), mapping the immune-modulatory effects of p53 hotspot mutations in other cancer types that have a high frequency of p53 aberrations may be an important avenue to pursue for better patient stratification. In lung cancer for example, patients with tumors expressing mutant p53 showed better responses to anti-PD-1 than p53-WT tumors in some studies[88-90], while others showed the opposite[91]. To understand its true prognostic value, it would therefore be of interest to correlate response to immune checkpoint inhibition to specific p53 mutations, rather than grouping all mutations together. Surely, using genomics datasets of immunotherapy-treated tumors would clarify whether such an association exists. Another interesting aspect of how mutant p53 affects anti-tumor immunity is the observation that T cell responses can be elicited against antigens derived from (mutant) p53. There have been clinical observations showing reactivity of intratumoral and peripheral blood $CD8^+$ and $CD4^+$ T cells to mutant p53 in patients of several cancer types[92-94]. In addition, mutational burden, a tumor characteristic that often correlates with response to immune checkpoint inhibition, has been reported to be higher in p53 mutant tumors than p53-WT tumors[83], as p53 can also affect genomic instability. Together, these studies show the wide array of effects that p53 can elicit on anti-tumor immune responses.

Targeting mutant p53 directly has been challenging, despite 40 years of extensive research into this protein[95]. Attempts have been made to use small molecules to re-activate or re-fold mutated p53 to revert its signaling back to the normal tumor suppressor function in cancer cells[96,97]. APR-246 is one such compound[98]: it restores WT function of p53 mutant proteins by chemically modulating the disrupted DNA binding domain, thus restoring its conformation, DNA binding and p53-mediated cell cycle arrest and pro-apoptotic functions in cancer cells. Phase 1 clinical trials with APR-246 have shown potent activation of p53 target genes, good tolerability and some (minor) anti-tumor effects in patients with leukemia and prostate cancer[99,100]. Currently, studies using this compound are being conducted for patients with ovarian cancer, prostate cancer and hematological malignancies (NCT03268382; NCT02098343; NCT00900614; NCT03072043). Interestingly, it was recently reported that APR-246 may improve responses to anti-PD-1 treatment in mouse melanoma and colon cancer cell line inoculation models[101]. A phase 1/2 clinical trial investigating the effects of APR-246 in combination with pembrolizumab (anti-PD-1) in several solid cancer types was initiated in 2020 (NCT04383938). Additionally, it was shown in transplanted melanoma and lymphoma cell line models that p53 reactivation, using intratumorally injected MDM2 inhibitor nutlin-3a, induced anti-tumor immunity[102]. Interestingly, this effect relied on p53 in both cancer cells and immune cells[102], hinting towards the complicating notion of the function of tumor suppressor genes (and oncogenes) in non-neoplastic cells[103]. Studying immune activation in clinical trials examining APR-246 and other agents that restore p53 function will be key in moving forward using p53 aberrations as targets for immunotherapeutic strategies.

Targeting WNT to relieve immunosuppression

We propose in **chapter 3** that rather than targeting p53 itself to limit systemic inflammation, we could target its downstream consequence: WNT secretion. In recent years important observations have been made on how tumor-intrinsic WNT/β-catenin activation can shape anti-tumor immune responses. In mouse models for melanoma and hepatocellular carcinoma, WNT pathway activation, either through mutations in the pathway or otherwise, impairs T cell priming through defective recruitment of dendritic cells (DCs)[104,105]. Consequently, this effect

impairs response to immunotherapy[104,105]. This WNT-mediated immunosuppression may hold true for many other cancer types, as β-catenin activation is strongly correlated with immune depletion across human cancers[106]. Utilizing the WNT pathway as an immunotherapeutic approach may therefore be an interesting avenue to pursue.

In our models, WNT is activated by deletion or mutation of p53 in cancer cells (**chapter 3**). We show that the inhibitor of WNT ligand secretion, LGK974, which is currently under phase 1 clinical investigation for several cancer types (NCT01351103), limits metastasis specifically in p53-null tumor-bearing mice by reducing systemic neutrophilic inflammation. This finding suggests that also for LGK974, stratification based on p53 status may be important to select patients who will benefit from this treatment. Of note, the way the WNT pathway is activated may also influence the efficacy of WNT inhibitors. In colorectal cancer mouse models with WNT pathway-activating mutations in the *APC* gene, response to Tankyrase inhibitors, which block WNT overactivation, depend on the specific type of *APC* mutation present in the tumor[107]. This suggests that while WNT activation is a strong biomarker for immune evasion across cancer types, successful targeting of WNT activation may depend on how the pathway is activated. It is also important to note that WNT targeting in patients is reported to induce major toxicities, for example in the gastrointestinal tract and bone, most likely through targeting of the stem cell niches[108]. Therefore, careful dose optimization will be important when targeting WNT as an immunity-stimulating therapy.

The potential of autophagy modulation as a therapeutic approach to enhance immunotherapy response

The effect of mutant p53 on the tumor immune environment of breast cancer is partly regulated by altered autophagy signaling. We show in **chapter 4** that some p53 mutations, via induction of autophagy, induce an immunologically 'hot' tumor, characterized by a high number of T cells, whereas other p53 mutants, which are low in autophagy, have a T cell-depleted phenotype. As a consequence, these autophagy-low p53 mutant tumors do not respond to immune checkpoint inhibitor anti-PD-1, whereas autophagy-high p53 mutant tumors do. Autophagy modulation may therefore prove to be another interesting approach to regulate p53-related immune phenotypes.

Autophagy is a homeostatic process that mediates the clearance of dispensable cytoplasmic content, such as protein aggregates or damaged organelles. It involves a large number of autophagy-related proteins (ATGs), which are highly conserved across evolutionary taxa, from yeast to humans[109]. The autophagy machinery is essential for organismal development, as demonstrated by embryonic or postnatal lethality in mice harboring full body knock-out of autophagy-related genes[110-112]. In adult organisms, it is essential for cellular homeostasis and is involved in a number of disease entities, including but not limited to cancer[113]. Using mice with tissue-specific deletion of autophagy-related genes, it was shown that autophagy-deficiency induced tumor initiation, but impairs growth of established tumors[114-117]. Interestingly, this effect is dependent on p53 in pancreatic tumor models, as tumors with p53 deletion grow faster upon loss of autophagy-genes[116]. This shows that the role of autophagy in cancer is dependent on stage of tumor development and oncogenic aberrations expressed by cancer cells.

The interplay of p53 and autophagy is determined by the subcellular localization of p53, as well as its mutational status: cytoplasmic, but not nuclear p53 inhibits autophagy, and p53 deletion consequently activates it[118]. We show in **chapter 4** that in breast cancer

some p53 mutants induce higher levels of ATG2B protein and autophagic flux compared to other mutants. When p53 is mutated, it cannot be degraded and accumulates in the cell. The aggregation of p53 protein that is observed upon mutation of this gene may impact autophagy signaling, as this cellular program removes protein aggregates. It would be of interest to examine whether the different p53 mutant forms assessed in **chapter 4** would accumulate in distinct manners (for example nuclear versus cytoplasmic). Conversely, as autophagy is required for the removal of protein aggregates, it would be interesting to check whether clearance of accumulated p53 is impacted in cancer cells that are defective in autophagy signaling, such as the *Atg2a/2b* knock-down cells used in **chapter 4**.

Gain-of-function mutations in p53 have been described to deactivate autophagy in a panel of cell lines of different cancer types[119], consistent with our findings in breast cancer. This study only examined the p53 mutants that we found to induce an immunologically 'cold' phenotype[119]. One could speculate that the 'cold' mutants have an altered autophagy-modulatory function, whereas the 'hot' mutants activate autophagy by loss-of-function similar to the p53-null cells[118], thus influencing immune signaling. Although we did not formally show this *in vivo* in **chapter 4**, the p53-autophagy link that underlies immunotherapy response in breast cancer could be due to negative regulation of the autophagy-suppressing pathway mTOR through a number of mechanisms[120]. The negative regulation of mTOR signaling may be alleviated in specific p53 mutants, which consequently suppresses autophagy. This will most likely be a consequence of protein complex formation by mutant p53, since we excluded direct chromatin binding (and thereby direct transcription regulation) by these mutants, but show a number of protein interactors that differ between 'hot' and 'cold' p53 mutants.

Having established a connection between p53 mutations, autophagy, immunity and cancer, how can we now use these insights for therapeutic proposes? It appears that cancer cell-intrinsic autophagy signaling can have opposing roles, limiting tumor onset but promoting progression. Considering just this insight, one would argue that therapeutic inhibition of autophagy would be beneficial in established tumors. However, as we show in **chapter 4** and others have reviewed extensively[121], autophagy positively affects anti-tumor immunity. It is involved in suppression of cancer-associated chronic inflammatory conditions and activation of antigen presentation, cytotoxic immune cell recruitment and T cell activation and survival[121]. Autophagy inhibition in cancer cells, as seen in some p53 mutant mammary tumors, could therefore have evolutionary benefits, as it helps tumors evade immune attack. In line with these findings, low expression of autophagy-related genes correlates with poor survival in p53-mutant breast cancer patients[119]. Furthermore, genetic or pharmacological blockade of autophagy in p53-mutant cell lines caused the accumulation of mutant protein, thus facilitating tumor growth[122].

It may therefore be more attractive to activate autophagy in cancer cells. However, no therapeutic agents have yet been developed to activate autophagy in a specific manner[113]. Some drugs can activate autophagy in an indirect manner, such as inhibitors of mTOR. We show in **chapter 4** that AZD8055, a small molecule mTORC1/2 inhibitor, can reactivate autophagy and rescue cytokine secretion by 'cold' p53 mutant cell lines *in vitro*. Inhibitors of mTOR are being used in the treatment of breast cancer, as these tumors often show activation of this pathway[123]. Of note, it is likely that *in vivo* mTOR inhibition will also have immunosuppressive consequences, as mTOR signaling has a key function in the activation of immunity as well[124], and mTOR inhibitors are widely used as immunosuppressants, for example for organ transplant patients[125].

Lastly, targeting autophagy as a systemic treatment may have a variety of undesired consequences for healthy tissue, given its essential role in cellular homeostasis. Therefore, using autophagy modulation to stimulate anti-tumor immunity in a therapeutic context will have to wait until more specific agents, that can be delivered intratumorally, are developed. Until that time, it is worthwhile to examine the relay signals between p53 mutants and autophagy to identify potential targets for therapeutic intervention.

Emerging oncogenic signaling pathways implicated in shaping the tumor immune landscape

Insights into oncogene- and tumor suppressor gene-modulated immune responses in the primary tumor microenvironment have only begun to scratch the surface. The collection of genetically engineered mouse models (GEMMs) for breast cancer examined in **chapter 3** have further revealed interesting genotype-immune phenotype relationships. For example, amplification of *Met* in the triple-negative breast cancer model *Wap-cre;Brca1$^{F/F}$;Trp53$^{F/F}$* augments p53-induced systemic neutrophilia (**Fig. 9.2a**). In that same model, it can be observed that *Myc* amplification dramatically reduces the amount of CD8$^+$ T cells in the tumor (**Fig. 9.2b**). And in mouse models for invasive lobular carcinoma, *Wap-cre;Cdh1$^{F/F}$;Akt^{E17K}* or *Wap-cre;Cdh1$^{F/F}$;Pik3ca^{E545K}*, a difference in the mutation activating the PI3K pathway is associated with a marked difference in intratumoral CD4$^+$ T cells (**Fig. 9.2c**). The translational relevance of these findings is evident considering that amplification in *MET* or *MYC* or activation of the PI3K pathway are highly common drivers of tumorigenesis in human breast cancer[126-129]. These are just some examples of oncogenic mutations or amplifications that may be of importance in shaping the tumor immune landscape, the mechanisms of which are currently being investigated (Duits *et al.*; Brambillasca *et al.*; van Weverswijk *et al.,* personal communication). Future studies must reveal the importance of these oncogenic drivers and their downstream effects for therapeutic targeting in breast cancer and beyond.

An emerging aspect of cancer cell-intrinsic mechanisms shaping the immune system is the epigenetic makeup of tumors, in the form of DNA- and histone-modifications[130]. Chromatin remodelers such as the SWI/SNF complex and the Polycomb repressor complex have been implicated in anti-tumor immunity and components of these complexes are also frequently mutated in cancer. Mutations in members of the SWI/SNF complex have been shown to induce anti-tumor immunity in mouse melanoma models[131] and correlate to beneficial responses to immune checkpoint inhibition in renal cell carcinoma[132], while in ovarian cancer patient samples and mouse models, perturbations in the SWI/SNF complex impair anti-tumor immune responses[133]. Proteins of the Polycomb repressor complex also affect anti-tumor immunity by silencing the antigen presentation machinery or impairing inflammatory cytokines[134-136]. Besides coding genes, the non-coding genome may play an important role in shaping anti-tumor immunity and immunotherapy response, as was shown for long non-coding RNA *LINK-A* in mouse and human triple-negative breast cancer[137]. These studies show that much work is to be done to find links between cancer (epi)genomes and immune phenotypes, and importantly, discern which ones are dominant in human tumors.

Approaches to study genotype–immune phenotype relations in cancer

Mouse-modelling based methodologies

The link between cancer cell-intrinsic genetic aberrations and immune responses in the tumor microenvironment and systemically is now evident. However, to be able to use this

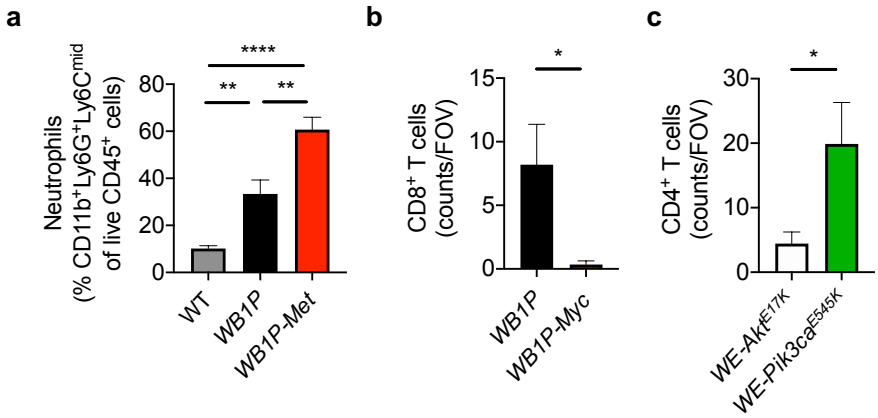

Figure 9.2. Genetics of murine mammary tumors linked to immune phenotypes. Data derived from analyses shown in chapter 3. **a.** Circulating neutrophil levels in tumor-bearing *Wap-cre;Brca1$^{F/F}$;Trp53$^{F/F}$* (*WB1P*), *Wap-cre;Brca1$^{F/F}$;Trp53$^{F/F}$;MET* (*WB1P-Met*) or non-tumor-bearing wild-type (WT) control mice, as determined by flow cytometry (n=6–9/group). **b.** Levels of intratumoral CD8$^+$ T cells in *Wap-cre;Brca1$^{F/F}$;Trp53$^{F/F}$* (*WB1P*), *Wap-cre;Brca1$^{F/F}$; Trp53$^{F/F}$;MYC* (*WB1P-Myc*) mice, as determined by immunohistochemistry (n=4/group). **c.** Levels of intratumoral CD4$^+$ T cells in *Wap-cre;Cdh1$^{F/F}$;Akt^{E17K}* (*WE-Akt^{E17K}*) or *Wap-cre; Cdh1$^{F/F}$;Pik3ca^{E545K}* (*WE-Pik3ca^{E545K}*) mice, as determined by immunohistochemistry (n=5–7/group). All data show mean ± s.e.m. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, as determined by one-way ANOVA with Tukey's correction (**a**) or Mann-Whitney U test (**b**, **c**).

information for therapeutic targeting or patient stratification, much research is needed to identify the relevant from the bystander phenotypes. There are several approaches one could take in trying to delineate the link between cancer genotypes and their immune phenotypes. One way is to compare a panel of mouse tumor models (such as GEMMs) that mimic human cancer pathology and profile their local and systemic immune environments, as was done in **chapter 3.** This type of approach is an extended version of what was used in earlier studies looking at the impact of one particular gene of interest by removing or overexpressing it in mice, which generated a wealth of information regarding individual genes such as *PTEN*[138,139], *MYC*[140] and *CTNNB1* (β-catenin)[104] in shaping the tumor immune landscape (discussed in **chapter 2**). One key advantage of using a larger panel of models is that individual models often harbor several genetic aberrations driving tumorigenesis, and one could therefore potentially find oncogenes and tumor suppressor genes that are dominant in dictating the observed phenotype. As human tumors also mostly harbor not one but many driver mutations, identifying these dominant phenotypes may be key in determining relevant genes for therapeutic targeting. As shown in **chapter 3**, loss of p53 drives WNT activation in cancer cells regardless of the other mutations that these tumors harbor (be it *Brca1, Cdh1, Myc* or otherwise). This exemplifies the power of using a panel of GEMMs for immunological research and will surely in the future help uncover novel therapeutic targets for breast cancer and beyond.

Comparison of a panel of mouse models has been successfully used to identify therapeutic targets for tumors bearing specific mutations. For example, comparing four prostate cancer models, Bezzi *et al.* revealed targetable cytokines that upon blockade could limit disease progression in tumors with specific genetic aberrations[79]. This notion can be extended to the systemic immune environment. In **chapter 3**, we identified WNT secretion as a target to modulate systemic inflammation, revealing a potential anti-metastatic therapy

specifically for p53-null tumors. Recently, the systemic immune environment of several orthotopically transplanted cell line models for different types of cancer was examined in detail and monitored over time and in response to surgical intervention[141]. This in-depth analysis demonstrated that systemic immunity was not only based on the tumor type and the mutations therein, and also depends on the tissue that is examined, time after transplantation and potentially other factors[141]. Interestingly, this work shows that tumor-induced systemic immune activation is partly reset upon resection of the tumor, as our lab has previously noted as well[25].

A pitfall of this mouse model-based approach is that it is limited by the availability of models, and that generation of particularly GEM models is time-consuming and costly, with no guarantee to identify relevant and targetable immune phenotypes. While genetic engineering in mice is generally based on frequently occurring mutations in the human equivalent of that particular cancer type, whether the immune phenotypes observed in mice also occur in human patients only becomes evident after the generation and characterization of the model. Moreover, throughput is another challenge, as in practice one can examine only a handful of genes at a time. This is partly overcome by the development of somatic modeling, in which tumor drivers can be ectopically expressed or deleted in tissue using a virus- or plasmid-based approach[142]. This type of technique allows for more rapid screening of oncogenes and tumor suppressor genes for their role in shaping the immune system, while still retaining the benefits of GEMMs in terms of growth kinetics, histological characteristics and co-evolution with host tissues.

Another challenge for the mouse model-based approach lies in the fact that different models often represent different cancer subtypes. As we discuss in **chapter 2**, and as also shown for example in lung cancer GEMMs[78], cancer subtype can strongly dictate tumor immune phenotypes. This also applies to the work shown in **chapter 4**: although p53 mutations occur in all breast cancer subtypes, the effect of p53 aberrations on immunity in the local tumor microenvironment can differ per subtype[143], and the prevalence of p53 mutations are strongly enriched in certain subtypes, such as basal-like breast cancer[128,144]. These subtype-specific differences may however be more strongly associated with local immune responses in the tumor microenvironment, than with systemic immune activation, as the latter appears to be more uniform between different subtypes (**chapter 3**) or even between different types of cancer[141]. However, the link between genetic makeup and systemic immunity has not been thoroughly examined in patients, and therefore still requires clinical validation.

Another critical aspect of using mouse models to study the immune system is tumor burden, which significantly impacts immune cell influx. Physiological aspects of tumors, such as pH and oxygen levels, drastically change with increasing size, thus influencing the immune system locally and systemically[141]. When comparing GEMMs with different pathologies and growth kinetics, normalizing to tumor burden may be an important aspect to exclude size- and burden-dependent phenotypes. This becomes evident in studies by Bezzi *et al.* and Busch *et al.*[78,79], in which models with vastly different growth kinetics are compared at set timepoints. In contrast to for example breast cancer models, where tumors are mostly easily measurable, visceral tumors are far more challenging to measure, and some type of normalization must be applied. This can be overcome by taking proper controls, such as using *in vivo* tracking methods for example based on luciferase expression to quantitatively compare tumor burden.

The tumor immune landscape is not a homogenous entity, and tumor heterogeneity

can also result in a heterogeneous immune microenvironment. To examine this heterogeneity, an adaptation to the GEMM-based immune phenotyping approach has been applied recently by expanding single cell clones derived from spontaneous murine pancreatic tumors[145]. By transplanting these monoclonal tumors back into mice, the tumor-intrinsic immune cell attractant mechanisms were identified for individual clones, with significant differences in immune infiltrate and response to immunotherapies[145]. However, since heterogeneity is inherent to tumors, clinical applicability of this type of study is hard to envision. Nonetheless, it lays open an avenue of research into how individual tumor clones shape microenvironments within microenvironments. Finding commonalities between clones may be a way to tackle heterogeneous clones within one tumor type.

In vivo screening-based approaches

While engineering genetic aberrations that drive tumorigenesis into mice and examining their effect on the immune system is an effective approach, other more unbiased methodologies have also proven fruitful. *In vivo* CRISPR-based screening approaches have been applied to identify cancer-intrinsic determinants of immune (de)activation, identifying genes such as *PTPN2*, *ADAR1*, and *PRKAR1*[146-148]. By using shRNA- or CRISPR-based screening approaches in cancer cell lines and transplanting these into either immunocompetent or immunodeficient mice, one could identify molecules that are required for anti-tumor immune responses. One major difference with the GEMM-based approach is that the genes identified here are not necessarily driver mutations, and therefore could be non-dominant. Where oncogenes and tumor suppressor genes have been selected through clonal evolution of the tumor by giving cancer cells growth advantage while also influencing the immune system, the regulators identified in such genetic screens will likely be more interesting in the therapeutic setting than in finding dominant drivers of immune phenotypes. A targeted screening approach focused on oncogenes and tumor suppressor genes of a given cancer type might be an interesting addition to these types of methodologies to be able to prioritize certain genes for further functional study. These screening approaches as used so far have been effective in identifying targetable molecules[149], as it provides functional information (*i.e.* disease outcome or treatment response) and not just descriptive correlation data. Also, expression of such targets can often be easily screened for in the clinic. So far, they have mainly been T cell-centered, and it would be of interest to identify what drives other immune cell types into the tumor microenvironment.

Assessing human tumor microenvironments

A more human-centered approach to identify genotype–immunophenotype relationships is based on assessment of RNA and DNA sequencing data from clinical studies and inferring immune cell levels, based on gene signatures for each immune cell type. These immune cell deconvolution methods, among which are CIBERSORT, TIMER and xCell[150-152], allow for estimation of immune infiltrate in tumors and, provided the data are available, following their dynamics over different disease stages, time or during treatment[153]. Large-scale genomics studies have led to the identification of certain commonalities among all cancer types in terms of immune landscape and correlates with oncogenic drivers[154]. One could also use this to correlate immune infiltrate with chemokines across cancer[155] or with specific molecular signaling pathways[156]. Of course, these types of analyses cannot inform on cellular heterogeneity and spatial information, which are also important aspects of anti-tumor immunity.

Moreover, these techniques are based on bulk tumors, often from biopsies of one part of the tumor. And surely, once made, these observations need to verified in relevant mouse model systems and proper immune cell measurements in clinical samples, as gene-based metrics of immune cells will only provide estimations. This is especially valid considering the different activation states cells might be in. Nevertheless, with the increasing generation of immune cell 'atlases' in different organs and cancer types using scRNAseq, these estimations are projected to become more and more robust. Another major drawback of this approach is the lack of information on systemic inflammation. However, as an increasing amount of data on diverse clinical parameters are being included in genomics datasets such as The Cancer Genome Atlas (TCGA), one could envision that information on systemic inflammation may be included in the future. I deem these approaches key to prioritize clinically relevant genes in shaping anti-tumor immunity, while simultaneously providing a translational rationale at the basis of the study.

Identifying clinically relevant changes in the immune system

Knowing how to address genotype-immunophenotype studies from the cancer-perspective is demanding enough, but unraveling which changes in the immune system are relevant for disease progression is a critical challenge in and of itself. If an increase in for example macrophages is observed in tumors with a certain mutation, does this mean that these cells are relevant for cancer development? Or is it only when these cells are in a particular activation state? Protein-based methods such as flow cytometry, mass cytometry and immunohistochemistry (single- or multiplexed) can generate a wealth of information concerning a large variety of cell types and their activation states, but will always be inherently biased towards certain markers and quality of antibodies. Dissecting immune heterogeneity using scRNAseq approaches has vastly expanded the way we describe cell types. This has revealed an unprecedented complexity in tumor microenvironments in terms of cell identity and cellular states of a given cell type. This wealth of information can also be daunting, because teasing out what is relevant becomes ever more difficult with increasing complexity. To find relevant players in complex tumor ecosystems, as we have discussed above, it may be of interest to examine cell-cell communication within the tumor microenvironment or systemically using single-cell-based approaches, either by sequencing physically interacting cells[73,74,157], by correlating ligands with known receptors on different cells[70,158] or by even profiling whole transcriptomes of immune infiltrates in a spatial fashion in intact tissue[159,160]. One could then prioritize immune cell types based on the extent of their crosstalk with other cells, be it cancer cells or tumor-antagonizing immune cells. As cell types with many communication partners will then likely function as hubs within a tissue or tumor, one could reason these must have a key role. Of course, these findings must subsequently be verified *in vitro* or in mouse intervention studies. One elegant study using such an approach in lung development revealed that basophils, which were not particularly numerous but engaged in extended network of communication with other cells, have a key role in the developing lung[71]. By unbiasedly assessing cellular crosstalk in the lung at several timepoints during embryonic development, followed by *in vitro* basophil manipulations and *in vivo* basophil depletion studies, the role of this cell type in lung development was uncovered[71]. These types of integrative systems approach, inferring cellular crosstalk and activation states and linking everything to tumor genotype, paired with functional studies, will be crucial in understanding what shapes the tumor immune landscape.

Outlook: The clinical utility of genetic makeup and immunity studies

The relationship between genetic aberrations in cancer cells and local and systemic immune activation may prove valuable for clinical practice once validated. This utility is two-fold: first, these could serve as biomarkers for immune activation. One could envision assessing biopsy material for certain markers, such as p53 mutational status or activated β-catenin, to inform on the use of immunotherapy treatment. When considering specific mutations, as we argue in **chapter 4** for p53, this may prove more time-consuming and costly, as sequencing will be involved. However, if p53 mutations are validated as dominant biomarkers for immunotherapy response in breast cancer and beyond, it might be worthwhile. As another example, neutrophil-targeting agents may be more useful for breast cancer patients with p53-mutant/null tumors than p53-WT tumors, so screening for p53 aberrations will also be useful in that regard. These insights may help guide decisions concerning immune-based treatments.

Secondly, these findings can be used to optimize response to immune-based therapies by using informed combinations of treatments. Immunotherapy might be improved by combination with targeted therapies, especially when those molecular targets in cancer cells influence the immune system as well. When β-catenin activation is observed before considering immunotherapy treatment, the potential limited efficacy will perhaps be improved by addition of WNT-targeting agents. One notion to take into account is that oncogenes and tumor suppressor genes also have functional roles in immune cells themselves[103]. For example, *PTEN* loss drives tumorigenesis and impairs T cell influx and immunotherapy response in tumors[139]. Targeted PI3K inhibitors may successfully kill *PTEN*-null cancer cells, but the PI3K pathway also has a crucial role in signaling downstream of T cell receptor activation[103]. In **chapter 4**, we show that for tumors with certain p53 mutations, mTOR activation correlates with an immunologically cold phenotype. However, as mentioned above, the immunosuppressive actions of the mTOR inhibitors on immune cells themselves may potentially annul the immune-stimulatory effect that mTOR inhibition may have when targeting this pathway just in cancer cells[124]. The same could be said for many other of these targeted agents. One could speculate that dosing and schedule of targeted agents could be optimized for optimal desired immune modulation, but this has to be tested in future studies.

With increasing knowledge on how oncogenic signaling shapes immune responses to tumors, we will have more information to estimate how a patient will likely respond to immunotherapy or what therapeutic additions may need to be put in place. While I have attempted to capture some of the complexities that arise when trying to make such estimations, far more complicating factors exist. There are numerous confounding elements in human populations, as tumor immune landscapes can be shaped by a large number of phenomena, such as patient age, treatment history, sex, obesity, microbiome composition, a history of smoking and so on. The hope lies in the fact that some aspects of cancer biology will be dominant in determining immune phenotypes, and identifying those should help the clinical utilization of these insights.

Concluding remarks

Tumors are complex ecosystems in which a given cell is interconnected with numerous others and each individual component constantly adapts according to internal, but also external, cues. This endless complexity and adaptability of tumors shows that to combat cancer is to combat evolution. Nonetheless, understanding cancer as a system yields the

understanding that in any system, interconnectedness between individual components creates interdependence. Exposing these interdependencies in the tumor microenvironment and in anti-cancer immunity will be essential in optimizing cancer treatments.

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