Immunosuppression in breast cancer: a closer look at regulatory T cells

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Discussion

The work described in this thesis sprouted from my fascination for the tumor-supportive role that the immune system can play in cancer progression, and studies this concept mainly from the angle of regulatory FOXP3$^+$CD4$^+$ T cells in breast cancer. First, the current knowledge and open questions regarding T_{reg} biology in breast cancer are reviewed in **chapter 2. Chapter 3** focusses on T_{regs} in the primary tumor context, and demonstrates the importance of macrophages for the intratumoral conversion of conventional CD4$^+$ T cells into T_{regs}. In **chapters 4** and **5**, the focus shifts to tissue-specific mechanisms of breast cancer metastasis. Specifically, research in **chapter 4** reveals a mechanism by which T_{regs} selectively promote lymph node metastasis, and **chapter 5** discusses how neutrophils enhance lung metastasis. **Chapter 6** lifts a corner of the veil regarding the potential detrimental role of T_{regs} in immunotherapy response in primary and metastatic breast cancer. Together, the work in this thesis aims to contribute to improved understanding of immunoregulatory mechanisms at play during diverse stages of breast cancer progression and immunotherapy response. Below, I detail how insights gained in this thesis:

1) fit within the current research literature.
2) may spark new research questions that when answered help improve our fundamental understanding of immune regulation in cancer metastasis.
3) may have therapeutic relevance in the form of T_{reg}-based clinical applications.

BYSTANDER OR ARCHITECT? T_{REGS} & BREAST CANCER PATHOGENESIS

T_{regs} abundantly populate primary breast tumors and metastases, as demonstrated in both patient tumor samples and preclinical mouse models. Over the past decade, this has raised significant interest to understand their functional role for disease progression. Based on the outcome of these studies (**chapter 2**), T_{regs} are often portrayed as undisputed benefactors of primary tumor growth. Based on findings in this thesis, I would like to suggest a nuance to this interpretation. So far, the link between T_{regs} and tumor progression stems from observations that show that targeting T_{regs} in mice bearing inoculated (GEMM-derived) cancer cell lines slows tumor growth by unleashing anti-tumor immunity[1–4]. However, these cell line-inoculated tumor models have key weaknesses that complicate the translation to human breast cancer. One issue is that syngeneic cell line-based tumors do typically not resemble the immunogenicity of human tumors from the same origin[5], thereby potentially setting unrealistic expectations for the efficacy of immune therapies in cold, non-inflamed tumor types. To illustrate this, human breast tumors are generally poorly immunogenic[6], have typically only modest infiltration of T cells[7] and score rather low in terms of mutational load[8]. On average, breast cancers harbour one somatic mutation per Mb: ten times less than what is considered sufficient to mount anti-tumoral T cell responses[9]. In contrast, two of the go-to murine tumor cell lines for breast cancer research which are classified as

triple-negative breast cancer (TNBC), E0771 and 4T1, have a similar mutational burden of 1-5 mutations per Mb[10,11], but are characterized by high intratumoral infiltration of T cells, expression of co-stimulatory receptors and neo-antigens[5,10–12]. In line, E0771 and 4T1 tumors are responsive to anti-PD-1 immune checkpoint blockade (ICB)[1,13], while the efficacy of PD-1/PD-L1 blockade in patients with TNBC is considered to be low[14]. Furthermore, T_{reg} depletion in 4T1 and E0771 tumors is sufficient to unleash anti-tumor immunity, suggesting T_{regs} are a key barrier to effective immunity in immunogenic breast tumor types[1,2,10,15]. In humans, TNBC is considered to have the relatively highest immunogenicity of all breast cancer subtypes, as TNBC harbours the highest frequency of neo-antigens and cancer germline antigens[16]. Counterintuitively, high T_{reg} infiltration is correlated to a favourable prognosis in TNBC (**chapter 2**), contrasting with the pro-tumoral role of T_{regs} in 4T1 and E0771 tumors.

Apart from disparities with human cancers, studying T_{reg} function in highly immunogenic models poses another complication. It was recently reported that the reduction in lung metastasis that is seen upon Treg depletion in 4T1-bearing mice is in fact a consequence of the primary tumor responding to Treg depletion, and not a direct effect of T_{regs} on metastatic colonies in the lung niche[2]. Thus, by using tumor models that are responsive to T_{reg} depletion such as 4T1, the effect of T_{reg} depletion on metastasis formation is obscured by its effect on the primary tumor. Together, this illustrates that insights into T_{reg} function deduced from popular cancer cell line-based mouse models may inaccurately reflect, and potentially overestimate, the importance of T_{regs} in human breast cancer.

In this thesis, we aimed to examine T_{regs} in a context that is more true-to-nature using genetically engineered mouse models (GEMMs) for mammary tumorigenesis. The use of GEMMs enables the study of tissue-specific, *de novo* tumor formation and progression of malignancies driven by clinically relevant mutations in an immune-proficient environment. Dependent on underlying genetic modifications, GEMMs can reflect the poorly immunogenic and chronic inflammatory state of human breast cancer, albeit with a lower mutational burden as observed in human tumors. The trade-off for superior cancer modelling is that the use of GEMMs is generally expensive, time-consuming and laborious due to extensive breeding costs, long-term tumor latencies and associated monitoring of animals. To circumvent the practical disadvantages of GEMMs, syngeneic cell lines derived from PyMT-MMTV and Pdx1-Cre;LSL-$Kras^{G12D}$;$Trp53^{F/F}$ GEMMs have been used to study T_{reg} function[3,17,18]. Despite producing rapid and reproducible results and the potential to easily introduce further genetic modifications, GEMM-derived cancer cell lines can show key differences in their immune landscape, with increased frequencies of T_{regs}, $CD8^+$ T cells and NK cells, as compared to *de novo* tumors[19]. As this may critically impact the outcome of immunological studies and thereby still reduce their clinical value as compared to GEMM-based models, this approach has been limited in this thesis.

In our studies in **chapter 3, 4 and 6**, we interrogated the impact of T_{regs} on breast cancer pathogenesis, utilising the lowly immunogenic *K14Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* (KEP) model for mammary tumorigenesis, reflective of human invasive lobular carcinoma[20]. To do so, we employed three different strategies to target T_{regs} in mice bearing spontaneous and orthotopically transplanted primary tumors.

1) Antibody-based depletion using an anti-CD25 antibody with enhanced binding to activating FcγRs for optimal intratumoral depletion[21].
2) Inducible ablation of FOXP3$^+$ cells by diphtheria toxin in *Foxp3$^{GFP\text{-}DTR}$* mice.
3) Indirect blockade of intratumoral T_{reg} accumulation via targeting of macrophages using anti-CSF1R.

Despite efficient intratumoral depletion of T_{regs}, none of the strategies affected primary tumor growth, thus contrasting with previous literature (**chapter 2**). Intriguingly, we did find intratumoral T_{regs} to be immunosuppressive *in vitro* and highly functional *in vivo*, as T_{reg} depletion increased the expression of inflammatory markers on myeloid cells, and strongly activated both CD4$^+$ and CD8$^+$ T cells (**chapter 4 and 6**). These functional results are consistent with previous observations in breast cancer models[1,3,15], and emphasize that T_{regs} are key orchestrators of the immunosuppressive tumor niche. Furthermore, T_{regs} in KEP tumors were validated to be enriched for a Tumor T_{reg} gene signature[22] that is conserved across species and tumor types, indicating T_{regs} in the KEP model do not display an atypical phenotype. Combined, these data suggest that highly immunosuppressive T_{regs} are not by definition indispensable for orchestrating immune escape of primary tumors in poorly immunogenic GEMMs. Instead, these findings are in line with the concept that different immunosuppressive cells including T_{regs}, tumor-associated macrophages, neutrophils, other suppressor cells of myeloid origin, cancer-associated fibroblasts, cancer cells and co-opted tissue-resident cells together construct an intricate immunoregulatory multi-layered network. Critically, this network is not breached by targeting single actors, as the multiple layers appear to be non-redundant, and can adapt to challenges through compensatory influx of immunosuppressive cells[23], or phenotypic adaptations (**chapter 3**). By dissecting separate layers of immunosuppression, like work in this thesis has aimed to do from the angle of T_{regs}, fundamental insights are gained that lay the foundation for the design of therapeutics which may, in the form of personalized combinations, dismantle cancer-associated immune suppression, and thereby set an important step towards anti-tumor immunity.

ONE OF THE GUYS: THE CLINICAL SIGNIFICANCE OF T_{REGS} AMONG THE SUPPRESSIVE TME

Keeping in mind T_{regs} are part of a greater immunosuppressive network, do they, as individual cell type, have a clearly defined clinical significance in the context of breast cancer? As described in **chapter 2**, high intratumoral T_{reg} infiltration correlates either with a poor or a favourable prognosis, dependent on the breast cancer subtype. Correlation with a favourable prognosis is observed in hormone (estrogen, progesterone) receptor negative, and triple-negative breast cancers (TNBC). High T_{reg} density strongly correlates with concurrent stromal and intratumoral $CD8^+$ T-, $CD4^+$ T- and B cell TILs[24,25], and the expression of inflammatory and immune-response related genes in TNBC[26], suggesting the favourable association of T_{regs} in TNBC is reflective of a broader lymphocyte-inflamed environment. This might be linked to the observation that TNBC tumors are relatively immunogenic[16], potentially driving T cell infiltration. As this observation contrasts with the immunosuppressive nature of T_{regs}, it would be of interest to test the functionality of T_{regs} isolated from these TNBC tumors, as chronic inflammatory conditions have been shown to induce IFN-γ-mediated T_{reg} dysfunction, and loss of suppressive phenotype[27]. Thus, sustained pro-inflammatory challenges like T cell responses to tumor-associated antigens, or therapeutic activation of innate cGAS-STING and inflammasome pathways may have the potential to relieve T_{reg} suppression in the TME. In hormone receptor positive tumors, the clinical significance of T_{regs} is more aligned with preclinical data: High T_{reg} density correlates with poor disease outcome (**chapter 2**). However, there is a catch in interpretation of these studies. The likelihood of finding high T_{reg} numbers is strongly associated to high tumor grade and incidence of lymph node metastasis, which are both negative prognostic indicators in itself, raising the question: Is the mere presence of T_{regs} sufficient to predict disease outcome? Several studies have shown that T_{regs} are a prognostic factor for disease outcome in univariate analyses, but not in multivariate analyses that include above-mentioned clinical variables, thereby showing that T_{regs} are likely not independently predictive of disease outcome (**chapter 2**). Even the discovery that the chemokine receptor CCR8 is uniquely expressed on tumor-associated T_{regs}, and detrimental for disease outcome in breast cancer, could not be substantiated in multivariate analysis[28]. A few studies have assessed the clinical relevance of T_{regs} in the context of the broader immunosuppressive TME. Interestingly, in patients with invasive ductal carcinoma, low T_{reg} infiltration was found to independently correlate with an increased overall survival in multivariate analysis with $CD8^+$ T cells, B cells and macrophages[29]. By assessing the predictive value of individual immune cell types using the deconvolution algorithm CIBERSORT on mixed breast cancer gene expression datasets, both M2-like macrophages and T_{regs} were associated with poor disease outcome[30]. A similar study using CIBERSORT showed that immune infiltrates are heterogeneous, and strongly differ per breast cancer subtype, but still identified T_{regs}, macrophages and mast cells to be amongst most detrimental immune cells[31]. The negative disease outcome associated to both macrophage and T_{reg} abundance is

reminiscent of work shown in **chapter 3**, which reveals that macrophages play an important role in the maintenance of T_{regs} in the breast TME. Notably, both these CIBERSORT-based studies found T_{regs} to be significantly associated to poor disease outcome in multivariate analysis, suggesting that measuring T_{reg} abundance relative to the immune infiltrate as a whole is potentially a more informative metric as opposed to single immunohistological assessment of absolute FOXP3 counts, which ignores other immunosuppressive cells within the TME. Currently, CIBERSORT on bulk RNAseq data classifies a biased number of cell types, and disregards (tissue-specific) cell phenotypes. Future use of deconvolution algorithms on single cell datasets of breast tumors may provide deeper insights into specific T_{reg} phenotypes that are associated with disease progression.

In addition to analyses that interrogate each cell type individually, another study performed a comprehensive analysis from the TME as a whole using CIBERSORT on breast cancer gene expression datasets, and analysed whether particular immune cell clusters are enriched in patients with poor disease outcome[32]. A pro-tumorigenic immune cluster was discovered, that correlated to poor prognosis across breast cancer subtypes. This cluster consisted of M2-like macrophages, resting mast cells, resting memory $CD4^+$ T cells, and γδ T cells. T_{regs} were not assigned to this cluster, potentially due to their contrasting roles in different breast cancer subtypes.

Taken together, the clinical significance of T_{regs} is influenced by a multitude of factors. While these factors include breast cancer subtype, and the broader immune infiltrate, the importance of tissue-specific T_{reg} phenotypes, or their spatial organisation within stroma or tertiary lymphoid structures, and cellular crosstalk is currently unclear. Looking forward, the emerging appreciation for breast cancer diversity in terms of ecotypes that take cancer- and immune cell heterogeneity into account, may further define the clinical significance of T_{regs} beyond traditional subtypes in the near future[33].

STOCKHOLM SYNDROME: T_{REGS} AND LYMPH NODE METASTASIS

Whereas the clinical significance of $FOXP3^+$ TILs on disease outcome is often conflicting and subtype dependent, one histopathological feature is consistently correlated to high intratumoral T_{reg} density: the presence of lymph node metastases[34]. From a clinical viewpoint, assessing lymph node involvement is paramount for evaluating prognosis and therapeutic follow-up, as regional lymph nodes are often the first site of metastasis[35]. Since breast cancer patients with lymph node metastasis have up to 40% lower five-year survival rate compared to node-negative patients[35–40], insights into this hallmark event that bridges local and metastatic disease are imperative to halt early-stage tumor spread. The correlation

between T_{regs} and lymph node metastasis has been validated in multiple independent meta-analyses that have probed the prognostic value of T_{reg} infiltration in breast cancer[34,41,42], which has triggered more in-depth assessments of T_{regs} in sentinel lymph nodes of breast cancer patients. On a quantitative level, T_{regs} were found to be increased in tumor-infiltrated lymph nodes compared to non-infiltrated lymph nodes of breast cancer patients[43], which associated with decreased maturation of dendritic cells[44]. Strikingly, others showed that T_{reg} accumulation and suppression of dendritic cells precedes detectable tumor-infiltration in lymph nodes[45,46], suggesting that T_{regs} might play a role in preparing the lymph node niche for tumor arrival. In line with this hypothesis, tumor-draining lymph nodes become heavily immune-suppressed during tumor progression, and switch from an inflammatory- to an inhibitory state, characterized by T cell exhaustion, reduced expression of IFN-γ, IL-17 and loss of T cell activation[44,45,47–49]. Moreover, qualitative assessment of breast cancer sentinel lymph nodes by flow cytometry revealed that functionally suppressive effector T_{regs}^{50} increase in invaded compared to non-invaded lymph nodes, and have increased expression of PD-1 and ICOS[51]. In a similar study, T_{regs} in metastatic lymph nodes were found to have increased expression of HLA-DR, PD-1, CD38, TIGIT and $CD45^{RO}$ and co-localised with $CD8^{+}$ T cells[49].

Despite the wealth of clinical data that point towards a role for T_{regs} in lymph node metastasis, mechanistic data are lacking. One study revealed that T_{regs} within tumor-draining lymph nodes potentiate distant cancer spread[52], but their role in loco-regional metastasis to lymph nodes has remained elusive. We addressed this open question in **chapter 4** and uncover a causal role for T_{regs} in the formation of lymph node metastasis. While we show that T_{regs} impair the function of anti-metastatic NK cells, we did not fully elucidate the mechanistic basis for this inhibitory interaction that occurs specifically in the lymph node niche. One possibility is that T_{regs} impair NK cell cytotoxicity by limiting the availability of IL-2, which is critical for NK cell function[53,54], plays a role in the expansion of T_{regs}^{55}, and is particularly abundant in lymph nodes[56]. Secondly, there are indications that T_{regs} restrain NK cell activation through suppression of lymph node dendritic cells[57,58], which would align with clinical data showing dendritic cells are suppressed in breast cancer sentinel lymph nodes[45]. Interestingly, LAG-3 expression by T_{regs} can limit dendritic cell activation via MHC-II engagement[59], and we observed *Lag3* to be uniquely upregulated in KEP T_{regs} in lymph nodes (**chapter 4**). Finally, emerging data show that tumor-draining lymph nodes turn acidic during cancer progression, which can impair T- and NK cell function[60,61]. In contrast, T_{regs} maintain their suppressive function in acidic conditions[62], and form a barrier for PD-1 blockade through upregulation of PD-1[63].

Besides breast cancer, there are indications that T_{regs} are associated with lymph node metastasis in other cancer types[48,64]. T_{regs} were found to be elevated in tumor-invaded, compared to non-invaded lymph nodes of patients with lung adenocarcinoma[65], melanoma[66],

cervical[67]- and gastric cancer[68], either in absolute numbers or as a ratio compared to conventional T cells. In patients with colon cancer, high T_{reg} density in lymph nodes was predictive of disease progession[69]. While still rather limited, these studies support the notion that T_{regs} are perhaps central regulators of lymph node metastasis in a multitude of cancer types. Again, mechanistic data are still lacking to substantiate this hypothesis, but there is a clear basis for future research to address this research question, thereby guiding the development of new therapeutic strategies to limit early metastatic spread. As an example: it was recently discovered in a GEMM for spontaneous lung adenocarcinoma that anergic $CD4^+$ T cells can differentiate into suppressive T_{regs} in tumor-draining lymph nodes[70], and it would be of interest to test the effect of T_{reg} depletion on lymph node metastasis in this model.

SUPPRESSED SOIL: THE ROLE OF IMMUNOSUPPRESSION IN ORGANOTROPISM OF METASTASIS

Chapter 4 of this thesis describes a mechanism by which tumor-educated T_{regs} promote metastasis to lymph nodes, but not lungs, surfacing a complex question about metastasis. Why are some tissues more prone to colonisation than others? Cancer cell-intrinsic mechanisms like subtype, gene-, and protein expression determine how compatible metastasizing cancer cells are with their new environment[71]. However, the destined local tissue and vasculature are not passive bystanders as cues from specialised resident cells can support metastatic outgrowth[72–74]. It is clear that highly specialised tissue-resident cells like brain astrocytes[75], lung fibroblasts[76] and hepatic stellate cells[77] engage in tissue-specific mechanisms to modulate metastasis formation in their own tissue, but how the immune system is involved in tissue-tropism of metastasis is only beginning to be understood.

Based on the findings in **chapter 4**, I propose that immunosuppressive pathways that support metastasis, have an important tissue-specific component. Across different organs, unique local cues dictate tissue-specific gene programs in resident and patrolling immune cell subsets. These tissue-specific adaptations may differentially impact immune crosstalk in response to tumor-derived signals, likely resulting in niche-specific immunoregulatory mechanisms[64,78,79] (**chapter 4**). In line with this concept, recent evidence shows that the systemic immune landscape is distinctly remodelled in a tissue-dependent fashion during primary tumor development in mice, causing systemic immune dysfunction[80]. Furthermore, metastases of breast and ovarian cancer were found to have distinct infiltrates of immunosuppressive cells in different tissues[81,82], and it is becoming increasingly clear that tissue-specific factors play an important role in shaping local tumor microenvironments and accompanying immunosuppressive features[74,83].

To add to this complexity, each tumor is unique, and primary tumors from different cell lines differentially impact the systemic immune landscape[80], suggesting cancer cell-intrinsic mechanisms can also affect distant immune suppression[84]. Tumor-secreted factors, acting in the TME or beyond can induce systemic immunosuppressive conditions, that allow distant metastasis formation (**chapter 4 & 5**). In particular the systemic mobilisation of myeloid cells in response to tumor-derived factors can support the preparation of a pre-metastatic niche, or a distant immunosuppressive environment[85]. For instance, research in our lab has shown that loss of the tumor suppressor gene p53 in mammary tumors kick-starts a cascade of CCL2 - TAM - Il-1β - γδ T cell – IL17 that drives the systemic accumulation of immunosuppressive neutrophils, which promote metastasis to lung and lymph nodes[86–88]. In addition, breast cancer-derived CCL2 has also been shown to enhance bone metastasis via the local recruitment and polarisation of monocyte-derived macrophages[89]. Although less well studied, tumor-derived factors can also engage systemic immune suppression mediated by the adaptive immune system, most prominently through activation of T_{regs} (**chapter 4**). While we did not identify which factors underlie systemic T_{reg} expansion and activation, others have identified galectin-1[90], IL-2[55,91] and IL-33[92,93] as potential candidates. Importantly, activated T_{regs} in the blood of breast cancer patients are predictive for poor disease outcome[94], highlighting the relevance of understanding how T_{regs} mediate systemic immune suppression during metastasis formation.

Another aspect of how cancer cell-intrinsic features may be intertwined with immunosuppression and organotropism relates to the finding that metastases in different tissues have a distinct genetic make-up[95]. Interestingly, lymph node metastases of colorectal cancer patients were found to be more polyclonal compared to distant metastases, potentially reflective of weaker evolutionary selection in nodular metastases[96]. As it has been shown that evolution of metastatic clones is in part shaped by immune pressure[97], two immune-related explanations for this phenomenon can be formulated. Either there is limited immune pressure in lymph nodes which would allow polyclonal, immunogenic metastatic clones to survive, or, as supported by preclinical and clinical data (**chapter 4**), there are strong immunosuppressive conditions that may limit immune editing in tumor-draining lymph nodes. This second hypothesis suggests that the adaptation of metastatic cells to a distant organ is, besides other determinants[34], dependent on the level of immunosuppression in the destined tissue. Weakly immunosuppressive conditions require cancer-cell intrinsic adaptations to evade immune recognition. In contrast, a high level of (tumor-induced) immunosuppression protects immunogenic metastatic clones from eradication by the immune system, and metastases are therefore not pressured to evolve poor immunogenicity. Thus, the immunogenicity of metastasizing cancer cells may be in part shaped by the potential of tumors to induce systemic immunoregulation, for example through co-option of neutrophils, TAMs and T_{regs}. From a therapeutic viewpoint, this suggests that tumors which succeed in creating systemic immunosuppressive conditions,

may have immunogenic metastatic clones, and may therefore be vulnerable to T cell-based immunotherapeutics, when combined with strategies that overcome immunosuppression. As we found in **chapter 4** that lymph node metastases, but not lung metastases were prone to NK cell-mediated killing, it would be of interest to analyse whether lymph node metastases express NK cell activating ligands, and whether the expression of these ligands is lost in lung metastatic clones.

Next to tissue-specific cues and cancer cell-intrinsic mechanisms, the homeostatic immune composition varies greatly across different organs[98], which has several consequences for local cancer-associated immunosuppression and organotropism of metastasis. Firstly, unique tissue-resident cell types may be prone to co-option by tumors, as demonstrated for lung-resident innate lymphoid type 2 cells and neutrophils (**chapter 5**), pulmonary alveolar macrophages, and central nervous system resident myeloid cells[99–101], which can locally suppress anti-metastatic immune responses in lungs and brain respectively. Secondly, tumor-associated immune suppression is typically not dependent on a single cell or pathway, but instead consists of multiple layers of interconnected and functionally redundant immunosuppressive mechanisms, that are also shaped by the local immune landscape[78,79], and can affect local effector cell function. To illustrate this, we demonstrate in **chapter 4** that NK cells in the lungs, but not the lymph nodes undergo a shift towards a more immature phenotype in tumor-bearing mice, that is independent of T_{regs}, reflective of a different immunosuppressive network.

Combined, organotropism of metastasis can be influenced by immunosuppression through tissue-specific cues, resident cells and immune composition, and cancer cell-intrinsic mechanisms in the form of genetic make-up and tumor-derived factors. Moreover, tissue-specific micro- and mycobiomes may further modulate local immunosuppression in the context of metastasis[102,103]. From the perspective of cytotoxic, anti-tumoral immune cells, getting effectively around distinct regulatory hurdles in different organs seems a daunting challenge. As such, in my view, overcoming tumor-associated immunosuppression will require a more tissue-focussed approach. Broader appreciation for tissue-specific mechanisms of immunoregulation may inspire novel approaches that selectively interfere with metastatic tropism to certain organs. Ultimately, combining these approaches may prove helpful to peel away the different layers of immunosuppression, allowing for anti-tumor immunity.

Going forward, as we are increasingly confronted with the complexity of metastatic disease, it will be important to develop sophisticated models that adequately recapitulate this complexity. This is not only important to increase our fundamental understanding of immunoregulatory mechanisms in cancer, but more so to improve the translation of promising preclinical findings into clinical success. This is an urgent need, as currently

only 0.1% of pre-clinical drug targets are ultimately approved for human use[104]. At the time of writing, a limited number of immunological studies have been performed in models that realistically recapitulate the metastatic cascade and spectrum as it occurs in patients. Experimental metastasis models based on intra-cardiac, intra-venous or intra-portal injections can reflect dissemination to clinically relevant organs (bone, brain, lung and liver), but by no means model the complexity related to heterogeneity, evolution and selection of highly metastatic clones[105]. On the other hand, popular model systems based on orthotopic inoculation of cancer cell lines such as 4T1, EO771 and GEMM-derived MMTV-PyMT more adequately model local tumor cell invasion and intravasation, but fail to recapitulate tissue-tropism of metastasis seen in the clinical setting, intratumoral heterogeneity, and the chronic and systemic inflammation that underlies *de novo* tumor development, progression and metastasis[106]. Because of this, most of current knowledge regarding the role of pro-metastatic, immunosuppressive immune cells and the formation of a pre-metastatic niche in breast cancer comes from single-organ metastasis systems like breast-to-lung[85]. While breast cancer in patients indeed often spreads to lungs, other important niches like lymph nodes, bone, brain and liver are heavily understudied, and it is unclear how interchangeable lung-specific mechanisms are to other tissues, or between different types of cancer. Therefore, more comprehensive models of systemic immunosuppression in the context of cancer are necessary to achieve clinical translation.

The development of *in vivo* somatic gene editing approaches through local delivery of viral vectors provides a cutting-edge approach to model tumorigenesis[107,108]. Excitingly, by replacing orthotopic transplantations of tumor fragments with somatic induction of *de novo* tumors, current spontaneous metastasis models can be updated to additionally reflect progression from initial neoplastic transformation to overt disseminated disease. Such a complete model of metastasis formation may also better reflect the full metastatic spectrum as it is observed in patients, which is still a caveat of GEMMs[105]. Interestingly, the *Cdh1$^{f/f}$Trp53$^{f/f}$;Foxp3$^{GFP\text{-}DTR}$* model described in **chapter 4** and **6** could potentially be used for this approach, and could be combined with genetic ablation of FOXP3^{+} cells to study their function in the progression of invasive breast cancer.

SUPRESSING SUPPRESSION: MANIPULATING T_{REGS} TO THE BENEFIT OF CANCER PATIENTS

The fundamental insights gained into T_{reg} biology in the context of cancer are moving towards clinical practice[109,110]. Importantly, therapeutic targeting of T_{regs} will always be a fine balance between mitigating immunoregulation to unleash anti-tumor immunity, and preserving peripheral tolerance to limit autoimmune-related toxicity. In order to limit toxicity related to the manipulation of T_{regs}, one approach that has been studied in cynomolgus monkeys

is to only partially deplete T_{regs} using anti-CD25, which is sufficient to enhance effector cell activation[111] but does not lead to catastrophic auto-immunity, as is observed upon sustained depletion of T_{regs} in $FOXP3^{DTR\text{-}GFP}$ mice[4,112]. Besides depleting T_{regs}, other approaches are possible that target specific aspects of T_{reg} biology, and can be fine-tuned to specific niches, such as targeting of chemokine receptors, cytokines or immunomodulatory proteins that are important for tumor-educated T_{regs}. The work described in this thesis may provide novel insights into therapeutic targeting of T_{regs} in the following three different contexts:

1) Primary breast cancer (**chapter 3**)
2) Lymph node metastasis (**chapter 4**)
3) Immune checkpoint blockade (**chapter 6**)

Primary breast cancer

In **chapter 3** we show that tumor-associated macrophages control two important independent facets regarding the conversion of $CD4^+$ T_{convs} into $FOXP3^+$ T_{regs}: release of TGF-β, and upregulation of PD-1 expression on $CD4^+$ T_{convs}. Thus, targeting macrophages using anti-CSF1R has the attractive collateral effect of reducing the intratumoral accumulation of peripherally induced T_{regs}, which have been implicated in suppressing antigen-specific anti-tumor immunity[70,113]. The benefit of such an indirect anti-T_{reg} approaches is that the reduction in T_{regs} is limited to the TME whereas systemic, indiscriminate targeting of T_{regs} may trigger severe widespread auto-immunity, due to their elemental role in immune tolerance[112]. Apart from the role of PD-1 in T_{reg} conversion, PD-1 expression is a hallmark of functionally exhausted intratumoral $CD4^+$ T_{helper} cells[114]. As our data show that macrophages promote PD-1 signalling on intratumoral $CD4^+$ T cells, it can be envisioned that macrophages thereby contribute to dysfunction of intratumoral $CD4^+$ T cells, which might be reversed upon macrophage depletion. However, evidence for this hypothesis and how exactly macrophages enhance PD-1 expression on $CD4^+$ T cells would require further study. Another benefit is that anti-CSF1R seems to hit two birds with one stone by reducing both immunosuppressive TAMs and T_{regs}. However, how many birds remain? Previous research using the KEP model has shown that targeting macrophages is not sufficient to induce anti-tumoral effects, due to the compensatory influx of immunosuppressive neutrophils in tumors upon anti-CSF1R, which suppress $CD8^+$ T cell function[23]. As such, targeting T_{regs}, whether these are of thymic- or peripheral origin **(chapter 3)**, is likely only part of the equation to achieve anti-tumor immunity in tumors with dominant immunosuppression. Excitingly, this realisation has already inspired interesting treatment combinations, and it has been shown that targeting multiple facets of this network simultaneously, combined with a T cell-activating treatment, can induce anti-tumor immunity against primary tumors in the KEP model ([23], Blomberg *et al;* personal communication).

Lymph node metastasis

In **chapter 4**, we demonstrate that targeting of T_{regs} impairs metastasis to tumor-draining lymph nodes, raising the question whether this may be therapeutically exploited in breast cancer patients. Our preclinical data show that immunosuppressive T_{regs} arise early during mammary tumorigenesis, indicating that T_{reg}-targeting strategies might be most beneficial to reduce cancer spread to lymph nodes in the neoadjuvant context. In support of this, the disappearance of lymph node metastasis in breast cancer patients treated with diverse neoadjuvant chemotherapy regimens was strongly associated with decreased intratumoral *CTLA4* gene expression and increased activity of peripheral NK cells[115]. Interestingly, there are early indications that neoadjuvant administration of ICB can drive major pathological responses in multiple patients groups[116–119], suggesting the pre-operative window might also be an attractive context to target T_{regs}. Below, I detail some exciting targets that may have relevance in the context of lymph node metastasis.

Most famously, the development of antibodies designed to block the co-inhibitory receptor CTLA-4 in patients has pioneered ICB for the treatment of cancer. CTLA-4 is highly expressed by tumor-educated T_{regs} in lymph nodes (**chapter 4**) and plays a key role in suppressing DC maturation and T cell priming by binding CD80/86 on DC's[109]. While there is ongoing controversy whether anti-CTLA4 antibodies deplete intratumoral T_{regs}[120–122], part of its efficacy is contributed to its inhibitory effect on T_{reg} function[123], which might be valuable to restrain T_{reg} activity in tumor-draining lymph nodes. As data in **chapter 6** show that anti-CTLA4 (combined with anti-PD1) increases the proliferation of T_{regs}, it will still be important to assess the net effect of blocking T_{reg} CTLA4 activity versus increased T_{reg} proliferation on lymph node metastasis formation.

A particularly promising target is the chemokine receptor CCR8. In breast cancer patients, CCR8 is selectively expressed on a clinically relevant population of highly suppressive T_{regs} found both in tumor[28] and blood[94]. In mice, single cell TCR clonotype analysis revealed that CCR8 is specifically expressed on clonally expanded T_{regs} in both tumor and tumor-draining lymph nodes, which are likely reactive to tumor-associated antigen[124]. Several groups have demonstrated that ablation of CCR8 selectively depletes intratumoral T_{regs} without induction of systemic toxicity, and improves tumor control of inoculated cancer cell lines[124–126], demonstrating the anti-tumor potential of this approach. Others showed that T_{regs} isolated from metastatic lymph nodes and tumors share expression of a gene signature consisting of *CCR8*, *CD80* and *HAVCR3*, which correlates to disease outcome in breast cancer patients[51]. Interestingly, both *Cd80* and *Havcr3* are part of the tissue-independent KEP T_{reg} gene signature described in **chapter 4**, indicating that the KEP model might be relevant to study the therapeutic potential of targeting these immunomodulatory receptors during metastasis formation. Finally, T_{reg}-derived TGF-β1 in tumor-draining lymph nodes potentiates distant metastatic spread[52], and blocking TGF-β might therefore be another

attractive approach. However, due to the pleiotropic and context-dependent role of TGF-β in cancer metastasis and immune regulation, clinical targeting of TGF-β has not yet matured[127]. Collectively, several exciting targets have been identified that, likely in combination with an approach that sustains T cell activation within tumors, may subvert immunosuppression by tumor-educated T_{regs} in breast cancer patients in the future.

Immune checkpoint blockade

Research in **chapter 6** describes how ICB inadvertently activates T_{regs}, thereby limiting its therapeutic benefit. Among the rapid development of novel therapeutics aimed at engaging T cells, this surfaces an important notion: T_{regs} are T cells. In fact, T_{regs} have been shown to express most, if not all, co-signalling receptors described to modulate the function of effector $CD8^+$ and conventional $CD4^+$ T cells in tumors. This includes proteins that widely attract clinical interest such as: PD-1, CTLA-4, TIGIT[128], ICOS[129], 4-1BB[130], CD27[131,132] and OX-40[133], for which antagonistic or agonistic monoclonal antibodies are in development. To optimally exploit these targets for anti-cancer benefit, it is critical to understand the net effect on immune activation of simultaneously engaging co-signalling receptors on T_{regs} and conventional $CD4^+$ and $CD8^+$ T cells. As observed in **chapter 6**, blockade of co-inhibitory receptors can increase T_{reg} proliferation and activation. These activated T_{regs} likely limit the intended activation of effector cells, since depletion of T_{regs} in the context of ICB mobilises $CD8^+$ T- and NK cells in blood. In line with this notion, it has been shown that anti-PD-1 reactivates dysfunctional $PD\text{-}1^+$ T_{regs}, which subsequently restrains concurrent $CD8^+$ T cell activation and negatively impacts immunotherapy response in patients with gastric cancer and non-small cell lung cancer[134,135]. Furthermore, CTLA-4 on T_{regs} inhibits the proliferation of effector cells by engaging CD28 on dendritic cells, but this same mechanism inhibits the proliferation of T_{regs} themselves[136]. Indeed, anti-CTLA-4 has been shown to expand T_{regs} in cancer patients[120,137], but it is unknown whether this impacts therapeutic benefit. Promisingly, blockade of so-called "second-tier"[128] co-inhibitory proteins TIGIT and TIM-3, but not LAG-3[138], does not appear to provoke T_{reg} activation, but instead reduces the suppressive activity of T_{regs}[139,140]. Therefore, in tumors that are abundantly populated by $PD\text{-}1^+$ or $CTLA\text{-}4^+$ $T_{regs,}$ an alternative approach to anti-PD-1/CTLA-4 therapy might be blockade of TIGIT and TIM-3.
On the other side of the co-signalling spectrum, therapeutic engagement of co-stimulatory receptors can also induce T_{reg} activation. Ligation of co-stimulatory receptor 4-1BB has been shown to activate both $CD8^+$ T cells[141] and T_{regs}[142]. In addition, CD27 co-stimulation expands T_{regs} in hyperlipidaemic mice[143], and ablation of CD27 on T_{regs} synergizes with anti-PD-1 therapy in mice bearing MC38 cell line tumors[144]. Finally, in mice, the co-stimulatory receptor ICOS has been shown to mark highly suppressive T_{regs}[129], and $ICOS^{KO}$ mice have impaired Th1 and Th2 responses, but also reduced T_{regs} in models for allergy and infection[145]. Combined, these findings indicate that pulling the brakes on co-inhibitory signalling does not only evoke (re)activation of beneficial effector cells, but engages immunosuppressive T_{regs} via the same mechanisms. Likewise, various co-stimulatory agonists appear to activate

both regulatory and conventional T cells. The intrinsic similarity in response to modulation of co-signalling between T_{regs} and conventional T cells on a cellular level, simultaneously activates opposing pro- and anti-inflammatory effector mechanisms. This may be a valuable built-in brake that limits excessive immune activation, but may also offset the benefit of therapeutic modulation of co-signalling receptors. Ultimately, this conundrum raises the question: which response takes the upper hand? As has been shown in the context of PD-1, this is likely dependent on the balance between cell types that express the receptor of interest, and the intensity of expression of both the receptor and its ligand[135], which might greatly differ in distinct niches. Indeed, different metastases within the same patient can have distinct expression of co-signalling molecules[81], demonstrating the relevance of this concept in cancer patients. Another consideration is that cells in distinct differentiation states of the same lineage can respond differently to immunomodulatory drugs. As we show in **chapter 3** and **6**, PD-1 plays a dual role on $CD4^+$ T cells. PD-1 signalling in intratumoral conventional $CD4^+$ T promotes their conversion into T_{regs}, and PD-1 blockade has been shown to inhibit this process in CT26 colorectal tumors[146]. On T_{regs}, PD-1 signalling has not been described to impact their differentiation, but is primarily linked to dysfunction[147]. It is possible that other co-signalling molecules also differently impact $CD4^+$ T cell plasticity, but this remains a topic of future research.

Going forward, a more comprehensive understanding of how therapeutic interference with co-signalling impacts regulatory and conventional T cells and its resulting effect on anti-tumor immunity may be key to improve clinical responses of these approaches. Promisingly, innovative treatment strategies could be employed that selectively activate conventional T cells, but not T_{regs}. As an example, anti-PD1-IL2 fusion proteins, consisting of a high affinity PD-1 antibody coupled to a non-T_{reg} binding IL-2 variant, have been shown to selectively expand tumor-specific T cells, but not T_{regs}, and are moving towards clinical development[91,148]. Another approach could be to develop bispecific antibodies which direct co-inhibitory agonists, or co-stimulatory antagonists to T_{regs}, using proteins abundantly expressed on T_{regs} like CD25 and GITR. Besides the detrimental role of T_{regs} in the context of ICB, little is known about the impact of T_{regs} on other T cell-based immunotherapy approaches, like adoptive T cell transfer using engineered chimeric antigen receptor (CAR) T cells or expanded TILs[149]. It would be of interest to investigate whether T_{regs} are present in these products, and antagonize the function of co-transferred conventional T cells. Combined, it is clear that T_{regs} are direct targets of immunomodulatory drugs, and should be regarded as such, in the design, validation, and clinical rollout of novel avenues of immunotherapy.

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