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# Multifaceted approaches to tumor microenvironment modulation and immune checkpoint targeting

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# CHAPTER 6

## Summary and Perspectives

# SUMMARY

The Tumor Microenvironment (TME) is composed of various cell types, extracellular matrix, and signaling molecules that can either support or hinder cancer development and metastasis and is a crucial determinant for the success of immunotherapies. For instance, immune cells infiltrated in the TME can either enhance or suppress immune responses and hereby shape the clinical and biological nature of cancers [1-3]. Therefore, design of specific therapeutic strategies that reprogram the TME and (re) activate anti-tumor immune responses is of considerable interest. Immune checkpoints can be inhibitory, such as PD-1/PD-L1 and CD300a, or stimulatory, such as CD27, and are crucial regulators of immune homeostasis. However, these signaling pathways are often exploited by tumors to evade immune surveillance, with different immune cells and signaling pathways contributing together to an immunosuppressive environment. Insight into such potential cooperative signaling pathways can reveal unique ways to activate anti-tumoral immune responses that are suppressed in the cancer setting.

In **chapter 2**, we investigated a bispecific Fc-based fusion protein, termed DSP502, that targets immune checkpoints PVR and PD-L1 as a novel immune checkpoint inhibitor (ICI). DSP502 (comprising the extracellular domain of TIGIT and PD-1) formed a stable heterodimer and bound specifically to PVR and PD-L1. Importantly, DSP502 bound significantly better to surfaces coated with both antigens compared to single antigen-coated surfaces. This synergistic effect, often described as an 'AND-gate' affinity, illustrates that the binding of DSP502 is most effective in the concurrent presence of both antigens, resembling the logic operation of an AND gate. In cellular assays, DSP502 exhibited dose-dependent binding to cancer cells expressing PVR and PD-L1, with a notable decrease in binding upon antigen blockade. Additionally, DSP502 could simultaneously bind CD56+ NK cells via FcγRIII interaction and triggered formation of NK -tumor cell doublets, an essential first step to allow for NK-mediated tumor killing. A transcriptomic analysis revealed that tumor-reactive CD8+ T cells in NSCLC tumors express both high levels of TIGIT and PD-1, positioning DSP502 as a potential candidate for immune re-activation in NSCLC. *In vitro* co-culture experiments with healthy donor PBMCs and NSCLC TILs confirmed DSP502's capacity to boost NK cell activation and enhanced the cytotoxic potential of T and NK cells. Notably, DSP502 also prevented the downregulation of the costimulatory receptor DNAM-1, a feature that could contribute to its therapeutic effect. *In vivo* studies reinforced the therapeutic potential of DSP502, with significant inhibition of tumor growth upon DSP502 treatment in two mouse models. Thus, DSP502 enhanced immune checkpoint blockade and simultaneously potentiated the cytotoxic activity of immune effector cells through FcR-mediated recruitment. Together, this multifold mode-of-action positions DSP502 as a promising candidate for cancer immunotherapy, especially in cancers like NSCLC, where dual PVR and PD-L1 blockade may be particularly beneficial.

In **chapter 3,** we investigated a strategy to provide targeted costimulation to T cells through the costimulatory receptor CD27, a receptor constitutively expressed on the majority of tumor-infiltrated T cells in epithelial cancers, as confirmed by a transcriptomic analysis. Specifically, we designed a bispecific antibody that on the hand targeted the broadly expressed carcinoma-associated antigen EGFR and on the other hand targeted CD27. We demonstrated enhanced EGFR-restricted activation of T cells and anti-tumor immune responses *in vitro*. Importantly, we measured the efficiency of T cell re-activation by secretion of IFNγ and found that CD27xEGFR selectively stimulated T cells to secrete high levels of IFNγ, indicating potent anti-tumor activity. An important aspect of the design of CD27xEGFR is that it contains a so-called silent Fc domain that does not interact with Fc receptors, which should reduce off-tumor toxicity. By the targeting of the bispecific to EGFR, effective activation of CD27 is restricted to the site of the cancer lesion, as CD27 signaling relies on higher-order cross-linking. Indeed, using an *in vitro* model of T cell activation in which ES-2 cancer cells were artificially expressing scFvCD3 to provide the necessary first signal for T cell activation, CD27xEGFR selectively activated T cells when exposed to EGFR+ but not EGFR– tumor cells. Thus, CD27xEGFR selectively activated T cells and potentiated anti-tumor activity, positioning CD27xEGFR as a potential therapeutic approach for most EGFR-expressing solid cancer with high TIL infiltration.

In **chapter 4**, we shifted focus to innate immune responses in B cell lymphoma by identifying and targeting of the receptor CD300a. CD300a is known to be expressed on phagocytic cells such as macrophages and neutrophils. In our study, CD300a was identified as a potentially relevant target in a large transcriptomic screen from publicly available microarray expression data of Diffuse Large B-Cell Lymphoma (DLBCL) samples, in which CD300a associated with poor prognosis in non-germinal center B-cell (non-GCB) DLBCL patients with high expression of CD47. Deconvolution methods applied to the bulk-RNAseq dataset identified that macrophages and neutrophils, and not DLBCL cells, were the cell populations with the highest expression of CD300a. This suggested that the association of CD300a with patient survival could be specifically related to regulation of innate immune responses. The validity of this finding was supported by *in vitro* membrane staining and gene expression analysis on primary tumor samples from patients, with high expression in macrophages and neutrophils vs. DLBCL cells. The known ligands for CD300a are PS and PE, with PS known to be a pivotal signal that dictates phagocyte activity. Blocking the interaction between CD300a and its ligands, PS and PE, with an anti-CD300a antibody significantly potentiated macrophage and neutrophil mediated phagocytic activity, specifically against the non-GCB subtype of DLBCL. The impact of CD300a blocking was corroborated using a F(ab')2 fragment of the anti-CD300a antibody, which lacks the Fc domain, thus excluding Fc/FcR-mediated cross-species signaling. The use of the F(ab')2 fragment resulted in an ~25% increase in trogocytosis by G-CSF stimulated neutrophils, indicating true checkpoint blockade activity upon CD300a inhibition. Moreover, when the CD300a F(ab')2 fragment was

used in conjunction with rituximab (RTX), it demonstrated similar enhancement of phagocytosis as the full-length CD300a antibody, suggesting that the F(ab')2 fragment could be a viable therapeutic modality. Importantly, a further retrospective analysis of patient expression data revealed that CD300a expression levels also correlated with worse prognosis in mantle cell lymphoma (MCL) and uveal melanoma (UM) patients. In line with this, the same anti-CD300a antagonistic antibody also significantly enhanced the phagocytosis of MCL and UM cell lines. Overall, our research demonstrated that CD300a may be a potential target for immunotherapy against specific tumors and subtypes, particularly for non-GCB DLBCL, MCL and UM.

In the final chapter of this thesis, **chapter 5** a literature review was performed to summarize the current knowledge on TME normalization strategies and the role of immune checkpoints in modulating the TME. Specifically, my focus was on understanding the complex mechanisms by which ICIs alter the behavior of immune cells and the vasculature within the TME. A crucial finding from the surveyed literature is that ICIs not only potentiate the immune response, but may also contribute to TME normalization, as seen in the clinic where their use has augmented the effectiveness of various cancer therapies. Evidence from preclinical models suggests that the normalization of blood vessels in ICI-responsive tumors, such as murine breast tumors, involves the critical participation of CD8+ T cells and IFNγ. This immune-vascular interaction, mediated by ICIs, points to a dual modality where IFNγ's concentration-dependent effects range from antiangiogenic to vascular normalization. Additionally, the intricate roles of different T cell subsets in vascular dynamics were explored, with CD8+ T cells possessing pro-angiogenic capacities, whereas CD4+ T cells may indirectly facilitate vascular normalization by promoting pericyte coverage and reducing hypoxia.

The insights gathered in **chapter 5** underscore the potential of TME normalization as a strategy for enhancing immunotherapy outcomes. Further, this chapter serves as a reflective account of my perspective on the significance of integrating TME normalization principles into the development of future ICI therapies. By combining the mechanistic understanding of ICIs in the TME with clinical observations, this chapter aimed to pave the way for novel therapeutic approaches that leverage the intricate connections between immune system and tumor vasculature.

# DISCUSSION AND PERSPECTIVES

## Strategizing PD-L1 and PVR Blockade and beyond

In **chapter 2**, I explored anticancer immunostimulatory activity of DSP502, a novel bifunctional molecule featuring an active IgG1 domain, that concurrently blocks both PD-L1 and PVR. In the *in vitro* model with the ES-2 ovarian cancer cell line, which intrinsically expresses PD-L1 and PVR, the enhancement in PBMC and TIL cytotoxicity was moderate with observed differences not surpassing a 10% increase. Although these results align with the outcomes associated with other PD-L1 and PVR inhibitors tested in similar *in vitro* conditions—accounting for equivalent target expression levels and protein structures—they may not be indicative of clinical efficacy [4-6]. For context, PD-(L)1 inhibitors, which are preclinically evaluated for their ability to stimulate IFNγ production, affinity to PD-(L)1, and receptor occupancy, have achieved a mean objective response rate (ORR) of 19.56% in clinical trials [7-10]. Consequently, the true clinical significance of a 10% augmentation in cytotoxicity *in vitro* will necessitate thorough assessment in clinical settings. Moreover, evaluations of efficacy in *in vivo* experiments should be done employing syngeneic models as their outcome is more predictive of clinical efficacy of ICIs [11]. Further, innovations in research methodologies, including the use of predictive and pharmacodynamic biomarkers [12, 13], may offer avenues to estimate the clinical potential of DSP502 more accurately.

Precedents in exploiting the PVR axis indicate that there are untapped combinatorial opportunities to augment the therapeutic efficacy of DSP502, possibly through the incorporation of associated checkpoint inhibitors. One example is the inhibitory checkpoint receptor PVRIG, which modulates T-cell activity by outcompeting DNAM-1's binding to PVRL2 on cancer cells. PVRIG, also known as CD112R, is an inhibitory receptor expressed on T and NK cells. It binds to its ligand PVRL2 (CD112) with a higher affinity than DNAM-1 and this interaction suppresses the activation and cytotoxic functions of immune cells. [14] In a preclinical study, the combination of TIGIT and PD-1 blockade with PVRIG had a superior antitumor effect compared to each individual pathway alone [15]. Moreover, preliminary results of NCT04570839 which evaluates the triple combination of PD-1, TIGIT, and PVRIG antibodies are suggestive of encouraging antitumor activity in patients with endometrial cancer including in patients refractory to PD-1 blockade [16]. Notably, in additional experiments not shown in this thesis, a co-culture of PBMCs with K562 cells led to a donor-dependent reduction in PVRIG expression on T cells in the presence of DSP502. This finding is in line with data by Whelan et al., who observed a decrease in PVRIG expression on T cells following initial activation but noted an increase after sustained activation on day 11 [17]. These observations indicate that an increased PVRIG expression may serve as an acquired resistance mechanism in certain donors, triggered by T cell reactivation induced by blocking PVR and PD-L1. Thus, the synergistic blockade of PVR and PD-L1 pathways with PVRIG is postulated to significantly enhance anti-tumor efficacy. An

innovative approach that could be explored to exploit the combination with PVRIG blockade involves engineering DSP502 to include an additional fusion site for PVRL2 attachment, potentially transforming DSP502 into a trispecific signaling protein (TSP). This could be achieved by genetically fusing a PVRIG-binding domain—derived from the extracellular domain of PVRL2—to the C-terminus of one of the heavy chains of the DSP502 construct. The design of this TSP could involve a linker sequence that optimizes the spatial orientation and accessibility of the PVRIG binding-domain, ensuring effective simultaneous interactions for all three targets. This TSP could simultaneously block inhibition and bridge PVRIG+ T and NK cells to PD-L1+PVR+ cancer cells thereby selectively activating DNAM1+ signaling, potentially surpassing the efficacy of DSP502 alone. Nonetheless, the production of complex structures such as TSPs, may encounter issues, including improper folding and aggregation, which could be addressed by optimizing factors such as production conditions, expression systems, or linker sequences.

In terms of TSP design, another potential add-on to the mode-of-action of DSP502 is a combination with CD96 co-stimulation. CD96 is usually co-expressed with TIGIT on TILs and also binds to PVR with an affinity that lies between TIGIT and DNAM-1. Although its role on T cells has been reported to be both inhibitory and stimulatory, there is a consensus that CD96 engagement can lead to co-stimulatory signaling, contingent on the crosslinking capacity of anti-CD96 mAbs used in various studies [18-21]. Preclinical models have demonstrated that an anti-CD96 mAb combined with blockade of PD-1/PD-L1 or TIGIT had enhanced antitumor efficacy compared to the single-agent blockades [20]. Building on this evidence, engineering a CD96-binding domain into DSP502 would enable it to use PVR and PD-L1 as anchoring domains, potentially facilitating the crosslinking of CD96 and enhancing T cell activation. This could be done in a similar approach to the addition of a PVRIG-binding domain, as described above, potentially synergizing with DNAM-1's co-stimulatory activity.

The feasibility of integrating PVRIG and CD96 into a TSP concept is best demonstrated by a similar approach that integrated the checkpoint LAG-3. LAG3 is a receptor usually upregulated on tumor-reactive T cells that has already been targeted in the context of TIGIT and PD-L1 co-blockade [22, 23]. Notably, the expression of LAG-3 is upregulated due to T cell exhaustion and is often associated with the expression of TIGIT and PD-1 in TILs [24]. In a multi-specific antibody targeting PD-L1, TIGIT and LAG-3, superior activity was detected compared to a PD-L1xTIGIT bispecific [22]. Importantly, unlike LAG-3, which is highly expressed on Tregs and Dendritic Cells, CD96 and PVRIG are mostly expressed on activated T cells and NK cells [25-29], thus likely providing a better therapeutic index by evading potential stimulatory effects towards immune inhibitory Tregs.

## Advancing CD27-Mediated Immunotherapeutics

In the realm of CD27 targeting, clinical and preclinical research has substantiated the therapeutic efficacy of CD27 engagement [4, 5]. However, effective CD27 receptor signaling in T cells requires at a minimum hexamerization of the receptor, which by itself presents a distinct challenge, as this process needs an engagement mechanism that emulates the receptor's natural cross-linking process. In **chapter 3**, I elucidated the feasibility of inducing hexamerization in T cells by leveraging a tumor-specific antigen, EGFR, to drive cross-linking of the anti-CD27 moiety of CD27xEGFR. This method of complex formation significantly amplified T cell activation and potentiated their cytotoxic capacity. Furthermore, the use of a silent Fc domain in CD27xEGFR precluded the attachment of Fc-receptor cells to the IgG domain of CD27xEGFR, which makes CD27xEGFR less likely to trigger off-tumor ADCP or ADCC. Nonetheless, this approach is constrained by the expression of tumor-associated antigens like EGFR that are also present in healthy tissues and may not be uniformly expressed across all tumor cells. Additionally, the efficacy of this strategy is contingent upon the prior infiltration of tumor-reactive T cells into the TME.

A prospective solution to the limitations of CD27 agonism in the context of CD27xEGFR, involves the integration of bispecific T cell engagers (biTEs). As delineated in Skokos et al.'s research, the synergistic effect of dual bispecifics – one being CD28xEGFR and the other, a biTE CD3xCD20– augmented the efficacy of CD3xCD20 by 34-fold *in vitro* [6]. Notably, CD28xEGFR exhibited limited activity on its own considering its dependency on the presence of tumor-reactive T cells for maximal efficacy, a phenomenon likely to be mirrored in CD27xEGFR. In addressing this, it becomes pertinent to target tumor associated antigens (TAAs) that are often upregulated in response to acquired resistance to EGFR-targeted therapies. TAAs such as HER2, HER3, and cMET, which are prominently activated in cetuximab-resistant cells, present compelling targets [7]. Consequently, a combination strategy involving CD27xEGFR with biTEs targeting these HER family members is expected to enhance tumor cell targeting, even in the context of EGFR resistance. This approach could provide a more comprehensive blockade of oncogenic signaling pathways, potentially leading to improved antitumor efficacy, and overcoming the resistance mechanisms that hinder current EGFR-based therapies. However, such combinatorial strategy would need to take into account the potential toxicity associated with TAA targeting such as HER family members. Specifically, HER-2 targeted therapies have been associated with cardio, gastrointestinal, and liver toxicities among other adverse effects [8]. Therefore, potential toxicities associated with HER2 targeting should be investigated in relevant humanized Her2-transgenic mouse models that recapitulate EGFR-resistant tumor characteristics, such as those observed in cetuximab-resistant tumors.

Of note, ionizing radiotherapy (RT) also represents a compelling adjunctive treatment that may exhibit synergy with CD27xEGFR in the modulation of TME. Ionizing

radiation has been observed to modulate the immune landscape of the TME, tipping the balance from immune suppression to activation and potentially promoting immune cell infiltration [9, 10]. Notably, EGFR expression was also increased following radiation and the cytotoxic impact of radiation appears to be inversely related to EGFR expression levels [11]. This evidence suggests that radiotherapy could act as a potent immunostimulant that could prime the TME for subsequent intervention with CD27xEGFR alone or in combination with a HER-based biTE. Preclinical data support this rational and has paved the way for the clinical development of RT and ICI combination therapy [12]. Moreover, preclinical studies on the synergy between RT and TNFSF receptor agonists of 41BB or CD40 further validate this approach [13, 14] Additionally, both preclinical and clinical data substantiate the abscopal effect, wherein localized radiation therapy triggers systemic antitumor responses across various tumor types, resulting in the regression of metastases beyond the irradiated area [15-17].

BsAbs such as CD27xEGFR offer a novel immunotherapeutic mechanism distinct from immunotherapies such as CAR-T cells, focusing on localized costimulation rather than direct cytolytic activity. This approach may result in a different adverse effect profile, potentially avoiding severe side effects like cytokine release syndrome and neurotoxicity associated with CAR-T therapies [18-20]. Despite their fundamental differences, combining CD27xEGFR with CAR-T therapy can offer a promising approach for enhancing solid tumor treatment efficacy. While CAR-T cells have revolutionized treatment for hematological cancers, their effectiveness in solid tumors has been comparatively limited, likely due to suppression within the TME [21]. Moreover, the complex and personalized nature of CAR-T cell manufacturing renders some patients ineligible for this treatment modality [22]. To circumvent these limitations, incorporating the CD27xEGFR construct into a CAR-T cell platform targeted towards specific TAAs, such as HER2, may help amplify the immune response against solid tumors. Moreover, the design of CAR-T cells to secrete bsAbs like CD27xEGFR only upon activation could ensure a tumor-localized enhancement of the immunity, combining the specificity of CAR-T targeting with the localized immunostimulatory effects of CD27xEGFR. Further including genetic modifications to mitigate toxicity—such as GM-CSF mutational inactivation—could enhance safety and maintain targeted cytokine production and immunomodulation within the TME [23, 24]. This combined strategy has the potential to surpass the therapeutic strength of CD27xEGFR alone, as it circumvents the dependency on the presence of endogenous tumor-reactive T cells or resistance phenomena like antigen loss, which is a challenge for both bsAbs and CAR-T therapies [25].

Further, as many tumor types overexpress PD-L1, another strategy that can enhance the selectivity of CD27 agonism with CD27xEGFR can be its combination with PD-L1/PD-1 blockade. Indeed, PD-L1 binding to PD-1 may be sufficient to inhibit T cell activation even in the presence of co-stimulation through CD27, indicating that PD-1 blockade combined with triggering of CD27 can act on the same individual cells to augment T

cell activation [26]. An illustrative example of this is CDX-527, a bispecific antibody combining PD-1 blockade with CD27 costimulation. CDX-527 potently inhibits PD-1 signaling and induces CD27-mediated T cell costimulation through PD-L1 cross-linking, yielding enhanced antigen-specific T cell immunity and antitumor activity in preclinical models [27]. Thus combining the selectivity of CD27xEGFR with PD-1/PD-L1 blockers may yield augmented efficacies. Further supporting this idea is the fact that combinational approaches of agonistic CD27 antibodies and PD-1 blockade have presented the highest preclinical efficacy, successfully eradicating tumors in preclinical models [5]. Of note, Koopmans et al. previously reported on PD-L1xEGFR bsAb, which enhanced tumor reactive T cell activity and had increased tumor uptake *in vivo*, highlighting the therapeutic potential of PD-L1 blockade in EGFR-positive tumors [28].

Similarly, the integration of EGFR-selective CD27 agonism with blockade of other immune checkpoints, like CD73, may enhance antitumor efficacy [29]. Notably, a preclinical study demonstrated that CD73, an ecto-enzyme involved in immune suppression, has a pivotal role in modulating the therapeutic response to 4-1BB agonistic antibodies. In this study, inhibiting CD73 not only augmented the anti-tumor efficacy of 4-1BB agonists but also induced tumor regression independently, underscoring the potential of combining TNFRSF member targeting with CD73 blockade for improved cancer treatment outcomes [30].

Although EGFR-restricted CD27 agonism provides clear proof-of-concept for targeted activation of CD27 signaling, identification of additional and perhaps better suited tumor antigens may optimize this approach. Specifically, antigens that not only confine CD27 co-stimulation to the lesion, but also aid in the infiltration of immune cells into the TME are of particular interest. As discussed in **Chapter 5**, combining ICIs with an anti-VEGFR2 antibody has demonstrated the potential to normalize tumor vessels through re-activation of CD4+ T cells. This suggests that antigens expressed on tumor-associated endothelial cells, such as VEGFR, may be especially relevant. Although EGFR is overexpressed in tumor-associated endothelial cells as well, its expression is not uniform, potentially limiting the efficacy of CD27xEGFR in certain tumor types [31-34]. While not covered in **Chapter 3**, evidence suggests that bispecific antibodies capable of recruiting T cells and inducing their extravasation through the endothelium can enhance T-cell infiltration, transforming immunologically "cold" tumors into "hot" tumors [35]. Consequently, although the dual targeting approach of CD27xEGFR may improve T-cell localization to tumors via targeted EGFR expression, its effectiveness may be constrained in tumor types with lower EGFR expression in the endothelial cells. To circumvent this limitation, the exploration of alternative endothelial tumor antigens could improve the strategy of directed CD27 agonism and T-cell recruitment. In the context of solid tumors characterized by angiogenesis, a more efficacious bispecific agent might be CD27xVEGFR, given the role of VEGF in reducing T-cell extravasation and its pronounced expression on the tumor endothelium [36, 37]. Therefore, while the

CD27xVEGFR bsAb has the potential to enhance T-cell infiltration and immunotherapy efficacy in solid tumors with active angiogenesis, it will be imperative to methodically investigate the specific impact of CD27xVEGFR on T-cell extravasation. Additionally, as CD27xVEGFR would likely concentrate its activity on the endothelial cells, it will be important to study whether CD27xEGFR can induce systemic immune activation within the tumor bed.

Innovative *in vitro* models such as microfluidic systems and organ-on-a-chip (OOC) technologies would be pertinent for elucidating the mechanisms of T-cell extravasation and the precise localization of CD27-costimulatory activity. For example, using a microfluidic 3D endothelium-on-a-chip model, researchers have been able to quantify and visualize T-cell transendothelial migration enhancements mediated by TNFα and CXCL12 in HIMEC-1 vessels [38]. Moreover, the evaluation of both the CD27 co-stimulatory effect and the extravasation potential of CD27xVEGFR or CD27xEGFR could be effectively modeled using a state-of-the-art OOC platform. A case in point is the Single-Flow MIVO® device, a recently developed system that enables quantification of interactions with matrix-embedded tumor cells, closely mimicking human pathology [39].

In addition to extravasation, the tumor-associated collagen architectures within the extracellular matrix (ECM) present a formidable barrier to T-cell infiltration. Notably, Type XX collagen is markedly upregulated in an array of cancers, underscoring its critical role in the ECM dynamics of oncogenesis and its viability as a biomarker [40]. An innovative therapeutic approach could involve engineering CD27xEGFR with a domain that binds to Type XX collagen. Ishihara et al. provided a foundation for this strategy, demonstrating that the conjugation of ICIs or IL-2 with a collagen-binding domain (CBD) facilitates their retention within the tumor stroma, enhancing local therapeutic effects. This methodology was validated across various murine models, yielding an enhancement in both efficacy and safety profiles when compared to free-form agents [41]. Consequently, the strategic targeting of Type XX collagen in conjunction with CD27 co-stimulatory signaling could potentiate anti-tumor immunity while concurrently mitigating systemic toxicity.

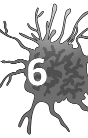
However, it is important to consider the limitations of the CBD-based tumor-targeting approach, particularly its dependency on tumors with adequate vascular permeability and angiogenesis. Moreover, confirming whether CBD can facilitate the delivery of CD27xEGFR to the tumor bed and effectively localize CD27 signaling at the T cell interface would be essential. Although Ishihara et al. demonstrated that CBD-conjugated αPD-L1 had an increased presence within the tumor, the precise localization and subsequent re-activation effect of CBD-αPD-L1 at the site where T cells engage tumor cells remains to be investigated. [41] Methodologies such as high-plex immunofluorescence imaging employing a fluorescently labeled CBD-CD27xEGFR,

could further delineate the microlocalization and functional consequences of this strategy *in vivo*, as suggested by a recent study [42].

## CD300a: A Multifaceted Target for Immunotherapy and Combination Treatment Strategies

In **chapter 4**, we identified CD300a as a potential inhibitory regulator of innate immunity in non-GCB, MCL and UM. In this study, *in vitro* treatment with a CD300a mAb or its derived F(ab')2 fragment increased phagocytosis by approximately 10% and 25%, respectively. This effect could be underestimated since factors within the tumor microenvironment (TME), such as hypoxia and inflammatory stimuli, are known to increase CD300a expression on myeloid cells [95, 96]. Additionally, CD300a expression is found to rise with age, in line with a wider trend where aging is linked to increased expression of inhibitory receptors [97-100].

Drawing parallels with other innate immunity inhibitors like CD47, which has shown variable phagocytosis enhancement with targeted therapies, our findings gain clinical relevance. For instance, *in vitro* studies of CD47-targeting therapies have reported variable phagocytosis enhancement that are in the same range with CD47 mAbs and their derived F(ab')2 fragments, with some studies showing no phagocytosis induction by CD47 F(ab')2 [30, 31]. These variations have also been considered in the pharmaceutical landscape; for example, TTI-621, which engages the IgG1 Fc region, has achieved an overall response rate (ORR) of 29% as monotherapy in DLBCL, whereas TTI-622, which features an IgG4 Fc domain designed to minimize ADCC and complement-dependent cytotoxicity, reports an ORR of 33% [32]. Notably, the presence of an Fc region, although conducive to phagocytosis, does not necessarily amplify the therapeutic impact of CD47 mAbs. Moreover, it is associated with adverse effects such as thrombocytopenia. For instance, TTI-621, featuring an active IgG1 Fc domain, has been linked to a 20% incidence of thrombocytopenia, whereas TTI-622, with the less active IgG4 Fc domain, has a reduced incidence rate of 5% [33-38]. Consequently, for future CD300a-targeting strategies, the development of therapeutics with inactive Fc domains might achieve comparable outcomes to those with an active IgG1 domain, potentially circumventing the side effects linked to Fc-mediated effector functions.



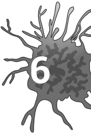

The application of cytokine therapy, such as GM-CSF, has been postulated as a means to modulate the immune response and potentially counteract the immunosuppressive effects of the TME [39]. In our study, GM-CSF-stimulated neutrophils treated with a CD300a F(ab')2 fragment, more effectively trogocytosed cancer cells, highlighting the therapeutic potential of such a combination. This observation aligns with the broader research demonstrating the efficacy of ICI combined with GM-CSF. For example, combining PD-1 inhibitors with radiotherapy and GM-CSF has yielded promising results as a salvage treatment in metastatic solid tumors [40]. Additionally, the induction of GM-CSF in dendritic cells via vaccination exerted a synergistic effect with PD-1

blockade [41-43]. Thus, the dual approach of targeting both the TME and the blockade of CD300a in tumor-associated neutrophils, could pave the way for more effective immunotherapeutic strategies. To restrict the targeting and blocking of CD300a to immune cells a bispecific approach might prove an interesting option. For example, Ring et al. with the development of an anti-CD70/SIRPα bispecific antibody, showed enhanced efficacy compared to the individual antibodies [44]. Given that CD70 is predominantly expressed in most B cell malignancies, such as DLBCL, and is associated with a worse prognosis, a bispecific antibody combining anti-CD70 and anti-CD300a is expected to potentiate the activation of myeloid cells without interfering with the potentially tumor-suppressive role of CD300a on cancer cells from solid tumors. [45]. In solid tumors, CD300a expression in cancer cells has been associated with an anti-oncogenic role, primarily through the downregulation of the Wnt/β-catenin pathway [46]. Here, the inclusion of SIRPα, another immune checkpoint molecule, as a second targeting domain in a CD300a bsAb could yield an immunotherapeutic strategy that maximizes efficacy on immune cells, while minimizing effects on cancer cells.

Our studies on CD300a were focused on phagocytic activity by neutrophils and macrophages, whereas we did not evaluate impact on NK cells that also highly express CD300a. On NK cells, CD300a acts as an inhibitory receptor that dampens ADCC responses by inhibiting degranulation and cytokine release [47, 48]. Existing studies in the literature indicate that targeting CD300a on NK cells has a clear effect, with mAb-mediated agonistic cross-linking of CD300a significantly inhibiting the inherent cytotoxic activity of NK cells [49]. Thus, the impact of antagonistic CD300a antibody treatment of NK cell should be evaluated in subsequent studies to gain a more complete understanding of therapeutic activity profile of this approach. CD300a is prominently expressed on the majority of blood NK cells, but at higher levels in the $CD56^{bright}$ subset, a finding that holds significant implications for the development of novel immunotherapies as this bright subset is enriched in the TME [49, 50]. Unlike their $CD56^{dim}$ counterparts, which are more cytotoxic, $CD56^{bright}$ NK cells are known for their robust production of cytokines such as IFN-γ upon activation [51, 52]. This ability to secrete cytokines makes the $CD56^{bright}$ subset particularly significant within the TME, where they can potentially influence the behavior of other immune cells and contribute to the shaping of anti-tumor responses. Interestingly, $CD56^{bright}$ NK cells also highly express NKG2A, another inhibitory receptor [53]. Thus, to improve NK cytotoxicity a strategy worthwhile to explore would be the development of a bifunctional protein that simultaneously block CD300a and NKG2A. NKG2A blockade with the monoclonal antibody monalizumab can restore NK cell cytotoxicity in chronic lymphoid leukemia [54], and combination NKG2A with PD-1 ICI yielded modest efficacies observed in several cancer types [55].

In the context of CD300a targeting, it also important to consider the role of CD300c, a molecule that shares high sequence homology (79.05%) with CD300a. This close

similarity is clinically relevant because mAbs developed against CD300a often cross-react with CD300c [56, 57]. CD300c is an activating receptor that upon binding to PS and PE promotes phagocytosis [56, 58, 59]. Therefore, therapeutic development of CD300a targeting strategies should exclude potential effects on CD300c. Further, the characteristics of CD300a and CD300c parallel the relationship observed in the KIR2DL-KIR2DS pair on NK cells, where KIR2DL functions as an inhibitory receptor and KIR2DS serves as a stimulatory receptor [50]. Co-expression of CD300c and CD300a is commonly noted in myeloid cells and CD4+ T cells, with their expression patterns on myeloid cells playing a role in determining these cells' inhibitory or activating capabilities [56, 58-60]. Notably, while these molecules are implicated in the regulatory balance of cell activation and inhibition, the degree to which they influence this balance may vary. The effects of targeting CD300a from **Chapter 4** have been relatively modest, indicating that while these receptors contribute to the cells' functional orientation, they are part of a broader and more complex regulatory network. Furthermore, similar to CD300a, the expression of CD300c is modulated by cytokines, such as IL-2 and IL-15, which are known to induce its expression in an immune-cell dependent manner [59, 61, 62]. However, a comprehensive understanding of the dynamic expression patterns of CD300a and CD300c, as well as the stimuli triggering this modulation, is essential for identifying novel therapeutic targets. Adding to this complexity, CD300c stimulatory activity may be limited to myeloid cells, as in T cells CD300c has been identified as a negative regulator of T cell immunity [63].




The reported ligands of CD300a, PS and PE, are bilayer phospholipids likely expressed in a dynamic process that depends on various metabolic factors, as cancer cells require more membrane for rapid cell proliferation [64-67]. Indeed, *de novo* fatty acid synthesis is associated with aggressive cancers and worse disease prognosis [68, 69]. Although, we did not correlate the levels of PS and PE in the outer leaflet of the membrane with CD300a blockade activity, it would be interesting to understand how the activity of CD300a blockade varies in relation to the level of exposure of PS and PE. In the context of the TME, PS and PE are implicated in immunosuppressive signaling, potentially contributing to a tolerogenic state that diminishes the activities of dendritic and NK cells and encourages a pro-tumoral M2 macrophage phenotype [70]. Antagonizing CD300a could, therefore, have the potential to alter this balance, shifting the immune landscape from a pro-tumoral to an anti-tumoral state. Further, combining CD300a blockade with chemotherapeutic agents could prove beneficial and might shift cell death from tolerogenic to immunogenic. This effect could be like the synergistic effects observed when 'don't eat me' signals like CD47 are targeted alongside chemotherapy, with development of T cell immunity being critical for preclinical anticancer activity [71]. Thus, CD300a blockade could become a relevant target in shifting the immune response within the TME, potentially potentiating the efficacy of chemotherapy by not only directly killing tumor cells, but also by modulating the TME to support innate immunity.

## Conclusion

In conclusion, the research presented within this thesis has offered proof-of-concept for several novel protein-based therapeutics in our model systems, laying the foundation for future exploration of their application in oncology. The innovative use of bsAbs, which simultaneously engage tumor antigens like EGFR and CD27, demonstrates a distinctive and tumor antigen-restricted mode of action. Likewise, co-targeting of PVR and PD-L1 increases selectivity and efficacy over single blockade as PVR blockade is restricted to tissue where PD-L1 is simultaneously expressed. Although a direct comparison to traditional mAbs was not conducted, these bifunctional proteins have a distinct mode-of-action that could potentially address limitations associated with current treatments. Moreover, targeting of CD300a on myeloid cells holds promise as a potential immune checkpoint and prognostic biomarker in several cancer types, with its inhibition increasing phagocytic uptake of cancer cells, which could promote the innate immune response and potentially adaptive anticancer immunity. The impact of these therapeutics on the remodeling of the TME remains to be investigated in more representative and complex models, including *in vivo* mouse models employing intravital microscopy to assess effects such as vascular normalization. The exploration of a multitude of options, as discussed in this chapter, will serve to refine these approaches, potentially driving them from disruptive technologies in the laboratory to offering improved treatment options for cancer patients.

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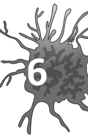

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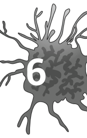
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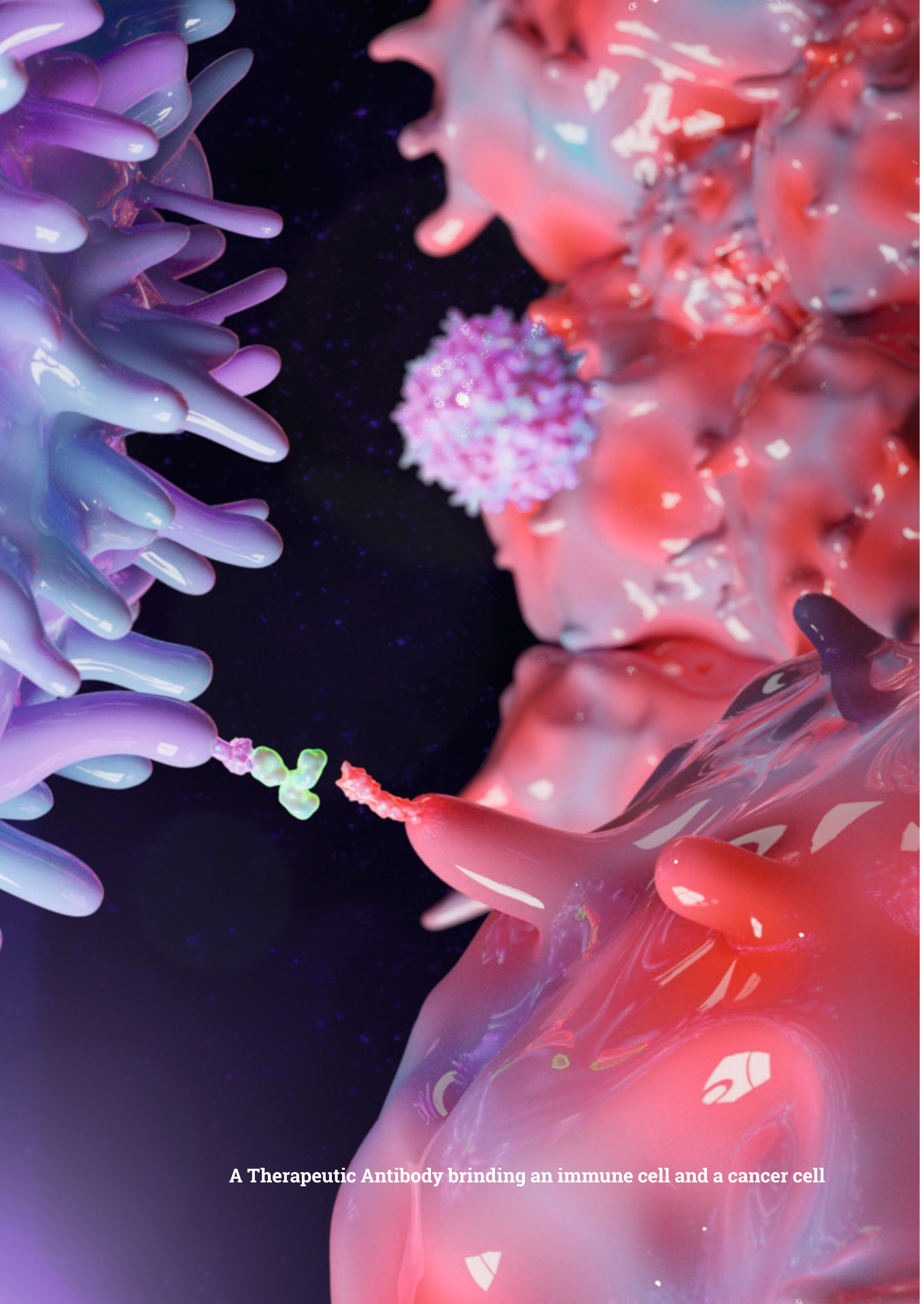
A Therapeutic Antibody brinding an immune cell and a cancer cell