Multifaceted approaches to tumor microenvironment modulation and immune checkpoint targeting

Melo Gallegos, Vinicio Alejandro

DOI:
10.33612/diss.1215629783

CHAPTER 1

Introduction to the Thesis

CANCER IMMUNOTHERAPY AS A MODALITY OF CANCER TREATMENT

Cancer is a major health challenge for humanity with the incidence of cancer projected to rise significantly in the coming decades. This projected increase is attributable to both the growth and aging of the global population, with forecasts suggesting an increase of over 45% of new cancer cases per year by the decade of 2040 - 2050 [1, 2].

To date, the standard of care mostly remains surgery, chemotherapy, and radiation therapy, with advances in these fields having contributed to increased overall survival and progression-free survival rates [3]. However, in the context of metastatic disease these modalities often have limited therapeutic activity and many patients succumb to the disease. Thus, there is an urgent need for alternative therapeutic modalities that can treat metastatic cancers and address issues like drug resistance, recurrence, and treatment-related toxicity. Among the novel therapeutic modalities that holds promise in this respect is so-called cancer immunotherapy in which the aim is to harness the body's immune response to fight the disease.

The first recorded evidence of cancer immunotherapy was Coley's toxin, developed by Dr. William B. Coley, who in 1891 treated a patient with sarcoma by injecting inactivated bacteria to stimulate an anti-tumor immune response [4]. This treatment led to regression of the tumor and has been explored as therapeutic option as recently as 2007 in a phase I clinical trial [5]. Coley's approach not only sparked numerous questions about the interactions between the immune system and tumor cells, but also laid the foundation for the development of modern immunotherapeutic strategies. From this work, it has become apparent that the foremost challenge to overcome in cancer treatment is what is termed 'tumor immune escape', which refers to the cancer's ability to evade or inhibit the body's immune system. Coley's methods were early attempts to overcome this specific obstacle. Similarly, Paul Ehrlich's introduction of the revolutionary 'magic bullet' concept in 1909 established foundational principles for targeted therapy, influencing various branches of treatment, including immunotherapy [6]. Ehrlich's idea, which anticipated the principles of passive immunotherapy, proposed creating therapeutic agents that could specifically target disease-causing cells without harming healthy tissues, a principle that has become integral to modern immunotherapeutic approaches.

Ever since and particularly in the past decades the field of cancer immunotherapy has evolved dramatically, with both so-called active and passive immunotherapeutic modalities enabling major advances in cancer treatment. Active immunotherapy involves triggering a new and/or improved immune response in the cancer patient to fight cancerous cells. Conversely, passive immunotherapy involves the direct administration of therapeutic agents that target cancer cells without relying on the body's immune

activation. The U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) has approved a growing number of immunotherapeutics, encompassing a wide range of modalities including immune checkpoint inhibitors (ICIs), bispecific antibodies (bsAbs), cytokines, toll-like receptor agonists, monoclonal antibodies (mAbs), radio-labeled antibodies, drug-conjugated antibodies, vaccines, oncolytic viruses, and chimeric antigen receptor T cell (CAR-T) therapy. Notable among these developments are ICIs due to their ability to unlock the immune system's natural capacity to fight cancer by inhibiting the mechanisms that allow cancer cells to evade immune detection. This groundbreaking approach has led to remarkable clinical successes across a spectrum of malignancies, fundamentally altering the landscape of cancer treatment and offering new hope to patients with previously untreatable forms of the disease.

ICIs are a class of drugs that specifically target regulatory pathways of the immune system that are often co-opted by cancer cells to avoid being attacked. These pathways, known as immune checkpoints (ICs), involve proteins like CTLA-4 and PD-1/PD-L1 that normally act as 'brakes' to prevent the immune system from over-activating and potentially causing damage to healthy cells. Cancer cells express these proteins to deceive the immune system into recognizing them as normal, thereby, evading immune detection. ICIs work by blocking these checkpoints, effectively lifting the 'brake' and allowing immune cells to recognize and destroy cancer cells. The success of ICIs has been particularly pronounced in cancers like melanoma and non-small cell lung cancer (NSCLC), leading to unprecedented long-term complete responses in previously end-stage patients [7, 8]. As of February 2024, seven ICIs targeting PD-(L)1 and CTLA-4 for various cancers are in clinical use, with expectations for FDA and EMA approval of additional ICIs as research progresses in identifying novel checkpoints.

Unfortunately, ICI responses are not consistent with up to 90% of patients either failing to respond or undergoing early relapse [9, 10]. Such resistance can arise from several factors intrinsic to the tumor's biology and its microenvironment. For instance, some tumors may not sufficiently express the targets of current ICIs, such as PD-(L)1 or CTLA-4. Other tumors may present fewer so-called neoantigens, new tumor-specific peptides that the cell machinery brings to the surface of cancer cells for the immune system to recognize as threats, or they may have defects in the machinery that presents these antigenic peptides. Additionally, tumors can create an immunosuppressive microenvironment that actively hinders immune cell function or can exclude immune cells altogether [11-13].

Importantly, PD-L1 and CTLA-4 are not the only ICs that can be targeted. Various other regulatory molecules that can act as ICs have been identified and identification of additional relevant checkpoints is being actively researched [14]. By exploring such additional checkpoints, strategies that can overcome the limitations of current therapies

can be developed. For instance, what is termed as 'cold' tumors, characterized by minimal immune activity, may be turned into 'hot' tumors—more inflamed and infiltrated by immune cells—making them more responsive to immunotherapy. This transformation can be facilitated by targeting checkpoints like the poliovirus receptor (PVR), which among others is expressed on the vascular tumor cells [15]. By inhibiting PVR, it is potentially possible to disrupt the immunosuppressive environment, enhancing the effectiveness of the immune response and converting 'cold' tumors to 'hot.' Such a strategy holds the promise of broadening the patient population that can benefit from ICI therapy.

OVERCOMING TUMOR MICROENVIRONMENT RESISTANCE: MECHANISMS, CHALLENGES, AND STRATEGIES IN CANCER IMMUNOTHERAPY

In cancer immunology, a variety of immune cells play critical roles in combating malignancies. Dendritic cells, as primary antigen-presenting cells (APCs), capture and present antigens from cancer cells and move to the lymph nodes where tumor antigen-specific T cells are activated and clonally expanded. T cells, once activated, are central to the adaptive immune response, targeting and destroying cancer cells with precision. Nevertheless, other immune cell types examined in this thesis, such as NK cells, macrophages, and granulocytes, also contribute to the anticancer immune response. For instance, NK cells can eliminate cancer cells through their innate ability to recognize cells that lack specific 'self' markers. Macrophages possess dual roles and can act as APCs, but can also directly engage in the destruction of cancer cells by engulfing them in a process known as phagocytosis. Importantly, macrophages can be antitumoral but can in the tumor microenvironment (TME) also be immunosuppressive, as they can be co-opted by tumors to promote growth and suppress T cell activity. Granulocytes destroy cancer cells among others by a process known as trogocytosis, which is the nibbling of membrane fragments from cancer cells. When immune cells migrate to the tumor site, overcoming the physical and immunosuppressive barriers posed by the TME to infiltrate the tumor they can become activated. Inside the tumor, T cells, supported by NK cells, macrophages, and granulocytes, recognize and engage cancer cells. This recognition is a critical step, as it enables the T cells to precisely target cancer cells for destruction.

The TME is a complex, dynamic system that plays a critical role in cancer progression and treatment response, consisting of cancer cells, immune cells, blood vessels, extracellular matrix, and signaling molecules [16]. One of the significant challenges in enhancing the efficacy of ICIs is the TME's variability in resistance mechanisms, particularly in differentiating between 'cold' and 'hot' tumors. Cold tumors are characterized by low immunogenicity and minimal immune cell infiltration, often resulting in limited effectiveness of ICIs due to the absence of a pre-existing immune

response to be amplified. Hot tumors, despite being more responsive to ICIs due to higher mutational burdens and the presence of tumor-infiltrating lymphocytes (TILs), also exhibit mechanisms of immune suppression [17, 18]. Notably, the expression of additional immunosuppressive molecules in hot tumors can contribute to immune suppression, effectively 'turning off' immune responses against the tumor. Thus, strategies to overcome TME resistance to ICIs must take into consideration the specific immunosuppressive mechanisms for each cancer phenotype.

Furthermore, the development of a functional vascular network is essential for immune cells to access and infiltrate tumor effectively [16]. In both hot and cold tumors, the absence of such a full formed network underscores the need for vascular normalization strategies, which has been clinically demonstrated to increase tumor sensitivity to ICIs [19]. In the context of cold tumors, these strategies not only enable a more effective infiltration of immune cells into the tumor parenchyma, but also improve the delivery of therapeutics. In the context of inflamed tumors, enhancing perfusion further supports the delivery of nutrients and oxygen, and the removal of immunoregulatory metabolic waste products which in turn improves the anti-tumor efficacy of TILs present within the TME. ICIs may also themselves play a role in normalizing the TME by activating immune responses that can lead to improved vascular function and, consequently, enhanced tumor perfusion [20, 21].

Whereas ICIs have the potential to improve outcomes by preventing or reversing T cell exhaustion, normalizing tumor vasculature, and enhancing T cell infiltration, their efficacy in many patients is limited by a spectrum of factors. This spectrum can roughly be subdivided into primary resistance mechanisms and acquired resistance mechanisms that evolve in response to ICI therapy.

Primary resistance mechanisms can for instance comprise the upregulated expression of a set of immune checkpoints, leading to the suppression of anti-tumor immunity upon ICI therapy. For instance, low levels of oxygen (hypoxia)—a prevalent condition in the TME—stabilizes hypoxia-inducible factors, which among others upregulate the expression of PD-L1 on cancer and immune cells [22]. Furthermore, hypoxia also upregulates other inhibitory checkpoints like CTLA-4 and VISTA and triggers secretion of immunoregulatory cytokines such as TGF-β and IL-10, further compounding immune suppression and complicating therapy [23, 24]. Additionally, biophysical forces such as elevated tumor interstitial fluid pressure is interlinked with hypoxia and can enhance the expression the aforementioned inhibitory molecules [25, 26]. Metabolic alterations within the TME, such as an increase in lactic acid produced during anaerobic glycolysis, generate an acidic environment that inhibits T cell activity and promotes immune checkpoint expression [27]. Moreover, resistance to ICI therapy can be driven by the presence of specific immune cells, such as myeloid-derived suppressor cells, which

exhibit increased PVR, CD80, B7-H1, or PD-L1 expression in carcinoma [28-31] and serve to create an immunosuppressive micro-environment.

Beyond primary resistance mechanisms posed by the TME, there are acquired resistance mechanisms that develop in response to ICI therapy. These acquired mechanisms can affect both innate and adaptive immune responses. For instance, upon ICI-mediated reactivation of the adaptive immune response, activated T cells release interferon-gamma (IFNγ). This very release of IFNγ can paradoxically promote resistance, e.g. by upregulating expression of CD47 on tumor cells, which facilitates tumor escape from immune surveillance and promotes metastasis [32]. In brief, within the innate immune system, cells such as natural killer (NK) cells, granulocytes, monocytes, dendritic cells, and macrophages express inhibitory molecules like signal regulatory protein alpha (SIRPα) [33] that interacts with CD47 to inhibit activation of innate immune cells.

An alternate acquired resistance mechanism during ICI therapy is the downregulation of major histocompatibility complex (MHC)–antigen complexes by tumor cells, which hinders the effective recognition by effector T cells [34, 35]. This evasion prevents the effective recognition of tumor cells by effector T cells, despite the T cells' tumor specificity [36, 37]. Additionally, TME factors can induce epigenetic modifications in TILs, leading to chromatin remodeling and sustained dysfunction of memory T cells present in the TME [38]. These alterations, coupled with the inadequate expansion of intra-tumoral memory T cells and defects in critical signaling pathways to coordinate the immune responses, contribute to a decrease in the efficacy of PD-1/PD-L1 blockade over time [39-41]. Moreover, the expression of other immune checkpoints, such as CTLA-4, TIM-3, LAG-3, and VISTA, are upregulated in response to PD-(L)1 therapy and play a crucial role in acquired resistance, suggesting that a multifaceted approach targeting multiple inhibitory receptors may be necessary for effective T cell re-activation [42-45].

Efforts to make the TME responsive to ICI therapy can be conceptualized through the lens of Le Chatelier's principle. This principle posits that if a dynamic equilibrium is disrupted by altering the conditions, the system will adjust to minimize the disturbance and restore equilibrium. Analogously, resistance to ICI monotherapy, as anticipated by Le Chatelier's principle, has been clinically observed, underscoring the need for a more integrative treatment strategy [44, 46]. Such a strategy should encompass a combination of immunotherapies to amplify therapeutic efficacy and surmount resistance mechanisms. This thesis proposes advancing research beyond the currently approved ICIs by identifying novel immune checkpoints and designing drugs that integrate these discoveries in innovative protein-based drug formats. This strategy may help to transition the TME from a state of resistance to one of responsiveness.

NOVEL IMMUNE CHECKPOINTS IN CANCER IMMUNOTHERAPY

This thesis has focused on the development of innovative immunotherapies beyond the established immune checkpoints. Consequently, research has been directed towards identifying and developing new agents capable of circumventing the acquired resistance encountered with current ICIs. This includes exploring innovative ICIs both as standalone treatments and in conjunction with anti-PD-L1 therapies. Specifically, in this thesis I have studied TIGIT/PVR, CD27, and CD300a as targets for cancer immunotherapy.

The TIGIT/PVR axis

PVR was initially discovered as a receptor for poliovirus. PVR plays a pivotal role in the entry in host cells and infection process of this virus. Beyond its virological significance, PVR has since been recognized as a critical component in the immune system, functioning as an immunoglobulin-like molecule involved in various cellular processes, including adhesion, migration, and immune modulation [47]. The almost exclusive overexpression of PVR in multiple solid tumors and low expression in healthy tissues as well as its involvement in immune escape strategies highlight it as a favorable candidate for cancer immunotherapy [48].

PVR expression increases following chemotherapy, and this increased expression correlates with adverse outcomes [49-53]. Additionally, PVR expression level is a predictive marker for the efficacy of PD-(L)1 immunotherapy, suggesting that PVR has a significant role in cancer progression and response to therapy [54, 55]. Accordingly, PVR and its cognate receptor TIGIT have been subject of intensive research in recent years [48, 56-75]. Importantly, a complementary ICI approach of PD-L1 targeted immunotherapy using Atezolizumab and TIGIT using Tiragolumab has in preliminary results yielded significant improvements in objective response rates compared to Atezolizumab monotherapy [76].

PVR interacts with TIGIT, CD96, and DNAM-1, which are expressed on T and NK cells. TIGIT is primarily recognized as an inhibitory checkpoint on T and NK cells, crucial for maintaining immune homeostasis and preventing overactivation [77]. CD96 generally acts as an inhibitory receptor on NK cells but can also provide a co-stimulatory role on T cells under specific conditions [78-80]. Conversely, DNAM-1 serves as a co-stimulatory molecule that enhances the activation of both T and NK cells [63, 81]. DNAM-1 also interacts with PVRL2, frequently co-expressed with PVR on cancer cells. Given that TIGIT and CD96 exhibit higher affinity for PVR than DNAM-1, blocking PVR emerges as a logical therapeutic approach, by blocking ICs and alleviating suppression of DNAM-1 costimulatory signaling. Notably, sustained engagement between PVR and DNAM-1 can reduce the expression of DNAM-1, an effect that may be negated by PVR-blocking therapies [82, 83]. Despite this, research on cancer treatments that target PVR directly

are limited [48], with no anti-PVR antibodies in clinical trials and only a limited number of preclinical studies exploring this strategy.

CD27

CD27 is a member of the tumor necrosis factor receptor (TNFR) superfamily and interacts with its ligand, CD70, to provide a co-stimulatory signal for T cell activation and expansion. This interaction is crucial for the generation and maintenance of effective T cell responses against cancer cells. However, overexpression of CD70 by cancer cells can have a detrimental effect as chronic CD27-CD70 interaction can trigger T cell apoptosis and exhaustion, and drive regulatory T cell expansion [84, 85]. CD27 is constitutively expressed on the majority of T cells and its expression is initially upregulated upon T cell activation, but is downregulated after several rounds of division and differentiation towards effector T cell [86, 87].

Agonistic mAbs targeting TNFR molecules like 4-1BB, OX40, GITR and CD27 have shown promising antitumor immunity in preclinical models and are under clinical investigation [88, 89]. Among these, Varlilumab, a CD27 agonistic antibody, has shown a modest efficacy both as monotherapy or in combination with PD-1 ICI in hematological and solid cancers in early clinical trials [90-92]. Some members of the TNFRSF, such as TNFR1 and CD40, can be activated by soluble ligand trimers alone, but other members such as CD27 require secondary cross-linking for optimal signaling, and are primarily activated by membrane bound TNFSF ligands rather than soluble forms. Indeed, effective CD27 signaling requires receptor oligomerization [93]. Thus, developing immunotherapies that exploit this property could in theory allow for the design of safe and effective immunotherapies. Specifically, triggering CD27 receptor hexamerization requires designing agonistic antibodies or ligands that bind and cluster CD27 on T cell surfaces. This process can mimic the interaction between CD27 with the natural ligand CD70 and initiate a potent co-stimulatory signal to enhance T cell activation, proliferation, and survival. However, it is important to note that first-generation therapeutics, such as monoclonal antibodies, often do not fulfill these criteria independently and require the presence of Fc receptor-positive (FcR+) innate immune cells to provide necessary cross-linking [90]. Further, ubiquitous FcR-mediated activation of TNFR mAb-mediated signaling by innate cells poses safety concerns, as it can lead to off-tumor toxicity, including severe liver toxicity [90].

Despite the potential of TNFRSF activation, such as CD27 for cancer therapy, evidenced by extensive preclinical and clinical evaluations with other TNFRs over the past two decades, only recombinant TNF-α (Beromun) has received clinical approval for treating soft tissue sarcoma via isolated limb perfusion [94]. This limited clinical efficacy of TNFR-stimulating therapeutics, especially antibodies can be attributed to several challenges. Key among these is the necessity for receptor oligomerization, which is crucial for efficient activation. However, not all TNFRs require this complex mechanism

to the same extent, and the variability in oligomerization needs can complicate the development of effective agonists [95].

CD300a

A third interesting yet under investigated candidate in the context of cancer immunotherapy is CD300a, a member of the CD300 family of receptors. This family is comprised of a group of type I transmembrane proteins with a single IgV-like extracellular domain that are primarily expressed on myeloid cells [96]. CD300a contains an ITIM motif through which it can regulate immune responses by inhibiting functions such as phagocytosis, cytokine release, and cell activation in both myeloid and lymphoid cells [96, 97]. This receptor is particularly notable for its regulatory effects on myeloid cell functions [97, 98]. CD300a and its highly homologous counterpart, CD300c, interact with shared ligands—phosphatidylserine (PS) and phosphatidylethanolamine (PE)—but exhibit opposite functions in immune regulation [97, 99], with CD300c being immunostimulatory.

A role of CD300a as immune checkpoint has already been established in various diseases, including arthritis and parasitic infections, highlighting its role in pathophysiology [100-103]. For instance, in leishmania-infected mice the specific blockade of CD300a increased macrophage and dendritic cell (DC) phagocytosis and increased the production of nitric oxide and pro-inflammatory cytokines [101, 103]. Further, CD300a blockade on DCs promoted differentiation of CD4+ or CD8+ T cells into a memory phenotype, contributing to the early clearance of parasites [101, 103]. Additionally, in models of ischemia in CD300a-deficient mice clearance of apoptotic cells was promoted, thus underscoring the critical regulatory role of CD300a on myeloid cell function [104]. Furthering this line of investigation, studies have shown that blocking the interaction between CD300a and its ligands PS and PE can lead to an increase in NK cell-mediated cytotoxicity [105, 106].

Of note, the lipid ligands PS and PE play significant roles across various physiological and pathological processes. Specifically, PS is widely recognized for its involvement in the clearance of apoptotic cells, by serving as an 'eat-me' signal when externalized on the extracellular leaflet of the cell membrane. This externalization facilitates the tolerogenic removal of dying cells, critical for maintaining immune homeostasis [107]. PE is similarly involved in cellular signaling and membrane dynamics, participates in apoptotic processes, and can influence cellular repair mechanisms [108]. Both lipids interact with a variety of receptors beyond CD300a, such as TIM-3, TIM-4, stabilin-2 and the TAM receptor, of which the integrated output helps dictate whether cell removal by myeloid immune effector cells is immunogenic or tolerogenic [97, 100]. Importantly, this promiscuous binding complicates the assessment of the functional impact of inhibiting CD300a.

CD300a and its ligands PS and PE, are prevalent across various cancer types including diffuse large B-cell lymphoma [109], gastric [110], ovarian [111], melanoma [112], prostate and breast cancers [113], among others [114-117]. The broad expression of CD300a in these cancers underscores the potential utility of targeting CD300a's recognition and binding mechanisms as a cancer therapeutic strategy. Additionally, the increase in CD300a expression in myeloid cells under hypoxic conditions bolsters the case for CD300a as a viable therapeutic target [118, 119]. Moreover, the engagement of CD300a by its ligands influences the immune landscape by modulating myeloid cell behavior, which could potentially boost the efficacy of existing treatments like Rituximab and/or experimental ones such as CD47 ICI therapy.

ADVANCED THERAPEUTIC STRATEGIES WITH BIFUNCTIONAL PROTEINS

To improve on the safety and efficacy of first generation immunostimulatory reagents such as monoclonal antibody-based ICIs, a new class of bispecific antibodies (bsAbs) and bifunctional proteins has been developed. These agents are engineered for dual specificity, allowing them to engage with two distinct targets simultaneously. The dual-targeting capability of bifunctional proteins could potentially circumvent primary and acquired resistance mechanisms encountered by single-target agents in cancer therapy as well as increase the safety by restricting ICI activity to the site of the tumor. Thus, by either targeting multiple immune checkpoints or modulating co-stimulatory activity, these proteins aim to enhance the precision and efficacy of cancer immunotherapy.

Tumor targeted immunomodulators

BsAbs represent the most prevalent form of bifunctional proteins, with more than a hundred bsAbs currently under clinical evaluation for cancer treatment [120]. These antibodies are engineered to possess two different recognition domains, enabling them to simultaneously bind to two distinct targets. BsAbs can be categorized into those that redirect T cell activity and those that modulate immune functions. An example of the former are Bispecific T-cell Engagers (BiTEs), which directly trigger T cell activity against tumors by binding to CD3zeta in the T cell receptor complex and a tumor-associated antigen (TAA), hereby, circumventing conventional TCR-dependent activation. Unlike ICIs that 'simply' lift inhibitory signals, BiTEs actively facilitate the formation of an immunological synapse between T cells and tumor cells, demonstrating effective tumor control in hematological malignancies [121]. However, in solid tumors, the efficacy of BiTEs has been limited due to 'off-tumor/on-target' toxicity, where activated T cells also harm healthy cells that express the tumor-associated target antigen, leading to significant adverse effects [122, 123]. Moreover, resistance mechanisms such as the upregulation of inhibitory molecules like PD-L1 and the loss of the TAA being targeted further challenge the long-term efficacy of BiTE therapy [124-126].

In a second approach, being pursued in this thesis, immunomodulatory bsAbs are engineered to enhance the immune response directly at the tumor site, reactivating exhausted T cells by either blocking inhibitory signals or promoting stimulatory signaling after binding to a TAA. A prevalent subclass of these bispecific antibodies, exemplified by TAAxPD-1 formats, specifically targets the PD-1 co-inhibitory pathway. Such bsAbs possess substantial antitumor efficacy both in preclinical and clinical settings. For instance, c-METxPD-1 has been shown to inhibit tumor growth by over 60% more effectively than control treatments, whereas VEGFxPD-1 has shown promising antitumor activity compared to monospecific antibodies or their combinations [127, 128]. This approach, particularly when used in conjunction with BiTEs, is currently under investigation with preclinical data showing enhanced therapeutic outcomes in solid tumors [122, 129]. For instance, the bsAb PD-L1xEGFR, targeting EGFR-expressing cancer cells, selectively reactivates anticancer T cells and significantly boosts the oncolytic activity of an EpCAM BiTE [130].

Furthermore, co-stimulatory bsAbs such as TAAxCD28 are gaining traction due to the possibility of targeted T cell activation, in the absence of the broad activation and toxicity associated as e.g. observed with CD28 superagonist [129, 131]. For example, PSMAxCD28 and EGFRxCD28 yielded superior anticancer efficacy in *in vitro* and *in vivo* compared to PD-1 blockade, without inducing systemic immune activation [129]. An important consideration is that these bsAbs show strong synergy with PD-1 blockade leading to complete tumor eradication in tumor models that are non-responsive to anti-PD-1 monotherapy. Building on this insight, using an immunomodulatory bsAb with the requirement of TNFRSFs like CD27 for effective signaling previously discussed, it could be possible to trigger T cell co-stimulation at the tumor site while minimizing systemic toxicity.

Dual immunomodulators

The second category of bifunctional proteins examined in this thesis are so-called dual immunomodulators. This innovative approach involves either inhibiting or activating two immune checkpoints simultaneously. Within this category, numerous bsAbs targeting various combinations of immune checkpoints have been developed, with promising results in both clinical and preclinical studies. In addition, a novel strategy developed by us are so-called Dual Signaling Proteins (DSPs) that utilize the natural ligand domains, instead of conventional antibody fragments for ICI activity. For instance, DSP107 (SIRPαx4-1BBL), the pioneering example of a DSP, targets CD47 on cancer cells using the SIRPα domain to block 'don't eat me' signal and promote phagocytic removal of cancer cells. Secondly, DSP107 engages 4-1BB on immune cells to trigger potent and localized costimulatory signaling using the ligand 4-1BBL, which is contingent upon DSP107's initial interaction with CD47. This dual engagement ensures that 4-1BB co-stimulation is finely tuned and occurs specifically at the tumor site. Such precise localization enhances the therapeutic index, minimizing systemic toxicity and

maximizing immune activation within the tumor microenvironment. Preclinical studies of DSP107 have demonstrated significant enhancements in macrophage-mediated phagocytosis and tumor growth inhibition, with its unique dual-action mechanism now being evaluated in several clinical trial [132, 133].

Whereas in DSP107 the ligands were genetically fused directly to each other, the inclusion of an active IgG1 domain in such bifunctional proteins can expand their therapeutic potential by enabling antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP), thereby, further leveraging both adaptive and innate immune responses against tumor cells.

RESEARCH SIGNIFICANCE AND SCOPE OF THIS THESIS

The development of novel immunotherapies that can help address the limitations of current treatments is evidently necessary. Expanding the arsenal of targeted checkpoints can uncover previously inaccessible pathways for immune activation and circumvention of acquired resistance, offering potential cures for a broader range of patients. Furthermore, it allows for the combination of therapies to synergistically enhance the anticancer immune response, while minimizing adverse effects.

In **chapter 2**, I start with the preclinical development of a bifunctional fusion protein termed "DSP502" in which two immune-inhibitory checkpoints, PVR and PD-L1, are blocked using the natural ligand-binding domains of TIGIT and PD-1. DSP502 comprises an active IgG1 as scaffold for these domains, allowing the additional recruitment of innate Fc-receptor positive immune cells. For the selection of a promising indication for DSP502 bioinformatic analyses highlighting elevated expressions of TIGIT, PD-1, PD-L1, and PVR, which have been associated with poorer survival outcomes, was evaluated yielding NSCLC as a potential indication. The strategic design of DSP502 aims to leverage these biomarkers to reactivate T and NK cells in the tumor microenvironment and upregulate key co-stimulatory signals such as DNAM-1. To assess the efficacy of DSP502 *in vitro* and humanized *in vivo* models were employed showing that DSP502 reactivated and enhanced the cytotoxic potential of T and NK cells specifically in cancer cells that express PVR and PD-L1.

In **chapter 3**, I present a novel therapeutic approach that aimed to restrict CD27 co-stimulatory activity to cancer cells that express EGFR. This antibody, termed CD27xEGFR, is engineered to bind selectively to both CD27 on T cells and EGFR on cancer cells, facilitating targeted activation and proliferation of T cells in the TME. This bispecific comprised a so-called silent Fc-domain that was designed to not bind and activate Fc-receptor positive innate cells and, hereby, minimize potential off-tumor toxicity due to ADCC and ADCP. In this study, preclinical studies provide evidence that CD27xEGFR enhances T cell proliferation, activation, and cytotoxicity against EGFR-expressing cancer

cells. Thus, CD27xEGFR may be a therapeutic strategy to improve anti-tumor immunity by leveraging the co-stimulatory CD27 signal in EGFR-expressing tumors.

In **chapter 4**, I present the identification and characterization of the blockade of CD300a as an immune checkpoint predominantly on myeloid immune responses. Based on previous work from our lab on the expression of CD47 as a determinant for the efficacy of Rituximab-CHOP in non-GCB DLBCL, other potential checkpoints that could define worse prognosis in non-GCB DLBCL patients treated with R-CHOP were explored. In a bioinformatics screen, we identified that CD300a had a significant negative impact on overall survival of non-GCB patients with high expression of CD47. Notably, CD300a was predominantly expressed by granulocytes, monocytes, and macrophages within the TME of non-GCB patients and not by lymphoma cells. Further, we preclinically explored blockade of CD300a with a monoclonal antibody for potentiation of cancer cell phagocytosis in DLBCL, mantle cell lymphoma and uveal melanoma. The findings in this chapter open up new opportunities for reactivating anticancer innate immunity by targeting CD300a.

In **chapter 5** I review how immunotherapies and most notably ICIs can induce the remodeling of the TME. By focusing on the remodeling of the TME, this review highlights the potential to enhance the efficacy of immunotherapies and improve patient outcomes. Specifically, we explored the mechanisms through which immunotherapies modulate immune and tumor cell functions, highlighting the challenge posed by the immunosuppressive nature of the TME. We discuss the significance of TME normalization, including vascular normalization and fibroblast reprogramming, as strategies to enhance the efficacy of immunotherapy. Finally, we discuss the complex interactions within the TME, underscoring the need for combination therapies that address both vascular abnormalities and immunosuppression to improve patient outcomes in cancer treatment.

In **chapter 6**, a comprehensive summary of the work of this thesis is presented and conclusions along with a discussion on the perspectives of the technologies developed in this thesis is presented. Notably, I discuss on how to optimize the activity of immunotherapies by inclusion of related immune checkpoints and novel fusion protein formats.

REFERENCES

1. Weir, H.K., et al., *Cancer Incidence Projections in the United States Between 2015 and 2050.* Prev Chronic Dis, 2021. **18**: p. E59.
2. Donnelly, D.W., et al., *Cancer Incidence Projections in Northern Ireland to 2040.* Cancer Epidemiol Biomarkers Prev, 2020. **29**(7): p. 1398-1405.
3. Dare, A.J., et al., *Surgical Services for Cancer Care*, in *Cancer: Disease Control Priorities, Third Edition (Volume 3)*, H. Gelband, et al., Editors. 2015: Washington (DC).
4. McCarthy, E.F., *The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas.* Iowa Orthop J, 2006. **26**: p. 154-8.
5. Karbach, J., et al., *Phase I clinical trial of mixed bacterial vaccine (Coley's toxins) in patients with NY-ESO-1 expressing cancers: immunological effects and clinical activity.* Clin Cancer Res, 2012. **18**(19): p. 5449-59.
6. Albrecht, H., *Role of Antibodies in Cancer Treatment (An Overview)*, in *Methods of Cancer Diagnosis, Therapy, and Prognosis: General Overviews, Head and Neck Cancer and Thyroid Cancer*, M.A. Hayat, Editor. 2010, Springer Netherlands: Dordrecht. p. 135-152.
7. Zhou, K., et al., *Mechanisms of drug resistance to immune checkpoint inhibitors in non-small cell lung cancer.* Front Immunol, 2023. **14**: p. 1127071.
8. Ziogas, D.C., et al., *Beyond CTLA-4 and PD-1 Inhibition: Novel Immune Checkpoint Molecules for Melanoma Treatment.* Cancers (Basel), 2023. **15**(10).
9. Cho, Y.H., et al., *Natural Killer Cells as a Potential Biomarker for Predicting Immunotherapy Efficacy in Patients with Non-Small Cell Lung Cancer.* Target Oncol, 2020. **15**(2): p. 241-247.
10. Haslam, A. and V. Prasad, *Estimation of the Percentage of US Patients With Cancer Who Are Eligible for and Respond to Checkpoint Inhibitor Immunotherapy Drugs.* JAMA Netw Open, 2019. **2**(5): p. e192535.
11. Carosella, E.D., et al., *A Systematic Review of Immunotherapy in Urologic Cancer: Evolving Roles for Targeting of CTLA-4, PD-1/PD-L1, and HLA-G.* Eur Urol, 2015. **68**(2): p. 267-79.
12. Castro, F., et al., *Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion.* Front Immunol, 2018. **9**: p. 847.
13. Binnewies, M., et al., *Understanding the tumor immune microenvironment (TIME) for effective therapy.* Nat Med, 2018. **24**(5): p. 541-550.
14. Kamali, A.N., et al., *Immune checkpoints and cancer immunotherapies: insights into newly potential receptors and ligands.* Ther Adv Vaccines Immunother, 2023. **11**: p. 25151355231192043.
15. Reymond, N., et al., *DNAM-1 and PVR regulate monocyte migration through endothelial junctions.* J Exp Med, 2004. **199**(10): p. 1331-41.
16. Kim, H.J., Y.R. Ji, and Y.M. Lee, *Crosstalk between angiogenesis and immune regulation in the tumor microenvironment.* Arch Pharm Res, 2022. **45**(6): p. 401-416.
17. Cho, Y.A., et al., *Relationship between the expressions of PD-L1 and tumor-infiltrating lymphocytes in oral squamous cell carcinoma.* Oral Oncol, 2011. **47**(12): p. 1148-53.
18. Chen, Y., et al., *The frequency and inter-relationship of PD-L1 expression and tumour mutational burden across multiple types of advanced solid tumours in China.* Exp Hematol Oncol, 2020. **9**: p. 17.

19. Lee, W.S., et al., *Combination of anti-angiogenic therapy and immune checkpoint blockade normalizes vascular-immune crosstalk to potentiate cancer immunity.* Exp Mol Med, 2020. **52**(9): p. 1475-1485.

20. Zheng, X., et al., *Increased vessel perfusion predicts the efficacy of immune checkpoint blockade.* J Clin Invest, 2018. **128**(5): p. 2104-2115.

21. Tian, L., et al., *Mutual regulation of tumour vessel normalization and immunostimulatory reprogramming.* Nature, 2017. **544**(7649): p. 250-254.

22. Shurin, M.R. and V. Umansky, *Cross-talk between HIF and PD-1/PD-L1 pathways in carcinogenesis and therapy.* J Clin Invest, 2022. **132**(9).

23. Hu, M., et al., *The regulation of immune checkpoints by the hypoxic tumor microenvironment.* PeerJ, 2021. **9**: p. e11306.

24. Deng, J., et al., *Hypoxia-Induced VISTA Promotes the Suppressive Function of Myeloid-Derived Suppressor Cells in the Tumor Microenvironment.* Cancer Immunol Res, 2019. **7**(7): p. 1079-1090.

25. Ou, Z., et al., *Pressure increases PD-L1 expression in A549 lung adenocarcinoma cells and causes resistance to anti-ROR1 CAR T cell-mediated cytotoxicity.* Sci Rep, 2022. **12**(1): p. 6919.

26. Rofstad, E.K., K. Galappathi, and B.S. Mathiesen, *Tumor interstitial fluid pressure-a link between tumor hypoxia, microvascular density, and lymph node metastasis.* Neoplasia, 2014. **16**(7): p. 586-94.

27. Wang, J.X., et al., *Lactic Acid and an Acidic Tumor Microenvironment suppress Anticancer Immunity.* Int J Mol Sci, 2020. **21**(21).

28. Ozbay Kurt, F.G., et al., *Enhancing immunotherapy response in melanoma: myeloid-derived suppressor cells as a therapeutic target.* J Clin Invest, 2023. **133**(13).

29. Mao, L., et al., *TIGIT/CD155 blockade enhances anti-PD-L1 therapy in head and neck squamous cell carcinoma by targeting myeloid-derived suppressor cells.* Oral Oncol, 2021. **121**: p. 105472.

30. Liu, Y., et al., *B7-H1 on myeloid-derived suppressor cells in immune suppression by a mouse model of ovarian cancer.* Clin Immunol, 2008. **129**(3): p. 471-81.

31. Nam, S., et al., *Analysis of the Expression and Regulation of PD-1 Protein on the Surface of Myeloid-Derived Suppressor Cells (MDSCs).* Biomol Ther (Seoul), 2019. **27**(1): p. 63-70.

32. Qu, S., et al., *Human lung adenocarcinoma CD47 is upregulated by interferon-gamma and promotes tumor metastasis.* Mol Ther Oncolytics, 2022. **25**: p. 276-287.

33. Seiffert, M., et al., *Human Signal-Regulatory Protein Is Expressed on Normal, But Not on Subsets of Leukemic Myeloid Cells and Mediates Cellular Adhesion Involving Its Counterreceptor CD47.* Blood, 1999. **94**(11): p. 3633-3643.

34. Luo, N., et al., *Melanoma response to anti-PD-L1 immunotherapy requires JAK1 signaling, but not JAK2.* Oncoimmunology, 2018. **7**(6): p. e1438106.

35. Taylor, B.C. and J.M. Balko, *Mechanisms of MHC-I Downregulation and Role in Immunotherapy Response.* Front Immunol, 2022. **13**: p. 844866.

36. Roberts, A., et al., *Ex vivo modelling of PD-1/PD-L1 immune checkpoint blockade under acute, chronic, and exhaustion-like conditions of T-cell stimulation.* Sci Rep, 2021. **11**(1): p. 4030.

37. Topfer, K., et al., *Tumor evasion from T cell surveillance.* J Biomed Biotechnol, 2011. **2011**: p. 918471.

38. Philip, M., et al., *Chromatin states define tumour-specific T cell dysfunction and reprogramming.* Nature, 2017. **545**(7655): p. 452-456.

39. Ribas, A., et al., *PD-1 Blockade Expands Intratumoral Memory T Cells.* Cancer Immunol Res, 2016. **4**(3): p. 194-203.

40. Shin, D.S., et al., *Primary Resistance to PD-1 Blockade Mediated by JAK1/2 Mutations*. Cancer Discov, 2017. **7**(2): p. 188-201.

41. Manguso, R.T., et al., *In vivo CRISPR screening identifies Ptpn2 as a cancer immunotherapy target*. Nature, 2017. **547**(7664): p. 413-418.

42. Shin, D.S. and A. Ribas, *The evolution of checkpoint blockade as a cancer therapy: what's here, what's next?* Curr Opin Immunol, 2015. **33**: p. 23-35.

43. Jiang, Y., Y. Li, and B. Zhu, *T-cell exhaustion in the tumor microenvironment*. Cell Death Dis, 2015. **6**(6): p. e1792.

44. Gide, T.N., et al., *Distinct Immune Cell Populations Define Response to Anti-PD-1 Monotherapy and Anti-PD-1/Anti-CTLA-4 Combined Therapy*. Cancer Cell, 2019. **35**(2): p. 238-255 e6.

45. Koyama, S., et al., *Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints*. Nat Commun, 2016. **7**: p. 10501.

46. Wilky, B.A., *Immune checkpoint inhibitors: The linchpins of modern immunotherapy*. Immunol Rev, 2019. **290**(1): p. 6-23.

47. Sloan, K.E., et al., *CD155/PVR plays a key role in cell motility during tumor cell invasion and migration*. BMC Cancer, 2004. **4**: p. 73.

48. Kucan Brlic, P., et al., *Targeting PVR (CD155) and its receptors in anti-tumor therapy*. Cell Mol Immunol, 2019. **16**(1): p. 40-52.

49. Niu, C., et al., *Low-dose bortezomib increases the expression of NKG2D and DNAM-1 ligands and enhances induced NK and gammadelta T cell-mediated lysis in multiple myeloma*. Oncotarget, 2017. **8**(4): p. 5954-5964.

50. Sun, Y., et al., *Combined evaluation of the expression status of CD155 and TIGIT plays an important role in the prognosis of LUAD (lung adenocarcinoma)*. Int Immunopharmacol, 2020. **80**: p. 106198.

51. Zhao, K., et al., *CD155 Overexpression Correlates With Poor Prognosis in Primary Small Cell Carcinoma of the Esophagus*. Front Mol Biosci, 2020. **7**: p. 608404.

52. Huang, D.W., et al., *CD155 expression and its correlation with clinicopathologic characteristics, angiogenesis, and prognosis in human cholangiocarcinoma*. Onco Targets Ther, 2017. **10**: p. 3817-3825.

53. Stamm, H., et al., *Targeting the TIGIT-PVR immune checkpoint axis as novel therapeutic option in breast cancer*. Oncoimmunology, 2019. **8**(12): p. e1674605.

54. Lee, J.B., et al., *Overexpression of PVR and PD-L1 and its association with prognosis in surgically resected squamous cell lung carcinoma*. Sci Rep, 2021. **11**(1): p. 8551.

55. Lee, B.R., et al., *Combination of PD-L1 and PVR determines sensitivity to PD-1 blockade*. JCI Insight, 2020. **5**(14).

56. Zhou, X., et al., *Repositioning liothyronine for cancer immunotherapy by blocking the interaction of immune checkpoint TIGIT/PVR*. Cell Commun Signal, 2020. **18**(1): p. 142.

57. Zhang, Q., et al., *Blockade of the checkpoint receptor TIGIT prevents NK cell exhaustion and elicits potent anti-tumor immunity*. Nat Immunol, 2018. **19**(7): p. 723-732.

58. Yu, X., et al., *The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells*. Nat Immunol, 2009. **10**(1): p. 48-57.

59. Stanietsky, N., et al., *The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity*. Proc Natl Acad Sci U S A, 2009. **106**(42): p. 17858-63.

60. Stamm, H., J. Wellbrock, and W. Fiedler, *Interaction of PVR/PVRL2 with TIGIT/DNAM-1 as a novel immune checkpoint axis and therapeutic target in cancer*. Mamm Genome, 2018. **29**(11-12): p. 694-702.

61. Stamm, H., et al., *Immune checkpoints PVR and PVRL2 are prognostic markers in AML and their blockade represents a new therapeutic option.* Oncogene, 2018. **37**(39): p. 5269-5280.

62. Shen, X., et al., *TIGIT-Fc Promotes Antitumor Immunity.* Cancer Immunol Res, 2021. **9**(9): p. 1088-1097.

63. Sanchez-Correa, B., et al., *DNAM-1 and the TIGIT/PVRIG/TACTILE Axis: Novel Immune Checkpoints for Natural Killer Cell-Based Cancer Immunotherapy.* Cancers (Basel), 2019. **11**(6).

64. Rui, S., et al., *The landscape of TIGIT target and clinical application in diseases.* MedComm – Oncology, 2022. **1**(2).

65. Rousseau, A., C. Parisi, and F. Barlesi, *Anti-TIGIT therapies for solid tumors: a systematic review.* ESMO Open, 2023. **8**(2): p. 101184.

66. Rotte, A., S. Sahasranaman, and N. Budha, *Targeting TIGIT for Immunotherapy of Cancer: Update on Clinical Development.* Biomedicines, 2021. **9**(9).

67. Mu, S., et al., *PD-L1/TIGIT bispecific antibody showed survival advantage in animal model.* Clin Transl Med, 2022. **12**(5): p. e754.

68. Manieri, N.A., E.Y. Chiang, and J.L. Grogan, *TIGIT: A Key Inhibitor of the Cancer Immunity Cycle.* Trends Immunol, 2017. **38**(1): p. 20-28.

69. Maas, R.J., et al., *TIGIT blockade enhances functionality of peritoneal NK cells with altered expression of DNAM-1/TIGIT/CD96 checkpoint molecules in ovarian cancer.* Oncoimmunology, 2020. **9**(1): p. 1843247.

70. Liu, L., et al., *Blocking TIGIT/CD155 signalling reverses CD8(+) T cell exhaustion and enhances the antitumor activity in cervical cancer.* J Transl Med, 2022. **20**(1): p. 280.

71. Kurtulus, S., et al., *TIGIT predominantly regulates the immune response via regulatory T cells.* J Clin Invest, 2015. **125**(11): p. 4053-62.

72. Kojima, M., et al., *Anti-human-TIGIT agonistic antibody ameliorates autoimmune diseases by inhibiting Tfh and Tph cells and enhancing Treg cells.* Commun Biol, 2023. **6**(1): p. 500.

73. Johnston, R.J., et al., *The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function.* Cancer Cell, 2014. **26**(6): p. 923-937.

74. Gorvel, L. and D. Olive, *Targeting the "PVR-TIGIT axis" with immune checkpoint therapies.* F1000Res, 2020. **9**.

75. Chiang, E.Y. and I. Mellman, *TIGIT-CD226-PVR axis: advancing immune checkpoint blockade for cancer immunotherapy.* J Immunother Cancer, 2022. **10**(4).

76. Cho, B.C., et al., *Tiragolumab plus atezolizumab versus placebo plus atezolizumab as a first-line treatment for PD-L1-selected non-small-cell lung cancer (CITYSCAPE): primary and follow-up analyses of a randomised, double-blind, phase 2 study.* Lancet Oncol, 2022. **23**(6): p. 781-792.

77. Harjunpaa, H. and C. Guillerey, *TIGIT as an emerging immune checkpoint.* Clin Exp Immunol, 2020. **200**(2): p. 108-119.

78. Chiang, E.Y., et al., *CD96 functions as a co-stimulatory receptor to enhance CD8(+) T cell activation and effector responses.* Eur J Immunol, 2020. **50**(6): p. 891-902.

79. Mittal, D., et al., *CD96 Is an Immune Checkpoint That Regulates CD8(+) T-cell Antitumor Function.* Cancer Immunol Res, 2019. **7**(4): p. 559-571.

80. Dougall, W.C., et al., *TIGIT and CD96: new checkpoint receptor targets for cancer immunotherapy.* Immunol Rev, 2017. **276**(1): p. 112-120.

81. Tahara-Hanaoka, S., et al., *Functional characterization of DNAM-1 (CD226) interaction with its ligands PVR (CD155) and nectin-2 (PRR-2/CD112).* Int Immunol, 2004. **16**(4): p. 533-8.

82. Braun, M., et al., *CD155 on Tumor Cells Drives Resistance to Immunotherapy by Inducing the Degradation of the Activating Receptor CD226 in CD8(+) T Cells*. Immunity, 2020. **53**(4): p. 805-823 e15.

83. Carlsten, M., et al., *Primary human tumor cells expressing CD155 impair tumor targeting by down-regulating DNAM-1 on NK cells*. J Immunol, 2009. **183**(8): p. 4921-30.

84. Jacobs, J., et al., *CD70: An emerging target in cancer immunotherapy.* Pharmacol Ther, 2015. **155**: p. 1-10.

85. Claus, C., et al., *CD27 signaling increases the frequency of regulatory T cells and promotes tumor growth.* Cancer Res, 2012. **72**(14): p. 3664-76.

86. Taraban, V.Y., et al., *CD27 costimulation contributes substantially to the expansion of functional memory CD8(+) T cells after peptide immunization.* Eur J Immunol, 2013. **43**(12): p. 3314-23.

87. Romero, P., et al., *Four functionally distinct populations of human effector-memory CD8+ T lymphocytes*. J Immunol, 2007. **178**(7): p. 4112-9.

88. Sanmamed, M.F., et al., *Agonists of Co-stimulation in Cancer Immunotherapy Directed Against CD137, OX40, GITR, CD27, CD28, and ICOS*. Semin Oncol, 2015. **42**(4): p. 640-55.

89. Melero, I., et al., *Agonist antibodies to TNFR molecules that costimulate T and NK cells.* Clin Cancer Res, 2013. **19**(5): p. 1044-53.

90. Dadas, O., A. Ertay, and M.S. Cragg, *Delivering co-stimulatory tumor necrosis factor receptor agonism for cancer immunotherapy: past, current and future perspectives*. Front Immunol, 2023. **14**: p. 1147467.

91. Ansell, S.M., et al., *Safety and activity of varlilumab, a novel and first-in-class agonist anti-CD27 antibody, for hematologic malignancies*. Blood Adv, 2020. **4**(9): p. 1917-1926.

92. Starzer, A.M. and A.S. Berghoff, *New emerging targets in cancer immunotherapy: CD27 (TNFRSF7).* ESMO Open, 2020. **4**(Suppl 3): p. e000629.

93. Wyzgol, A., et al., *Trimer stabilization, oligomerization, and antibody-mediated cell surface immobilization improve the activity of soluble trimers of CD27L, CD40L, 41BBL, and glucocorticoid-induced TNF receptor ligand.* J Immunol, 2009. **183**(3): p. 1851-61.

94. Eggermont, A.M., J.H. de Wilt, and T.L. ten Hagen, *Current uses of isolated limb perfusion in the clinic and a model system for new strategies*. Lancet Oncol, 2003. **4**(7): p. 429-37.

95. Kucka, K. and H. Wajant, *Receptor Oligomerization and Its Relevance for Signaling by Receptors of the Tumor Necrosis Factor Receptor Superfamily.* Front Cell Dev Biol, 2020. **8**: p. 615141.

96. Borrego, F., *The CD300 molecules: an emerging family of regulators of the immune system.* Blood, 2013. **121**(11): p. 1951-60.

97. Simhadri, V.R., et al., *Human CD300a binds to phosphatidylethanolamine and phosphatidylserine, and modulates the phagocytosis of dead cells*. Blood, 2012. **119**(12): p. 2799-809.

98. Kim, E.J., et al., *CD300a and CD300f differentially regulate the MyD88 and TRIF-mediated TLR signalling pathways through activation of SHP-1 and/or SHP-2 in human monocytic cell lines*. Immunology, 2012. **135**(3): p. 226-35.

99. Dimitrova, M., et al., *CD300c is uniquely expressed on CD56 bright Natural Killer Cells and differs from CD300a upon ligand recognition.* Sci Rep, 2016. **6**: p. 23942.

100. Cao, Y., et al., *CD300a and CD300f molecules regulate the function of leukocytes.* Int Immunopharmacol, 2021. **93**: p. 107373.

101. Singh, R., et al., *CD300a Receptor Blocking Enhances Early Clearance of Leishmania donovani From Its Mammalian Host Through Modulation of Effector Functions of Phagocytic and Antigen Experienced T Cells.* Front Immunol, 2021. **12**: p. 793611.

102. Valiate, B.V.S., et al., *The immunoreceptor CD300a controls the intensity of inflammation and dysfunction in a model of Ag-induced arthritis in mice.* J Leukoc Biol, 2019. **106**(4): p. 957-966.

103. Anand, A., et al., *Leishmania donovani induces CD300a expression to dampen effector properties of CD11c(+) dendritic and antigen activated CD8(+) T cells.* Acta Trop, 2023. **239**: p. 106826.

104. Nakahashi-Oda, C., et al., *CD300a blockade enhances efferocytosis by infiltrating myeloid cells and ameliorates neuronal deficit after ischemic stroke.* Sci Immunol, 2021. **6**(64): p. eabe7915.

105. Lankry, D., et al., *The interaction between CD300a and phosphatidylserine inhibits tumor cell killing by NK cells.* Eur J Immunol, 2013. **43**(8): p. 2151-61.

106. Vitalle, J., et al., *CD300a inhibits CD16-mediated NK cell effector functions in HIV-1-infected patients.* Cell Mol Immunol, 2019. **16**(12): p. 940-942.

107. Birge, R.B., et al., *Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer.* Cell Death Differ, 2016. **23**(6): p. 962-78.

108. Patel, D. and S.N. Witt, *Ethanolamine and Phosphatidylethanolamine: Partners in Health and Disease.* Oxid Med Cell Longev, 2017. **2017**: p. 4829180.

109. Jiang, L., et al., *Suppression of CD300A inhibits the growth of diffuse large B-cell lymphoma.* Oncotarget, 2015. **6**(31): p. 31191-202.

110. Woehlecke, H., et al., *Enhanced exposure of phosphatidylserine in human gastric carcinoma cells overexpressing the half-size ABC transporter BCRP (ABCG2).* Biochem J, 2003. **376**(Pt 2): p. 489-95.

111. Dong, H.P., et al., *Evaluation of cell surface expression of phosphatidylserine in ovarian carcinoma effusions using the annexin-V/7-AAD assay: clinical relevance and comparison with other apoptosis parameters.* Am J Clin Pathol, 2009. **132**(5): p. 756-62.

112. Kirszberg, C., et al., *Simultaneous tissue factor expression and phosphatidylserine exposure account for the highly procoagulant pattern of melanoma cell lines.* Melanoma Res, 2009. **19**(5): p. 301-8.

113. Radaic, A., et al., *Phosphatidylserine-Gold Nanoparticles (PS-AuNP) Induce Prostate and Breast Cancer Cell Apoptosis.* Pharmaceutics, 2021. **13**(7).

114. De, M., et al., *A Novel Therapeutic Strategy for Cancer Using Phosphatidylserine Targeting Stearylamine-Bearing Cationic Liposomes.* Mol Ther Nucleic Acids, 2018. **10**: p. 9-27.

115. Luo, Z.K., et al., *The Roles And Signaling Pathways Of Phosphatidylethanolamine-Binding Protein 4 In Tumors.* Onco Targets Ther, 2019. **12**: p. 7685-7690.

116. Marien, E., et al., *Non-small cell lung cancer is characterized by dramatic changes in phospholipid profiles.* Int J Cancer, 2015. **137**(7): p. 1539-48.

117. Xu, Z.J., et al., *Pan-cancer analysis identifies CD300 molecules as potential immune regulators and promising therapeutic targets in acute myeloid leukemia.* Cancer Med, 2023. **12**(1): p. 789-807.

118. Raggi, F., et al., *Identification of CD300a as a new hypoxia-inducible gene and a regulator of CCL20 and VEGF production by human monocytes and macrophages.* Innate Immun, 2014. **20**(7): p. 721-34.

119. Nissim Ben Efraim, A.H., et al., *The inhibitory receptor CD300a is up-regulated by hypoxia and GM-CSF in human peripheral blood eosinophils.* Allergy, 2013. **68**(3): p. 397-401.

120. Wei, J., et al., *Current landscape and future directions of bispecific antibodies in cancer immunotherapy.* Front Immunol, 2022. **13**: p. 1035276.

121. Kamakura, D., R. Asano, and M. Yasunaga, *T Cell Bispecific Antibodies: An Antibody-Based Delivery System for Inducing Antitumor Immunity.* Pharmaceuticals (Basel), 2021. **14**(11).

122. Warwas, K.M., et al., *Co-Stimulatory Bispecific Antibodies Induce Enhanced T Cell Activation and Tumor Cell Killing in Breast Cancer Models.* Front Immunol, 2021. **12**: p. 719116.

123. Goebeler, M.E. and R.C. Bargou, *T cell-engaging therapies - BiTEs and beyond.* Nat Rev Clin Oncol, 2020. **17**(7): p. 418-434.

124. Krupka, C., et al., *Blockade of the PD-1/PD-L1 axis augments lysis of AML cells by the CD33/CD3 BiTE antibody construct AMG 330: reversing a T-cell-induced immune escape mechanism.* Leukemia, 2016. **30**(2): p. 484-91.

125. Tumeh, P.C., et al., *PD-1 blockade induces responses by inhibiting adaptive immune resistance.* Nature, 2014. **515**(7528): p. 568-71.

126. Herrmann, M., et al., *Bifunctional PD-1 x alphaCD3 x alphaCD33 fusion protein reverses adaptive immune escape in acute myeloid leukemia.* Blood, 2018. **132**(23): p. 2484-2494.

127. Sun, Z.J., et al., *A novel bispecific c-MET/PD-1 antibody with therapeutic potential in solid cancer.* Oncotarget, 2017. **8**(17): p. 29067-29079.

128. Zhao, Y., et al., *AK112, a novel PD-1/VEGF bispecific antibody, in combination with chemotherapy in patients with advanced non-small cell lung cancer (NSCLC): an open-label, multicenter, phase II trial.* EClinicalMedicine, 2023. **62**: p. 102106.

129. Waite, J.C., et al., *Tumor-targeted CD28 bispecific antibodies enhance the antitumor efficacy of PD-1 immunotherapy.* Sci Transl Med, 2020. **12**(549).

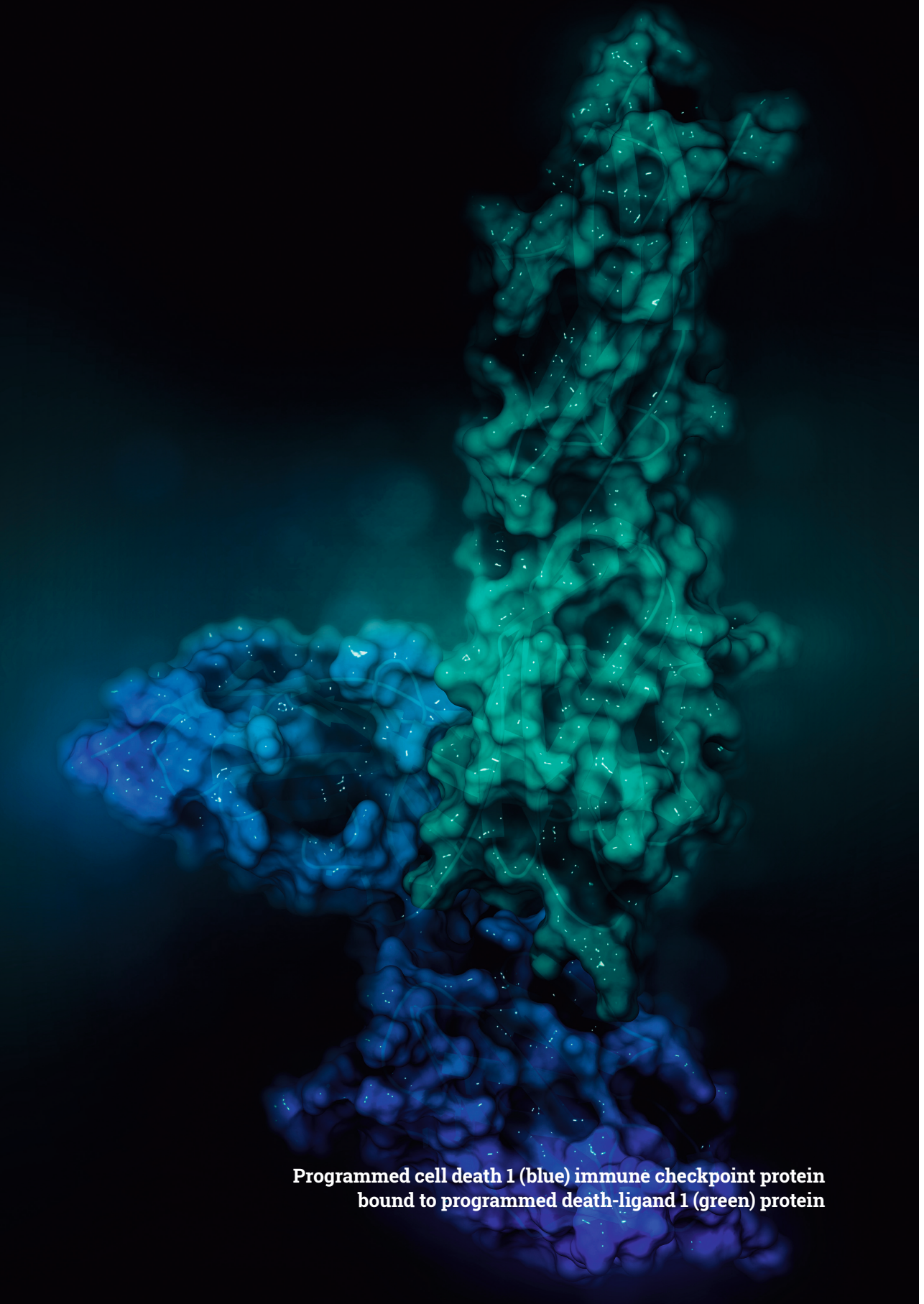
130. Koopmans, I., et al., *A novel bispecific antibody for EGFR-directed blockade of the PD-1/PD-L1 immune checkpoint.* Oncoimmunology, 2018. **7**(8): p. e1466016.

131. Skokos, D., et al., *A class of costimulatory CD28-bispecific antibodies that enhance the antitumor activity of CD3-bispecific antibodies.* Sci Transl Med, 2020. **12**(525).

132. Cendrowicz, E., et al., *DSP107 combines inhibition of CD47/SIRPalpha axis with activation of 4-1BB to trigger anticancer immunity.* J Exp Clin Cancer Res, 2022. **41**(1): p. 97.

133. Saeed, A., et al., *Phase 1 dose escalation study of DSP107, a first-in-class CD47 and 4-1BB targeting fusion protein, in combination with atezolizumab in patients with advanced solid tumors.* Journal of Clinical Oncology, 2023. **41**(16_suppl): p. 2632-2632.


1



Programmed cell death 1 (blue) immune checkpoint protein bound to programmed death-ligand 1 (green) protein