Novel antibody-based drugs for PD-L1 and TRAIL-R targeted cancer immunotherapy

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Djoke Hendriks

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Table of contents

CHAPTER 1

Introduction to the thesis

General introduction

In the general public domain, cancer is often believed to be a 'modern' and rapidly increasing disease. However, the earliest medical records describing cases of cancer are papyri dating back as early as 2500 B.C.[1-3] Indeed, cancer has been detected in Egyptian mummies using modern medical technology.[4, 5]

However, in ancient times the incidence of cancer appears to have been relatively low compared to current numbers. This may be due to a significant increase in life expectancy and radical changes in life style and environmental factors.[5] Nowadays, cancer is among the leading causes of death worldwide and the number of new cancer cases is expected to increase with 70% in the next two decades.[6] Fortunately, the understanding of cancer has tremendously increased. In addition to well-known fundamental characteristics of cancer cells such as genetic instability, chronic proliferation and replicative immortality,[7] it has become increasingly clear that the immune system is involved in both tumor suppression and progression.[8]

According to the initial cancer immunosurveillance concept proposed by Paul Ehrlich and further developed by Burnett and Thomas, the immune system continuously scans the body to identify and eliminate transformed cells before they can develop into an uncontrollable disease. During tumor progression, malignant cells accumulate mutations some of which may directly affect their immunogenicity. Presentation of tumor-specific antigens (peptides) may result in the induction of humoral (antibodies) and/or cellular anticancer immune responses.[9] Indeed, 'spontaneous' regressions have been described in cancer patients,[10] particularly in patients that were suffering from fulminant infections with pyogenic microorganisms. It is thought that such an infection boosts the natural anticancer immune response. This phenomenon inspired the development of various rudimentary cancer immunotherapies, with a history spanning hundreds of years.[11] A prominent example is 'Coley's toxin', a cancer vaccine containing killed bacteria that was developed in the late 1800s.[12, 13]

However, cancer cells and tumors arise in the presence of an apparently fully functional immune system. Apparently, cancer cells have or acquire the ability to modulate and eventually escape elimination by the immune system. The current paradigm for this process, termed the immunoediting concept, builds on the immune surveillance paradigm and integrates the immune system's dual role in cancer development. The immunoediting concept consists of 3 phases: elimination, equilibrium, and escape.[8] Essentially, the elimination phase resembles the cancer immunosurveillance concept. Herein innate and adaptive immunity work together to detect and destroy transformed cells long before they become clinically apparent. However, some cancer cell variants may not be completely eliminated in this phase and may subsequently enter into the equilibrium phase in which the immune system is able to control tumor outgrowth. In this phase, cancer cell populations are kept in a state of functional dormancy through constant interaction with cells of the immune system. However, immune effector cells provide selection pressure for the genetically unstable cancer cells by effectively recognizing and eliminating cancer cells with highly immunogenic antigens. Thus, cancer cells may gradually acquire mutations that result in loss of tumor antigens or defects in antigen processing/presentation

machinery, yielding cancer cell variants that are no longer effectively recognized by the immune system. Ultimately, these mutations may allow certain cancer cell variants to enter the escape phase, where cancer cells escape from immune prosecution and progress to a clinically apparent disease.

Tumor progression may additionally promote the induction of an immunosuppressive and/or tumor-promoting inflammatory microenvironment. In the latter case innate immune cells such as tumor-associated macrophages appear to stimulate cancer cell proliferation and survival, and suppress adaptive immune responses.[14] Accordingly, presence of such myeloid suppressor cells associates with poor prognosis in several cancer types.[15, 16] It has therefore become increasingly clear that immune suppression in the tumor microenvironment can hamper the efficacy of therapeutic strategies aimed to improve anticancer immunity. Therefore, many approaches to overcome immune suppression at the tumor site have been developed in recent years. Especially immunotherapeutic agents targeting so-called immune checkpoint interactions have yielded unprecedented response rates and even cure, particularly in melanoma.[17]

However, immune checkpoint molecules are not exclusively expressed on cancer cells and ubiquitous (re)activation of immune cells can therefore associate with severe autoimmune-related side effects.[18] In addition, conventional cytotoxic therapies such as radio- and chemotherapy, that remain the basis of cancer treatment in advanced stage cancer patients, frequently cause severe systemic side effects as they basically target all rapidly dividing cells.[19] Fortunately, the improved understanding of cancer biology and breakthroughs in biotechnology have led to the development of therapeutic strategies that selectively target cancer cells using antibodies. In this thesis, we pre-clinically evaluated various novel antibody-based agents that were designed to improve the tumor-selective action of cancer immunotherapy.

Outline of the thesis

In **Chapter 2,** we review recent developments in the field of antibody-based cancer therapy. Due to their high binding affinity and selectivity, antibodies are prominent candidates for targeted cancer therapy. Indeed, more than 20 antibodies have been approved for cancer therapy in the US and Europe, and this number is expected to rapidly expand in the near future. However, tumor antigens that are exclusively present on cancer cells are rare. Hence, most (if not all) currently available antibodies are directed against tumor-associated antigens rather than to tumor-specific antigens, which may associate with on-target/off-tumor side effects.

With the advent of recombinant DNA engineering, a large variety of advanced rationally-designed molecular formats of antibody-derived agents have become available, including antibody fragments, CAR-transfected T cells, antibody-cytokine fusion proteins and recombinant bispecific antibodies. As detailed in **Chapter 2**, these novel antibody formats can have enhanced tumor selectivity. For example, a bispecific antibody directed against HER-3 and the insulin-like growth factor I receptor, showed superior anticancer activity compared to control monospecific or combination antibody treatment in pre-clinical studies.[20]

Bispecific antibodies can also be designed to redirect immune effector cells to cancer cells, thereby exploiting their intrinsic anticancer activity. Currently, two bispecific antibodies that combine specificity for CD3-epsilon (part of the T cell receptor signaling complex) with a tumor-associated cell surface antigen have been approved for clinical use. Additionally, many other bispecific antibodies are currently in (pre)clinical development.[21]

Alternatively, antibody-based agents can be exploited to selectively deliver immune effector molecules such as cytokines to the tumor site. Cytokines are major regulators of the immune system and can have significant anticancer effects. For example, treatment with recombinant IL-2 induces complete remission in a subset of patients with melanoma and renal cell carcinoma.[22] However, since most cytokines have no intrinsic selectivity for cancer cells, the amount of e.g. non-targeted IL-2 that needs to be injected to achieve adequate anticancer activity is relatively high and associates with severe side effects.[23]

To overcome this issue, antibody-cytokine fusion proteins have been generated with the aim of combining the high target selectivity of antibodies with tumor-localised stimulation of the immune system.[24] In this respect, TNF-related Apoptosis Inducing Ligand (TRAIL) is a particularly interesting candidate for cancer therapy as it can selectively induce programmed cell death (apoptosis) in cancer cells while sparing normal cells.[25] TRAIL is expressed on the surface of various immune effector cells and can bind to 4 TRAIL receptors (TRAIL-R). TRAIL-R1 and TRAIL-R2 are capable of inducing cell death, while TRAIL-R3 and TRAIL-R4 are thought to have a decoy function. The anticancer efficacy of both recombinant soluble TRAIL and agonistic antibodies specific for TRAIL-R1 or TRAIL-R2 has been evaluated in early stage clinical trials where favorable safety profiles were observed.[26] However, these first-generation TRAIL-R agonists had limited clinical efficacy, possibly due to intrinsic resistance to TRAIL and/or acquired resistance upon treatment with TRAIL-R agonists. Furthermore, TRAIL receptors are ubiquitously expressed throughout the body,[27] limiting the accumulation of TRAIL-R agonists at the tumor site. Additional issues include a short half-life and rapid clearance from the circulation of recombinant soluble TRAIL.[28, 29]

Of note, unlike membrane-bound TRAIL, soluble TRAIL requires cross-linking to efficiently activate TRAIL-R2. Similarly, conventional TRAIL-R2 targeted antibodies require additional cross-linking by Fc-receptor positive cells for effective induction of apoptosis.[30] scFv:TRAIL fusion proteins, in which antibody fragments targeting a tumor antigen are genetically fused to soluble TRAIL, can provide the required cross-linking to induce TRAIL-R2 mediated apoptosis. In this format, binding of the antibody fragment to the target antigen converts the essentially inactive soluble TRAIL domain to a membrane-like form of TRAIL that effectively induces apoptosis. Similarly, bispecific antibodies can selectively deliver a TRAIL-R2 targeted antibody fragment to a tumor antigen and provide cross-linking upon binding to the target antigen.

In **Chapter 3**, we describe such a novel bispecific antibody-based strategy that may improve the efficacy of TRAIL-R2 targeted therapy. We constructed MCSPxDR5, a bispecific tetravalent antibody that comprises a scFv fragment derived from tigatuzumab, a clinically evaluated agonistic antibody targeting TRAIL-R2 (DR5), a high affinity scFv

antibody fragment targeting the melanoma-associated chondroitin sulfate proteoglycan (MCSP), and a human IgG1 Fc domain. MCSP is overexpressed on most melanomas and promotes adhesion, growth and tissue invasion of melanoma cells.[31] Bispecific antibody (bsAb) MCSPxDR5 was designed to selectively induce TRAIL-R2-mediated apoptosis in MCSP-positive melanoma cells. In line with this, bsAb MCSPxDR5 induced potent MCSP-restricted TRAIL-R2 mediated apoptosis in a panel of melanoma cell lines and primary patient-derived melanoma cells. Moreover, cross-linking of its IgG domain, using either artificial cross-linker or Fc receptors on immune effector cells, significantly enhanced its anticancer activity. Taken together, bsAb MCSPxDR5 has promising MCSP-directed and DR5-restricted anticancer activity, which warrants its further pre-clinical development for the treatment of melanoma and other MCSP-positive malignancies.

Alternatively, the efficacy of soluble TRAIL can be enhanced using the aforementioned scFv:TRAIL fusion protein format. Our group has previously validated this concept with TRAIL fusion proteins directed against several tumor antigens including MCSP, EGFR or EpCAM, which selectively deliver TRAIL to the cancer cell surface.[32-34] Treatment with such scFv:TRAIL fusion proteins triggered target antigen-selective binding and triggered apoptosis *in vitro* and *in vivo*. Depending on the biology of its respective target antigen, a TRAIL fusion protein can have additional anticancer effects such as blockade of EGFR- or MCSP-mediated protumorigenic signaling[32, 33] or blockade of CD47-mediated "don't eat me" signaling.[35] Alternatively, the scFv:TRAIL fusion protein format can be used to arm immune effector cells with exogenous membrane-bound TRAIL. We have previously shown that CD3- and CD7-targeted TRAIL fusion proteins greatly enhance the anticancer efficacy of T cells, both *in vitro* and in mouse tumor models.[36] Importantly, CD3-targeted TRAIL formulations potently activate CD3-signalling in T cells, thereby enhancing their anticancer activity.

In this thesis, we explored whether TRAIL fusion proteins can be exploited to block the PD-L1/PD-1 immune checkpoint. This immune checkpoint is hijacked by cancer cells to suppress and overcome natural anticancer immunity[17] (Figure 1).

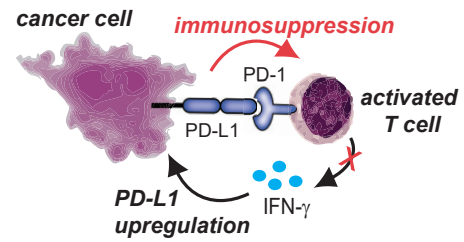

Figure 1: The interaction between Programmed Death Ligand 1 (PD-L1) and its receptor PD-1 on activated T cells normally ensures timely down-regulation of an immune response to prevent collateral damage of healthy cells.[37] However, cancer cells can also upregulate PD-L1 expression, either via oncogenic signaling pathways or in response to IFN-γ produced in the tumor microenvironment.[38, 39] Interaction between PD-L1 and PD-1 suppresses the antitumor activity of tumor-infiltrating T cells, allowing cancer cells to escape from the immune system.[38, 40]

Correspondingly, expression of PD-L1 on cancer cells often correlates with an unfavorable prognosis.[41, 42] Antibodies that block the PD-L1/PD-1 interaction can restore the anticancer activity of functionally impaired tumor-infiltrating lymphocytes (TILs), specifically cytotoxic T cells. Although treatment with such antibodies has led to unprecedented response rates, particularly in advanced melanoma,[43, 44] there is ample room for improvement as these responses are limited to a minority of cancer patients and a select set of malignancies.

In **Chapter 4**, we describe a bi-functional scFv:TRAIL fusion protein, designated anti-PD-L1:TRAIL, that comprises a PD-L1 blocking scFv antibody fragment genetically fused to soluble TRAIL. Fusion protein anti-PD-L1:TRAIL was designed to selectively induce TRAIL-mediated apoptosis in PD-L1-positive cancer cells while simultaneously reactivating anticancer immunity via blockade of the PD-L1/PD-1 immune checkpoint. Indeed, we demonstrated that fusion protein anti-PD-L1:TRAIL selectively induced apoptosis in PD-L1-positive cancer cell lines and in primary patient-derived melanoma cells. At the same time, anti-PD-L1:TRAIL enhanced anticancer activity and IFN-γ production of T cells via blockade of the PD-L1/PD-1 interaction. Since IFN-γ increases PD-L1 expression and sensitizes cancer cells to TRAIL-mediated apoptosis,[39, 45, 46] anti-PD-L1:TRAIL may trigger a feed-forward loop of increasing IFN-γ, increasing PD-L1 expression and increasing TRAIL sensitivity. In line with this, IFN-γ enhanced anti-PD-L1:TRAIL-mediated apoptosis in cell lines and in primary patient-derived melanoma cells. Furthermore, anti-PD-L1:TRAIL converted PD-L1-expressing and thus potentially immune suppressive myeloid cells into TRAIL-displaying effector cells that induced TRAIL-mediated apoptosis in cancer cell lines. In conclusion, fusion protein anti-PD-L1:TRAIL has promising multifold and mutually reinforcing therapeutic effects comprised of PD-L1 checkpoint blockade and simultaneous tumor-selective induction of TRAIL-mediated apoptosis. This new fusion protein may provide possibilities to enhance the efficacy of therapeutic PD-L1/PD-1 checkpoint inhibition alone or in combination with other immunotherapeutic strategies.

Since PD-1 and PD-L1 are broadly expressed on normal tissues, antibodies blocking either PD-1 or PD-L1 are not inherently tumor-selective. Indeed, autoimmune-related adverse effects have been observed for PD-L1 blocking antibodies.[43] In **Chapter 5**, we describe a novel bispecific antibody-based strategy to improve the selectivity of PD-L1/PD-1 checkpoint blockade. Selectively delivery of PD-L1/PD-1 blockade to a tumor-associated target antigen may improve the tumor-selective action of PD-L1/PD-1 blocking immunotherapy, potentially with increased efficacy and safety. Hereto, we constructed a human IgG1 bispecific antibody, PD-L1xEGFR, that comprises a PD-L1 blocking scFv antibody fragment and an epidermal growth factor receptor (EGFR) targeted scFv antibody fragment. EGFR is a well-established tumor target antigen that is overexpressed by various malignancies in which it correlates with poor prognosis.[47, 48] Aberrant EGFR-signaling plays crucial roles in the pathogenesis of cancer by initiating the early stages of tumor development, sustaining tumor growth, promoting infiltration, and mediating resistance to therapy.[49] Moreover, mutations that activate EGFR-signaling were recently reported to induce PD-L1 overexpression in lung cancer,[50, 51] making it an interesting tumor target for

tumor-selective PD-L1-blocking immunotherapy.

Bispecific antibody PD-L1xEGFR was designed to selectively target PD-L1/PD-1 checkpoint inhibition to EGFR-positive cancer cells. On EGFR-positive cells, bsAb PD-L1xEGFR inhibited the PD-L1/PD-1 interaction with similar efficacy as a conventional PD-L1 antibody. However, our data showed that treatment of EGFR/PD-L1-positive cancer cells with bsAb PD-L1xEGFR resulted in EGFR-directed blocking of PD-L1, which resulted in both enhanced anticancer activity and IFN-γ production of T cells. Additionally, bsAb PD-L1xEGFR enhanced NK cell-mediated Antibody Dependent Cellular Cytotoxicity (ADCC) towards cancer cells via its human IgG1 Fc domain and reduced the viability of EGFR-positive cancer cells by blocking oncogenic EGFR-signaling. Indeed, bsAb PD-L1xEGFR selectively enhanced the anticancer activity of T cells towards EGFR-positive cells and as such outperformed a PD-L1-blocking antibody that is in clinical use. In conclusion, the promising multi-fold EGFR-restricted anticancer activity of bsAb PD-L1xEGFR may provide possibilities to improve clinical efficacy and reduce side effects compared to conventional PD-L1/PD-1 antibodies.

Finally, **Chapter 6** provides a summary of the work presented in this thesis and provides perspectives on further development of tumor-selective blockade of the PD-L1/PD-1 checkpoint axis and improving the tumor-selective efficacy of PD-L1 and TRAIL-R targeted agents.

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CHAPTER 2

Antibody-based cancer therapy: successful agents and novel approaches

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Abstract

Since their discovery, antibodies have been viewed as ideal candidates or 'magic bullets' for use in targeted therapy in the fields of cancer, autoimmunity and chronic inflammatory disorders. A wave of antibody-dedicated research followed, which resulted in the clinical approval of a first generation of monoclonal antibodies for cancer therapy such as rituximab (1997) and cetuximab (2004), and infliximab (2002) for the treatment of autoimmune diseases. More recently, the development of antibodies that prevent checkpoint-mediated inhibition of T cell responses invigorated the field of cancer immunotherapy. Such antibodies induced unprecedented long-term remissions in patients with advanced stage malignancies, most notably melanoma and lung cancer, that do not respond to conventional therapies. In this chapter, we will recapitulate the development of antibody-based therapy, and detail recent advances and new functions, particularly in the field of cancer immunotherapy. With the advent of recombinant DNA engineering, a number of rationally-designed molecular formats of antibodies and antibody-derived agents have become available, and we will discuss various molecular formats including monoclonal antibodies, bispecific antibodies, antibody-cytokine fusion proteins, and T cells genetically modified with chimeric antigen receptors. With these exciting advances, new antibody-based treatment options will likely enter clinical practice and pave the way towards more successful control of malignant diseases.

Outline

Introduction

Antibodies are key components of the humoral immune response and are characterised by a high specificity and binding affinity for a specific antigenic epitope. Antibodies have been held to be of therapeutic interest, already since their original postulation by Paul Ehrlich in the late 1800s. Indeed, antibodies have been viewed as ideal candidates or 'magic bullets' for the use of targeted therapy in various fields of medicine, with initial studies on the clinical application of antibodies performed with polyclonal antisera, e.g. for the treatment of infectious diseases and of snake- and spider-bites (Calmette 1895). Also in the areas of stem cell- and organ transplantation and in the treatment of anaplastic anaemia, polyclonal anti-Lymphocyte- and anti-Thymocyte- sera and anti-IgG helped to prevent graft versus host disease (Finke et al. 2009). The subsequent discovery of a production method for monoclonal antibodies (mAbs) in 1975 (Kohler et al. 1975) started the exploration of antibodies as highly specific targeting and effector molecules for cancer therapy. The first attempts at mAb-based cancer therapy made use of murine mAbs, but often showed disappointing activity in clinical trials (reviewed by (Vaickus et al. 1991)). This outcome was frequently due to the development of neutralising antibodies against the therapeutic antibody produced by the host. However, with the advent of antibody engineering many modifications to mAbs became possible and significant steps forward were made.

The first major step was the development of chimeric antibodies in which a human Ig Fc-domain was fused to murine antigen recognition domains (LoBuglio et al. 1989). By virtue of this human Fc-domain, chimeric antibodies resemble human antibodies more closely and interact better with effector cells to trigger their anticancer activity. Further, chimeric mAbs are less immunogenic, less likely inactivated by neutralising host antibodies, and have a serum half-life closer to that of fully human antibodies, typically ranging from 2 to 4 weeks. Chimeric mAbs were the first clinically successful antibodies in oncology as illustrated by the FDA-approval of the CD20 antibody rituximab for the treatment of B cell lymphomas (1997), which opened the field of antibody-based cancer therapy (Maloney et al. 1997). Rituximab is a chimeric human IgG1 containing monoclonal antibody (Reff et al. 1994) that binds to the tetraspanin CD20, an integral transmembrane protein expressed on the surface of normal and malignant B-lineage cells. Soon afterwards, the chimeric EGFR antibody cetuximab was approved for the treatment of colorectal cancer (Cunningham et al. 2004). The growth factor receptor EGFR is expressed on epithelial cells and is often overexpressed in epithelial malignancies. Blocking of this receptor with the antibody deprives the cancer cell of essential growth factor signals needed for its survival. At present (July 2016), more than 47 mAbs are approved for therapy in the US and Europe (Reichert 2015).

Many other antibody modifications and antibody-derived therapeutic agents have been developed, including Fc-engineered antibodies with optimised effector functions, bispecific antibodies, bispecific fusion proteins of antibody-fragments, antibody fragments carrying toxins or cytokines, and tri- or tetra-specific antibody-derivatives. Finally, in Chimeric Antigen Receptors (CAR)-transfected T cells, antibody fragments are used to

redirect T cells to malignant target cells.

Here we review some of the major molecular formats of antibody-derived molecules, including mAbs, antibody-cytokine fusion proteins, bispecific antibodies and CAR T cells. We will review the use of agents designed in these molecular formats for direct targeting of malignant cells and for the recruitment of immune effector cells to initiate innate and/or adaptive anticancer immunity. With the latter approach, remarkable progress has been achieved in the past few years, with new antibodies that target so-called immune checkpoint inducing previously unheard of long-term remissions in hard-to-treat cancers such as melanoma (Brahmer et al.2012, Topalian et al. 2012). Additionally, the use of CAR-transfected T cells for B-lineage Acute Lymphoblastic Leukaemia has resulted in unprecedented clinical responses in early clinical trials (Maude et al. 2014).

Monoclonal antibodies

Antibodies are composed of distinct functional domains (Figure 1A), with the variable (Fab) domains being responsible for high affinity binding to the target antigen. The constant Fc domain is responsible for binding to and interaction with components of the immune system.

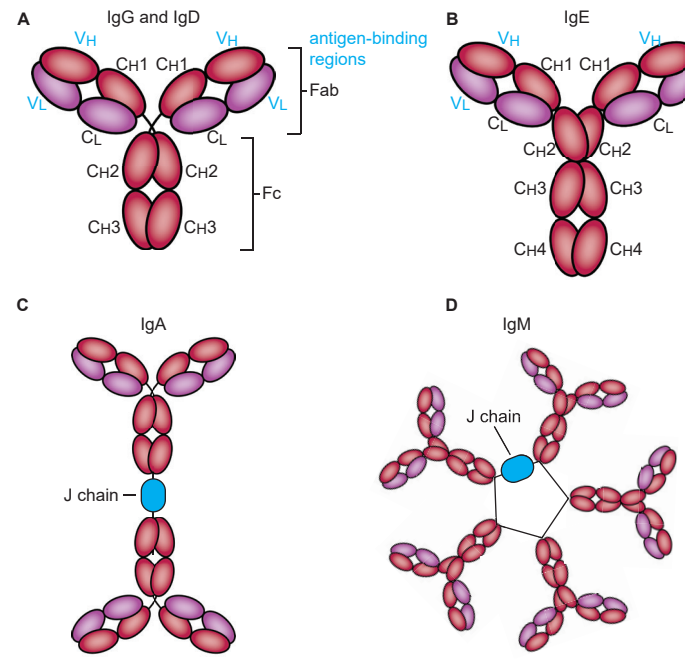

Figure 1: Antibody classification. Antibodies comprise a variable (Fab) domain that is responsible for antigen recognition and binding, and a (Fc) domain that interacts with components of the immune system. There are different classes of antibodies being: IgG, IgD, IgE, IgA or IgM, which differ in abundancy, structure, and function. **A)** IgG and IgD contain the general heavy (H) chain constant domain defined as CH1-CH2-CH3. **B)** IgE contains an additional CH4 domain. **C)** IgA has the same H chain constant domain structure as IgG and IgD, but forms dimers, that are connected via the joining (J) chain. **D)** IgM has a similar amount of H chain constant domains as IgE, but appears in a pentameric form, connected via the J chain.

In a cancer patient, the therapeutic effects of mAbs can be achieved by several mechanisms, mainly Antibody Dependent Cellular Cytotoxicity (ADCC) (Clynes et al. 1998), Antibody Dependent Cellular Phagocytosis (ADCP) (Horton et al. 2008, Gül et al. 2014), and Complement Dependent Cytotoxicity (CDC) (Introna et al. 2009). Furthermore, depending on the antigen, inhibition or activation of intracellular signalling mediated through the target antigen can be a major effector mechanism. The relative contribution of each of these effector mechanisms is difficult to dissect in patients and likely varies for different antibodies. Indeed, even different mAbs targeting the same target antigen can have distinct modes-of-action. Further, the contributions of these mechanisms is likely further influenced by patient characteristics such as individual allelic variants of Fc receptors. Nevertheless, the relative contributions of these mechanisms can be separately studied *in vitro* by laboratory experimentation.

The most frequently used constant Fc domain for therapeutic mAbs is derived from the IgG1 subclass of human Igs, although other immunoglobulin heavy chain subclasses also offer features of interest for mAb-based therapy. In brief, antibodies can be of the IgM, IgD, IgA, IgE or IgG class (Figure 1A-D), with the IgG class comprising four different isotypes (IgG1, IgG2, IgG3 and IgG4). These isotypes differ in their amino acid sequences in the Fc domain and the hinge region, which leads to functional differences that can be exploited for the design of mAb-based therapy (reviewed in Jefferis et al. 2012). For instance, the IgG1 isotype can efficiently trigger ADCC and CDC, making it the isotype of choice for targeting malignant cells for destruction, e.g. by the CD20 antibody rituximab (Dall'Ozzo et al. 2004). In contrast, the IgG4 isotype has a strongly reduced capacity to induce ADCC and CDC. Therefore, the IgG4 Fc-domain is preferentially used for the design of immunomodulatory antibodies that block or activate receptors on immune effector cells to avoid elimination of these effector cells by ADCC/CDC (Wang et al. 2014). Prominent examples are PD-1 targeting antibodies, such as nivolumab, which block inhibitory signalling through PD-1 on the surface of tumor-reactive T cells.

Effector mechanisms and activity of tumor targeting antibodies

Antibody Dependent Cellular Cytotoxicity

Antibody Dependent Cellular Cytotoxicity (ADCC) is triggered when Fc-receptor- (FcR-) bearing effector cells recognise a target cell that has been opsonised by antibodies. The main effector cells mediating ADCC are NK cells, although other FcR-bearing cells such as monocytes/macrophages, granulocytes and γδ T cells also contribute to ADCC-mediated elimination of target cells (Dall'Ozzo et al. 2004, Lefebvre et al. 2006, Hernandez et al. 2003, Tokuyama et al. 2008, Capietto et al. 2011, Gogoi et al. 2013, Seidel et al. 2014). Briefly, binding of the Fc domain of a mAb, typically an IgG1, activates the effector cell to release cytokines such as IFN-γ and cytotoxic molecules such as perforin and granzymes which lyse the target cell (Figure 2A) (Bowles et al. 2005).The IgG Fc domain triggers ADCC by binding to the activating receptors FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa and FcγRIIIb (Bruhns et al. 2009). Conversely, binding to FcγRIIb creates an inhibitory signal (Clynes et al. 2000, Nimmerjahn et al. 2008). The binding profile of individual IgG subclasses to these FcγRs differs and thus can impact the outcome of signalling and is

an important consideration for the design of therapeutic mAbs.

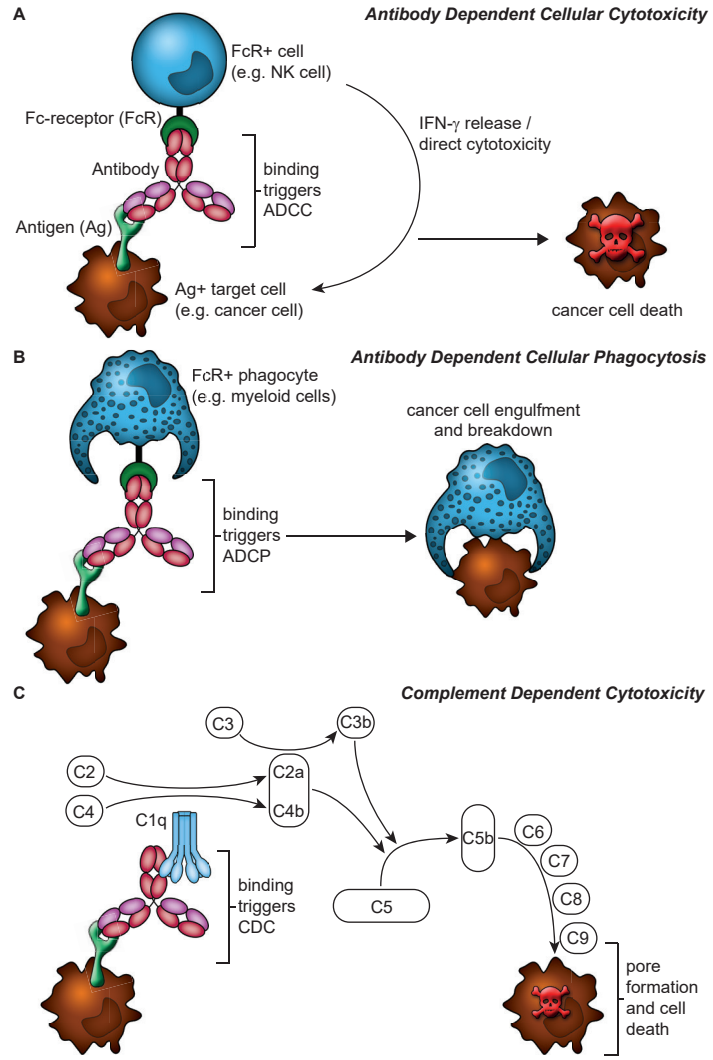

Figure 2: Antibody effector functions. **A)** Target cells that have been opsonised by antibodies can be killed via Antibody Dependent Cellular Cytotoxicity (ADCC). During ADCC, the Fc domain of the antibody binds to activating Fc receptors (FcR) on FcR-positive immune cells like NK cells, monocytes, macrophages, and granulocytes. This antibody-FcR interaction triggers release of cytokines such as IFN-γ as well as cytotoxic molecules such as perforin and granzymes that lyse the targeted cell. Of note, clustering of mAb is required for complement activation and binding to low affinity FcR. **B)** During Antibody Dependent Cellular Phagocytosis (ADCP), the interaction between the Fc domain of the antibody and activating FcRs on phagocytes triggers engulfment of the opsonised cells, which eventually leads to degradation of the target cell. **C)** Complement Dependent Cytotoxicity (CDC) is triggered upon binding of complement factor C1q to antibody opsonised cells, followed by formation of the C1 complex that initiates the complement cascade. Briefly, cleavage of C4 and C2 results in formation of the C4b-C2a complex that acts as C3-convertase. Binding of C3b to the C4b-C2a complex leads to formation of C5b, the initiator protein for the so-called membrane-attack complex (MAC). The MAC comprises C5b, C6, C7, C8 and C9, and forms a pore in the cell membrane that promotes lysis of the target cell.

Antibody Dependent Cellular Phagocytosis

Although most of the cellular cytotoxic activity induced by mAbs has been attributed to ADCC, Antibody Dependent Cellular Phagocytosis (ADCP) is also an important mechanism for the removal of cancer cells during mAb therapy. In brief, ADCP is initiated upon recognition of the Fc domain of an antibody opsonising a target cell by FcR-bearing effector cells, such as monocytes, macrophages and neutrophils (Braster et al. 2014). Subsequently, the opsonised cells or cellular fragments are taken up by phagocytes leading to their final degradation (Figure 2B). The importance of ADCP has been illustrated by studies in mouse models in which the removal of malignant B lymphoma cells by therapeutic CD20 mAbs depended on the presence of liver macrophages (Oflazoglu et al. 2007). Further, depletion of macrophages, but not of NK cells (important for ADCC), inhibited removal of lymphoma cells treated with a CD30 mAb. These experimental findings are consistent with the observation that a polymorphism in FcγRIIa, expressed on myeloid effector cells but not NK cells, was correlated with clinical responses to rituximab (Weng et al. 2003). Importantly, although macrophages can also trigger ADCC, recent observations suggest that phagocytosis of opsonised cancer cells and their fragments likely is a major effector mechanism of mAb treatment contributed by macrophages. It is still unclear, however, whether macrophages first engulf entire cancer cells and only then fragment them intracellularly, or whether they first fragment them in the extracellular space and subsequently engulf fragments (Gül et al. 2014, Gül et al. 2015). Specifically, depletion of rituximab-treated leukemic B-lineage cells from the circulation in mice was mainly due to rapid uptake of opsonised cancer cells and their fragments by Kupffer cells, a liver-resident subset of macrophages (Montalvao et al. 2013). Kupffer cells also efficiently removed opsonised circulating tumor cells and prevented formation of liver metastases in a mouse melanoma model (Gül et al. 2014). Along the same line, treatment with the CD38 antibody daratumumab triggered macrophage-mediated depletion of multiple myeloma (MM) cells in 11 of 12 samples of primary cells from MM patients and a similar depletion contributed to the *in vivo* therapeutic effect of this antibody towards xenografted leukemic cells in mice (Overdijk et al. 2015).

The relative importance of neutrophils in ADCP in patients under mAb therapy is less clear, because the IgG1 Fc domain typically used in mAbs only poorly triggers neutrophil-mediated ADCP. In contrast, Fc domains of the IgA isotype trigger very effective ADCP through their cognate Fc alpha receptor (Huls et al. 1999). Furthermore, engineering of Fc domains can augment neutrophil-mediated phagocytosis. An example hereof is the CD20 antibody obinutuzumab, a glycoengineered IgG1, which triggers more effective phagocytosis of opsonised cancer cells than rituximab (Golay et al. 2013). Of note, induction of ADCP by therapeutic mAbs may also provide a crucial link to adaptive immune responses. Specifically, uptake of cancer cells and their fragments by macrophages and dendritic cells (DCs) can lead to the presentation of tumor-derived peptides on MHC class II, thereby priming specific antitumor responses by CD4-positive helper T cells (Gül et al. 2014). Moreover, cross-presentation of tumor-derived peptides on MHC class I can prime protective immunity by cytotoxic CD8 T cells.

Protective T cell responses developing under mAb therapy have been observed in a

mouse model of xenografted B-lineage leukaemia cells, where treatment with a CD20 mAb triggered protective helper- and cytolytic T cell immunity (Abes et al. 2010). DCs are the major type of professional antigen presenting cells (APC) that are capable of priming immune responses by naive T cells (Banchereau et al. 1998, Amigorena 2002, Dhodapkar et al. 2008), although neutrophils may also provide MHC-I mediated cross-priming of naive T cells with induction of specific CD8 T cell responses reported in a mouse model (Beauvillain et al. 2007).

Complement Dependent Cytotoxicity

Complement Dependent Cytotoxicity (CDC) in mAb-based therapy is initiated by binding of the complement protein C1q to the Fc domain of mAbs opsonising a target cell (Introna et al. 2009). Binding of C1q triggers the activation of the complement cascade and leads to the formation of the membrane attack complex (MAC), which forms pores causing the lysis of target cells (Figure 2C). CDC is effectively triggered by the Fc-domains of IgM and IgG1 antibodies, but poorly by the IgG2 isotype and not at all by antibodies of the IgG4 isotype.

The exact contribution of CDC to therapeutic effects in patients under therapy with mAbs is unclear. For instance, depending on the mouse model the depletion of B cells by CD20 mAbs can be both CDC-independent (Uchida et al. 2004) and CDC-dependent (Di Gaetano et al. 2003). Nevertheless, the ability of mAbs to trigger CDC is relevant for therapy, as illustrated by the next-generation CD20 antibody ofatumumab, which generates greater CDC activity than rituximab (Teeling et al. 2004). Activation of CDC by rituximab can be augmented by selective targeting and down-modulation of CD46, a membrane-bound regulatory protein of complement activation that blocks CDC at the level of C3 (Zell et al. 2007). Briefly, treatment with the small protein Ad35K++ selectively down-modulated surface-expressed CD46 and augmented CDC *in vivo* triggered by rituximab (Richter et al. 2016). Therefore, Ad35K++ acted as an adjuvant to enhance the CDC activity of rituximab, a potentially useful approach also for other therapeutic mAbs. This advantage may however be restricted to particular situations, such as the case described for rituximab, as complement activation is usually accompanied by pro-inflammatory responses that may yield detrimental off-target effects.

Signalling modulation

Binding of a therapeutic mAb to its target can also activate or inhibit signalling if the target antigen is a signalling receptor. A prominent example is rituximab, which binds to the extracellular loop of CD20, a tetraspanin protein with no known receptor or ligand (reviewed in Pescovitz et al. 2006). The physiological role of CD20 is poorly understood although it has been reported to among others act as a calcium channel (Koslowski et al. 2008). Binding of rituximab to CD20 can directly trigger apoptosis in malignant B cells and is reportedly associated with a relocalisation of CD20 to membrane microdomains (Figure 3A) (Deans et al. 2002). This direct pro-apoptotic effect of rituximab may also occur in patients, because malignant B-lymphoid cells freshly isolated from rituximab-treated patients displayed features of apoptosis (Byrd et al. 2002). Interestingly, CD20

antibodies targeting different epitopes than Rituximab that do not trigger relocalisation of CD20 to rafts are more effective in triggering apoptotic cell death (Teeling et al. 2004). A second prominent example is the targeting of the EGFR with cetuximab, a mAb blocking EGF binding to EGFR, whereby pro-survival mitogenic signalling through this receptor is abrogated (Figure 3B) (Cunningham et al. 2004). *In vitro*, cetuximab treatment strongly inhibits growth of EGFR-bearing cancer cells (Prewett et al. 2002). In patients treated with cetuximab, the contribution of EGFR-blockade to the overall therapeutic effect relative to other effects, such as ADCC and ADCP, is still unclear. A third example are antibodies targeting agonistic TRAIL receptors, either TRAIL-R1 or TRAIL-R2, which induce apoptosis in many types of cancer cells after activation (Figure 3C) (reviewed in Ashkenazi et al. 2008). Binding of these mAbs to their target cross-links the receptor and initiates the extrinsic pathway of apoptosis, which can be augmented through the Fc domain of the therapeutic protein. This effect is achieved through further cross-linking of the TRAIL-receptors on the target cell in trans mediated by Fc-receptors carried by effector cells.

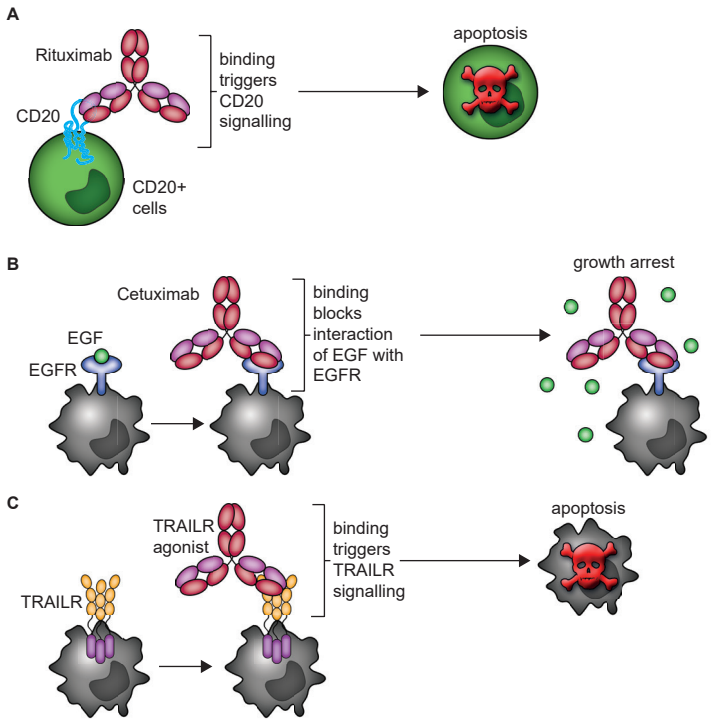

Figure 3: Antibody-mediated modulation of target antigen signalling. **A)** Binding of rituximab to CD20 can directly trigger apoptotic elimination of malignant B cells. **B)** Antibodies can be used to inhibit tumourigenic signalling as provided by for instance epithelial growth factor (EGF). By binding to the EGF receptor (EGFR), cetuximab blocks the interaction between EGF and EGFR, thereby inducing growth arrest. **C)** Agonistic antibodies targeting the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor (TRAILR) induce cross-linking of agonistic TRAILRs which triggers the induction of cancer cell apoptosis.

Clinical experience

The initial intent of mAb-based cancer therapy was the direct targeting and elimination of malignant cells, and the drug approval of rituximab (anti-CD20) and trastuzumab (anti-HER2) delivered important proof-of-concept (Maloney et al. 1997, Piccart-Gebhart et al. 2005). Combination of rituximab with established chemotherapeutic regimens such as CHOP (Cyclophosphamide, Hydroxydaunorubicin, Oncovin Prednisone) enhanced the clinical efficacy in various malignancies, including follicular lymphoma (FL) (Oers et al. 2006), chronic lymphocytic leukaemia (CLL) (Hillmen et al. 2014), diffuse large B cell lymphoma (DLBCL) (Coiffier et al. 2002), mantle cell lymphoma (MCL; (Kluin-Nelemans et al. 2012), and aggressive malignancies such as Burkitt's lymphoma (Barnes et al. 2011). Furthermore, maintenance therapy with rituximab was effective in the long-term control of FL (Oers et al. 2010) and CLL (Abrisqueta et al. 2013). Rituximab also binds to and depletes non-malignant B cells, but the toxicity of rituximab treatment in cancer patients is tolerable, especially with regard to infectious complications. Indeed, the depletion of non-malignant B cells mediated by rituximab has been successfully implemented in the treatment of various B-cell-mediated diseases, including rheumatoid arthritis (RA) (reviewed in Liossis et al. 2008).

The success of rituximab inspired the development of more advanced antibody formats, such as the fully humanised CD20 antibodies ofatumumab and obinutuzumab, receiving FDA approval in 2009 and 2013 for the treatment of CLL and FL, respectively, and finally of fully human antibodies generated by the immunization of mice transgenic for human immunoglobulin genes or by panning of libraries of fully human antibody fragments with the appropriate antigen. Ofatumumab has higher CDC activity than rituximab (Teeling et al. 2004) and is effective in FL and CLL patients previously treated with rituximab (Coiffier et al. 2008, Hagenbeek et al. 2008). Obinutuzumab was also designed to induce potent CDC and was superior to rituximab in patients with newly diagnosed CLL treated with Chlorambucil (Goede et al. 2014) and in patients with indolent non-Hodgkin lymphoma (Sehn et al. 2016). Further, obinutuzumab triggered more effective neutrophil-mediated phagocytosis through enhanced binding to FcγRIIIb (Golay et al. 2013).

Beyond CD20 various other leukaemia-associated antigens, such as CD38, CD52 and SLAMF7, are being explored for direct mAb-based therapy. Recently, the antibody daratumumab, that targets CD38 on the surface of MM cells, was found to have potent anti-MM activity as a single-agent in heavily pretreated MM patients (Lokhorst et al. 2015). Additional studies are ongoing to evaluate its potential benefits in combination treatments with currently available chemotherapeutic agents. The paradigm for beneficial combinatorial effects between antibodies and chemotherapeutic agents is the treatment of B-CLL in younger patients with fludarabine and cyclophosphamide combined with rituximab (Weiner et al. 2010, Beck et al. 2010, Scott et al. 2012).

mAb-based therapy of solid tumors began in 1998 with the approval of trastuzumab for the treatment of breast cancer that overexpresses the HER2 surface antigen. Trastuzumab was used after adjuvant chemotherapy for one year, which improved disease-free survival but produced no benefit in overall survival (Piccart-Gebhart et al. 2005). Unfortunately, a significant proportion of patients treated with trastuzumab did not respond to

the treatment. Further, trastuzumab was associated with cardiotoxicity in up to 0.5 percent of treated women, especially when combined with anthracyclines (Piccart-Gebhart et al. 2005). Another antibody widely used in the treatment of solid tumors is cetuximab, a chimeric antibody targeting the epidermal growth factor receptor EGFR (Jonker et al. 2007). Cetuximab received FDA approval in 2004 for patients with advanced colorectal cancer (Jonker et al. 2007). Since its initial approval, cetuximab has been included in the treatment of triple negative breast cancers (Carey et al. 2012) and in chemo-radiotherapy regimens for head and neck cancers (Bonner et al. 2006). In colorectal cancers, cetuximab is mostly effective in patients without mutations in K- or N-RAS (Karapetis et al. 2008). In addition to cetuximab, the fully human EGFR antibody panitumumab was recently approved by the FDA for the same indications. Panitumumab and cetuximab appear to have a similar activity profile in patients, but panitumumab has a more favorable toxicity profile (Price et al. 2014).

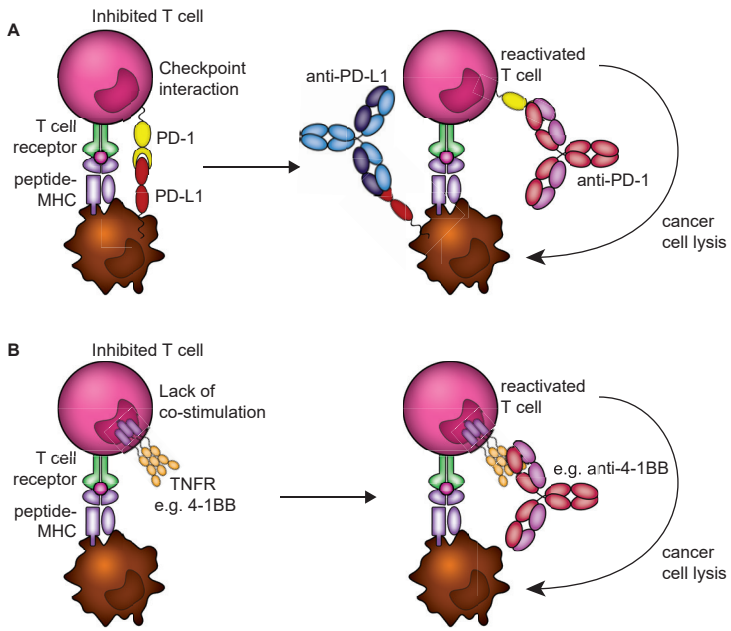

Figure 4: Immunomodulatory antibodies. **A)** The PD-1/PDL-1 checkpoint interaction counteracts activation of T cells and this inhibitory pathway is overactivated in PDL-1 overexpressing tumors. Therefore, PD-1/PDL-1 blocking antibodies reactivate T cells, inducing cancer cell lysis. **B)** Members of the tumor necrosis factor receptor (TNFR) superfamily provide important co-stimulatory signals required for the induction of antitumor immunity. In the absence of co-stimulation, T cell activation is inhibited. Hence, TNFR agonists such as 4-1BB antibodies reactivate T cells, inducing cancer cell lysis.

Immunomodulatory antibodies

In recent years, major progress in mAb-based therapy has been made through the use of immunomodulatory mAbs, most notably those targeting immune checkpoints. Immune checkpoints are receptor/ligand pairs that transmit inhibitory signalling to effector cells and antibodies that block these checkpoints (checkpoint inhibitors) can (re)activate tumor-specific T cell immunity (Figure 4A). Of note, such antibodies typically contain IgG4 Fc domains with low binding affinity for Fc-receptors on myeloid effectors and NK cells, designed to prevent elimination of the T cells by other effector cells through ADCC or CDC. Reversely, immunomodulatory antibodies can be targeted to activate co-stimulatory members of the TNF-receptor (TNFR) superfamily (Figure 4B). This TNFR superfamily provides important co-stimulatory signals required for the induction of adaptive immunity at all stages of the immune responses and targeted activation of these signals can augment anti-tumor activity.

Checkpoint blocking mAbs

The first immune checkpoint targeted antibody to enter clinical practice was directed against the protein CTLA4. CTLA4 is an inhibitory receptor expressed on activated T cells that inhibits T cell co-stimulation by competing with CD28 for CD80/86 interaction on APCs (Krummel et al. 1995). CTLA4 has a higher affinity than CD28 and thus competitively inhibits CD28-mediated co-stimulatory signalling (Linsley et al. 1994). The CTLA4-blocking antibody ipilimumab was the first checkpoint inhibitor to be approved by the FDA (in 2011) for the treatment of melanoma. Ipilimumab triggered strong durable responses in patients with previously treated metastatic melanoma, with 1 and 2 year survival rates of 46% and 24% respectively (Hodi et al. 2010). Similarly, combination of ipilimumab with paclitaxel and carboplatin improved progression free survival in chemotherapy-naïve NSCLC patients (Lynch et al. 2012).

A second prominent example of immune checkpoints is PD-1, an inhibitory receptor expressed on T cells, which inhibits T cell activity after binding to its ligand PD-L1 on the surface of a cancer cell or on antigen presenting cells (APCs) (Freeman et al. 2000). Antibodies blocking this inhibitory interaction restored the antitumor activity of T cells in murine models (Figure 4A) (Wang et al. 2014). Under homeostatic physiological conditions the PD-1/PD-L1 receptor/ligand pair ensures a timely shut-down of T cell responses (reviewed in Keir et al. 2008). During inflammation, expression of PD-L1 is induced on APCs, such as DCs, and on myeloid suppressor cells by locally produced IFN-γ (Freeman et al. 2000, Loke et al. 2003, Kuang et al. 2009). At the same time, activated T cells upregulate PD-1 (Nishimura et al. 1999), whereby T cell activity is inhibited via PD-1/PD-L1 interactions (Freeman et al. 2000).

Many types of cancer cells (over)express PD-L1 either constitutively, through oncogenic signalling pathways, or in response to tumor micro-environmental IFN-γ (Taube et al. 2012, Dong et al. 2002). Due to the PD1/PD-L1 interaction, infiltrating T cells are silenced in the tumor micro-environment, which allows the escape and growth even of immunogenic cancers such as melanoma (Ahmadzadeh et al. 2009, Taube et al. 2012).

Consequently, elevated expression of PD-L1 correlated with poor prognosis (Thompson et al. 2004, Massi et al. 2014). Both PD-1 and PD-L1 blocking antibodies can restore the ability of functionally impaired T cells to eliminate cancer cells (Figure 4A). Treatment with the PD-1 blocking antibody nivolumab achieved unprecedented objective response rates of >30% (Larkin et al. 2015). A PD-L1 blocking antibody produced objective responses in up to 17% of patients with advanced stage cutaneous melanoma (Brahmer et al. 2012), although long term remissions were obtained only for a minority of patients. Response rates in other malignancies, especially in haematological malignancies, were sub-optimal, although new results point to a promising activity in patients with Hodgkin's lymphoma refractory to the antibody-drug conjugate brentuximab vedotin (Ansell et al. 2015, Armand et al. 2016, Westin et al. 2014).

Importantly, treatment of melanoma patients with a combination of the PD-1-blocking antibody nivolumab and ipilimumab significantly enhanced response rates (Larkin et al. 2015). Therefore, the use of combination therapies may significantly improve the impact of checkpoint inhibition as a treatment modality for selected patients. The increased response rates however were also accompanied by a sharp increase in systemic toxicity, and therefore, combination therapies with several different checkpoint inhibiting antibodies may present benefits only for restricted subgroups of patients. Various other checkpoints have been identified, including TIM-3, TIGIT, LAG3, and CD28, and are currently explored as potential targets for mAb-based therapy. These may also help to expand the impact and scope of (combinatorial) checkpoint inhibition.

Immunostimulatory mAbs that target the TNFR superfamily

The TNFR superfamily provides crucial co-stimulatory signals in a spatiotemporally regulated manner that drives development of effective immune responses (Croft 2009, Sanmamed et al. 2015). These co-stimulatory receptors are either constitutively expressed, such as CD27, or expression is induced upon antigen recognition as is the case for 4-1BB, GITR, OX40. Most of these receptors are being evaluated as targets for cancer immunotherapy, with a few prominent examples being highlighted below.

A prominent immunotherapeutic TNFR target on T cells is 4-1BB. T cells responding to their cognate antigen rapidly and transiently upregulate 4-1BB on their cell surface. Interaction of T cell expressed 4-1BB with 4-1BBL on activated APCs induces proliferation, cytokine production as well as up-regulation of survival genes and inhibition of activation-induced cell death in T cells (DeBenedette et al. 1997, Arch et al. 1998, Starck et al. 2005). Thus, 4-1BB activation enables T cell expansion and persistence. Of note, 4-1BB signalling triggers a stronger proliferative response in CD8 cytotoxic T cells than in CD4 T-helper cells, particularly *in vivo* (Shuford et al. 1997). 4-1BB is therefore considered to be a bona fide CD8 T cell activating molecule (Takahashi et al. 1999), with 4-1BB expression specifically detected on tumor infiltrating T cells (Ju et al. 2005). Furthermore, T cells responding to cognate antigen upregulate 4-1BB, yielding co-stimulatory signalling for antigen-specific CD8 T cell responses (Choi et al. 2014). All these characteristics make 4-1BB an interesting target for cancer immunotherapy. Indeed, already over a decade ago treatment of mice with a soluble 4-1BB agonist was found to induce tumor

regression of established and poorly immunogenic sarcoma (Melero et al. 1997). To date, a large body of preclinical data supports the use of 4-1BB agonists for cancer immunotherapy, both as single agents and in combination with e.g. vaccine-based strategies (Sharma et al. 2010). Agonistic antibodies that target 4-1BB re-activate and restore T cell mediated antitumor immunity in various animal studies (Melero et al. 1997, Kim et al. 2001) (Figure 4B). However, ubiquitously active agonistic antibodies that trigger co-stimulatory TNFR signalling frequently associate with dose-limiting toxicities. For example, treatment of patients with the 4-1BB antibody urelumab produced severe and fatal liver toxicity at the highest dose tested, leading to termination of a phase II clinical trial (reviewed in Bartkowiak et al. 2015). Nevertheless, a subsequent dose-escalation study with a 4-1BB antibody has been performed (NCT01471210) and 4-1BB mAbs are currently evaluated in clinical trials focused on combination strategies, e.g. combinations with PD-1 antibodies (NCT02179918) or with agonistic antibodies that target the co-stimulatory TNFR OX40 (NCT02315066).

A second TNFR target is OX40. OX40 is transiently upregulated on antigen stimulated CD4 and CD8 T cells, and its ligand OX40L is transiently expressed on activated APCs (reviewed in Croft et al. 2009). OX40 signaling is involved in T cell survival and the generation and reactivation of memory T cells (Rogers et al. 2001, Gramaglia et al. 2000). Combination of OX40 agonists and antibodies targeting PD-1 or CTLA4 induced potent antitumor immunity in preclinical models (Redmond et al. 2014, Guo et al. 2014).

A third TNFR being targeted with agonistic mAbs is GITR. GITR is highly expressed on regulatory T cells, and while low levels are present on naïve and memory T cells, GITR expression is rapidly upregulated upon activation (Gurney et al. 1999, Zhan et al. 2004). Its ligand, GITRL is predominantly expressed on activated APCs (Tone et al. 2003). GITR agonists have promising antitumor activity *in vivo* by expanding effector T cells, while simultaneously inhibiting regulatory T cells (Kim et al. 2015a; Coe et al. 2010). Similarly, single agent dose-escalation studies of GITR as well as combinations with PD-1 antibodies are currently ongoing in solid malignancies including melanoma (NCT01239134) (reviewed in Knee et al. 2016).

The final TNFR target discussed here is CD40, which is expressed on APCs and interacts with CD40L on CD4 T-helper cells. This interaction is crucial for induction of adaptive immune responses as the absence of CD40 signalling can induce anergy or lead to formation of regulatory T cells (reviewed in Melief et al. 2008). Agonistic CD40 antibodies strongly induced DC maturation and antitumor activity in preclinical models (reviewed in (Khong et al. 2012) and although dose-limiting toxicities have been observed, CD40 agonists can be effective at tolerable doses (Johnson et al. 2015b, Vonderheide et al. 2007). Clinical trials evaluating CD40 agonists alone (NCT02482168, NCT02376699) or in combination with CTLA4 antibody (NCT01103635) are currently ongoing.

As outlined above, various therapeutic regimens that combine checkpoint targeting antagonistic antibodies with agonistic TNFR antibodies are currently being explored. This strategy is appealing as it may boost antitumor immunity by complementing blockade of inhibitory signalling with simultaneous co-stimulation. However, since both TNFR agonists and checkpoint blocking antibodies are associated with immune-adverse related

toxicities, combinatorial strategies need to be carefully designed to avoid greatly increased toxicities.

Of note, the therapeutic effect of checkpoint blocking antibodies and TNFR agonists may also partly be independent of signalling modulation. For example, the efficacy of ipilimumab is co-defined by Fc-dependent depletion of regulatory T cells by macrophages in the tumor microenvironment (Simpson et al. 2013). Similar modes of action may contribute to TNFR agonists efficacy as TNFRs CD27, GITR, OX40 and 4-1BB are expressed on regulatory T cells (Sanmamed et al. 2015). Therefore, optimal design of therapeutic agents and combinatorial strategies should take requirement of FcR interactions into account.

Alternative Antibody Formats

Antigen binding fragments

Recombinant DNA technology has enabled scientists to design many different antibody formats based on conventional mAbs (Figure 1A), including bispecific antibodies, dia-, tri- and tetra-bodies, triplebodies, minibodies, nanobodies, Fabs, F(ab')$_2$ and scFv-fragments (reviewed in Spiess et al. 2015 & Carter et al. 2006), some of which will be discussed below. Of particular relevance to the field is the so-called single chain fragment of variable regions (scFv) antibody fragment (Glockshuber et al. 1990). In brief, an scFv is a single polypeptide comprising the VH and VL regions of an antibody connected through a flexible linker (Figure 5A). The scFv antibody fragment has been used as a 'building block' in many different antibody-based drug formats discussed later in this chapter, including bispecific antibodies, immunocytokines and CAR T cells. An additional engineered antibody domain of interest is the so-called antigen-binding Fc fragment (Fcab), in which the C-terminal loops of the IgG1 Fc domains have been engineered to bind antigens (Figure 5B) (Wozniak-Knopp et al. 2010). An Fcab thus comprises only the Fc domain and is smaller in size, more stable, and can be used as a building block for engineering of bispecific antibodies. Differently engineered Fcabs can have increased or reduced affinity for Fc receptors, which can be used to optimise specific effector functions (Kainer et al. 2012). Nanobodies, the smallest naturally-occurring antigen-binding domains, are derived from 'heavy-chain-only antibodies' expressed by camelids and sharks (reviewed in Muyldermans 2013). Nanobodies consist of heavy chain subunits only and are almost 10-times smaller than regular mAbs (mass of ~15 kDa vs. 150 kDa, respectively) (Figure 5C). Nanobodies are stable and have good solubility and are interesting building blocks for the design of bi/tri or multi-specific antibody-based drugs (reviewed in Steeland et al. 2016).

Bispecific antibodies

Bispecific antibodies (bsAbs) combine the binding specificities of two antibodies (Figure 5D) and can be designed to retarget immune effector cells to cancer cells to trigger target cell elimination. Further, bsAbs can be used for dual targeting of cancer cells, whereby e.g. signalling by two growth factors can be simultaneously blocked (reviewed in Kontermann et al. 2015, Spiess et al. 2015). Approximately 30 different bsAbs and

bispecific fusion proteins of mAb-fragments are currently in clinical evaluation. In the following section, we will review recent advances in both immune retargeting and tumor targeting bsAbs and bispecific fusions of mAb-fragments, and highlight some of the successes and challenges.

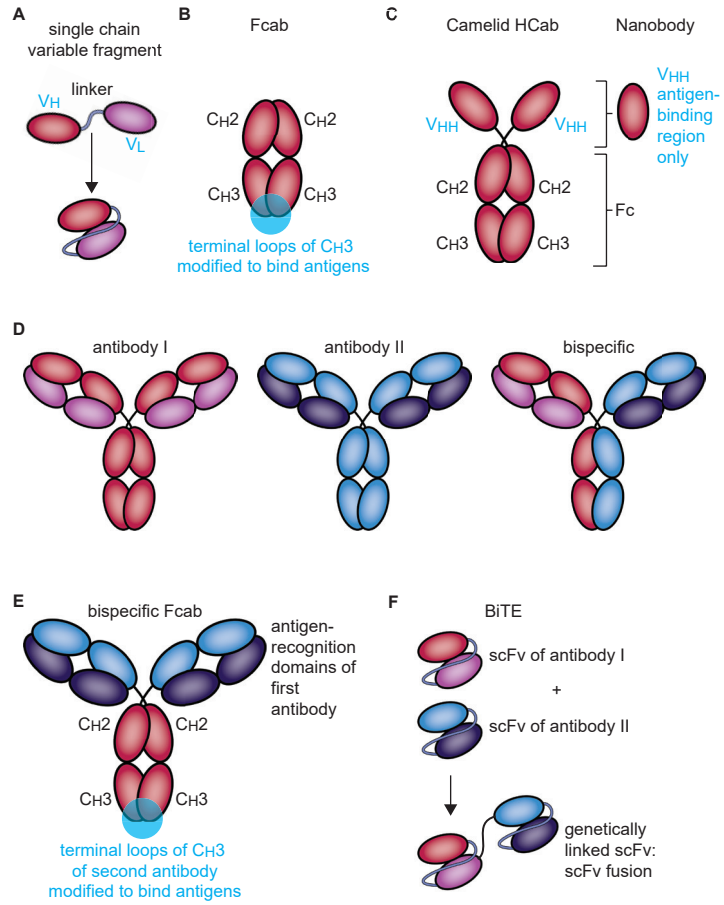

Figure 5: Alternative antibody formats. **A)** A single chain fragment of variable regions (scFv) antibody fragment is a single polypeptide, comprising the VH and VL regions of an antibody connected through a flexible linker. **B)** Antigen-binding Fc fragments or Fcabs, are engineered antibodies in which the C-terminal loops of the IgG1 Fc domain have been modified to bind antigens. **C)** Nanobodies are the smallest naturally-occurring antigen-binding fragments normally expressed by camelids. Nanobodies solely comprise heavy chain subunits and are almost a factor 10 smaller than regular mAbs (size of ~15kDa vs. 150kDa, respectively). **D)** A bispecific antibody is constructed by merging two different antibodies together. It contains two heavy and two light chains, one each from two different antibodies, and thereby has specificity for two antigens. **E)** Fcab antibodies can be constructed in a bispecific format, containing an engineered terminal CH3 loop to induce the ability to bind antigens. **F)** A Bispecific T cell Engager (BiTE) contains an scFv antibody fragment specific for CD3 and a second scFv recognising a tumor antigen connected by a flexible peptide linker. These scFvs allow the antibody to induce T cell activation via CD3-mediate cross-linking and specifically recruit the activated T cells to the tumor by virtue of the tumor-targeting scFv.

Immune retargeting bsAbs

Although initial studies with bispecific antibodies were hampered by issues with heterogeneity of antibody formulations, the use of quadroma technology permitted the production, purification and clinical development of hybrid (rat-mouse) bsAbs (Lindhofer et al. 1995). The first clinically approved bsAb, catumaxomab (Seimetz et al. 2010), was such a chimeric rat-mouse antibody comprising a murine antibody domain (one H- and one L-chain) specific for CD3 and a rat antibody domain specific for EpCAM. The mouse IgG2a and rat IgG2b H-chains carried in the hetero-dimer can bind to the activating human Fcγ receptors FcγRI, FcγRIIA and FcγRIII. Thus, catumaxomab re-targets CD3-positive T cells to EpCAM whereupon an immunological synapse is formed. Cross-linking of CD3 activates the T cell and directs its full cytolytic potential towards the target cell, including the release of perforin and granzymes, FasL and the release of cytokines such as IFN-γ, leading to elimination of the target cell. In addition, catumaxomab recruits FcγR-bearing effector cells including NK cells and macrophages that induce ADCC and ADCP. This multi-component mode-of-action was very effective in pre-clinical and clinical studies, and led to drug approval of catumaxomab in the EU for the treatment of malignant ascites in patients with EpCAM-positive carcinomas in 2009 (Seimetz et al. 2010). However, the chimeric rat-mouse Fc-domain triggered the production of neutralising human anti-mouse (HAMA) and human anti-rat antibodies (HARA) in most patients, which limited its clinical usefulness, although HAMA/HARA responses were reported not to be associated with safety concerns or reduced clinical activity (Ruf et al. 2010). Production of HAMAs after treatment with catumaxomab was positively correlated with clinical outcome in patients with malignant ascites, likely reflecting an active immune response (Ott et al. 2012). A second bsAb of this format, FBTA05 or Lymphomun, was recently evaluated in a clinical trial with patients suffering from refractory B cell malignancies. Nine of ten patients showed a clinical response, including 5 complete remissions (Schuster et al. 2015). Finally, a third bsAb of the same format, ertumaxomab, selectively targets the breast carcinoma antigen HER2 and produced antitumor responses in a clinical trial in 5 out of 15 evaluable patients with manageable toxicity (Kiewe et al. 2006). However, clinical development of ertumaxomab was halted due to non-scientific reasons.

Additional and newer bispecific molecular formats of antibody-derived agents have been developed and some have proven to be successful in clinical trials. Front-runner in this field is the so-called Bispecific T cell Engager (BiTE) format. A BiTE comprises an scFv antibody fragment recognising CD3 and a second scFv fragment recognising a tumor antigen, connected through a flexible peptide linker which allows the scFv domains to rotate and bind their targets (reviewed in Huehls et al. 2015) (Figure 5F). BiTEs do not contain Fc domains and therefore selectively recruit $CD3^+$ T cells to the antigen-bearing cell, where an immunological synapse is formed and the target cell is killed. The retargeted T cells, still decorated by the BiTE protein, can move on to a next target and perform serial lysis of up to a dozen target cells (Hoffman et al. 2005). The BiTE-induced synapse resembles a standard immunological synapse generated through MHC/TCR interaction (Offner et al. 2006). Further, the antitumor activity of a BiTE crucially depends on the cell-to-cell distance resulting from binding of the CD3- and tumor-specific scFvs. In this

respect, the melanoma antigen MCSP proved to be too bulky to permit the formation of a sufficiently tight and productive synapse between the melanoma-target and effector cells (Bluemel et al. 2010). This spatial requirement for the formation of a functional synapse likely holds true for other molecular formats of bispecific agents. Thus, antigen size and the position of the epitope within the antigenic surface protein are important considerations for the design of T cell retargeting agents.

Various other BiTEs have been evaluated in pre-clinical and some in clinical studies, including a CD33-directed BiTE called AMG 330, designed to target Acute Myeloid Leukaemia (AML) (Mills et al. 2015), an MCSP-directed BiTE to target melanoma (Bluemel et al. 2010), EpCAM- and CEA-specific BiTEs to target carcinoma (Schlereth et al. 2005), a PSMA-directed BiTE to target prostate cancer (Friedrich et al. 2012) and a CD19-specific BiTE to target B-lineage leukaemia and lymphoma (Sheridan et al. 2015). The latter BiTE, termed blinatumomab, retargets T cells to CD19-bearing tumor cells and accomplished a 43% complete remission rate in a phase II clinical trial in patients with B-lineage Acute Lymphoblastic Leukaemia (B-ALL) (Topp et al. 2015, Zugmaier et al. 2015). Grade 3 and 4 adverse effects occurred in a subset of patients, including neurotoxicity and cytokine-release syndrome (Topp et al. 2015). Nevertheless, overall results were sufficiently positive to lead to fast-track approval of blinatumomab by the US FDA for second-line treatment of relapsed or refractory B-ALL in 2014, which has since been converted into a definitive approval. Blinatumomab is further being evaluated for the treatment of Non-Hodgkin Lymphoma (NHL) and Diffuse Large B cell lymphoma (DLBCL) (Zugmaier et al. 2015). Of note, the small size of BiTEs results in improved tumor penetration, but also in a short serum half-life of less than 2 hrs, as determined for blinatumomab (Zugmaier et al. 2015). For comparison, the Fc-containing bsAb catumaxomab has a serum half-life of 2.5 days (Seimetz et al. 2010), and IgGs have half-lives of approx. 20 days in healthy human individuals.

Efforts to improve on the BiTE format have been made by using so-called "Dual Affinity Retargeting T cell" engaging agents (DARTs). DARTs consist of 2 covalently linked VH/VL polypeptide chains, which upon heterodimerization form the two antigen binding domains. Compared to BiTEs, DARTs have a more flexible configuration and contain an additional inter-chain disulfide bond to increase stability (Moore et al. 2011). In a side-by-side evaluation of blinatumomab with a DART carrying the corresponding scFvs, the DART agent had a higher affinity. Further, the DART mediated a strongly improved cytolytic activity of T cells against B-lineage leukaemia target cells, with an approximately 60-fold reduction in EC50 using freshly isolated peripheral blood mononuclear cells as the source of effectors (Moore et al. 2011). Many DARTs have been generated and some are currently in preclinical and early clinical evaluation, including a DART targeting the AML marker CD123 (Al-Hussaini et al. 2016, Chichili et al. 2015) and a DART targeting the colorectal carcinoma marker gpA33 (NCT 02248805).

Other interesting molecular formats of bispecific agents include the so-called Duobody format (Labrijn et al. 2014), the Crossmab format (Klein et al. 2016), the DVD-IgG format (Gu et al. 2012), and the single-chain triplebody format (Kugler et al. 2010, Braciak et al. 2013, Roskopf et al. 2016). This growing field has been periodically reviewed, most

recently by Kontermann & Brinkmann and Spiess, Zhai & Carter. A duobody exploits single amino acid mutations in the IgG1 domain to trigger controlled Fab arm exchange to yield a homogeneous IgG1 bsAb preparation (Labrijn et al. 2014). An agent of this duobody class has recently advanced into clinical evaluation (NCT02758392).

In a triplebody, three scFv domains are genetically fused in tandem, with the central domain being either a CD3-specific scFv to recruit T cells or a CD16-directed scFv to recruit NK- and γδ T cells as effectors. This triplebody format enables dual-targeting of two tumor associated target antigens on the same cancer cell, to which T cells or NK cells can be preferentially directed. Proof-of-concept for this approach was recently reported for a CD19-CD3-CD33 triplebody, which induced preferential elimination of CD19/CD33 double-positive biphenotypic B/myeloid leukaemia cells over CD19 or CD33 single-positive cells (Roskopf et al. 2016). Further, the triplebody 19-16-19, carrying two scFv binding sites for CD19 and one for CD16, was recently reported to recruit γδ T cells as potent cytolytic effectors to malignant B-lineage target cells *in vitro* (Schiller et al. 2016).

Other effector cells, most notably NK cells, are also evaluated in the context of tumor retargeting. NK cells are very potent cytotoxic lymphocytes critically involved in tumor immunosurveillance and have among others been retargeted to CD133, a proposed cancer stem cell marker, using the so-called Bispecific Killer Engager (BiKE) approach (Schmohl et al. 2016). A BiKE has a similar tandem diabody format as BiTEs, but instead of a CD3-directed scFv contains a CD16-specific scFv to recruit and activate NK cells. The CD133 BiKE effectively triggered NK cell mediated lysis of target cells *in vitro* and *in vivo*. Similarly, an EpCAM BiKE strongly potentiated NK cytotoxicity towards EpCAM-expressing cells *in vitro* (Vallera et al. 2013). An additional NK cell retargeting therapeutic, designated AFM 13, is a tetravalent construct with two scFv binding domains each for CD30 and CD16 (Rothe et al. 2015).

As already mentioned above, the triplebody format can also be used to retarget NK cells and γδ T cells, by incorporating a CD16-specific scFv. Using a CD19-CD16-HLA-DR triplebody, NK cells were successfully redirected *in vitro* to trigger a preferential elimination of CD19/HLA-DR double-positive cancer cells over single-positive leukemic cells present in the same reaction environment (Schubert et al. 2012). This triplebody format can thus be used to enhance tumor-selectivity, by selection of target antigen combinations that are unique to a certain malignancy, such as CD33- and CD123-double-positive AML. Using this approach, off-target toxicity towards normal single antigen-positive cells is expected to be reduced. In addition, retargeting of other effector cell populations such as monocytes and T cells are being evaluated, although EGFR-retargeting of FcγRI-positive effector cells failed to yield clinical benefit in a phase I trial (Fury et al. 2008).

A final bispecific format of interest to mention is based on the Fcab format (Figure 5E) (reviewed in Lobner et al. 2016). For instance, fusion of an Fcab with specificity for HER2 with an scFv specific for CD3 resulted in the bispecific Fcab fusion FcabCD3 (Wang et al. 2013). This agent induced specific T cell-mediated tumor cell lysis *in vitro*, which was more potent than treatment with either trastuzumab or the parental Fcab alone. Also in animal studies, FcabCD3 reduced tumor growth.

Of note, it was recently found that immune-inhibitory mechanisms in the tumor microenvironment can inhibit the therapeutic activity of T cell retargeting by bispecific agents. For instance, in a blinatumomab resistant patient with B cell acute lymphoblastic leukaemia (B-ALL), the percentage of PD-L1 expressing B-ALL blasts was strongly increased (Kohnke et al. 2015). This increase in PD-L1 expression also strongly reduced the efficacy of blinatumomab-mediated lysis of target cells ex vivo. In line with this, treatment of primary AML cells with the CD33-directed BiTE AMG 330 ex vivo triggered strong up-regulation of PD-L1 on the AML blasts (Krupka et al. 2016). Combination treatment with this BiTE and a PD-L1 checkpoint inhibiting antibody induced synergistic tumor cell lysis *in vitro*. Similar results were obtained for a CEA-specific BiTE (Osada et al. 2015). In line with these findings, *in vivo* activity of a full-length HER2/CD3 bsAb was limited by expression of PD-L1, an effect abrogated by treatment with a PD-L1 mAb (Junttila et al. 2014). Along the same line, the activity of CD20- and EGFR-targeted bsAbs was optimised by inhibition of CTLA4 using ipilimumab (Yano et al. 2014). Similarly, combination treatment with a BiTE-agent and a co-stimulatory antibody targeting CD28 or 4-1BB also augmented T cell-mediated tumor cell lysis (Laszlo et al. 2015, Aliperta et al. 2015). Using an EpCAM-directed BiTE, additional inhibitory mechanisms including IDO expression on the target cells and secretion of TGF-β were identified (Deisting et al. 2015). Since signalling through co-inhibitory and co-stimulatory pathways may affect the therapeutic efficacy of antibody-derived agents relying on immune effector cells for their therapeutic activity, this issue is an important consideration in the design of combinatorial treatment strategies.

Direct tumor targeting with bsAbs

In addition to redirecting immune effector cells, a variety of bsAbs inhibit growth factor receptor signalling via dual targeting of cancerous cells. For instance, based on the finding that HER3 signalling represents an important resistance mechanism for HER2 inhibitors, bispecific agent MM-111 comprising HER2 and HER3 targeted scFvs and human serum albumin was generated (McDonagh et al. 2012). MM-111 was found to overcome HER2 resistance and block HER3 signalling through formation of an inactive MM-111/HER2/HER3 complex. MM-111 synergised with HER2 inhibitor trastuzumab in several xenograft models and has been evaluated in early phase clinical trials (NCT01304784/NCT01774851). However, disappointing preliminary results of a phase II study in gastric cancer, possibly due to low HER2 expression, prompted abandonment of MM-111 for this indication.

Targeting of HER2 has also been explored using MM-141, comprising scFvs targeting HER3 and insulin-like growth factor I receptor (IGFR-IR) fused to an IgG1 constant domain, with superior activity compared to control single or combination antibody therapy in preclinical studies (Fitzgerald et al. 2014). Recent preliminary reports on clinical activity in patients with advanced solid cancer were suggestive of clinical activity, with patients having the potential eligibility biomarker of elevated serum IGF-I remaining on treatment longer. Further, IGF-IR and HER3 expression levels decreased upon treatment with MM-141 (Isakoff et al. 2015). A more clinically advanced HER3 targeting bispecific agent is duligotuzumab (MEHD7945A), a so-called dual-action Fab (DAF) antibody. A

DAF is created by mutagenesis of a Fab of a monospecific antibody into a dual recognition antibody, in the case of duligotuzumab for EGFR and HER3 (Eigenbrot et al. 2013, Schaefer et al. 2011). Duligotuzumab overcomes acquired resistance to EGFR and radiation in preclinical models (Huang et al. 2013) and had comparable antitumor activity to cetuximab in patients with head and neck cancer, although the risk of adverse effects was slightly increased (Fayette et al. 2014). Clinical trials in epithelial cancers and head and neck cancer are ongoing (NCT01911598/ NCT01207323).

Several other formats for direct tumor-targeted bispecific agents are in clinical development including the so-called DVD-Ig format, in which the variable domains of two monoclonal antibodies are genetically fused, yielding a tetravalent bispecific antibody (Gu et al. 2012). For instance, simultaneous binding of a DVD-Ig that selectively targets CD20 and HLA-DR on B cell leukaemic cells potently induced both ADCC and CDC (Zeng et al. 2015).

Bispecifics can also be designed to overcome two immune-inhibitory mechanisms associated with mAb-based therapy. An example hereof is a bispecific tetravalent antibody that blocks CD47 by selective delivery to B cell expressed CD20 (Piccione et al. 2015). CD47 is an important don't eat me signal on tumor cells that inhibits phagocytic removal, hence CD47 blocking mAbs can enhance phagocytic removal of cancer cells and synergise with other antibodies like rituximab (Chao et al. 2010). The CD20/CD47 bispecific antibody had an increased selectivity for CD20/CD47 double-positive cancer cells and augmented their phagocytic removal *in vivo* (Piccione et al. 2015).

Finally, we recently reported application of the bispecific format to enhance the tumor-selective activity of TRAIL-receptor 2 antibody-based therapy, using a tetravalent bispecific agent comprised of an scFv targeting MCSP and an scFv derived from TRAIL-R2 agonist tigatuzumab (He et al. 2016, Chapter 3 this thesis). TRAIL-R2 mAbs such as tigatuzumab are well tolerated in patients (reviewed in (Fox et al. 2010), but have minimal clinical benefit. This lack of activity may be due to the widespread expression of TRAIL-R2, limiting tumor accretion, and the fact that TRAIL-R2 mAbs require additional cross-linking by Fc-receptor positive cells for effective induction of apoptosis. In this respect, a tetravalent nanobody (TAS266) did not require such secondary cross-linking for induction (Huet et al. 2014), but this nanobody yielded unexpected hepatotoxicity in a phase I clinical trial in patients with solid malignancies leading to termination of the trial (Papadopoulos et al. 2015). Thus, ubiquitous maximal cross-linking and signalling by TRAIL-R2 may associate with unwanted toxicity. In contrast, MCSPxDR5 had high binding specificity for MCSP-positive melanoma cells and potently triggered apoptosis only in MCSP-positive cancer cells, including primary patient-derived melanoma cells (He et al. 2016). Similarly, a FAP-targeted TRAIL-R2 bispecific agent, RG7386, was reported to have potent antitumor activity superior to conventional non-targeted TRAIL-R2 mAb. Apoptotic activity of RG7386 strictly depended on binding to FAP and did not require additional FcR-mediated cross-linking (Brunker et al. 2016). This increase in tumor-selectivity as well as the increased target antigen-restricted agonist activity is anticipated to augment the therapeutic potential of TRAIL receptor agonist antibody-based approaches.

Antibody-cytokine fusion proteins

Cytokines are major regulators of the immune system and were among the first success stories in cancer immunotherapy. Specifically, two of the most researched cytokines, interferon-α (IFN-α) and interleukin-2 (IL-2), were found to have a significant positive impact on progression free and overall survival in several clinical trials in melanoma and renal cell carcinoma (RCC) (Rosenberg et al. 1994, Kirkwood et al. 2001). Consequently, recombinant IL-2 and IFN-α were FDA-approved for these malignancies in the 1990s and have since become an important part of the treatment protocol. However, cytokines have a multifaceted impact on many components of the immune system. Therefore, it has proven difficult to harness the biological effect of many promising cytokines, such as IL-12 and IL-15, due to toxicity in humans in spite of promising pre-clinical efficacy in animal models. Indeed, as cytokines have no intrinsic selectivity for the tumor, the concentration of non-targeted cytokine needed for antitumor activity is typically too high to achieve without also causing dose-limiting toxicity.

To overcome this drawback, antibody-cytokine fusion proteins have been generated with the aim of combining the high target selectivity of antibodies with tumor-localised activation of immunity by the selected cytokine (reviewed in Young et al. 2014). In the following section of the manuscript, we will first discuss antibody-based targeting of key cytokines such as interferons and interleukins. Second, we will discuss studies aimed at exploiting anticancer signalling through the prominent immunomodulatory Tumor Necrosis Factor (TNF) ligand and TNF receptor superfamily. These TNF ligands hold considerable promise for activation of anti-cancer immunity and, as will be detailed below, are ideally suited for incorporation into antibody-based targeting strategies.

Interferons

IFN-α and β are type I interferons, a class of cytokines that can stimulate antitumor immunity via distinct mechanisms. For instance, IFN-α drives differentiation of monocytes into DCs (Hervas-Stubbs et al. 2011) and enhances cross-presentation of tumor-derived antigenic peptides in MHC-I by Dendritic Cells (DCs) (Gallucci et al. 1999, Ito et al. 2001). Further, IFN-α directly stimulates CD8 T cell expansion and acquisition of effector function (Hervas-Stubbs et al. 2010 & 2012). Additionally, IFN-α negatively regulates proliferation of regulatory T cells and blocks immunosuppressive activity of MDSCs (Pace et al. 2010, Zoglmeier et al. 2011). Furthermore, IFNs have direct antitumor effects such as inhibition of proliferation and induction of apoptosis (reviewed in Parker et al. 2016). Type I IFNs are of clear importance for immunosurveillance, as e.g. demonstrated in murine models of carcinogen-induced cancer (Dunn et al. 2005). In line with this, melanoma metastases spontaneously infiltrated with T cells also express a set of type I IFN regulated genes (Fuertes et al. 2011). In mouse models, this type I IFN signalling, specifically of IFN-β, was required for spontaneous cross-priming of tumor antigen-specific CD8 T cells by DCs. Thus, type I IFNs link innate immunity to development of adaptive immunity. Reversely, type I IFN can also induce expression of immune checkpoint ligand PD-L1 on monocytes, DCs, and endothelial cells, as demonstrated for IFN-β (Schreiner

et al. 2004). This latter effect may actually suppress T cell immune responses. The potential importance of type I IFNs in patients was further recently highlighted by the positive correlation of IFN expression levels with clinical immune responses against cancer (Fuertes et al. 2011).

Of note, type I IFN signalling also impacts on the therapeutic efficacy of direct tumor targeting mAbs, such as those targeting HER2 (Yang et al. 2014). Specifically, blocking of type I IFN signalling intratumorally inhibited the antitumor activity of HER2 mAb in a mouse model, suggesting that type I IFNs may be essential cytokines for the antitumor efficacy of antibodies. Correspondingly, cancer cells resistant to EGFR mAb treatment did not produce type I IFNs. Thus, combination of type I IFNs with therapeutic mAbs could help improve clinical outcome. Although recombinant IFN-α was the first cytokine approved for treatment of human cancer (Quesada et al. 1986), identification of the optimal treatment regimen for IFN-α has proven difficult in view of its dose-limiting toxicity and modest activity in a variety in human cancers (reviewed in Parker et al. 2016). Specific problems are the short serum half-life of IFN-α (only 5 hours in pre-clinical models), with only 0.01% of the injected dose reaching the tumor site (Suzuki et al. 2003).

To overcome the shortcomings of non-targeted recombinant IFN-α, IFN-α has been genetically fused to the C-terminus of an HER2 antibody (Huang et al. 2007), as well as a CD20 antibody (Xuan et al. 2010). Such antibody-cytokine constructs have reduced IFN-α activity compared to non-targeted IFN-α *in vitro*. However, anti-proliferative activity of anti-CD20-IFN-α towards a CD20-positive tumor cell line is increased approximately 1000-fold compared to non-targeted IFN-α. Correspondingly, treatment with anti-CD20-IFN-α cured established tumors of human NHL in all of the mice. Similarly, a murine anti-CD20-IFN-α eliminated established xenografts, whereas a non-targeted IFN-α fusion protein combined with a CD20 mAb did not significantly improve survival compared to anti-CD20 treatment alone (Xuan et al. 2010). A CD20-targeted human IgG1 IFN-α fusion protein (IGN002) is currently evaluated in a phase I clinical trial in relapsed or refractory NHL patients (NCT02519270). Additionally, antibody-based targeting of IFN-α is explored in Multiple Myeloma (MM), where treatment of MM xenograft models with an anti-CD138-IFN-α immunocytokine in combination with proteasome inhibitor bortezomib achieved cure in the vast majority of mice (Vasuthasawat et al. 2016).

Antibody-mediated targeting of IFN-β has also been pre-clinically validated, most notably for EGFR (Yang et al. 2014). An anti-EGFR-IFN-β immunocytokine accreted to EGFR-positive tumors in mice and was strongly retained intra-tumorally for up to 7 days post-injection. Furthermore, no significant toxicity was detected, with no increase in AST and ALT serum levels and only a minor increase in circulating levels of pro-inflammatory cytokines IFN-γ and MCP-1. Importantly, anti-EGFR-IFN-β more effectively inhibited tumor growth than treatment with EGFR mAb alone and facilitated DC-mediated cross-priming and induction of adaptive CD8-positive T cell immunity. Indeed, induction of adaptive immunity was required for the therapeutic effect of anti-EGFR-IFN-β. This finding is in line with a study in which the *in vivo* therapeutic activity of HER2 mAbs was found to be crucially dependent on type I IFN signalling, as well as on IFN-γ–producing CD8 T cells (Stagg et al. 2011). Similar to EGFR-targeted IFN-β, a murine anti-CD20

fusion protein containing mouse IFN-β proved effective in xenografted tumors with lower interferon-α/β receptor expression that were resistant to non-targeted IFN-α (Trinh et al. 2013). Thus, both type I IFNs may be used in antibody-based targeting strategies to enhance the intra-tumoral concentration, reduce off-target activity, and augment the efficacy of therapeutic mAbs. In this respect it is interesting to note that type I IFNs can also directly upregulate expression of certain tumor-associated membrane antigens, such as Carcinoembryonic Antigen (CEA) (Greiner et al. 1984), which may be of use for rational design of new type I IFN-containing immunocytokines.

In addition to the standard antibody formats described above, alternative engineered mAb formats are also being explored for selective delivery of type I IFNs. For instance, using a dock-and-lock system an anti-CD20 immunocytokine containing 4 IFN-α units was constructed (Rossi et al. 2009). This immunocytokine, termed 20-2b, effectively depleted lymphoma cells from human blood and was more effective in treatment of lymphoma xenografts than parental CD20 mAb veltuzumab and a non-targeted IFN-α-mAb immunocytokine. In a similar dock-and-lock approach, an anti-HLA-DR IFN-α immunocytokine was generated, essentially yielding an analogous, but HLA-DR-restricted therapeutic profile (Rossi et al. 2011).

Interleukins

Interleukin-2 (IL-2) is normally secreted by activated CD4 and CD8 T cells in response to antigen as well as by NK cells. Binding of IL-2 to the high affinity IL-2 receptor among others expands CD8-positive effector T cells. Treatment with recombinant IL-2 induces complete remission in a subset of patients with melanoma and renal cell carcinoma (Rosenberg et al. 1994 & 1998, Dillman et al. 1993). However, relatively high doses are needed to obtain clinical responses, which is associated with severe side effects such as VLS (Atkins et al. 1999, Vial et al. 1992). Systemic use of IL-2 in cancer therapy is further hindered by its short serum half-life and associated rapid clearance (Lotze et al. 1985), as well as by the expansion of regulatory T cells upon IL-2 therapy (Ahmadzadeh et al. 2006). Efforts to improve its circulation time include incorporation into micelles (Miki et al. 2014). Further, so-called superkines that have high affinity for IL-2Rβ and circumvent the functional requirement of CD25 receptor for normal IL-2 signalling have been designed to improve activity (Levin et al. 2012).

In order to increase tumor-selectivity, various antibody-targeted IL-2 immunocytokines have been developed, including a CD20 targeted IL-2 fusion protein (Gillies et al. 2005). This immunocytokine had superior activity towards xenografts of B cell leukaemia compared to a combination of non-targeted IL-2 and rituximab (Gillies et al. 2005), a combinatorial approach that was previously reported to yield superior antitumor activity in patients (Friedberg et al. 2002). Of note, fusion of IL-2 to the C-terminus of the antibody did not affect ADCC activity or induction of CD20-dependent apoptosis, but did inhibit induction of CDC. Similarly, an EpCAM-targeted IL-2 immunocytokine, comprised of an EpCAM mAb and two copies of IL-2, had an acceptable toxicity profile in a phase I trial at doses at which IL-2-mediated biological effects such as increased NK cell counts and activity were detected (Ko et al. 2004). In a follow-up phase Ib study in advanced car-

cinoma patients, the acceptable safety profile of this immunocytokine was confirmed, although no clinical responses were detected in this study (Connor et al. 2013). In line with this, treatment of melanoma patients with a GD2-targeted IL-2 immunocytokine was similarly associated with manageable toxicities (King et al. 2004). Treatment with anti-GD2-IL-2 was characterised by activation of immunity, with e.g. increased NK cell activity, NK cell numbers and elevated levels of soluble interleukin-2 receptor, leading to stable disease in a subset of patients. These results were corroborated in a phase II trial where out of 14 patients, 1 patient had a partial response and 4 had stable disease (Albertini et al. 2012).

In addition to full mAb-IL-2 fusions, scFv-based IL-2 fusion proteins have been evaluated. For instance, the immunocytokine F16-IL-2, which targets an alternatively spliced variant of extracellular matrix protein Tenascin-C using the scFv antibody fragment F16. This Tenascin-C variant is expressed in the stroma of various tumors, but not in normal tissues. F16-IL2 selectively localized to xenografted breast cancer cell line MDA-MB-231 and had promising antitumor activity when combined with doxorubicin or paclitaxel (Marlind et al. 2008). In a subsequent dose escalation study, the combination of F16-IL2 with doxorubicin was associated with an acceptable toxicity profile and promising antitumor activity (Catania et al. 2015). Similarly, targeting of an alternatively spliced extracellular domain B of fibronectin, specifically found in tumor vasculature and stroma, was evaluated using IL-2 fused to scFv L19. Treatment with L19-IL-2 was associated with typical IL-2 toxicities that were manageable and transient. Importantly, although objective responses were not reported, L19-IL-2 treatment associated with stable disease in 51% of all patients and in 83% of patients with advanced RCC (Johannsen et al. 2010). In all of these trials, the circulation time of IL-2 immunocytokines was much improved compared to the 20 min serum half-life of non-targeted IL-2, ranging from 2-3 h for L19-IL-2, 4 h or more for EpCAM-IL-2 and GD2-IL-2 to up to 13 h for F16-IL-2.

To further improve IL-2 immunocytokines, engineering of recombinant IL-2 to increase selectively for the high affinity IL-2 receptor, expressed on activated T and NK cells, has been performed. The rationale for this approach lies in the fact that signalling through the intermediate βγ IL-2 receptor, e.g. on circulating cells in the vasculature, is thought to be at least one of the underlying causes for VLS (Shanafelt et al. 2000). In a recent study, mutation of the aspartic acid to threonine at position 20 was found to highly increase selectivity of mAb-targeted IL-2 for the high affinity αβγ IL-2R (Gillies et al. 2011). Of note, this aspartic acid is part of a three amino acid toxin-like domain of IL-2 thought to be responsible, at least in part, for its vascular toxicity (Baluna et al. 1999). This IL-2-D20T-based immunocytokine retained prominent antitumor activity in murine models and had an improved toxicity profile in cynomolgus monkeys.

IL-2 cytokines were also generated by fusing IL-2 to the C-terminus of the light chain instead of the typical fusion to the heavy chain of the antibody (Gillies et al. 2013). Such a melanoma-targeted IL-2 immunotoxin had a longer half-life and retained ADCC and CDC activity compared to standard HC-based IL-2 fusion proteins. Importantly, activation of the high affinity αβγ IL2R was retained, whereas activation of the intermediate affinity βγ IL2R was reduced. The latter was attributed to steric hindrance of binding of the IL-2

Asp20 residue to the β-chain of the intermediate affinity IL2R in this format. Of note, in a recent study with three different immunocytokines, where IL-2 was fused to the IgG light-chain, it was found that these cytokines did not have improved tumor homing or recruitment of effector functions such as ADCC, but that efficacy of the immunocytokines was related to the increased circulation time (Tzeng et al. 2015). Thus, improved circulation time may prove to be the key determinant for IL-2-based immunotherapy. In this respect, conjugation of IL-2 to 6 releasable polyethylene glycol (PEG) chains was recently reported to yield sustained low levels of active IL-2 conjugates by virtue of the slow release of PEG chains (with a reported half-life of ~20 hours for each release step *in vitro*) (Charych et al. 2016). Using this approach, the sustained release of active IL-2 increased tumor accumulation of IL-2 and induced superior antitumor activity in mouse melanoma models, with synergistic activity in combination with anti-CTLA4 treatment. In addition, depletion of regulatory T cells significantly potentiated IL-2-induced anticancer immune responses in a mouse model of colon adenocarcinoma (Imai et al. 2007).

Other interleukins of interest for antibody-based targeting include IL-15, which like IL-2 binds to IL-2 receptor β and γ, but has unique binding to IL-15Rα (Budagian et al. 2006). Unlike IL-2, IL-15 does not trigger expansion of regulatory T cells, but is required for the generation and maintenance of CD8 memory T cells as well as NK cells (Berger et al. 2009). Furthermore, treatment with high dose IL-15 did not associate with VLS in mice and IL-15 treatment of macaques revealed an improved toxicity profile compared to IL-2 (Munger et al. 1995, Berger et al. 2009). Based on these favorable characteristics, IL-15 was targeted to the tumor vasculature-associated EDB domain of fibronectin, using the above-described scFv F19. F19-IL-15 triggered potent antitumor activity in immunocompetent mice that was dependent on CD8 T cell activity and was superior to mock scFv-IL-15 fusion protein (Kaspar et al. 2007). In addition, targeting to melanoma marker GD2 was explored using an IL-15 fusion construct containing the so-called sushi domain of human IL-15Rα (Vincent et al. 2013). This IL-15/IL-15Rα fusion, termed RLI, was previously established to have more potent immunostimulatory effects than IL-15 alone (Mortier et al. 2006, Bessard et al. 2009). The GD2-RLI antibody-cytokine retained cytokine activity, as well as melanoma-selective binding activity and was able to activate ADCC through the GD2 mAb. In addition, GD2-RLI proved to have superior activity compared to combination of RLI and anti-GD2 in mouse syngeneic tumor models.

To further improve on IL-15-based immunotherapy, a tri-functional antibody fusion protein comprising an antibody fused to RLI and the extracellular domain of 4-1BBL was generated (Kermer et al. 2014). This immunocytokine reduced metastasis formation more effectively than corresponding tumor-targeted RLI and 4-1BBL fusion proteins in a murine melanoma model. More recently, IL-15 was incorporated into the so-called BiKE format, yielding a so-called trispecific killer engager (TriKE) molecule comprised of a CD16 scFv to recruit NK cells, IL-15 to activate NK cells, and a CD33 scFv to target AML (Vallera et al. 2016). This CD33-targeted TriKE was more effective than an analogous anti-CD16/anti-CD33 BiKE in activating NK cell-mediated ADCC towards primary AML cells. Importantly, the CD33-targeted TriKE also triggered the proliferation of NK cells in post haematopoietic stem cell transplant (HSCT) samples, which may help overcome the

reduced NK cell functionality after HSCT (Foley et al. 2011).
An additional interleukin of particular interest is IL-21, which proved to be well tolerated in patients in early clinical trials, with evidence of clinical activity including a complete response in MM and four partial responses in RCC (Thompson et al. 2008). Interestingly, long-term disease-free survival upon trastuzumab treatment in breast cancer patients depends on innate and adaptive immune signalling (Park et al. 2010), and was recently found to associate with increased IL-21 receptor (IL-21R) expression (Mittal et al. 2016). In line with this, treatment with recombinant IL-21 and HER2 mAb in mice yielded superior antitumor activity towards primary tumors and metastases (Mittal et al. 2016), with expression of IL-21R on CD8 T cells being important for HER2 mAb treatment efficacy. In line with this, combination treatment of indolent B cell lymphomas with rituximab and recombinant IL-21 yielded clinical activity in 42% of patients, with some responses being more durable than that of previous rituximab treatment (Timmerman et al. 2012). Thus, combination strategies of therapeutic mAbs with IL-21 or inclusion of this cytokine into an immunocytokine strategy appear promising. In this respect, a CD20-targeted IL-21 fusion protein possessed enhanced antitumor efficacy compared to rituximab or IL-21 combinations *in vitro* and may thus yield synergistic activity.
Various other interleukins are of interest for incorporation into the immunocytokine format, including IL-12 for which among others fibronectin and CD30-targeted immunocytokines are already in (pre)clinical development (Lo et al. 2007, Heuser et al. 2003, Rudman et al. 2011), and IL-7 which is currently in clinical trials for several cancers including breast cancer (Tredan et al. 2015).

Tumor Necrosis Factor superfamily ligands

The superfamily of Tumor Necrosis Factor (TNF)-related proteins (TNFSF) comprises 27 members mediating multiple regulatory functions in the immune system, ranging from induction of apoptosis in superfluous cells to providing co-stimulatory signalling at various stages of the immune response. All TNFSF-members contain the hallmark TNF homology domain (THD) in the extracellular region and typically exist as non-covalent homotrimeric type II transmembrane proteins (reviewed in Bodmer et al. 2002). However, most of the TNSF members can be processed into a soluble ligand comprising the extracellular domain. TNF ligands bind to cognate members of the TNF receptor (TNFR) superfamily, typically type I transmembrane proteins characterised by extracellular cysteine-rich domains (CRDs).
One of the functions of the TNFSF family is the induction of apoptotic cell death in target cells, which is governed by a subgroup of TNFSF members termed the Death Inducing Ligands (reviewed in Walczak et al. 2013). This subgroup consists of TNF, FasL and TRAIL and activates the extrinsic pathway of apoptosis through cognate TNFRs called Death Receptors (DRs). A second pivotal function of the TNFSF is the temporally coordinated induction of co-stimulatory signals that drive various stages of the adaptive immune response. Here, binding of a TNFSF to a cognate TNFR induces the recruitment of so-called TNF receptor associated factors (TRAFs) that subsequently trigger a multitude of pro-inflammatory signalling pathways, such as NFκB, PI3K, and PKB.

Membrane TNFSF ligands effectively trigger activation of cognate receptors (Figure 6A). In contrast, soluble TNFSF ligands typically bind their cognate receptor(s), but are less effective in activation of downstream receptor signalling (Figure 6A). Importantly, as demonstrated using recombinant affinity tagged sTNFSF members, this soluble and inactive TNFSF ligand can gain full signalling activity by secondary cross-linking, e.g. with tag antibodies. Similarly, agonistic TNF receptor antibodies can directly bind to and cross-link TNF receptors, which typically required FcR-mediated (cellular) cross-linking for effective downstream signaling (Figure 6A). This differential activity profile of soluble vs. membrane TNFSF can be exploited for immunotherapy by genetic fusion of a soluble TNFSF ligand to a high affinity scFv antibody fragment (reviewed in Bremer 2013). In brief, high affinity binding of the scFv to its target antigen leads to display of the TNFSF on the cell membrane. This 'membrane-like' TNFSF can now provide the requisite cross-linking to achieve optimal activation of the cognate TNFRSF. Importantly, the soluble scFv:TNFSF ligand is relatively inactive in solution while 'en route', thus reducing the chance of systemic toxicity (Figure 6B). This concept has been applied to antibody-based targeting of a variety of TNF ligands, including FasL and co-stimulatory TNFSF ligands, like CD40L and 4-1BBL (not included here), and TRAIL as discussed below.

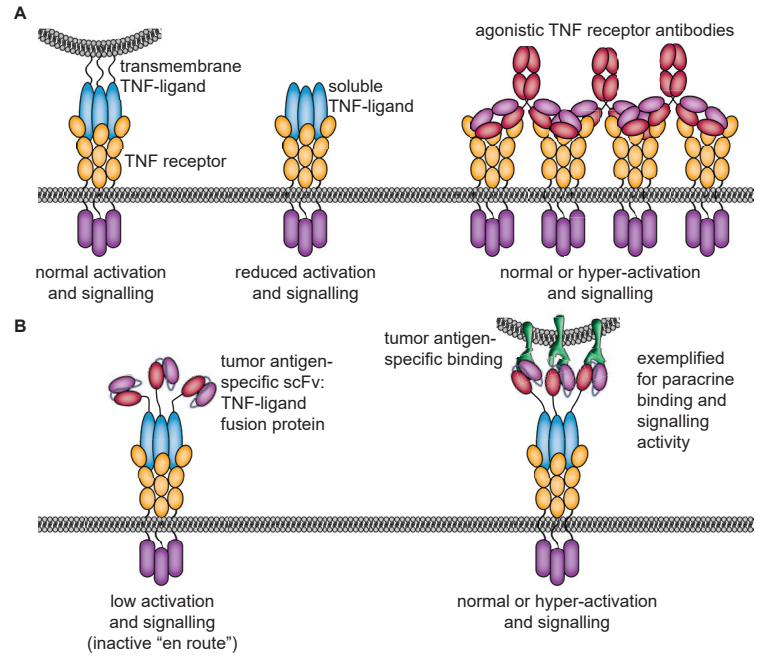

Figure 6: Antibody-based targeting of TNF superfamily members. **A)** Binding of TNF ligands to their receptors induces receptor cross-linking, which triggers potent death signalling. In contrast, soluble TNF ligands typically bind their cognate receptor(s), but are less effective in activation of downstream receptor signalling. However, agonistic TNF receptor antibodies do induce sufficient cross-linking of the TNF receptors inducing normal or hyper-activation of death signalling. **B)** TNF-ligand fusion proteins comprising a tumor specific scFv genetically fused to a TNF ligand show low activity en route, as binding of the TNF ligand only is insufficient for triggering receptor cross-linking and activation. After binding of the scFv to its recognising antigen expressed on tumor cells, the soluble TNF ligand is converted into membrane bound TNF. Thereby, the TNF receptor can be efficiently be cross-linked, leading to normal or hyper-activation and signalling.

TNF-related apoptosis inducing ligand (TRAIL)

TNF-related apoptosis inducing ligand (TRAIL) is an important immune effector molecule, among others involved in peripheral tolerance, NK and T cell cytotoxicity and immunosurveillance of circulating cancer cells (Kayagaki et al. 1999, Martinez-Lorenzo et al. 1998). TRAIL is unique among the Death Inducing Ligand subgroup of TNFSF as it selectively induces apoptosis in cancer cells, with no to minimal activity towards healthy normal cells. TRAIL induces caspase-8 mediated apoptosis via activation of its agonistic receptors TRAIL-R1 and TRAIL-R2. TRAIL-R2 has a higher affinity for TRAIL, but cannot be effectively activated by soluble TRAIL as it requires secondary cross-linking (Wajant et al. 2001). In contrast, TRAIL-R1 is efficiently activated by soluble TRAIL (Wajant et al. 2001). TRAIL additionally interacts with antagonistic receptors TRAIL-R3 and TRAIL-R4, which are believed to function as decoy receptors, and the soluble receptor osteoprotegrin (Almasan et al. 2003). TRAIL-R1 and TRAIL-R2 are highly expressed in many malignancies (Strater et al. 2002, Spierings et al. 2003, Kurbanov et al. 2005), which combined with the apparent tumor-selective activity of sTRAIL makes this regulatory axis an attractive target for antibody-based cancer therapy.

In line with the lack of toxicity in preclinical studies, dulanermin, a recombinant form of TRAIL, was well tolerated in patients in phase I/II clinical trials with no dose-limiting toxicity (reviewed in Fox et al. 2010). In a multicentre clinical trial, dulanermin induced partial responses and stable disease at higher doses of 8 mg/kg and yielded 2 complete and 1 partial response in Non-Hodgkin Lymphoma patients when combined with antibody rituximab (Ashkenazi,A. 2008a & 2008b). The latter is in agreement with the synergy observed upon *in vitro* treatment with rituximab and TRAIL (Daniel et al. 2007). Unfortunately, dulanermin lacked activity in a phase II trial in non-small cell lung cancer patients and is not being further developed at the moment (Soria et al. 2011). This disappointing outcome may among others be due to instability and rapid clearance (Kelley et al. 2001, Herbst et al. 2010), as well as intrinsic and acquired resistance to TRAIL as e.g. observed after treatment with suboptimal doses of an TRAIL-R2 antibody (Li et al. 2006). Several strategies have been developed to increase the therapeutic potential of TRAIL-based drugs (reviewed in de Miguel et al. 2016). These include the use of zipper motifs (Ganten et al. 2006) and oligomerization domains (Berg et al. 2007), whereby the trimeric conformation is stabilised, as well as covalent linkage to polyethylene glycol (PEG) (Chae et al. 2010). Other recent strategies include immobilizing TRAIL onto the surface of nanoparticles or encapsulation of TRAIL inside nanoparticles (de Miguel et al. 2015, Kim et al. 2011). To increase tumor-selectivity, nanoparticles can be further equipped with scFv antibody fragments (Bae et al. 2012, Seifert et al. 2014). Furthermore, nanoparticles containing drugs that synergise with TRAIL, such as doxorubicin, greatly enhanced therapeutic efficacy in mouse models in the absence of systemic toxicity (Jiang et al. 2011). Interestingly, combined treatment of cancer cells with recombinant TRAIL and TRAIL-R2 agonist also yielded a strong synergistic effect comparable to the effect of isoleucine zipper-TRAIL in preclinical studies (Tuthill et al. 2015), highlighting a non-redundant role for different TRAIL-R agonists.

TRAIL has also been used by various groups as effector moiety for an immunocytokine

approach, typically by fusion of sTRAIL to a high affinity scFv antibody fragment or peptides that selectively deliver TRAIL to the tumor cell surface. Such fusion proteins convert soluble TRAIL into membrane-bound TRAIL via high affinity and tumor selective binding of the antibody fragment, inducing tumor-selective apoptosis via activation of both TRAIL-R1 and TRAIL-R2 (Wajant et al. 2001, de Bruyn et al. 2013). Of note, upon target antigen binding to a cancer cell, such an scFv:TRAIL fusion protein can also trigger apoptosis in neighbouring target antigen-negative cells via the so-called bystander effect, thereby potentially reducing the risk of escape of target-antigen negative cancer cells (Bremer et al. 2004).

Importantly, rational choice for an antibody fragment with an intrinsic tumoricidal signalling activity can be used to optimise the therapeutic effect of scFv:TRAIL fusion proteins. For instance, a blocking EGFR antibody fragment can trigger inhibition of EGFR pro-mitogenic signalling and simultaneously activate TRAIL-mediated apoptosis (Bremer et al. 2005, Bremer et al. 2008, Bremer et al. 2008), leading to prominent *in vitro* and *in vivo* antitumor activity. Similarly, an MCSP-blocking antibody fragment can deliver pro-apoptotic TRAIL while simultaneously inhibiting pro-metastatic MCSP signalling (de Bruyn et al. 2010). Furthermore, inhibition of CD47/SIRPα don't eat me signalling using a CD47-blocking antibody fragment triggers CD47-selective apoptosis while simultaneously optimising phagocytic removal of cancer cells (Wiersma et al. 2014). In addition, scFv-mediated targeting of TRAIL to TNFRs overexpressed on cancer cells, such as CD70 and CD40, induced CD70- and CD40-selective apoptosis in malignant cells (Trebing et al. 2014, El-Mesery et al. 2013). Although not investigated, targeting of these immunostimulatory receptors may also yield immunomodulatory effects on antitumor immune responses.

In order to further increase the activity and stability of scFv:TRAIL fusion proteins, highly stable single polypeptide chain TRAIL variants (scTRAIL) have been generated. In scTRAIL, three monomers have been genetically fused, which upon production leads to formation of a single polypeptide TRAIL trimer (Schneider et al. 2010). Such scTRAIL constructs have also been used in scFv-based fusion proteins, e.g. by targeting the EGFR, and have potent tumoricidal activity. An important distinction between the scFv:scTRAIL and scFv:TRAIL format is the presence of a single targeting scFv vs. three targeting scFvs, which in case of the latter may yield an increase in avidity. In addition, fusion proteins that dimerize TRAIL trimers have been developed using diabodies (Siegemund et al. 2012) and Fc-mediated dimerization (Gieffers et al. 2013, Seifert et al. 2014), leading to optimal cross-linking and activation of TRAIL-receptor signalling. Additional efforts to further increase the tumor-selective activity of TRAIL include the use of immunoglobulin E (IgE) heavy-chain domain 2 (EHD2) to covalently link and homodimerize scTRAIL with an EGFR scFv. The concomitant tetravalent scTRAIL molecule had increased thermal stability, solubility and antitumor activity compared to non-targeted EHD2-scTRAIL (Siegemund et al. 2016).

Of note, scFv-based targeting of TRAIL can also be used to selectively target immune effector cells to enhance their tumoricidal activity. Delivery of TRAIL to CD3 or CD7 on T cells for instance enhanced the cytotoxic activity of T cells >500-fold *in vitro* and

increased survival ~6-fold *in vivo* (de Bruyn et al. 2011). Of note, targeted delivery of TRAIL to CD3 also triggered additional T cell activation, thereby yielding a dual signalling effect of scFvCD3:TRAIL. In a similar fashion, granulocytes were armed with CLL1-targeted TRAIL, which enhanced both the induction of TRAIL-mediated apoptosis in CLL-1 positive cancer cells and augmented induction of ADCP by therapeutic antibodies such as rituximab (Wiersma et al. 2015).

Recently, we generated another immunomodulatory scFv:TRAIL fusion protein by using an scFv antibody fragment targeting PD-L1 (Hendriks et al. 2016, Chapter 4 this thesis). This PD-L1:TRAIL fusion protein directly triggered TRAIL-mediated apoptosis in PD-L1$^+$ tumor cells, but also armed PD-L1-expressing myeloid cells, such as DCs and macrophages. Hereby, PD-L1:TRAIL converted possibly immunosuppressive myeloid cells into pro-apoptotic tumoricidal platforms. PD-L1:TRAIL additionally inhibited PD-1/PD-L1 interaction and thereby augmented the anticancer activity of T cells. An interesting feature of PD-L1:TRAIL is the fact that by virtue of PD-L1/PD-1 inhibition, T cell secretion of IFN-γ secretion is augmented, which on tumor cells also increased expression of PD-L1, a well-established effect of IFN-γ (Dong et al. 2002). Further, IFN-γ sensitised tumor cells to TRAIL-mediated apoptosis, another well-established effect of IFN-γ (Langaas et al. 2001). Thus, PD-L1:TRAIL treatment essentially creates a feed-forward loop, with increased target antigen and increased sensitivity to apoptotic elimination.

Although TRAIL fusion proteins have not yet been tested in clinical trials, a favourable toxicity profile of such fusion proteins is anticipated, with optimal target antigen-restricted activation of apoptosis, and an improved serum half-life due to the increase in molecular weight compared to soluble TRAIL. Furthermore, combinatorial regimes with chemotherapeutic drugs that sensitise cancer cells to TRAIL-mediated apoptosis further increase their potential. In this respect, combination of bortezomib and an EGFR-targeted scTRAIL fusion protein had promising tumor-selective activity in hepatocellular carcinoma, with no apparent hepatotoxicity (Wahl et al. 2013). Similarly, combination of CD33-targeted TRAIL with histone deacytelase inhibitors also synergistically induced apoptosis (ten Cate et al. 2009).

In conclusion, antibody-cytokines can solve many of the issues frustrating the application of non-targeted cytokines in cancer therapy, including severe systemic toxicity and short serum half-life. Correspondingly, some immunocytokines have already shown promising activity in clinical trials. Unlike antibody-drug-conjugates (reviewed in Polakis 2016), antibody-cytokines do not require target antigen-mediated internalisation for their efficacy. Therefore, the repertoire of target antigens to choose from includes tumor microenvironmental as well as immune effector cell antigens. The choice for a particular target antigen will depend on both the intended target cell and the anticipated antitumor effect, i.e. direct antitumor activity vs. induction of antitumor immunity. Further, depending on target and cytokine, immunocytokines may bridge innate and adaptive immunity and may combine direct antitumor activity with activation of adaptive immunity, as e.g. illustrated for the PD-L1:TRAIL immunocytokine.

CAR-transfected T cells

An additional exciting development in the field of antibody-based therapy are so-called Chimeric Antigen Receptor (CAR)-modified T cells. A CAR is comprised of an extracellular scFv domain, a transmembrane domain, and intracellular T cell co-stimulatory domains. CAR-modified T cells are generated by transducing isolated peripheral blood T cells from a patient with the CAR construct. Hereby, the entire T cell repertoire is redirected to cancer cells (Figure 7A). Upon binding to the specific tumor-associated antigen, the CAR T cells proliferate and mount an immune response, leading to killing of the tumor cells. Thus, CAR T cells harness the power of both antibody-mediated target antigen recognition and selective delivery of the full cytotoxic T cell armament. The currently used and clinically active CARs are of the so-called second generation and comprise an extracellular scFv antibody fragment, a transmembrane domain, the CD3ζ chain, and either the CD28 or 4-1BB co-stimulatory signalling domain (Figure 7A) (Maude et al. 2014, Kochenderfer et al. 2015). Which of these co-stimulatory signalling domains is superior, and whether further engineering of co-stimulatory domain composition can increase activity, remains to be determined. In this respect, third generation CARs containing CD28 and 4-1BB or OX40 co-stimulatory domains are being evaluated (pre)clinically (Pule et al. 2005).

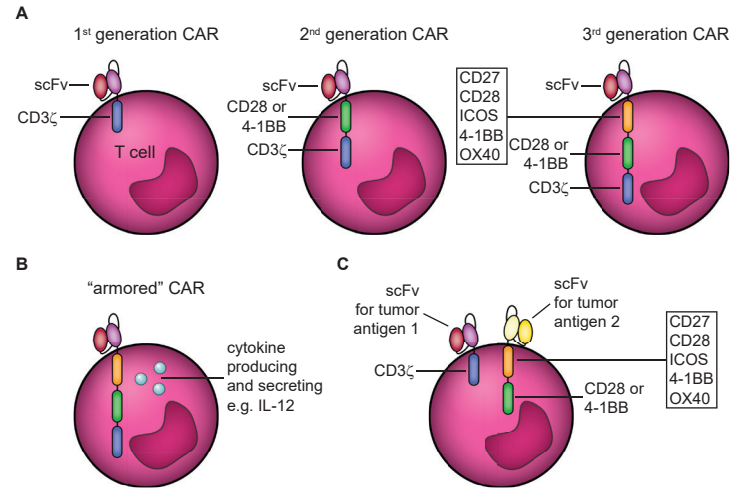

Figure 7: Chimeric antibody receptor (CAR) T cells. **A)** Chimeric antigen-receptors (CAR) T cells comprise a tumor specific scFv to redirect the T cells to target antigens expressed on tumor cells. First generation CARs only contained such a scFv fused to the CD3-zeta transmembrane and endodomain. However, second generation CAR T cells that are currently being evaluated in the clinic are CARs that also comprise a co-stimulatory signalling domain like CD28 or 4-1BB. Third generation CAR T cells contain co-stimulatory molecules two instead of one, which will provide the CAR with additional activation and survival signalling. **B)** The tumor micro environment is generally immune silencing, which may hamper the efficacy of CAR T cells. To overcome this problem, additionally "armed" CAR T cells that are engineered to secrete cytokines have been developed. **C)** Targeting of a single tumor antigen with CAR T cells can lead to relapse of target antigen-negative disease. To overcome this problem, CAR T cells with dual specificity (TanCARs) have been developed.

Initial striking successes have been made in the field of B cell malignancies using CARs that recognise B cell marker CD19. In patients with relapsed and refractory B-ALL, treatment with CD19 CAR T cells resulted in a complete response in 27 out of 30 patients (90%) 1 month after infusion, with 19 patients having a sustained remission (Maude et al. 2014). CD19-targeted CAR T cells have also been used in patients with other B cell malignancies, such as relapsed and refractory CLL yielding a 57% response rate with 4 complete and 4 partial responses out of 14 patients (Porter et al. 2015). Additionally, complete remissions were reported in 8 out of 15 patients with DLBCL (Kochenderfer et al. 2015) and a sustained complete response was reported in a patient with advanced MM receiving CD19 CAR T cells together with autologous transplantation (Garfall et al. 2015). Of note, sustained clinical responses have been thought to be associated with persistence of CD19 CAR T cells, although persistence does not necessarily correlate with response in certain trials (Maude et al. 2014, Lee et al. 2015).

Many other target antigens on haematological malignancies are being considered, including a CD30-targeted CAR to treat patients with Hodgkin lymphoma and CD30-positive non-Hodgkin lymphoma (NCT01316146), a CD138-targeted CAR for MM that induced stable disease in four out of five patients (Guo et al. 2016), and AML targeted CARs directed at CD33 and CD123 (NCT01864902, NCT02159495).

Although less clinically advanced, CAR T cells are also being evaluated against many solid tumor targets, including mesothelin, HER2, CEA and EGFR (reviewed in Jackson et al. 2016). Of interest here are CARs directed at EGFRvIII (Johnson et al. 2015a, Morgan et al. 2012), a tumor-specific mutant of the EGFR among others expressed on glioblastoma. EGFRvIII-targeted CARs are being evaluated in clinical trials of glioblastoma and other brain tumors (NCT02209376, NCT01454596). Since EGFRvIII is a tumor-specific antigen, EGFRvIII-targeted CAR T cells are likely to have a good safety profile. Indeed, safety is a particularly important issue for CAR-based therapy of solid cancers such as epithelial malignancies, as many of the targets are overexpressed on cancerous cells but also present on healthy epithelial cells. Indeed, major risks of CAR T cell treatment include on-target/off-tumor activity toward healthy antigen-expression cells (reviewed in Brudno et al. 2016). CAR T cell therapy may also associate with on-target/on-tumor toxicity such as tumor lysis syndrome (TLS) or cytokine release syndrome (CRS), although the latter also appears to correlate with clinical activity for CAR-based therapy (Davila et al. 2014). In line with this, treatment-related deaths have been reported for second generation CD19 CAR T cells containing the CD28 domain and third generation HER2 CAR T cells containing CD28 and 4-1BB co-stimulatory domains (Figure 8A) (Park et al. 2010, Morgan et al. 2010).

Thus, identifying the optimal window for antitumor activity in the absence of immunopathology is critical and inclusion of mechanisms to ensure timely shut-down of CAR T cells are being explored in order to improve safety. Specifically, various types of suicide gene approaches enable timed elimination of CARs. Of particular interest is the inducible caspase-9 (iCasp9) system (Di Stasi et al. 2011), which upon treatment with a caspase-9 inducer drug yields a 90% reduction in CD20 CAR T cell numbers within 12 h (Budde et al. 2013). Various iCasp9-expressing CAR T cells are currently being evaluated, including a GD2-specific iCasp9-expressing CAR (GD2-iCAR) (Gargett et al. 2015) which is currently tested a phase I trial in neuroblastoma patients (NCT01822652).

CAR transduced T cells are, particularly in solid cancers, still subjected to immune inhibitory pathways present in the tumor micro-environment. Indeed, mouse studies with established tumors identified a multifactorial inhibition of infiltrating CAR T cells, due to upregulation of intrinsic T cell inhibitory enzymes, such as diacylglycerol kinase, the expression of inhibitory receptors, such as PD-1, LAG3 and TIM3 (Moon et al. 2014), and immunosuppressive mediators, such as prostaglandin E2 (PGE2) (Newick et al. 2016). These immunosuppressive effects can be abrogated by CAR engineering, whereby therapeutic activity is augmented in preclinical models (Newick et al. 2016). An additional approach to overcome this problem are the so-called armoured CAR T cells that are engineered to secrete cytokines or express ligands that prevent immune silencing, such as secretion of interleukin-12 (IL-12), or co-expression of CD40L or 4-1BBL (reviewed in Yeku et al. 2016) (Figure 7B). Alternatively, CAR T cells can be combined with checkpoint inhibitor antibodies targeting e.g. PD-1, to augment therapeutic efficacy. In this respect, a CAR construct in which extracellular domain of PD1 was fused to the transmembrane and cytoplasmic co-stimulatory CD28 domain has recently been described (Liu et al. 2016). Hereby, PD-L1/PD-1 interaction is converted into a co-stimulatory signal, which enhanced CAR infiltration into the tumor and induced tumor regression. Such PD-1:CD28 CAR T cells were less susceptible to silencing in the tumor micro-environment.

Another important consideration for CAR T cells is that retargeting of patient T cells towards a single tumor target antigen using a high affinity CAR may lead to relapse of target antigen-negative disease, as for instance reported for CD19 CAR therapy (Grupp et al. 2013). Although this seems to be a relatively rare event for CD19, it is an important issue when targeting solid cancers where target antigen expression can be highly variable between disease stage, tumor subtype, and most importantly within lesions of a single patient (Sottoriva et al. 2013, Navin et al. 2011, Thirlwell et al. 2010). Therefore, the targeted delivery of CAR-transfected T cells to a single target antigen is likely to eliminate only a subset of malignant cells. To overcome this issue, a dual specificity CAR, termed tandem CAR (TanCAR) that simultaneously targets CD19 and HER-2 was developed in a proof of concept study (Figure 7C) (Grada et al. 2013). This CAR redirected T cell reactivity to either of the antigens and induced synergistic cytolytic T cell activity upon simultaneous binding to CD19 and HER2. Similarly, a TanCAR directed against HER2 and IL-13Rα2 displayed superior activity upon binding to both antigens and effectively eliminated glioblastoma in a mouse model (Hegde et al. 2016). Alternatively, CARs that recognise universal tags such as fluorescein isothiocyanate (FITC) and biotin have been developed (Tamada et al. 2012, Urbanska et al. 2012). FITC-targeted CAR T cells could be selectively targeted to folate receptor-overexpressing cancers using FITC-labelled folate (Kim et al. 2015b) and could be dual targeted to CD19 and CD22 (Ma et al. 2016), as well as HER2 and CD20 (Tamada et al. 2012). For heterogeneous cancers, simultaneously targeting of two or more antigens with a universal CAR may be crucial to increase the therapeutic efficacy.

Finally, selection of specific T cell populations to transduce with a CAR construct is an

important step to achieve optimal activity with CAR-based therapy (Golubovskaya et al. 2016). For instance, a subset of CD19 CAR-transfected T cells that is characterised by CD8/CD45RA/CCR7 expression, resembling so-called T memory stem cells, is associated with *in vivo* expansion of CD19 CAR T cells in patients. This phenotype could be selectively induced *in vitro*, using cytokines IL-7 and IL-15, and was associated with improved function and survival in pre-clinical CAR T cell infusion models (Xu et al. 2014). In an alternative approach, so-called induced pluripotent stem cell clones (iPSCs) were used to generate CD19-targeted CAR T cells, which yielded potent cytotoxic activity toward $CD19^+$ cancer cells (Themeli et al. 2013). Intriguingly, a recent paper reported the generation of an allogeneic universal CAR T cell formulation, by using the CRISPR/Cas9 system to disrupt multiple genomic loci to yield CAR T cells deficient in endogenous TCR, HLA class I (HLA-I) and PD-1 (Ren et al. 2015). Such allogeneic engineered CAR T cells did not induce GVHD and may thus provide a universal platform for CAR T cells as an alternative to autologous CAR T cells.

In conclusion, a combinatorial approach including selection of specific T cell populations to generate universal and optimally active allogeneic CAR T cells and CAR design to circumvent tumor escape and tumor immune-silencing is anticipated to improve the therapeutic applicability of CAR-based cancer immunotherapy.

Conclusions

Antibody-based therapy has clearly come of age, with all the different approaches described in this review having yielded lead candidates that show promising clinical activity, mostly in trials with heavily pretreated and refractory patients. The continued progress in design, engineering and insights in the tumor-immune interaction will aid further optimisation of antibody-based approaches. One of the important challenges in the upcoming years is to incorporate the advances in antibody-engineering into optimal combinations with standard-of-care treatment such as chemo and/or radiotherapy, to achieve curative treatment. In this respect, it is important to consider that patients treated with high-dose chemotherapy are likely to have only low levels of immune effector cells, which may at least in part explain the limited success of mAb treatment in AML. This also highlights the importance of identification of optimal timing and dosing strategies for each individual therapeutic combination. Importantly, rational choice of chemotherapy may help to augment antitumor immunity, with e.g. doxorubicin inducing so-called immunogenic cell death (ICD) that can induce T cell immune responses (Fucikova et al. 2011). Thus, combining ICD inducers with immunomodulatory antibodies such as CTLA-4 antibody ipilimumab may provide a 'natural' vaccination effect that can (re)educate and steer antitumor immunity (NCT01524991). In addition, anticancer immune responses may be enhanced by depletion of immunosuppressive regulatory T cells. This approach (using CD25-targeted daclizumab) has proven beneficial in the clinic without the development of autoimmunity when applied in parallel with tumor antigen vaccination (Rech et al. 2009 & 2012).

Similarly, combination therapy with immune-modulatory drugs (IMiDs), such as the thalidomide analogue lenalidomide, can enhance the therapeutic effect of tumor-targeting

mAbs, as evidenced by augmented NK cell-mediated ADCC upon combination treatment of lenalidomide with daratumumab (van der Veer et al. 2011). Such synergy can also be achieved by combining tumor-targeting mAbs such as rituximab with immunomodulatory mAbs such as 4-1BB mAb urelumab (Kohrt et al. 2013). Combinations of checkpoint inhibitors and immunostimulatory mAbs are already evaluated in clinical trials and are anticipated to augment anticancer immunity. Furthermore, combinations of CAR T cells with immunomodulatory antibodies and cytokines can help to ensure induction of curative immunity even in the immunosuppressive tumor microenvironment.

In the future, these advances are anticipated to lead to the development of effective new antibody-based therapeutic approaches for a growing number of patients and an expanding range of cancer types.

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CHAPTER 3

Melanoma-directed activation of apoptosis using a novel bispecific antibody directed at MCSP and TRAIL receptor 2/Death Receptor 5

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Abstract

Agonistic anti-DR5 antibodies, alone or in combination with other treatment modalities, show promising activity towards a variety of cancer types, including malignant melanoma. However, the therapeutic efficacy of current anti-DR5 antibodies is potentially limited as they indiscriminately interact with DR5 that is broadly present on normal cells. Therefore, we developed a novel bispecific antibody-based approach that promotes melanoma-directed pro-apoptotic activation of DR5. We engineered a novel recombinant bispecific antibody, designated MCSPxDR5, which combines high binding affinity for the melanoma-associated antigen MCSP with potent agonistic activity towards DR5. The mode of action of MCSPxDR5 involves high-affinity binding to tumor cell surface-expressed MCSP with concomitant locally enhanced cross-linking of DR5. MCSPxDR5 showed potent MCSP-directed pro-apoptotic activity towards MCSP-positive melanoma cells with essentially no or minimal toxicity towards normal cells. The antitumor activity by MCSPxDR5 was enhanced after secondary cross-linking of its IgG domain by either an artificial cross-linker or by Fc receptors present on myeloid immune effector cells. Importantly, MCSPxDR5 potently induced apoptosis in primary patient-derived melanoma cells that was further enhanced after secondary cross-linking of its human IgG1 Fc domain. Taken together, we present a melanoma-directed DR5 agonistic bispecific antibody in which high-affinity binding to MCSP results in melanoma cell-localized activation of pro-apoptotic DR5 signaling. This novel antibody-based approach may provide a new avenue to unlock the therapeutic potential of DR5-targeted cancer therapy, in particular for targeted treatment of melanoma and other MCSP-expressing malignancies.

Introduction

Malignant melanoma is the most lethal type of skin cancer and its incidence continues to increase at an alarming rate.[1] When diagnosed at an early stage, localized malignant melanoma can be cured by radical removal of the lesion, resulting in excellent survival rates. However, once progressed to the metastatic stage, melanoma is extremely difficult to cure.[2] Currently, ipilimumab (CTLA4 blockade), BRAF inhibitors (vemurafenib and dabrafenib) and high-dose IL-2 are used as first line agents for stage IV melanoma. Newly emerging treatment options include antibodies that target the PD-1/PD-L1 checkpoint axis (e.g. nivolumab), oncolytic viruses, adoptive T cell transfer and dendritic cell vaccines.[3]

In this respect, Tumor Necrosis Factor-related apoptosis-inducing ligand (TRAIL) appears to be a promising anticancer agent as it induces apoptosis in a wide range of refractory malignancies including melanoma.[4] TRAIL is an immune effector protein that induces apoptosis in virus-infected cells and cancer cells by activating death receptor-4 (DR4) and/or death receptor-5 (DR5) without deleterious activity towards DR4/DR5-expressing normal cells.[5] Consequently, DR4/DR5 agonists have been regarded as promising anticancer agents. Indeed, treatment with "first-generation" DR4/DR5-targeted therapeutics, such as recombinant human soluble TRAIL (rhTRAIL) and agonistic DR4/DR5 antibodies was well tolerated, but had disappointing clinical efficacy.[4] For instance, in a phase I dose-escalation study in patients with relapsed or refractory carcinoma, the DR5-agonistic antibody tigatuzumab only induced stable disease in selected patients.[6] Similarly, in a phase II trial in non-small cell lung cancer patients, combined treatment with rhTRAIL and chemotherapy had no added benefit compared to chemotherapy treatment alone.[7]

However, advances in the understanding of death receptor signaling revealed that first-generation DR4/DR5 agonists do not fully exploit the unique signaling characteristics of TRAIL receptor-mediated cancer cell death.[4,8] Specifically, DR4 and DR5 have distinct cross-linking requirements for the induction of apoptosis. DR4 is activated upon binding of rhTRAIL (or conventional DR4 antibodies), whereas apoptotic DR5 signaling requires membrane-bound TRAIL or secondarily cross-linked rhTRAIL.[8,9] Indeed, to gain therapeutic activity, agonistic DR5 antibodies such as tigatuzumab appear to require cross-linking by Fc-receptors as present on myeloid effector cells.[10-12] Further, rhTRAIL and conventional agonistic DR4/5 antibodies have no tumor-selective binding activity, since TRAIL receptors are ubiquitously expressed on normal tissue.[13] Consequently, a massive target antigen sink formed by DR5 expressed on normal cells may hamper the efficacy of agonistic anti-DR5 antibodies.

To overcome these limitations, we engineered a recombinant bispecific antibody, designated MCSPxDR5, with high binding affinity for Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP) and potent agonistic activity towards DR5, complemented with a human IgG1 Fc domain. MCSP is highly overexpressed on the cell surface of over 90% of cutaneous and uveal melanomas and is a well-established target for melanoma immunotherapy. MCSP expression in normal tissues is largely restricted to cells of the melano-

cyte lineage.[14-15] MCSPxDR5 was designed to selectively bind with high affinity to tumor cell surface-expressed MCSP and concomitantly trigger localized activation of apoptosis by DR5 cross-linking.

Material and Methods

Antibodies and reagents

mAb 9.2.27, a high-affinity murine IgG2a antibody directed against an extracellular epitope on MCSP was purchased from Abcam. Anti-DR5 antibody DJR2-4 was purchased from Enzo Life Sciences. Recombinant TRAILR2:Fc protein was from ALEXIS. Goat-anti-human IgG (Southern Biotech); goat anti-human IgG-PE (Santa Cruz biotechnology); mouse anti-human IgG-488 and mouse anti-human IgG-647 (ImmunoTools,) and FITC-conjugated goat anti-mouse (Invitrogen) were used for flow cytometry. BRAF inhibitor vemurafenib (APExBio) and caspase inhibitor zVAD-fmk (Calbiochem) were dissolved at 10 mM in DMSO. Histone deacetylase inhibitor valproic acid (VPA) (Sigma-Aldrich) was dissolved at 2.5 mM in DMSO. The proteasome inhibitor Bortozomib (Millennium Pharmaceuticals) was dissolved at 10 mM in DMSO; Vybrant DiD cell-labeling reagent (Invitrogen); Easy-Titer Human (gamma chain) kit (Thermo Scientific), were used according to the manufacturer's recommendations.

Cell lines

Human cell lines A375M, MM-RU, SK-MEL-28 ($MCSP^{pos}$ cutaneous melanomas); DLD-1, HCT 116 (colorectal adenocarcinomas) and Jurkat (T-ALL leukemia) were purchased from American Tissue Culture Collection (ATCC, Rockville, MD). Human $MCSP^{pos}$ uveal melanoma cell line OCM3 was kindly provided by Prof. G.P.M. Luyten, Department of Ophthalmology, LUMC, Leiden, Netherlands. The $MCSP^{neg}$ human melanoma parental cell line M14 and the $MCSP^{pos}$ transfectant cell line M14.MCSP were kindly provided by Prof. James McCarthy, University of Minnesota, Minneapolis, USA. Cell line HEK293.CD64 stably expressing human CD64 (high-affinity IgG Fc-receptor) was generated by transfecting parental HEK293 cells with pCMV/hygroCD64 (Sino Biological Inc). HEK293.CD64 cells were cultured in DMEM plus 10% dialyzed fetal calf serum (dFCS), supplemented with 500 µg/ml Hygromycin B (Life technologies). Human cells were cultured in either RPMI 1640 or DMEM culture medium with 10% FCS at 37°C in a humidified 5% CO_2 atmosphere. Cells lines were authenticated by commercial Short Tandem Repeat analysis and routinely tested for mycoplasma infection.

Primary patient-derived melanoma cells and primary human hepatocytes (PHH)

Tumor samples were collected during conventional surgical resection procedure after informed consent. Tumor samples were minced and cultured in RPMI 1640 supplemented with 10% FCS and allowed to adhere to culture flasks. Non-adherent cells were discarded and phenotyping of remaining adherent cells was performed by flow cytometry using antibodies directed against CD14, DR5 and MCSP, respectively. Primary patient-derived melanoma cells used in this study were CD14 negative, DR5 and MCSP positive and were used before passage 4.

Cryopreserved human hepatocytes were purchased from Tebu-bio bv. Hepatocytes were cultured in a 48 well plate to a density of $2.5x10^4$ cells per well before use.

Isolation of white blood cells and lymphocytes

White blood cells (WBCs) were obtained from venous blood from healthy volunteers after informed written consent. Briefly, blood was diluted ten times in cold isotonic ammonium chloride lysis buffer and incubated at 4°C for 10-15 min until red blood cells had lysed. Leukocytes were harvested by centrifugation (1000g, 10 min), washed with PBS and re-suspended in RPMI 1640/10% FCS.
Peripheral blood lymphocytes (PBLs) were obtained from venous blood of healthy volunteers after informed written consent using standard density gradient centrifugation (Lymphoprep).

Analysis of cell surface expression of DR5 and MCSP

Cell surface expression levels of DR5 and MCSP were assessed by flow cytometry. In short, cells were harvested and washed with PBS and re-suspended at 1×10^5 cells in 200 µl fresh culture medium containing anti-DR5 mAb (DJR2-4) or mAb 9.2.27 according to the manufacturer's recommendations. Specific binding of these antibodies was detected using secondary conjugated antibodies (FITC-conjugated for DR5 staining and APC-conjugated for MCSP staining). All antibody incubations were carried out for 45 min at 0°C followed by 3 washes with PBS.

Construction of MCSPxDR5

Antibody fragment scFvMCSP was constructed using published VH and VL sequence data of mAb 9.2.27 by applying standard antibody engineering technologies. Similarly, scFvDR5 was constructed using published VH and VL sequence data of agonistic anti-DR5 antibody tigatuzumab. For construction and production of MCSPxDR5 we used eukaryotic expression plasmid pEE14-bsAb, which is equipped with an enhanced CMV promoter to drive recombinant protein expression and an N-terminal leader peptide for excretion of MCSPxDR5 protein in the culture medium. The pEE14-bsAb contains 3 consecutive multiple cloning sites (MCS#1, MCS#2 and MCS#3, respectively). MCS#1 and MCS#2 are interspersed by a 22 amino acid flexible linker derived from a CH1 domain. MCS#1 was used for directional and in-frame insertion of the scFvDR5 encoding DNA fragment. MCS#2 was used for directional and in-frame insertion of the scFvMCSP encoding DNA fragment. MCS#3 of pEE14-bsAb was used for in-frame insertion of DNA fragments encoding either a human IgG1 or IgG4 domain. Of note, hinge regions and flexible spacers were inserted between the different antigen recognizing parts of the bispecific antibody (bsAb) to allow each part of the molecule to function independently. Moreover, the codon usage of the various protein encoding DNA sequences mentioned above were optimized for expression in human (HEK) and hamster (CHO) production cell lines and were ordered from a certified gene synthesis services (Genscript). This procedure yielded plasmids pEE14-MCSPxDR5-IgG1 and the corresponding IgG4 isotype variant pEE14-MCSPxDR5-IgG4.

Production of MCSPxDR5 in CHO cells

Plasmids pEE14-MCSPxDR5-IgG1 and pEE14-MCSPxDR5-IgG4 were stably transfected in CHO production cells using the FuGene-HD reagent (Promega), after which clones with amplified recombinant protein production (10 µg/ml and 7 µg/ml for MCSPxDR5-IgG1 and MCSPxDR5-IgG4, respectively) were selected by the glutamine synthetase selection method as described previously.[16]

Concentrations of MCSPxDR5 and MCSPxDR5-IgG4 were determined using the Human Easy-Titer kit (Thermo Scientific) according the manufacturer's recommendations.

MCSP-selective binding of MCSPxDR5

MCSP-selective binding of MCSPxDR5 was assessed by flow cytometry using CHO.MCSP cells and MCSP-negative parental CHO cells. In short, cells were incubated with MCSPxDR5 (1 µg/ml) in the presence or absence of an excess amount of the competing parental anti-MCSP antibody mAb 9.2.27 (10 µg/ml). Detection of cell surface-bound MCSPxDR5 was analyzed by flow cytometry using anti-human IgG1-PE.

MCSP-directed induction of apoptosis by MCSPxDR5

A panel of $MCSP^{pos}$ and $MCSP^{neg}$ tumor cell lines was treated with MCSPxDR5. Briefly, tumor cells were seeded in 48-well plates at a concentration of $2x10^4$ cells/well and treated with MCSPxDR5 (1 µg/ml) overnight, after which apoptosis was assessed using Annexin-V staining according to manufacturer's instructions (Immunotools). Briefly, cells were washed once with cold binding buffer (140 mmol/L NaCl, 2.5mmol/L $CaCl_2$ and 10 mmol/L HEPES, pH 7.4) and resuspended in binding buffer containing Annexin-V-FITC. After incubation for 10-15 min at 4°C, apoptosis was assessed by flow cytometry. Alternatively, tumor cells were treated with MCSPxDR5 (2.5 µg/ml) on ice for 40 min, after which unbound MCSPxDR5 was removed by washing twice with cold PBS. After overnight incubation, apoptosis was assessed using Annexin-V staining. Finally, where indicated, tumor cell lines were treated with MCSPxDR5 for 24-72h, after which cell viability was assessed using an MTS-based calorimetric assay (CellTiter 96 AQueous One Solution Cell Proliferation, Promega) at 490 nM using a Victor V3 multi-label plate counter (Perkin Elmer). Absorbance of maximum death (treatment with 70% ethanol for 15 min) was subtracted from all values, after which cell viability was calculated as percentage of medium control.

To determine toxicity of MCSPxDR5 to normal cells, human hepatocytes, lymphocytes and HUVECs were treated with MCSPxDR5 (2.5 µg/ml) overnight, after which apoptosis was assessed using Annexin-V staining.

Assessment of synergy between MCSPxDR5 and clinical antitumor drugs

$MCSP^{pos}/DR5^{pos}$ melanoma cell lines (A375M, MM-RU and OCM-3) were treated with MCSPxDR5 (0.25 µg/ml) in the presence or absence of proteasome inhibitor velcade (1 nM), HDAC inhibitor VPA (2.5 µM) or BRAF-inhibitor vemurafenib (10 µM). After 18h, apoptosis was assessed using Annexin-V staining. Briefly, cells were washed once with cold binding buffer (140 mmol/L NaCl, 2.5mmol/L $CaCl_2$ and 10 mmol/L HEPES, pH 7.4)

and resuspended in binding buffer containing Annexin-V-FITC. After incubation for 10-15 min at 4°C, apoptosis was assesed by flow cytometry. Synergy was determined using the cooperativity index (CI), in which the sum of apoptosis induced by single-agent treatment is divided by apoptosis induced by combination-treatment. When CI<1, treatment was termed synergistic.

Colony forming assay

In short, a single-cell suspension of tumor cells was prepared in 0.6% low gelling temperature (LGT) agarose dissolved in pre-warmed complete medium (RPMI 1640 with 20 % FCS). Tumor cells ($1.0x10^4$ cells per well) were placed in 24-well plates containing a bottom layer of solidified 4% LGT agarose in complete medium. Once the tumor cell-containing agarose top layer had solidified, a final 0.5 ml of complete medium was added. In this assay, $MCSP^{pos}$ melanoma cells A375M, SK-MEL-28, MM-RU and HT1080 were treated with MCSPxDR5 (final concentration up to 250 ng/ml) for 14 days, after which colony formation was evaluated by light microscopy. In control experiments, identical treatment was performed in the presence of an excess amount of TRAIL-R2-Fc (5 µg/ml) or the pan-caspase inhibitor zVAD-fmk (10 µM). Colony forming assays were performed in quadruplicates. The number and size of colonies were quantified using image processing software (ImageJ). The percentage of colony formation was calculated according to the formula: percentage of colonies formed = (number of colonies in experimental condition) / (number of colonies in medium control) x 100%.

siRNA-mediated knockdown of DR5 expression

SK-MEL-28 cells were pre-seeded at a concentration of $1x10^5$ cells/well in 12-wells plates, 24h before treatment with either DR5-selective siRNA or scrambled siRNA according to manufacturer's recommendations (DharmaFECT 2 transfection reagent (Thermo Scientific)). siRNA knockdown DR5 protein expression was evaluated after 72h by flow cytometry using anti-DR5 antibodies. Antibody incubations were carried out for 45 min at 0°C and were followed by 3 washes with PBS. Subsequently, DR5-silenced SK-MEL-28 cells were seeded and subjected to treatment as indicated.

Assessment of MCSPxDR5 antitumor activity upon cross-linking

The following assays were used to assess whether MCSP-directed pro-apoptotic activity of MCSPxDR5 towards cancer cell lines and primary patient-derived melanoma cells could be further enhanced by secondary cross-linking of its IgG1 Fc domain.

Artificial cross-linking: In short, cancer cells were seeded at $3x10^4$/well in 96-well micro-culture plates and subsequently treated with increasing doses of MCSPxDR5 in the presence or absence of an Fc cross-linker (0.5 µg/ml of goat-anti-human IgG), hereafter indicated as CL. Apoptosis and cell viability were evaluated after 24h and 72h, respectively. To investigate whether the observed apoptotic activity of MCSPxDR5 was dependent on DR5 signaling, MM-RU cells ($3x10^4$/well) were co-treated with MCSPxDR5 and CL in the presence or absence of recombinant TRAILR2-Fc (5 µg/ml) or pan-caspase inhibitor zVADfmk (5 µM) for 24h, after which cell viability was assessed by MTS. Additionally,

we used MCSP^{pos} SK-MEL-28 melanoma cells in which DR5 expression was knocked-down using DR5-specific siRNA silencing technology as described previously.[17] Subsequently, DR5-silenced SK-MEL-28 cells and wt SK-MEL28 cells were treated with MCSPxDR5 (1 µg/ml) or anti-TRAILR2 monoclonal antibody HGT-ETR2 (1 µg/ml), after which apoptosis was assessed using Annexin-V staining.

Cell-based cross-linking: To mimic cell-based Fc cross-linking of MCSPxDR5, we used HEK293.CD64 cells ectopically expressing human CD64, also known as the high-affinity IgG receptor FcγRI. In short, DiD-labeled MM-RU cells were co-cultured with HEK293. CD64 cells at a cellular ratio of 5:1 in the presence or absence of MCSPxDR5 (250 ng/ml). After 24h, apoptosis in DiD-labeled MM-RU cells was evaluated. Analogously, various effector cells (PBLs, leukocytes (WBCs) or NK cells) were used as natural cell-based Fc cross-linkers[18] of MCSPxDR5. In short, DiD-labeled MM-RU target cells were treated with MCSPxDR5 in the presence of various ratios of myeloid effector cells (E:T cell ratios were 20:1, 5:1 and 20:1 for PBLs, WBCs and NK cells, respectively). To discriminate ADCC from DR5-cross-linking antitumor activity we used MCSPxDR5-IgG4, a human IgG4-isotype variant of MCSPxDR5 that has only marginal ADCC activity.[19]

Statistical analysis

The results reported in this study are mean values ± standard deviation of the mean of at least three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer post-test or by two-sided unpaired Student's t-test. $p<0.05$ was defined as a statistically significant difference.

Results

MCSPxDR5 has MCSP-directed pro-apoptotic activity

BsAb MCSPxDR5 (Fig. 1A) was designed to bind to MCSP on cancer cells and subsequently induce apoptosis in targeted cancer cells by local ligation of DR5. In line with this, flow cytometric analysis showed that MCSPxDR5 strongly bound to MCSP^{pos}/DR5^{pos} SK-MEL-28 melanoma cells (Fig. 1B), but not to MCSP^{neg}/DR5^{pos} DLD-1 carcinoma cells (Fig. 1C). Binding of MCSPxDR5 to SK-MEL-28 cells was blocked by co-incubation with excess amounts of epitope-competing anti-MCSP mAb 9.9.27 (Fig. 1B), indicating that MCSPxDR5 indeed selectively binds to cell surface-expressed MCSP. Correspondingly, treatment of tumor cells incubated with MCSPxDR5 for 40 min at 4°C, after which any unbound antibody was removed by washing, selectively induced apoptosis in a panel of MCSP^{pos}/DR5^{pos} melanoma cells, but not in MCSP^{neg}/DR5^{pos} HCT116 carcinoma cells (Fig. 1D). Treatment in the continued presence of MCSPxDR5 (no washing) induced apoptosis in both MCSP^{pos}/DR5^{pos} and MCSP^{neg}/DR5^{pos} cancer cells (Fig. 1E), albeit in the latter cancer cells at much lower levels. Moreover, MCSPxDR5 treatment was associated with reduced tumor cell viability in MCSP^{pos}/DR5^{pos} MM-RU, SK-MEL-28 and OCM3 cells, but not in MCSP^{pos}/DR5^{neg} DLD-1 cells (Fig. 1F).

MCSP-selective pro-apoptotic activity of MCSPxDR5 was also evaluated using primary patient-derived melanoma cells that expressed MCSP and DR5 (Fig. 1G). These primary tumor cells were sensitive to MCSPxDR5 treatment, with an >40% increase in apoptosis

compared to medium control (Fig. 1H). Thus, MCSPxDR5 selectively binds to MCSP and has MCSP-directed pro-apoptotic activity towards MCSP^{pos}/DR5^{pos} cancer cell lines and primary patient-derived melanoma cells.

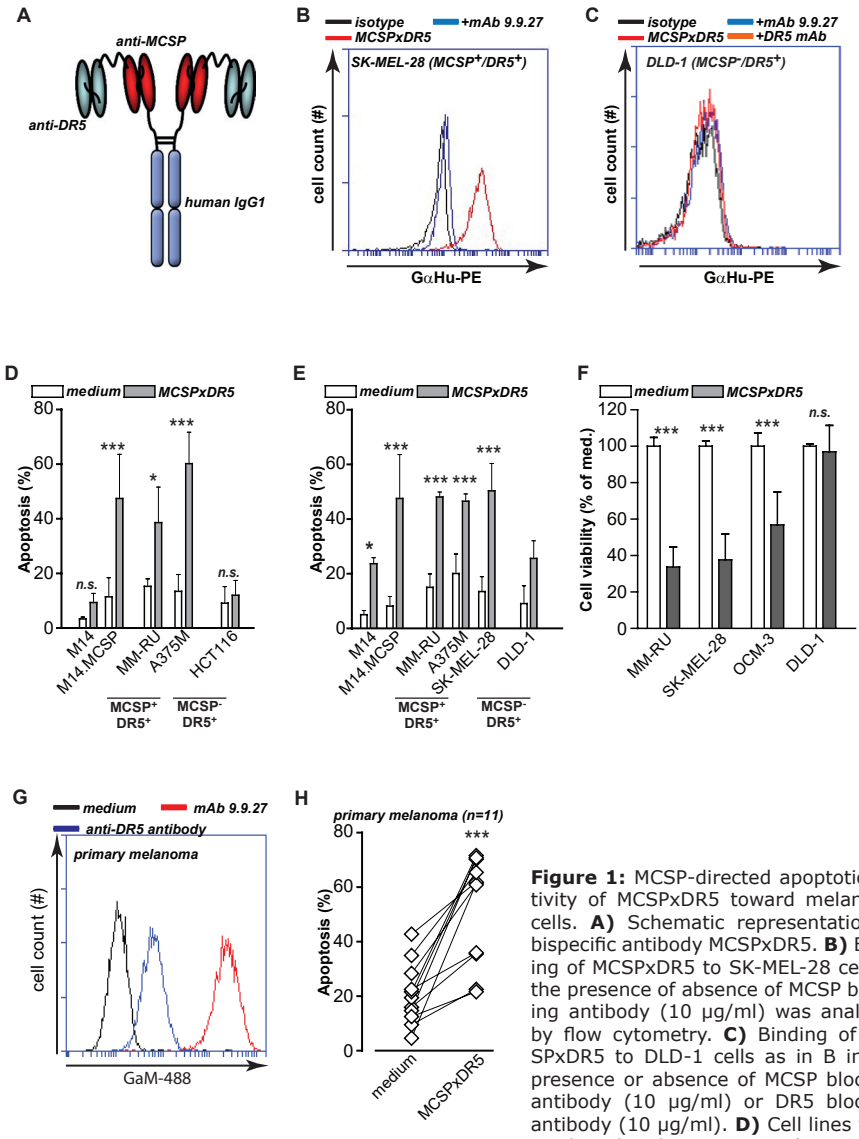

Figure 1: MCSP-directed apoptotic activity of MCSPxDR5 toward melanoma cells. **A)** Schematic representation of bispecific antibody MCSPxDR5. **B)** Binding of MCSPxDR5 to SK-MEL-28 cells in the presence of absence of MCSP blocking antibody (10 µg/ml) was analyzed by flow cytometry. **C)** Binding of MCSPxDR5 to DLD-1 cells as in B in the presence or absence of MCSP blocking antibody (10 µg/ml) or DR5 blocking antibody (10 µg/ml). **D)** Cell lines were incubated with MCSPxDR5 (2.5 µg/ml) at 4 °C for 40 min followed by 2 washes with excess cold phosphate buffered saline. Subsequently, cells were incubated for 18h at 37°C, after which apoptosis was measured by flow cytometry using Annexin-V/PI staining. **E)** Both MCSP^{pos} and MCSP^{neg} cancer cells were treated with or without MCSPxDR5 (1 µg/ml) for 18h, after which apoptosis was assessed using Annexin-V staining **F)** Cells were treated as in E for 72h, after which cell viability was determined by MTS. **G)** Representative histogram of MCSP and DR5 expression of a primary patient-derived melanoma cells. **H)** Patient-derived melanoma cells were treated with MCSPxDR5 (1 µg/ml) or left untreated for 24h, after which apoptosis was analyzed by flow cytometry with Annexin-V staining. Statistical analysis was performed using two-sided unpaired Student t test (D-F) or the Mann-Whitney U test (H). *P < 0.05; **P < 0.01; ***P < 0.001.

MCSPxDR5 inhibits of colony formation of MCSP^{pos}/DR5^{pos} cancer cells

Next, the effect of MCSPxDR5 on anchorage–independent growth of MCSP^{pos} melanoma was evaluated using a soft agar colony forming assay. Treatment with MCSPxDR5 for 14 days led to a significant reduction in colony number in MCSP^{pos} melanoma cell lines (Fig. 2A). Further, as illustrated for A375M, residual colonies after MCSPxDR5 treatment were reduced in size by >90% (Fig. 2B). This MCSPxDR5-mediated effect was completely blocked by either co-treatment with recombinant anti-TRAILR2-Fc or by pan-caspase inhibitor zVADfmk, indicating that the inhibitory effect of MCSPXDR5 on colony formation is caspase-mediated and dependent on DR5 (Fig. 2C). Of note, MCSPxDR5 also reduced colony number of MCSP^{pos}/DR5^{pos} HT1080 sarcoma cells by ~90%, whereas control non-targeted anti-DR5 antibody HGS-ETR2 only reduced colony number by ~50% (Fig. 2D). Taken together, MCSPxDR5 selectively inhibits colony forming capacity of MCSP^{pos}/DR5^{pos} cancer cells.

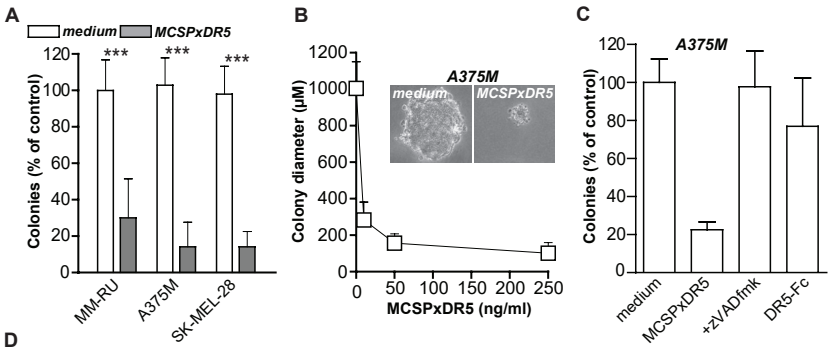

Figure 2: MCSPxDR5 inhibits colony formation of melanoma cells. **A)** MCSP^{pos}/DR5^{pos} cell lines were treated with MCSPxDR5 (250 ng/ml) or left untreated in colony-forming assays for 14 days, after which the number of colonies was determined by counting three fields of view per condition. Number of colonies was represented as percentage of colonies compared to medium control. **B)** Representative light microscopic images of colony size of A375M cells in medium control versus MCSPxDR5-treated conditions in colony-forming assay and dose-response curve of colony size upon MCSPxDR5 treatment. **C)** MCSP^{pos}/DR5^{pos} A375M cells were treated with MCSPxDR5 (50 ng/ml) in the presence or absence of pan-caspase inhibitor zVADfmk (5 µM) or recombinant TRAILR2-Fc (5 µg/ml) for 14 days, after which the number of colonies was determined. **D)** MCSP^{pos}/DR5^{pos} HT1080 cells were treated with MCSPxDR5, MCSP:sTRAIL or HSG-ETR2 (250 ng/ml) or left untreated in colony-forming agar assays for 14 days, after which the number of colonies was determined. Statistical analysis was performed using two-sided unpaired Student t test. *P < 0.05; **P < 0.01; ***P < 0.001.

Efficacy of MCSPxDR5 is enhanced by cross-linking of its Fc domain

MCSPxDR5 was designed to trigger apoptosis through MCSP-directed cross-linking of DR5. Since the antitumor activity of tigatuzumab appears to requires cross-linking of its IgG1 Fc domain through binding to FcγRs on immune effector cells.[10,12] Therefore, cross-linking of tumor cell-bound MCSPxDR5 via its IgG domain may further augment its pro-apoptotic activity.

To investigate this in more detail, we exploited MCSP^{neg}/DR5^{pos} Jurkat cells as indicator cells for effective DR5 cross-linking. Treatment of Jurkat cells with MCSPxDR5 alone did not induce apoptosis. However, in the presence of a human IgG cross-linking reagent, MCSPxDR5 strongly induced apoptosis in Jurkat cell up to almost 100%, with apoptotic activity of MCSPxDR5 already apparent at doses as low as 5 ng/ml (Fig. 3A). Similarly, secondary cross-linking of MCSPxDR5 enhanced its pro-apoptotic antitumor activity in a panel of MCSP^{pos}/DR5^{pos} melanoma cell lines (Fig. 3B and 3C). Furthermore, secondary cross-linking of MCSPxDR5 also increased the level of apoptosis induced in primary patient-derived melanoma cells, with a mean increase in apoptosis from 48% to 70% (Fig. 3D). Although the apoptotic activity of MCSPxDR5 was enhanced by cross-linking, its activity was still abrogated by co-treatment with recombinant DR5-Fc or pan-caspase inhibitor zVADfmk (Fig. 3E). Moreover, siRNA-mediated DR5 silencing in A375M cells significantly decreased DR5 expression (Fig. 3F) and strongly reduced the apoptotic activity of both MCSPxDR5 and the DR5-agonistic antibody HGS-ETR2 (Fig. 3G). Thus, Fc-mediated cross-linking augments the pro-apoptotic antitumor activity of MCSPxDR5 through enhanced DR5 signaling.

Efficacy of MCSPXDR5 is augmented by FcR-mediated cross-linking

Next, the effect of Fc cross-linking of MCSPxDR5 by surface expressed Fc receptors was evaluated using HEK293.CD64 cells ectopically expressing the high-affinity Fc receptor CD64. HEK293 cells are resistant to TRAIL receptor-mediated apoptosis and lack intrinsic cytolytic activity (data not shown). Treatment of mixed cultures of MM-RU target cells and parental HEK293 cells with an increasing dose of MCSPxDR5 only induced apoptosis in ~30% of MM-RU cells. However, in mixed cultures of MM-RU with HEK293.CD64, MCSPxDR5 treatment resulted in >70% apoptosis (Fig. 4A). To further confirm this cellular FcR-mediated cross-linking effect and exclude contributions of antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) to MCSPxDR5 activity, experiments were performed with isotype variant MCSPXDR5-IgG4, containing the human IgG4 Fc domain that is known to be largely devoid of ADCC and CDC activity.[19] Treatment of MM-RU/HEK293.CD64 mixed cultures with MCSPxDR5-IgG4 induced up to 60% apoptosis, similar to MCSPxDR5-IgG1 treatment (Fig. 4B).

Importantly, Fc receptor cross-linking also induced MCSP-independent DR5-mediated activation in MCSP^{neg}/DR5^{pos} M14 melanoma cells (Fig. 4C). However, Fc receptor-mediated cross-linking on MCSP^{pos} cells was abrogated when treatment was performed in the presence of MCSP-competing mAb 9.9.27 (Fig. 4D). In mixed culture experiments with freshly isolated PBLs, MCSPxDR5 dose-dependently reduced the viability of MM-RU cells to down to ~40% (Fig. 4E). Moreover, treatment with an increasing dose of MCSPxDR5 reduced MM-RU cell viability down to ~20% in the presence of leukocytes (Fig. 4F). Similarly, MCSPxDR5 significantly enhanced NK cell-mediated killing of MCSP^{pos}/DR5^{pos} A375M cells, but not of MCSP^{neg}/DR5^{pos} DLD1 cells (Fig. 4G). Thus, the MCSP-directed pro-apoptotic activity of MCSPxDR5 is further augmented by FcR-mediated cellular cross-linking and is able to induce ADCC through interaction with Fc receptor-expressing immune effector cells.

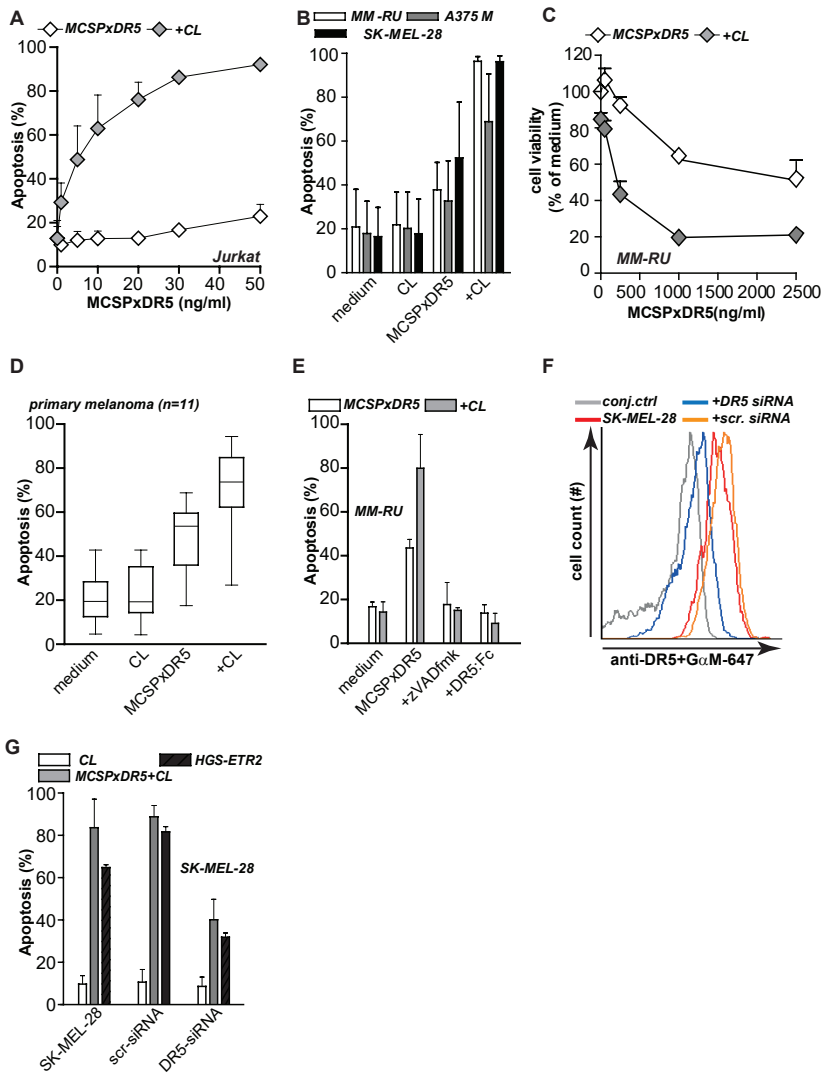

Figure 3: Cross-linking of its Fc domain enhances antitumor activity of MCSPxDR5. **A)** MCSP^{neg}/DR5^{pos} Jurkat cells were treated with MCSPxDR5 in the presence or absence of goat-anti-human IgG (cross-linker [CL]; 0.5 mg/ml). Apoptosis was measured after 18h by flow cytometry using Annexin-V. **B)** MCSP^{neg}/DR5^{pos} melanoma cell lines were treated with MCSPxDR5 (250 ng/ml) in the presence or absence of CL for 18h, after which apoptosis was measured by flow cytometry using Annexin-V. **C)** MCSP^{neg}/DR5^{pos} melanoma MM-RU cells were treated with an increasing dose of MCSPxDR5 in the presence or absence of CL for 72h, after which cell viability was determined by MTS assay. **D)** Primary patient-derived melanoma cells were co-treated with MCSPxDR5 (0.25 mg/ml) and CL. Apoptosis was measured after 18h. **E)** MM-RU cells were treated with MCSPxDR5 in the presence or absence of pan-caspase inhibitor (zVAD-fmk, 5 μM) or recombinant human DR5:Fc (5 μg/ml) for 18h, after which apoptosis was evaluated by flow cytometry using Annexin-V/PI staining. **F)** RNA silencing was used to selectively knock down DR5 expression in SK-MEL-28 cells, which was confirmed by flow cytometry using an anti-DR5 mAb. **G)** SK-MEL-28 cells were treated with either CL alone, MCSPxDR5 plus CL, or DR5 agonistic antibody HGS-ETR2 for 18h, after which apoptosis was evaluated by flow cytometry using Annexin V/PI staining.

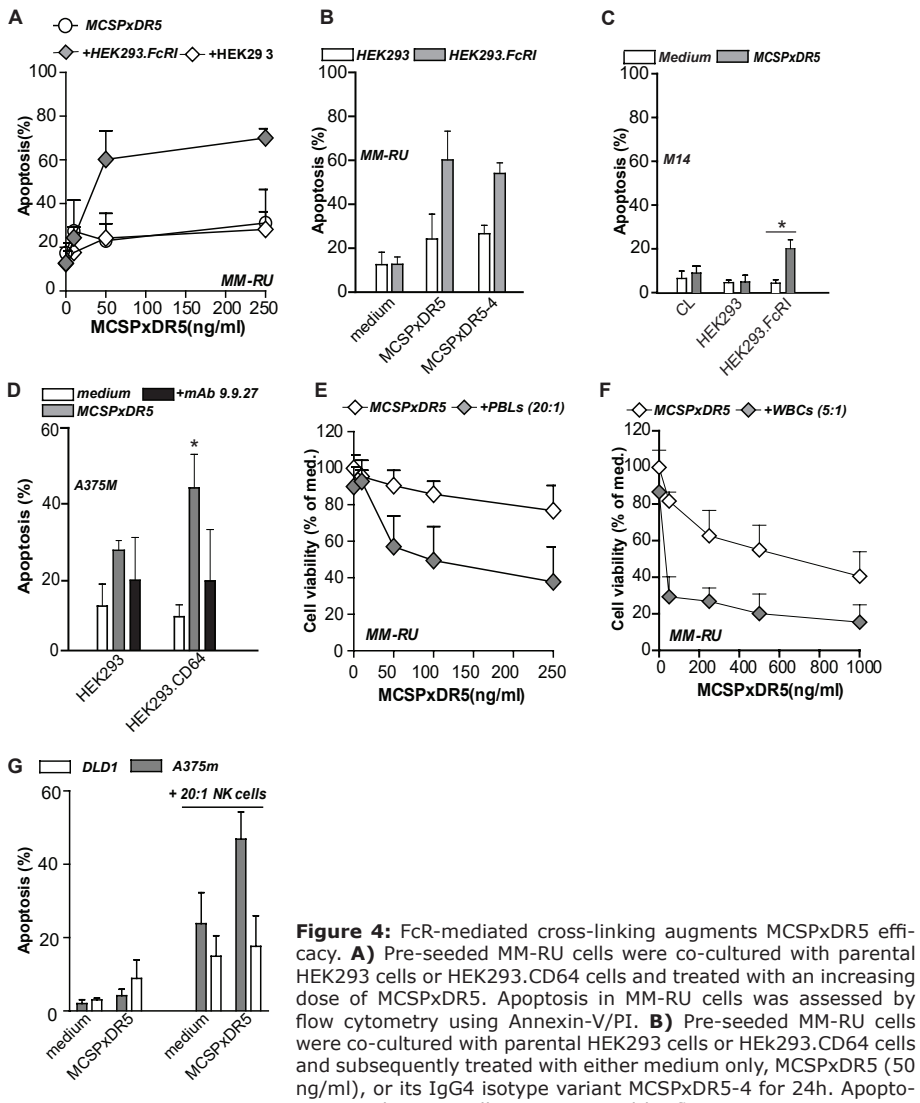

Figure 4: FcR-mediated cross-linking augments MCSPxDR5 efficacy. **A)** Pre-seeded MM-RU cells were co-cultured with parental HEK293 cells or HEK293.CD64 cells and treated with an increasing dose of MCSPxDR5. Apoptosis in MM-RU cells was assessed by flow cytometry using Annexin-V/PI. **B)** Pre-seeded MM-RU cells were co-cultured with parental HEK293 cells or HEk293.CD64 cells and subsequently treated with either medium only, MCSPxDR5 (50 ng/ml), or its IgG4 isotype variant MCSPxDR5-4 for 24h. Apoptosis in melanoma cells was assessed by flow cytometry using Annexin-V/PI. **C)** Pre-seeded M14 cells (MCSP^{neg}/DR5^{pos}) were co-cultured with parental HEK293 cells or HEK293.CD64 cells and subsequently treated with either medium only or MCSPxDR5 (50 ng/ml). Apoptosis in melanoma cells was assessed by flow cytometry using Annexin-V/PI. **D)** (DiD)-labeled A375M cells were treated with either culture medium or MCSPxDR5 (1.5 μg/ml) in the presence or absence of anti-MCSP mAb 9.9.27. Unbound antibodies were removed by repeated washing steps. Subsequently, parental HEK293 cells or HEK293.CD64 cells were added, after which apoptosis was assessed in DID-labeled cells by flow cytometry using Annexin V/PI staining. **E)** Peripheral blood lymphocytes (E) were added to pre-seeded MM-RU target cells (T) at an E:T ratio of 20:1 and treated with an increasing dose of MCSPxDR5 for 48h. Subsequently, the non-adherent immune cells were carefully removed, and the cell viability of MM-RU cells was assessed by MTS assay. **F)** As in E, WBCs (E) were added to pre-seeded MM-RU cells (T) at an E:T ratio of 5:1. **G)** Pre-seeded MM-RU cells (T) were co-cultured with NK cells of healthy donors (E) at E:T ratio of 20:1 in the presence or absence of MCSPxDR5 (250 ng/ml) for 6h after which cell viability of MM-RU cells was assessed by MTS assay. Statistical analysis was performed using two-sided unpaired Student t test. *P < 0.05; **P < 0.01; ***P < 0.001.

MCSPxDR5 shows no or limited toxicity toward normal cells

Treatment of DR5-expressing normal primary human hepatocytes and immortal human hepatocytes (IHH) with MCSPxDR5 induced no or minimal apoptosis (Fig. 5A). Moreover, treatment of HUVECs and normal lymphocytes with MCSPxDR5 (2.5 µg/ml) did not induce apoptosis (Fig. 5B). This indicates that MCSPxDR5 has selective tumoricidal activity with no or only limited toxicity towards normal cells. This is in line with a previous phase I clinical study that showed that tigatuzumab was well-tolerated with dose-limiting toxicity (DLT) reached and no drug-related grade 3 or 4 hepatic or hematological toxicity observed.[20]

Anticancer drugs synergize with anticancer activity of MCSPxDR5

Melanoma-relevant drugs (bortezomib, valproic acid and vemurafenib) were evaluated for their ability to synergize the antitumor activity of MCSPxDR5. Co-treatment of MCSP^{pos}/DR5^{pos} melanoma cells with MCSPxDR5 and proteasome inhibitor velcade triggered synergistic induction of apoptosis in A375M cells and MM-RU cells but not in OCM-3 cells (Fig. 6A). MCSPxDR5-mediated apoptosis showed strong synergy with HDAC inhibitor VPA in A375M cells, as apoptosis increased up to 60% compared to 20% for single treatment with either MCSPxDR5 or VPA alone. A synergistic treatment effect with VPA was also observed in MM-RU and SK-MEL-28 cells (Fig. 6B). Interestingly, we found that vemurafenib not only enhanced MCSPxDR5-mediated apoptosis in V600-mutant cell lines OCM-3 and SK-MEL-28, but also induced apoptosis up to 60% in V600-wt melanoma cell line MM-RU compared to single treatment with MCSPxDR5 (Fig. 6C).

Discussion

Recombinant TRAIL formulations (e.g. dulanermin) and agonistic anti-DR4/5 antibodies (e.g. conatumumab and tigatuzumab) have been extensively evaluated in pre-clinical models and in a number of clinical studies.[6-7,21] Results from early-stage clinical studies indicated that both recombinant TRAIL and agonistic anti-DR antibodies are well tolerated in patients with various cancers. However, the therapeutic efficacy of current DR agonists is rather disappointing. This appears to be related to the fact that these agonists indiscriminately interact with DRs that are broadly present on normal cells,[13,20,22] thereby precluding sufficient accumulation of such agonists in malignant lesions. Moreover, current DR agonists have limited capacity to activate pro-apoptotic DR5 signaling in cancer cells in the absence of secondary cross-linking.[12]

To overcome these limitations, we developed a novel bispecific antibody-based approach that promotes melanoma-directed pro-apoptotic activation of DR5. Hereto, we engineered bispecific antibody MCSPxDR5 that combines high binding affinity for the melanoma-associated antigen MCSP with potent agonistic activity towards DR5. The mode-of-action of MCSPxDR5 involves high-affinity binding to tumor cell surface-expressed MCSP with concomitant localized enhanced cross-linking of DR5. The antitumor activity of tumor-bound MCSPxDR5 was further enhanced by secondary cross-linking of its human IgG Fc domain by either an artificial cross-linker or by Fc receptors on myeloid immune effector cells. Moreover, various melanoma-relevant drugs (bortezomib, valproic

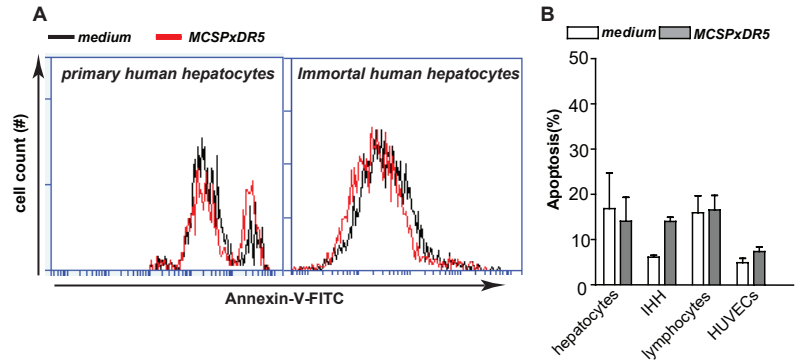

Figure 5: MCSPxDR5 has limited toxicity towards normal cells. **A)** Binding of MCSPxDR5 to primary human hepatocytes and immortal human hepatocytes (IHH) was assessed by flow cytometry. **B)** Normal human cells were treated with MCSPxDR5 (1 µg/ml) for 18h, after which apoptosis was determined by Annexin-V/PI staining.

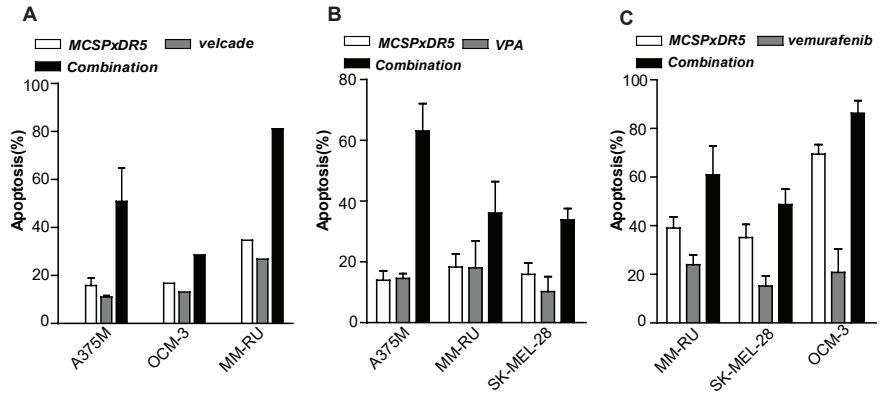

Figure 6: MCSPxDR5 shows synergy with anticancer drugs **A)** Pre-seeded melanoma cells were co-treated with MCSPxDR5 (0.25 µg/ml) and velcade (2.5 µM) for 24h. **B)** Pre-seeded melanoma cells were co-treated with MCSPxDR5 (0.25 µg/ml) and VPA (2 µM) for 24h. **C)** Pre-seeded melanoma cells were co-treated with MCSPxDR5 (1 µg/ml) and vemurafenib (10 µM) for 24h. In A-C, apoptosis was determined by flow cytometry using Annexin-V/PI.

acid and vemurafenib) synergistically enhanced the antitumor activity of MCSPxDR5. Importantly, MCSPxDR5 potently induced apoptosis in primary patient-derived melanoma cells, which was further enhanced after secondary cross-linking of its IgG1 Fc domain.

The anti-DR5 antibody fragment we used in bsAb MCSPxDR5 is derived from the agonistic DR5 antibody tigatuzumab. The *in vivo* therapeutic activity of tigatuzumab appears to fully rely on cross-linking of its Fc domain that may occur after binding to FcγRs as expressed on myeloid effector cells.[10-12] In contrast, selective binding of MCSPxDR5 to MCSP already triggered effective activation of membrane DR5 in melanoma cells, indicating that on MCSP^{pos} target cells MCSPxDR5 can at least partially circumvent the need for FcR-mediated cross-linking. Importantly, the antitumor activity of MCSPxDR5 could be further enhanced by FcR-expressing immune effector cells, via FcR-mediated cross-linking as well as by induction of ADCC.

Recently, a similar DR5-targeted bispecific antibody, RG7386, targeting the fibroblast-associated protein (FAP) on cancer-associated fibroblasts was described.[23] RG7386 induced apoptosis in FAP-positive cells and, when combined with irinotecan or doxorubicin, induced tumor regression in patient-derived xenograft models.[23] Our *in vitro* data largely corroborates these observations, indicating a common mode-of-action for RG7386 and MCSPxDR5.

MCSP is expressed in 90% of melanoma lesions[15] and other malignancies, e.g. sarcomas and gliomas.[24-25]. MCSP overexpression plays a significant role in melanoma progression by influencing tumor cell adhesion/spreading, migration, invasion, and metastasis. It has been reported that binding of mAb 9.2.27 to MCSP already in its own right can partially inhibit various oncogenic features of MCSP expression.[26] Indeed, we previously reported on a MCSP-targeting TRAIL fusion protein that not only induced MCSP-directed TRAIL-mediated apoptosis in melanoma cells, but also inhibited MCSP-mediated tumorigenic signaling.[27] The MCSP-directed antibody fragment used in bsAb MCSPxDR5 is derived from mAb 9.2.27. Although not formally studied here, it is tentative to speculate that bsAb MCSPxDR5 may have retained the potential to (partially) inhibited MCSP-tumorigenic signaling that may add to or even synergize with its pro-apoptotic anticancer activity.

Co-treatment of agonistic anti-DR5 antibodies or soluble TRAIL with various chemotherapeutics or (experimental) small inhibitory molecules has been evaluated in order to mutually augment their antitumor activities towards various forms of cancer, including carcinoma, breast cancer, lung cancer and metastatic melanoma.[21,28-30] Here, we found that co-treatment of HDAC inhibitor VPA synergistically enhanced the pro-apoptotic anticancer activity of MCSP in MCSP^{pos}/DR5^{pos} melanoma cells. This synergy is likely attributable to the fact that HDAC inhibitors are known to down-regulate c-FLIP and thereby increase sensitivity to treatment of DR5-based agonists.[31] Interestingly, we found that BRAFV600E inhibitor vemurafenib enhanced MCSPxDR5-induced apoptosis in both wild-type and V600E-mutant melanoma cells. This non-specific synergy with vemurafenib and TRAIL was also published by others[32] but the underlying mechanism remains unclear.

Our current data indicates that MCSPxDR5 has potent MCSP-directed pro-apoptotic activity towards MCSP^{pos}/DR5^{pos} melanoma cells with essentially no or minimal toxicity towards various normal cells. However, more in-depth *in vivo* studies are needed to establish whether this will ultimately also translate in a safe toxicity profile when applied in melanoma patients.

Taken together, we present a novel bispecific antibody-based approach that promotes melanoma-directed pro-apoptotic activation of DR5. This novel approach may be of value for the targeted treatment of melanoma and other MCSP-expressing malignancies.

Acknowledgements

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Conflict of interest

The authors state no conflict of interest.

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CHAPTER 4

Programmed Death Ligand 1 (PD-L1)-targeted TRAIL combines PD-L1-mediated checkpoint inhibition with TRAIL-mediated apoptosis induction

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Abstract

Antibodies that block PD-L1/PD-1 immune checkpoints restore the activity of functionally-impaired antitumor T cells. These antibodies show unprecedented clinical benefit in various advanced cancers, particularly in melanoma. However, only a subset of cancer patients responds to current PD-L1/PD-1-blocking strategies, highlighting the need for further advancements in PD-L1/PD-1-based immunotherapy. Here, we report on a novel approach designed to combine PD-L1 checkpoint inhibition with the tumor-selective induction of apoptosis by TNF-related Apoptosis Inducing Ligand (TRAIL). In brief, a new bi-functional fusion protein, designated anti-PD-L1:TRAIL, was constructed comprising a PD-L1 blocking antibody fragment genetically fused to the extracellular domain of the pro-apoptotic tumoricidal protein TRAIL. Treatment of PD-L1-expressing cancer cells with anti-PD-L1:TRAIL induced PD-L1-directed TRAIL-mediated cancer cell death. Treatment of T cells with anti-PD-L1:TRAIL augmented T cell activation, as evidenced by increased proliferation, secretion of IFN-γ and enhanced killing of cancer cell lines and primary patient-derived cancer cells in mixed T cell/cancer cell culture experiments. Of note, elevated levels of IFN-γ further up-regulated PD-L1 on cancer cells and simultaneously sensitized cancer cells to TRAIL-mediated apoptosis by anti-PD-L1:TRAIL. Additionally, anti-PD-L1:TRAIL converted immunosuppressive PD-L1-expressing myeloid cells into pro-apoptotic effector cells that triggered TRAIL-mediated cancer cell death. In conclusion, combining PD-L1 checkpoint inhibition with TRAIL-mediated induction of apoptosis using anti-PD-L1:TRAIL yields promising multi-fold and mutually reinforcing anticancer activity that may be exploited to enhance the efficacy of therapeutic PD-L1/PD-1 checkpoint inhibition.

Introduction

Programmed Death Ligand 1 (PD-L1) and its cognate receptor PD-1 represent an immune checkpoint of great interest for cancer immunotherapy. Antibodies that block PD-L1/PD-1 interaction restore the anticancer activity of functionally impaired tumor infiltrating lymphocytes (TILs), specifically cytotoxic T cells. Treatment with these antibodies has transformed the landscape of cancer immunotherapy, yielding long-term remission and cure in a subset of advanced stage melanoma patients.[1, 2]

The PD-L1/PD-1 immune checkpoint normally ensures timely shut-down of immune responses to prevent collateral damage or autoimmunity (reviewed in [3]). In brief, PD-L1 expression is upregulated on antigen-presenting cells during inflammation by locally produced IFN-γ and is expressed by myeloid suppressor cells.[4-6] Simultaneously, expression of PD-1 increases on activated T cells[7], which upon interaction with PD-L1 dampens the cytolytic activity of T cells[4]. Interestingly, activated T cells not only express PD-1, but upon activation also upregulate PD-L1.[8, 9] Antibody-mediated cross-linking of PD-L1 on T cells triggers co-stimulatory signaling and ultimately leads to induction of apoptosis[10], indicative of an immunoregulatory role for PD-L1 on T cells.

Various cancer types upregulate PD-L1 expression either constitutively via oncogenic signaling pathways or in response to IFN-γ produced in the tumor environment.[11, 12] Consequently, antitumor T cells are inhibited via PD-L1/PD-1 interaction, which allows cancer cells to evade the immune system even in highly immunogenic malignancies such as melanoma.[12, 13] Hence, the expression of PD-L1 on cancer cells often correlates with unfavorable prognosis.[14, 15] Although PD-1 and PD-L1-blocking antibodies have triggered breakthrough curative anticancer immunity, most notably in advanced melanoma,[1, 2] the benefit of these antibodies is still restricted to a minority of cancer patients.

To expand the clinical effects of immune checkpoint therapy, various combinatorial approaches have been attempted in order to identify opportunities for synergistic activity. For instance, treatment with PD-1-blocking antibody nivolumab was combined with CTLA-4 antibody ipilimumab, which significantly enhanced response rates in melanoma patients.[16] Similarly, combination of PD-1/PD-L1 blockade with induction of cytotoxic cancer cell death by radiotherapy proved more effective and enhanced activation of anticancer immunity.[17, 18] These selected examples highlight that more efficacious PD-L1/PD-1 targeted therapy can be achieved by rational design of combinatorial therapeutic approaches.

In this respect, we and others have previously reported on a class of bi-functional fusion proteins that comprise an scFv antibody fragment genetically fused to the tumoricidal protein tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) (reviewed in [19]). TRAIL is a homotrimeric death-inducing ligand of the TNF superfamily with well-documented tumor-selective pro-apoptotic activity that has been proven safe in clinical trials (reviewed in [20]). Antibody fragment-mediated delivery of soluble TRAIL optimizes its target cell-selective accretion and, moreover, triggers enhanced TRAIL-receptor mediated apoptosis in targeted cancer cells.[21, 22] Importantly, use of an scFv antibody fragment with antagonistic activity equips the scFv:TRAIL fusion protein with addi-

tional tumoricidal activity, e.g. by inhibition of EGFR-mitogenic signaling[23] or by blocking tumor-expressed CD47 and thereby augmenting neutrophil-mediated phagocytosis of cancer cells[24]. Furthermore, scFv-mediated display of TRAIL on the surface of T cells or granulocytes augments the cytolytic activity of these immune effector cells.[25, 26]
Based on this bi-functional TRAIL-based fusion protein format, we constructed and pre-clinically evaluated an anti-PD-L1:TRAIL fusion protein comprised of a PD-L1 blocking antibody fragment genetically fused to human soluble TRAIL. This anti-PD-L1:TRAIL fusion protein was designed to combine PD-L1 checkpoint inhibition with simultaneous TRAIL-mediated activation of cancer cell death.

Materials & methods

Antibodies, Reagents, Inhibitors

The following antibodies were used in this study; anti-CD279-PE (PD-1, clone MIH4, eBioscience), anti-CD274-APC (PD-L1, clone 29E.2A3, BioLegend), anti-CD83-PE (clone HB15e, eBioscience), anti-CD206-PE (clone 19.2, eBioscience), anti-CD3-PerCP-Cyanine5.5 (clone OKT-3, eBioscience), anti-TRAIL/TNFSF10-PE (clone 75402, R&D systems), NG2-FITC (anti-MCSP, clone LHM2, Santa Cruz Biotechnology), polyclonal Goat-anti-Human-PE (SouthernBiotech), Goat-anti-Mouse IgG (H+L) Secondary Antibody (Alexa Fluor 488 conjugate, Thermo Scientific) and anti-CD4-APC (clone MEM-241), anti-CD8-FITC (clone HIT8a), anti-CD56-PE (clone B-A19), anti-CD14-FITC (clone MEM-15), anti-CD11b-FITC (clone MEM-174), anti-HLA-DR-PE (clone MEM-12), anti-CD86-FITC (clone BU63), Mouse IgG2b-APC, Annexin-V-FITC (all Immunotools). Recombinant human IFN-γ, GM-CSF, M-CSF, IL-4, IL-10, TGF-β1, anti-CD3 (clone UCHT-1) and anti-IFN-γ (clone B27) were purchased from ImmunoTools. LPS (Lipopolysaccharides from E. coli 0111:B4) was purchased from Sigma-Aldrich. Recombinant human PD-1:Fc was purchased from R&D systems. Pan-caspase inhibitor z-VAD-fmk, TRAILR1 (clone DJR1) and TRAILR2 (clone DJR2-4) antibodies were purchased from Enzo Life Sciences. TRAIL-neutralizing mAb 2E5 was purchased from Life Technologies. Recombinant CMV protein pp65 was purchased from Miltenyi Biotec. A PD-L1 neutralizing murine antibody was purchased from BPS Bioscience.

Cell Lines

DLD-1, HCT-116, SK-MEL-28, A2058 and CHO-K1, NCI-H1975, ES-2, MDA-MB-231 were obtained from the American Type Culture Collection (ATCC). TRAIL-resistant cell line HCT-116.cFLIPs was kindly provided by Prof. dr. Harald Wajant (University of Würzburg, Würzburg, Germany). All cell lines were cultured in RPMI-1640 or DMEM (Lonza) supplemented with 10% fetal calf serum (FCS, Thermo Scientific). DLD-1.PD-L1 cells were generated by transfection of parental DLD-1 cells with eukaryotic expression plasmid pCMV6-PD-L1 using Fugene-HD (Promega). Stable transfectants were generated using Hygromycin B selection (Life technologies). All cells were cultured at 37°C, in a humidified 5% CO_2 atmosphere. Cell numbers were quantified using a cell counter (Sysmex). For experiments, tumor cells were cultured in 48-wells plates at a density of $2x10^4$ cells/well.

For up-regulation of PD-L1, cells were pre-treated for 24 hours with 20 ng/ml IFN-γ. PD-L1 expression was analyzed with an Accuri C6 flow cytometer (BD Biosciences) using PD-L1-APC antibody or appropriate isotype control. Relative PD-L1 expression levels are listed in Supplementary Table 1. TRAIL receptor expression was determined by flow cytometry using TRAILR1 and TRAILR2 antibodies with secondary Goat-anti-Mouse-488 conjugate staining. Relative TRAIL receptor expression levels are listed in Supplementary Table 2.

Primary patient-derived melanoma cells and Tumor Infiltrating Lymphocytes

Fresh melanoma and appendix carcinoma tissue was collected during surgical resection after informed consent (local approval nr. METc2012/330). Tissue was minced and cultured in RPMI 1640 with 10% FCS. Adherent cell phenotype was analyzed by flow cytometry using fluorescently labeled CD14, PD-L1 and MCSP antibodies. Primary patient-derived melanoma cells used in this study were CD14-negative and MCSP-positive and were used before passage 4. For generation of Tumor Infiltrating Lymphocytes (TILs), minced tissue fragments were cultured in RPMI 1640 with 10% FCS supplemented with 50 IU/ml IL-2 (Proleukin, Novartis). TIL phenotype was analyzed by flow cytometry for CD3, CD4, CD8 and CD56.

Production of TRAIL fusion proteins

Anti-PD-L1:TRAIL was constructed by insertion of an anti-PD-L1 mAb 3G10-derived scFv into Sfi1 and Not1 restriction sites into the previously described plasmid pEE14-scFv:TRAIL.[27] Briefly, CHO-K1 cells were transfected with eukaryotic expression plasmid pEE14scFv:sTRAIL using the Fugene-HD reagent (Promega) and stable transfectants were generated by the glutamine synthetase selection method. Stable transfectants were cultured at 37°C in serum-free CHO-S SFM II suspension medium (Gibco, Life Technologies) for up to 7 days after which supernatant was harvested (1,500g, 10 min) and stored at −20°C until further use. Fusion protein concentration in culture supernatant was determined by TRAIL ELISA (Abcam). Anti-EpCAM:TRAIL and anti-MCSP:TRAIL were described before.[22, 27]

PD-L1 specific binding of anti-PD-L1:TRAIL

Tumor cells were incubated with anti-PD-L1:TRAIL (1 μg/ml) for 1 hour at 4°C, washed twice with PBS (1000g, 5 min), stained with anti-TRAIL-PE for 30 minutes at 4°C, washed twice with PBS, and analyzed for binding by flow cytometry. Where indicated tumor cells were pre-incubated with excess (10 μg/ml) PD-L1 blocking mAb.

PD-1/PD-L1 blocking by anti-PD-L1:TRAIL

DLD-1 and DLD-1.PD-L1 cells were pre-incubated with indicated concentrations of anti-PD-L1:TRAIL for 1 hour at 0ºC, after which cells were washed twice (1000g, 5 min) and incubated with 4 μg/ml PD-1:Fc for 1 hour at 0ºC. Subsequently, cells were washed twice (1000g, 5 min) and stained with Goat-anti-Human-PE for 30 min at 0°C and washed twice (1000g 5 min). PD-1:Fc binding was evaluated by flow cytometry.

Apoptosis assay

Tumor cells were treated with anti-PD-L1:TRAIL or anti-EpCAM:TRAIL and, where indicated, in the presence of PD-L1 blocking mAb (10 µg/ml), pan-caspase inhibitor z-VAD-FMK (10 µM) or TRAIL-neutralizing mAb 2E5 (1 µg/ml). After 18 hours, apoptosis was assessed by flow cytometry using Annexin-V staining according to manufacturer's protocol (Immunotools). Where indicated, cells were co-treated with 1 µg/ml cycloheximide (CHX, Sigma-Aldrich).

Spheroid assay

DLD-1, DLD-1.PD-L1 or NCI-H1975 cells (1-5x10^3 cells/well) were seeded in low adherence 96-well plates (Costar) in DMEM supplemented with MITO+ Serum Extender (Corning). Cells were treated with anti-PD-L1:TRAIL, anti-EpCAM:TRAIL or anti-MCSP:TRAIL as indicated. After 72 hours, cell viability was determined using MTS (CellTiter 96 AQueous One Solution Cell Proliferation, Promega) at 490 nM using a Victor V3 multi-label plate counter (Perkin Elmer). Absorbance of the maximum death control (treatment with 70% ethanol for 15 min) was subtracted from all values, after which cell viability was calculated as percentage of medium control. Light microscopy images were acquired at 10x magnification using the EVOS XL core cell imaging system (Life Technologies) and colony number was counted manually in three separate fields-of-view per condition in triplicates. Pre-formed spheroids of DLD-1 and DLD-1.PD-L1 cells were generated by 96 hours culture in low adherence flasks (Costar), after which spheroids were transferred to low adherence plates for experiments.

PBMC stimulation assays

Peripheral blood mononuclear cells (PBMCs) were obtained from venous blood of healthy volunteers after informed written consent using standard density gradient centrifugation (Lymphoprep). PBMCs (1.25x10^5/well) were cultured in a 48-well plate in the presence of 0.5 µg/ml agonistic CD3 mAb (UCHT-1) and indicated concentrations anti-PD-L1:TRAIL, anti-EpCAM:TRAIL or PD-L1 mAb. After 72 hours, total cell number was quantified using an automated cell counter (Sysmex) and culture supernatants were stored at -20°C. IFN-γ levels in culture supernatant were determined by IFN-γ ELISA (eBioscience). Where indicated, freshly isolated PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE) (CellTrace CFSE Cell Proliferation Kit, Invitrogen), and after 72 hours of respective treatment the cell proliferation was analyzed by flow cytometry within the live PBMCs (defined by FSC/SSC gating).

For CMV specific responses, freshly isolated PBMCs from CMV negative and positive donors were cultured in 96-well plates (1.5x10^5/well) in the presence of CMV pp65 according to manufacturer's instructions (Miltenyi Biotec). After 96 hours, culture supernatants were stored at -20°C and secreted IFN-γ was determined by IFN-γ ELISA.

Antitumor reactivity assay

Tumor cells were labeled with DiD (Vybrant Cell-Labeling Solution, LifeTechnologies). Subsequently, 2x10^4 tumor cells were co-cultured with freshly isolated PBMCs or $CD3^+$ T cells (98% purity, using the human Pan T Cell Isolation Kit (Miltenyi Biotec)) in the presence of 0.5 µg/ml agonistic CD3 mAb (UCHT-1) or CD3/CD28 beads (Dynabeads Human T-Activator CD3/CD28, Thermo Fischer) at a bead-to-cell ratio of 1:10, respectively. Mixed cultures were further treated with 0.5 µg/ml anti-PD-L1:TRAIL, anti-EpCAM:TRAIL or PD-L1 mAb. After 48 hours, loss of mitochondrial membrane potential (Δψ) in DiD labeled tumor cells was analyzed by DiOC6 staining (Eugene) as previously described.[27] After harvesting, PBMCs were stained with fluorescent CD4 and CD8 antibodies whereupon the number of $CD4^+$ and $CD8^+$ T cells within the PBMC gate was analyzed by flow cytometry.

Alternatively, 5x10^4 DLD-1 cells were pre-seeded 24 hours before addition of freshly isolated PBMCs. Tumor cells were mixed with PBMCs at indicated E:T ratio's and co-treated with 50 ng/ml BIS-1 (anti-EpCAM:anti-CD3 bispecific antibody previously described in[28]) and 0.5 µg/ml anti-PD-L1:TRAIL or anti-MCSP:TRAIL. After 24 hours, non-adherent cells were carefully washed away and cell viability was determined using MTS as described above.

Patient-derived melanoma and appendix carcinoma cells were co-cultured with autologous TILs in E:T ratio of 2:1. After 48 hours, apoptosis was assessed by Annexin-V staining and IFN-γ levels in culture supernatant were determined by ELISA.

Isolation and generation of myeloid-derived cell types

Monocytes: PBMCs were subjected to magnetic-activated cell sorting (MACS) with anti-CD14-beads and MS columns (Miltenyi Biotec). ***Macrophages:*** monocytes (1x10^6/ml) were treated with 50 ng/ml M-CSF for 6 days, yielding M0 macrophages. M0 macrophages were subsequently stimulated with 50 ng/ml LPS and 20 ng/ml IFN-γ to generate M1 macrophages or 20 ng/ml IL-4, IL-10 and TGF-β1 to generate M2 macrophages. Macrophage phenotype was confirmed by flow cytometric analysis of CD14, CD206 and PD-L1, as described before.[29] ***Dendritic cells:*** immature DCs (iDCs) were generated by treatment of monocytes (3x10^6/ml) with 500 U/ml GM-CSF and 1000 U/ml IL-4 for 7 days. Mature DCs (mDCs) were generated by treatment of iDCs with 1 µg/ml LPS for 3 days. DC phenotype was confirmed by flow cytometric analysis of PD-L1, CD83, CD14, HLA-DR and CD86.

For mixed culture experiments, myeloid cell types were mixed with 2.5x10^3 DLD-1 cells (at E:T ratio 4:1) with the indicated concentrations of anti-PD-L1:TRAIL in presence or absence of PD-L1 mAb. After 18 hours, DLD-1 cells were analyzed for apoptosis by flow cytometric analysis of Annexin-V staining. DLD-1 cells were separately analyzed by excluding $CD14^+$ (monocytes and macrophages) or $CD11b^+$ (DCs) cells.

Statistical analysis

Statistical analysis was performed by two-way ANOVA followed by Tukey-Kramer post-test, one-way ANOVA followed by Tukey-Kramer post-test, Wilcoxon matched pairs test or two-sided unpaired Student t test as indicated using Prism software. $P<0.05$ was defined as a statistically significant difference. Where indicated * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$.

Results

anti-PD-L1:TRAIL induces PD-L1 restricted TRAIL-mediated apoptosis in cancer cells

The anti-PD-L1:TRAIL fusion protein was designed to bind to PD-L1 on cancer cells and subsequently trigger TRAIL-mediated apoptosis by activating agonistic TRAIL-receptors. In line with this, anti-PD-L1:TRAIL strongly and dose-dependently bound to carcinoma cell line DLD-1.PD-L1 that ectopically overexpress PD-L1 (Fig. 1A). In contrast, anti-PD-L1:TRAIL did not bind to parental DLD-1 cells (Fig. 1B and Suppl. Fig. 1A). Binding of anti-PD-L1:TRAIL to DLD-1.PD-L1 was abrogated by co-incubation with molar excess of epitope-competing anti-PD-L1 monoclonal antibody (Fig. 1A). Treatment of DLD-1.PD-L1 with anti-PD-L1:TRAIL triggered dose-dependent TRAIL-mediated apoptosis, whereas similar treatment of DLD-1 cells did not trigger apoptosis (Fig. 1C). Thus, anti-PD-L1:TRAIL triggers cell death specifically after binding to cell surface-expressed PD-L1. Of note, treatment of DLD1.PD-L1 with PD-L1-blocking antibody alone and anti-EpCAM:TRAIL alone induced ~20% and 45% apoptosis, respectively (Fig. 1D). Combined treatment with PD-L1-blocking antibody and anti-EpCAM:TRAIL additively enhanced apoptosis to ~65% (Fig. 1D). However, treatment with anti-PD-L1:TRAIL at the same concentration induced up to 90% apoptosis (Fig. 1D). The apoptotic activity of anti-PD-L1:TRAIL was abrogated when cells were treated in the presence of excess epitope-competing PD-L1 monoclonal antibody (Fig. 1E). Further, apoptotic activity was TRAIL-mediated since TRAIL-neutralizing monoclonal antibody or total caspase-inhibitor z-VAD-fmk blocked apoptosis induction in DLD-1.PD-L1 (Fig. 1E). Of note, DLD-1 and DLD.PD-L1 are equally sensitive to TRAIL-mediated apoptosis since control EpCAM-targeted anti-EpCAM:TRAIL, that binds equally well to both cell lines (data not shown), induced apoptosis to a similar extent in both cell lines (Fig. 1D). In contrast, a control non-targeted fusion protein, anti-MCSP:TRAIL, only minimally induces apoptosis in either cell line (Fig. 1E). In a small panel of cancer cell lines that naturally express PD-L1 (Suppl. Table 1), treatment with anti-PD-L1:TRAIL also induced apoptosis (Fig. 1F). Anti-PD-L1:TRAIL treatment further abrogated spheroid formation in DLD-1.PD-L1, but not DLD-1 cells (Fig. 1G-H), and strongly reduced viability of established spheroids (Fig. 1I). In spheroid forming assays, anti-PD-L1:TRAIL also significantly reduced cell viability of NCI-H1975 cells that endogenously express PD-L1 (Suppl. Fig 1B-C). Thus, apoptotic activity of anti-PD-L1:TRAIL is dependent on PD-L1 specific binding to target cells and subsequent induction of TRAIL-mediated apoptosis.

anti-PD-L1:TRAIL blocks PD-1/PD-L1 interaction and enhances T cell activation

Since TRAIL is a naturally occurring homotrimer the anti-PD-L1:TRAIL fusion protein contains three PD-L1 blocking scFvs, which should allow for effective inhibition of PD-L1/PD-1 interaction. Indeed, anti-PD-L1:TRAIL dose-dependently inhibited binding of recombinant PD-1:Fc to DLD-1.PD-L1 cells, whereas similar treatment with anti-EpCAM:TRAIL did not affect PD-1:Fc binding (Fig. 2A). In line with the PD-L1/PD-1 blocking effect of anti-PD-L1:TRAIL, treatment of peripheral blood mononuclear cells (PBMCs) with anti-PD-L1:TRAIL and an agonistic CD3 mAb increased proliferation and cell number 2-fold

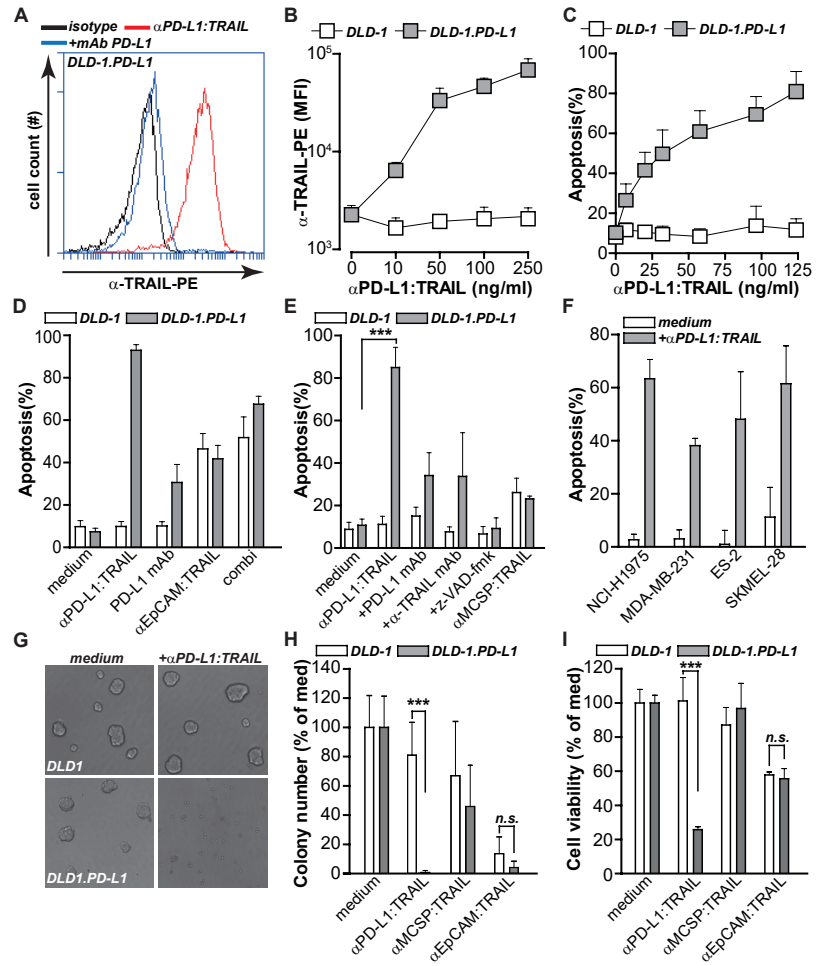

Figure 1: anti-PD-L1:TRAIL induces PD-L1-restricted TRAIL-mediated apoptosis in cancer cells. **A)** Binding of anti-PD-L1:TRAIL to DLD-1.PD-L1 cells in the presence or absence of excess PD-L1 blocking antibody (10 μg/ml) was analyzed by flow cytometry. **B)** DLD-1.PD-L1 or DLD-1 cells were incubated with an increasing dose of anti-PD-L1:TRAIL and binding was assessed by flow cytometry. **C)** DLD-1.PD-L1 or DLD-1 cells were treated with an increasing dose of anti-PD-L1:TRAIL for 18 hours, after which apoptosis was measured by flow cytometry using Annexin-V staining. **D)** DLD-1.PD-L1 or DLD-1 cells were treated with anti-PD-L1:TRAIL (250 ng/ml), anti-EpCAM:TRAIL (250 ng/ml) or PD-L1 antibody (1 μg/ml). Apoptosis was assessed by Annexin-V staining after 18 hours. **E)** DLD-1.PD-L1 or DLD-1 cells were treated with anti-PD-L1:TRAIL (250 ng/ml) in the presence or absence of PD-L1 blocking antibody (10 μg/ml), TRAIL-neutralizing mAb (1 μg/ml) or total caspase inhibitor z-VAD-fmk (10 μM). DLD-1.PD-L1 or DLD-1 cells were also treated with anti-MCSP:TRAIL(250 ng/ml). Apoptosis was assessed by Annexin-V staining after 18 hours. **F)** PD-L1-expressing cell lines were co-treated with cycloheximine (CHX, 1 μg/ml) and anti-PD-L1:TRAIL (1 μg/ml). Apoptosis was determined by Annexin-V staining after 18 hours. **G)** Representative light microscopy images of spheroid size of DLD-1.PD-L1 cells or DLD-1 cells in medium control versus anti-PD-L1:TRAIL-treated conditions after 72 hours. **H)** Spheroid formation of DLD-1.PD-L1 or DLD-1 cells was assessed in the presence or absence of 100 ng/ml anti-PD-L1:TRAIL, anti-MCSP:TRAIL or anti-EpCAM:TRAIL. Number of spheroid colonies was determined after 72 hours by counting three fields-of-view per condition in triplicates. **I)** Established spheroids of DLD-1.PD-L1 cells or DLD-1 cells were treated with 500 ng/ml anti-PD-L1:TRAIL, anti-MCSP:TRAIL or anti-EpCAM:TRAIL. Cell viability was determined by MTS after 72 hours. All graphs represent mean±SD. Statistical analysis was performed using two-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001, n.s. not significant).

compared to treatment with the agonistic CD3 mAb alone (Fig. 2B, C). Furthermore, anti-PD-L1:TRAIL dose-dependently increased secretion of IFN-γ (Fig. 2D), to a level similar to that induced by a monoclonal antagonistic anti-PD-L1 antibody (Fig. 2E). This T cell stimulatory effect of anti-PD-L1:TRAIL was also detected in mixed cultures of PBMCs with DLD-1 cells (Suppl. Fig. 1D). Importantly, co-treatment with anti-EpCAM:TRAIL did not increase T cell proliferation or T cell number compared to CD3 activation alone (Fig. 2B, C). To subsequently investigate pro-inflammatory activity of anti-PD-L1:TRAIL in an antigen-specific reaction, a model system using Cytomegalovirus (CMV)-specific T cells was used. To this end, PBMCs from CMV^{pos} and CMV^{neg} donors were loaded with CMV protein pp65 in combination with anti-PD-L1:TRAIL or anti-EpCAM:TRAIL. Loading of PBMCs with pp65 in the presence of anti-PD-L1:TRAIL significantly increased IFN-γ secretion by CMV^{pos} donor cells (Fig. 2F), whereas no effect on IFN-γ secretion was detected in T cells from CMV^{neg} donors.

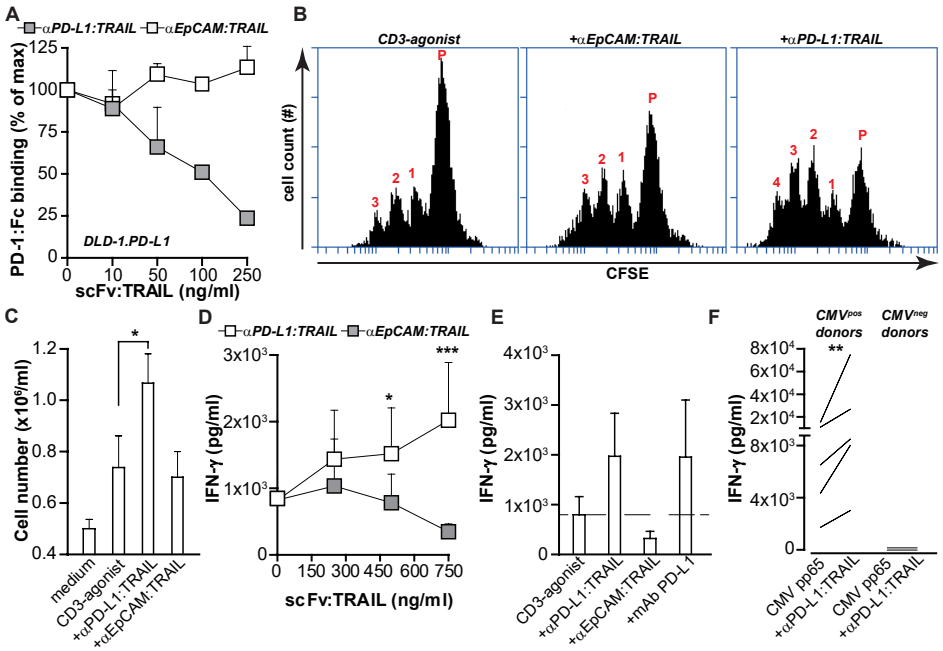

Figure 2: anti-PD-L1:TRAIL blocks the PD-1/PD-L1 interaction and enhances T cell activation. **A)** Binding of PD-1:Fc (4 µg/ml) to DLD-1.PD-L1 cells in the presence of an increasing dose of anti-PD:L1:TRAIL or anti-EpCAM-TRAIL was analyzed by flow cytometry. **B)** Representative histograms of CFSE-labeled PBMCs co-treated with agonistic CD3 mAb (0.5 µg/ml) and 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL. After 72 hours, cell proliferation was analyzed by flow cytometry. **C)** PBMCs were treated with 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL in the presence of agonistic CD3 mAb (0.5 µg/ml). After 72 hours, cell number was quantified using an automated cell counter. **D)** PBMCs were co-treated with agonistic CD3 mAb (0.5 µg/ml) and an increasing dose of anti-PD-L1:TRAIL or anti-EpCAM:TRAIL. After 72 hours, IFN-γ levels in culture supernatant were determined by ELISA. **E)** PBMCs were co-treated with agonistic CD3 mAb (0.5 µg/ml) and 500 ng/ml anti-PDL1:TRAIL, anti-EPCAM:TRAIL or mAb PD-L1 for 72 hours. IFN-γ levels in culture supernatant were determined by ELISA. **F)** PBMCs from CMV-positive or CMV-negative donors and were treated with 500 ng/ml anti-PD-L1:TRAIL in the presence of CMV protein pp65 for 96 hours. IFN-γ levels in culture supernatant were determined by ELISA. All graphs represent mean±SD. Statistical analysis was performed using unpaired two-sided Student t test (C), two-way ANOVA (D) or Wilcoxon matched pairs test (F) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant).

Importantly, anti-EpCAM:TRAIL only minimally induces IFN-γ secretion (Suppl. Fig. 1E). Taken together, anti-PD-L1:TRAIL potentiates T cell proliferation and IFN-γ production via blockade of PD-1/PD-L1 interaction.

PD-L1:TRAIL enhances anticancer T cell activity

Next, we analyzed whether enhanced T cell activation by anti-PD-L1:TRAIL could augment anticancer T cell activity. In mixed cultures of A2058 melanoma cells and PBMCs, treatment with CD3 agonist at sub-optimal doses triggered apoptosis in ~30% of cancer cells, whereas treatment with anti-PD-L1:TRAIL alone did not induce apoptosis (Fig. 3A). However, combination treatment with CD3 agonist and anti-PD-L1:TRAIL synergistically enhanced apoptosis in A2058 to over 60% (Fig. 3A). Further, co-treatment with anti-PD-L1:TRAIL also significantly increases IFN-γ secretion (Suppl. Fig. 1F). Correspondingly, in these mixed culture experiments the expression of PD-L1 on A2058 increased 3-fold (Suppl. Fig. 1G), whereas PD-1 expression on T cells increased 6-fold (Suppl. Fig. 1H). Importantly, treatment of activated T cells with PD-L1:TRAIL did not induce apoptosis in T cells (Suppl. Fig. 1I). Anti-PD-L1:TRAIL also significantly increased production of IFN-γ in mixed culture experiments with primary melanoma patient-derived TILs and autologous melanoma cells (Fig. 3B), to a level similar to that induced by anti-PD-L1 blocking antibody. PD-L1:TRAIL treatment of autologous primary cancer/TIL mixed culture experiments also increased apoptotic cell death in autologous cancer cells, both in melanoma and appendix carcinoma cells (Fig. 3C). The enhanced cytotoxicity of PBMCs upon CD3 agonist and anti-PD-L1:TRAIL treatment was blocked to medium levels by co-incubation with TRAIL-neutralizing antibody (2E5) (Fig. 3D) and was, therefore, dependent on TRAIL/TRAIL-receptor interaction. Correspondingly, anti-PD-L1:TRAIL induced apoptosis in mixed cultures of HCT-116 and PBMCs, but not in TRAIL resistant HCT-116.cFLIPs (Suppl. Fig. 1J), demonstrating that cell death upon anti-PD-L1:TRAIL treatment is TRAIL-mediated.

Of note, treatment with anti-EpCAM:TRAIL, PD-L1 blocking antibody or a combination did not significantly enhance the cytotoxic activity of PBMCs towards A2058 cells (Fig. 3A, D) nor did it increase the number of T cells (Fig. 3E). In these mixed cultures, the number of T-cells significantly increased upon anti-PD-L1:TRAIL treatment (Fig. 3E). Furthermore, in mixed cultures of A2058 and isolated $CD3^+$ T-cells, only anti-PD-L1:TRAIL treatment synergistically enhances apoptosis in A2058 to >80% (Fig. 3F) and significantly increases IFN-γ secretion (Fig. 3G). Thus, anti-PD-L1:TRAIL augments cytolytic activity of T cells in mixed culture experiments with T cells and tumor cells.

To mimic antigen-specific T cell activation, DLD-1 cancer cells were treated with T cell retargeting bispecific antibody (bsAb) anti-EpCAM:anti-CD3 (28). This bsAb retargets T cells to EpCAM-positive cancer cells and, in mixed cultures of DLD-1 and PBMCs, triggered cell death in an E:T ratio-dependent manner (Fig. 3H). Importantly, co-treatment with anti-EpCAM:anti-CD3 and anti-PD-L1:TRAIL significantly increased apoptotic elimination of DLD-1 cells leading to ~80% loss in cell viability after 24h of treatment at an E:T ratio of 5:1. (Fig. 3H). In contrast, control fusion protein anti-MCSP:TRAIL did not potentiate the cytotoxic effect of anti-EpCAM:anti-CD3 retargeted T cells (Fig.3H). In these mixed

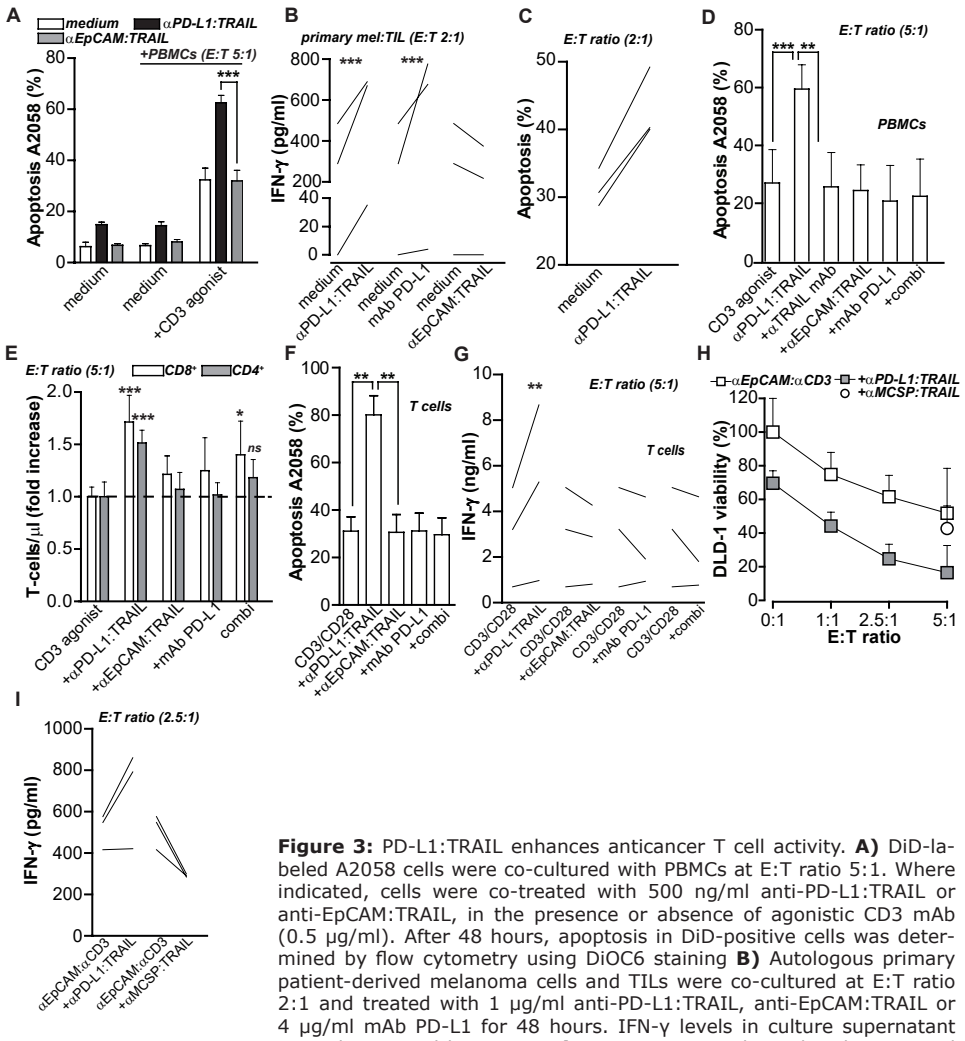

Figure 3: PD-L1:TRAIL enhances anticancer T cell activity. **A)** DiD-labeled A2058 cells were co-cultured with PBMCs at E:T ratio 5:1. Where indicated, cells were co-treated with 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL, in the presence or absence of agonistic CD3 mAb (0.5 μg/ml). After 48 hours, apoptosis in DiD-positive cells was determined by flow cytometry using DiOC6 staining **B)** Autologous primary patient-derived melanoma cells and TILs were co-cultured at E:T ratio 2:1 and treated with 1 μg/ml anti-PD-L1:TRAIL, anti-EpCAM:TRAIL or 4 μg/ml mAb PD-L1 for 48 hours. IFN-γ levels in culture supernatant were determined by ELISA. **C)** Primary patient-derived melanoma and appendix carcinoma cells were co-cultured with autologous TILs at E:T ratio 2:1 and treated with 1 μg/ml anti-PD-L1:TRAIL for 48 hours, after which apoptosis was assessed by Annexin-V staining. **D)** DiD-labeled A2058 cells were co-cultured with PBMCs at E:T ratio 5:1 in the presence of agonistic CD3 mAb (0.5 μg/ml). Cells were co-treated with 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL, where indicated cells were co-treated with TRAIL-neutralizing antibody (1 ug/ml). After 48 hours, apoptosis in DiD-positive cells was determined by flow cytometry using DiOC6 staining. **E)** In mixed cultures of PBMCs and A2058 as described in D, the PBMC population was stained with fluorescent CD4 and CD8 antibodies whereupon the number of $CD4^+$ and $CD8^+$ T cells within the PBMC gate was analyzed by flow cytometry. **F)** DiD-labeled A2058 cells were co-cultured with isolated $CD3^+$ T-cells at E:T ratio 5:1 in the presence of CD3/CD28 beads at a bead-to-cell ratio of 1:10. Cells were co-treated with 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL and after 48 hours, apoptosis in DiD-positive cells was determined by flow cytometry using DiOC6 staining. **G)** IFN-γ levels in culture supernatant of F were determined by ELISA. **H)** DLD-1 cells were pre-seeded 24 hours before PBMCs were added at indicated E:T ratios in the presence of anti-EpCAM:anti-CD3 (50 ng/ml) with or without 500 ng/ml anti-PD-L1:TRAIL or anti-MCSP-TRAIL. Cell viability was determined by MTS after 24 hours. **I)** IFN-γ levels in culture supernatant of H were determined by ELISA. All graphs represent mean±SD. Statistical analysis was performed using two-way ANOVA (A), one-way ANOVA (D) or unpaired two-sided Student t test (B, F) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant).

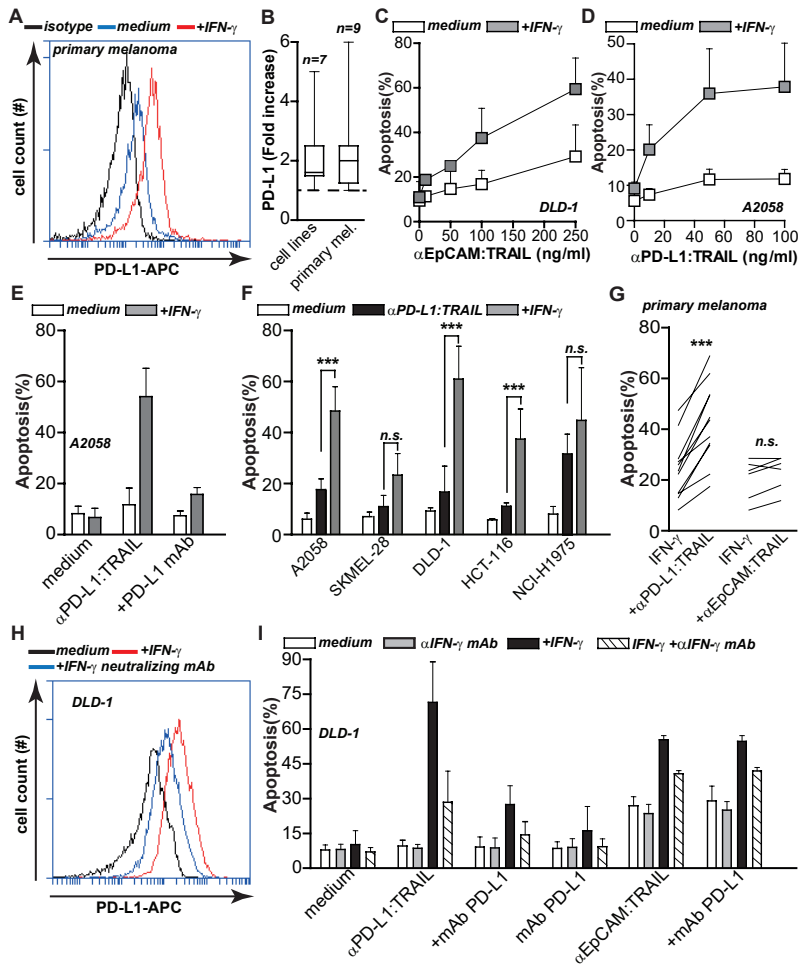

Figure 4: IFN-γ upregulates PD-L1 expression and sensitizes cancer cells to TRAIL-mediated apoptosis. **A)** Primary patient-derived melanoma cells were treated with or without 20 ng/ml IFN-γ for 24 hours after which PD-L1 expression was analyzed by flow cytometry. **B)** 7 cancer cell lines and 9 primary patient-derived melanoma cell cultures were treated with or without 20 ng/ml IFN-γ for 24 hours after which PD-L1 expression was analyzed by flow cytometry. Fold increase was calculated compared to non-treated cells. **C)** IFN-γ pre-treated or non-treated DLD-1 cells were incubated with an increasing dose of anti-EpCAM:TRAIL for 18 hours, after which apoptosis was assessed by flow cytometry using Annexin-V staining. **D)** IFN-γ pre-treated or non-treated A2058 cells were incubated with an increasing dose of anti-PD-L1:TRAIL. Apoptosis was assessed by Annexin-V staining after 18 hours. **E)** IFN-γ pre-treated or non-treated A2058 cells were incubated with 500 ng/ml anti-PD-L1:TRAIL in the presence or absence of PD-L1 blocking mAb (10 μg/ml). Apoptosis was determined by Annexin-V staining after 18 hours. **F)** A small panel of cancer cell lines were pre-treated with or without IFN-γ (20 ng/ml), followed by treatment of anti-PD-L1:TRAIL (500 ng/ml) for additional 18 hours. Apoptosis was determined by Annexin-V staining. **G)** IFN-γ pre-treated primary patient-derived melanoma cultures were treated with 1 μg/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL for 48 hours. Apoptosis was determined using Annexin-V. **H)** DLD-1 cells were treated with or without 20 ng/ml IFN-γ in the presence or absence of 8 μg/ml IFN-γ neutralizing mAb. After 24 hours, PD-L1 expression was analyzed by flow cytometry. **I)** DLD-1 cells were pre-treated with or without 20 ng/ml IFN-γ in the presence or absence of 8 μg/ml IFN-γ neutralizing mAb. After 24 hours, cells were treated with anti-PD-L1:TRAIL (250 ng/ml) in the presence or absence of PD-L1 blocking mAb (10 μg/ml), anti-EpCAM:TRAIL (250 ng/ml) or mAb PD-L1 (1 μg/ml). All graphs represent mean±SD. Statistical analysis was performed using two-way ANOVA (F) or Wilcoxon matched pairs test (G) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant).

cultures, IFN-γ production increased upon co-treatment with anti-EpCAM:anti-CD3 and anti-PD-L1:TRAIL when compared to co-treatment with anti-MCSP:TRAIL (Fig. 3I).

IFN-γ enhances PD-L1 expression and sensitizes cancer cells to TRAIL-mediated apoptosis

IFN-γ up-regulates PD-L1 expression on cancer cells,[11] a finding confirmed here in a panel of 7 cancer cell lines and 9 primary patient-derived melanoma cell cultures (Fig. 4A, B). Since pro-apoptotic activity of anti-PD-L1:TRAIL is dependent on PD-L1 specific binding to cells, this upregulation of PD-L1 may sensitize cancer cells to anti-PD-L1:TRAIL-mediated killing. Further, IFN-γ is known to sensitize cancer cells to TRAIL-mediated apoptosis, e.g. through down-regulation of cFLIP.[30, 31] Correspondingly, apoptotic activity of control fusion protein anti-EpCAM:TRAIL on DLD-1 cells was increased by IFN-γ pre-treatment (Fig. 4C). This effect was due to TRAIL sensitization and not due to EpCAM upregulation as IFN-γ does not affect EpCAM expression.[32] Thus, IFN-γ both upregulates PD-L1 and sensitizes cancer cells to TRAIL, which may augment anti-PD-L1:TRAIL activity. In line with the above, pre-treatment of A2058 cells with IFN-γ dose-dependently sensitized A2058 cells to anti-PD-L1:TRAIL-mediated apoptosis (Fig. 4D), as well as a further panel of 5 cancer cell lines (Fig. 4F) and 11 primary melanoma cell cultures (Fig. 4G). This apoptotic activity of anti-PD-L1:TRAIL was still abrogated by co-treatment with excess PD-L1 blocking antibody (Fig. 4E). Additionally, co-treatment with IFN-γ neutralizing antibody inhibited IFN-γ mediated PD-L1 upregulation on DLD-1 cells (Fig. 4H) and abrogated anti-PD-L1:TRAIL-mediated apoptosis (Fig. 4I). Thus, the apoptotic activity of anti-PD-L1:TRAIL is enhanced by IFN-γ, likely due to both upregulation of PD-L1 expression on cancer cells and simultaneous sensitization of cancer cells to TRAIL-mediated apoptotic signaling.

Anti-PD-L1:TRAIL converts PD-L1 expressing myeloid cells into pro-apoptotic tumoricidal effector cells

Within the tumor micro-environment, various types of infiltrated myeloid cells, such as M2 macrophages and DCs, are known to express PD-L1 and to suppress antitumor immunity.[33, 34] On these cells, PD-L1 expression is further elevated by tumor localized IFN-γ.[4, 5] Previously, we demonstrated that direct arming of myeloid effector cells using a TRAIL fusion protein that binds to CLL-1 on granulocytes, enhanced the pro-apoptotic activity of such myeloid cells.[26] To assess whether potentiation of myeloid effector cell activity might also contribute to anti-PD-L1:TRAIL activity, we generated various myeloid effector cell populations, i.e. monocytes, M0, M1 and M2 macrophages, immature DCs (iDCs) and mature DCs (mDCs). All of these effector cells expressed PD-L1 to a varying degree, with monocytes having lowest and mDCs having the highest expression (Fig. 5A). Further, M1 macrophages had higher PD-L1 expression compared to the immunosuppressive M2 macrophages, a finding in line with an earlier report.[29] PD-L1 expression was upregulated by IFN-γ pre-treatment, as illustrated for monocytes (Fig. 5B). In subsequent mixed culture experiments of monocytes and DLD-1 target cells, such IFN-γ pre-treated monocytes alone did not significantly induce apoptosis in DLD1

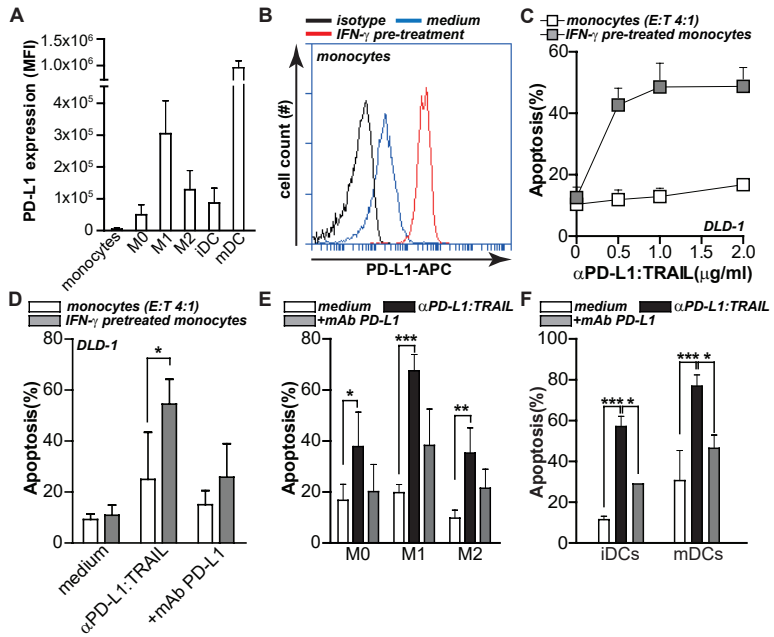

Figure 5: Anti-PD-L1:TRAIL converts PD-L1 expressing myeloid cells into pro-apoptotic tumoricidal effector cells. **A)** PD-L1 expression levels of monocytes, M0, M1, M2 macrophages, immature and mature DCs were determined by flow cytometry. Isotype control MFI was subtracted from original MFI. **B)** Monocytes were treated with or without 20 ng/ml IFN-γ for 24h after which PD-L1 expression was analyzed by flow cytometry. **C)** Monocytes were pre-treated with or without 20 ng/ml IFN-γ for 24 hours, washed twice with PBS after which DLD-1 cells were added at E:T ratio 4:1 in the presence of an increasing dose of anti-PD-L1:TRAIL. After 18 hours, apoptosis was assessed by Annexin-V staining. **D)** As in C with 500 ng/ml anti-PD-L1:TRAIL with or without PD-L1 blocking mAb (10 μg/ml). **E)** M0, M1 or M2 macrophages were co-cultured with DLD-1 cells at E:T ratio 4:1 in the presence of 500 ng/ml anti-PD-L1:TRAIL with or without PD-L1 blocking mAb (10 μg/ml). After 18 hours, apoptosis was assessed by Annexin-V staining. **F)** Immature or mature DCs were co-cultured with DLD-1 cells at E:T ratio 4:1 in the presence of 500 ng/ml anti-PD-L1:TRAIL with or without PD-L1 blocking mAb (10 μg/ml). After 18 hours, apoptosis was assessed by Annexin-V staining. All graphs represent mean±SD. Statistical analysis was performed using two-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant).

cells (Fig. 5C). However, treatment with anti-PD-L1:TRAIL dose-dependently increased apoptosis in DLD-1 cells (Fig. 5C). This increase was blocked by co-treatment with molar excess of PD-L1 blocking mAb (Fig. 5D). Similarly, M0, M1 or M2 macrophages and immature or mature DCs alone minimally induced apoptosis of DLD-1 cells in mixed culture experiments (Fig. 5E, F). However, addition of anti-PD-L1:TRAIL to these mixed cultured significantly triggered apoptosis in DLD-1 cells (Fig. 5E, F), with e.g. an >50% increase in apoptosis in DLD-1 cells in mDC mixed culture experiments (Fig. 5F). In all mixed culture experiments, PD-L1 blocking mAb strongly inhibited the apoptotic activity of anti-PD-L1:TRAIL (Fig. 5D-F). Of note, background apoptosis in DLD-1 cells was reduced from ~20 to ~10% in the presence of M2 macrophages, a finding in line with their pro-tumorigenic role (Fig. 5E). Further, although M1 macrophages express higher levels of PD-L1 (Fig. 5A) and were intrinsically more cytolytic (Fig. 5E), the potentiating effect of anti-PD-L1:TRAIL was most pronounced for M2 macrophages, with a 4-fold increase in apoptosis with M2 vs. a 3-fold increase with M1 macrophages (Suppl. Fig. 1K).

Taken together, these data demonstrate that anti-PD-L1:TRAIL binds to PD-L1 on myeloid cells, whereupon these normally immunosuppressive cells trigger TRAIL-mediated apoptotic cell death in cancer cells.

Discussion

Here, we describe a new PD-L1/PD-1 checkpoint inhibitor approach that combines PD-L1 checkpoint blockade with targeted delivery of the pro-apoptotic tumoricidal protein TRAIL. Fusion protein anti-PD-L1:TRAIL has a multi-fold therapeutic effect, depicted in Fig. 6, comprised of 1. induction of TRAIL-mediated cancer cell death after binding to tumor-expressed PD-L1, 2. reactivation of antitumor T-cells by blocking of PD-L1/PD-1 interaction, 3. converting suppressive monocytes/macrophages/DCs into pro-apoptotic effector cells that trigger TRAIL-mediated cancer cell death and 4. enhancement of IFN-γ production by immune effector cells, leading to simultaneous PD-L1 upregulation and sensitization of cancer cells to TRAIL.

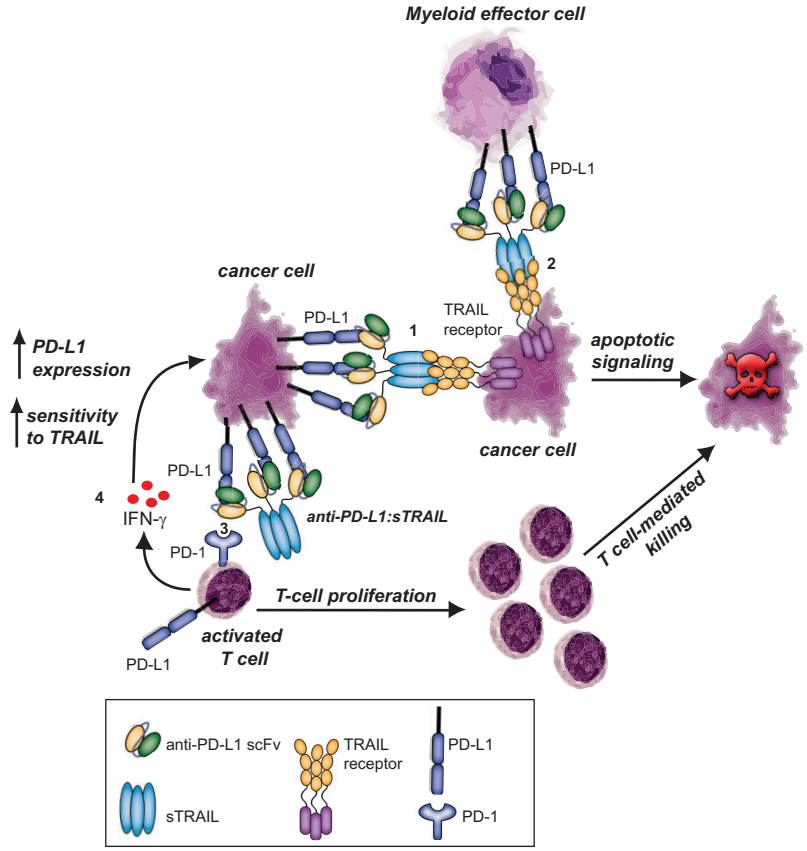

Figure 6: Proposed mechanism of action for anti-PD-L1:TRAIL. anti-PD-L1:TRAIL induces TRAIL-mediated cancer cell death after binding to tumor-expressed PD-L1 **(1)** or after binding to PD-L1 on myeloid effector cells **(2)**, restores proliferation and antitumor activity of T cells by blocking PD-L1/PD-1 interaction **(3)** and enhances IFN-γ production of T cells, leading to simultaneous PD-L1 upregulation and sensitization of cancer cells to TRAIL **(4)**.

Blockade of the PD-L1/PD-1 axis by anti-PD-L1:TRAIL enhanced T cell activation, proliferation and IFN-γ production, an effect similar to that reported for anti-PD-L1 monoclonal antibodies,[35] and triggered TRAIL-mediated apoptosis in cancer cells. TRAIL has strong pro-apoptotic activity towards various cancers in the absence of deleterious activity towards normal cells.[36] Clinical evaluation of first-generation recombinant human TRAIL (rhTRAIL, brand name "dulanermin") and TRAIL receptor antibodies typically yielded low to absent toxicity towards normal cells and tissues (reviewed in[37]), with a maximum tolerable dose (MTD) not being reached for dulanermin.[38] Of note, dulanermin did not have toxicity towards immune cells in these studies.[37, 38] Thus, the use of TRAIL as additional effector domain is anticipated to have no or only minimal side-effects when combined with PD-L1 blockade strategies. In line with this, we did not detect any negative effects of the TRAIL domain of PD-L1:TRAIL on T cells, with no increase in apoptosis in activated T cells. A recent study did report suppression in T cell activation and proliferation when T-cells were co-stimulated with recombinant TRAIL and CD3/CD28 antibodies.[39] However, this suppressive effect was only observed at a concentration of recombinant TRAIL of 50 µg/ml, which is ~50 fold higher than the highest concentration of 1 ug/ml typically used in literature and by us here for PD-L1:TRAIL.

Of note, early clinical trials in various types of malignancies yielded only limited clinical benefit for dulanermin with stable disease being the best-reported activity.[38, 40, 41] However, it has become evident that first generation TRAIL receptor-agonists do not optimally exploit the unique apoptotic signaling characteristics of the various TRAIL receptors (reviewed in[42-44]). Most notably, apoptotic signaling via TRAIL-R2, one of the two agonistic TRAIL receptors, is not efficiently achieved by soluble homotrimeric rhTRAIL, as TRAIL-R2 requires binding of membrane-bound TRAIL or secondarily cross-linked rhTRAIL.[21, 45, 46] Since TRAIL-R2 is often highly expressed on cancer cells it forms an important target for TRAIL-based therapy. Importantly, we and others have previously shown that tumor-directed scFv:TRAIL fusion proteins effectively activate TRAIL-R2 on targeted cancer cells only. In this process high affinity and tumor-selective binding via its scFv domain converts a soluble and essentially inactive scFv:TRAIL fusion protein into membrane-bound TRAIL with highly potent agonistic activity towards both TRAIL-R1 and TRAIL-R2.[21, 47, 48] Thus, anti-PD-L1:TRAIL has combined and mutuallyreinforcing PD-L1-blocking and TRAILR agonistic activities within one therapeutic anticancer fusionprotein, which outperforms combined treatment with PD-L1-blocking antibody and TRAIL.

Importantly, in mixed culture experiments using primary patient-derived melanoma cells and autologous TILs, treatment with anti-PD-L1:TRAIL enhanced IFN-γ production and augmented TIL-mediated cancer cell apoptosis. Additionally, when T cells were redirected to carcinoma cells using an anti-EpCAM:anti-CD3 bispecific antibody, anti-PD-L1:TRAIL synergistically enhanced their pro-apoptotic antitumor activity. A similar potentiating effect of PD-L1 blockade has been previously reported for T cell retargeting BiTEs that target CEA or CD33.[49, 50] This suggests that anti-PD-L1:TRAIL may be exploited to augment tumor-specific activity of T cells. In line with this, anti-PD-L1:TRAIL also increased IFN-γ secretion in a model antigen-specific reaction where T-cells from CMV-positive donors were stimulated with CMV protein pp65.

Tumor cells that do not constitutively express PD-L1 can rapidly upregulate PD-L1 when the tumor micro-environment is infiltrated by T cells. As previously reported, this up-regulation is likely due to IFN-γ generated by T cells upon tumor cell recognition and attempted elimination.[12] In the context of anti-PD-L1:TRAIL, this IFN-γ production may contribute to its antitumor efficacy as IFN-γ not only sensitizes cancer cells to TRAIL but also upregulates PD-L1 expression.[11, 30, 31] Thus, anti-PD-L1:TRAIL may trigger a feed-forward loop of increasing IFN-γ, increasing PD-L1 expression and increasing TRAIL sensitivity.

In the tumor microenvironment, various myeloid cells such as macrophages and DCs, also express PD-L1 and hereby can suppress antitumor immunity.[33, 34] The presence of these suppressive cell types correlated with disease progression and reduced survival in HCC and breast cancer patients.[6, 51] Here, we showed that anti-PD-L1:TRAIL can arm PD-L1-expressing monocytes, DCs and macrophages with TRAIL, turning these suppressive cell types into pro-apoptotic effector cells whilst simultaneously blocking potential PD-L1-mediated immunosuppressive effects. The obvious promise of this arming strategy is illustrated by reports where PD-L1 blockade alone significantly improved the *in vivo* antitumor activity of T cells treated with suppressive DCs or monocytes.[6, 33]

Interestingly, based on the known receptor interactions of PD-1 ligands (reviewed in[3]), PD-1 antibodies may have distinct biological activities from PD-L1 antibodies and their activities may not be redundant, depending on the dominant interaction for a particular cancer. In this respect, simultaneous blockade of PD-1 and PD-L1 maximized cytolytic T cell activity of tumor-directed bispecific T cell engaging antibodies.[49]. These findings suggest that combining anti-PD-L1:TRAIL with PD-1 blocking antibodies may further optimize checkpoint inhibitor-based therapy.

In conclusion, fusion protein anti-PD-L1:TRAIL has promising multi-fold and mutually reinforcing therapeutic effects comprised of PD-L1 checkpoint blockade and simultaneous induction of TRAIL-mediated apoptosis. This new fusion protein may provide possibilities to enhance the efficacy of therapeutic PD-L1/PD-1 checkpoint inhibition alone or in combination with other immunotherapeutic strategies.

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Conflict of interest

The authors state no conflict of interest.

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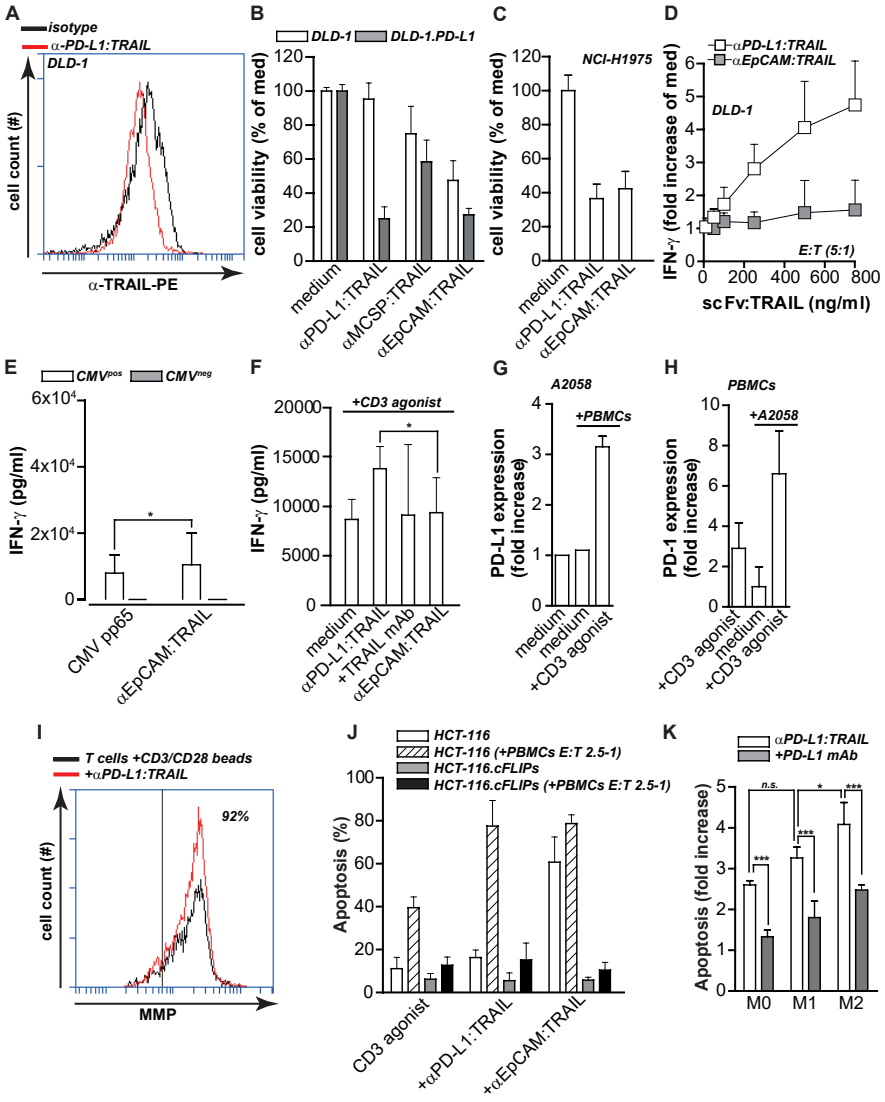

Supplementary Figure 1: A) Binding of anti-PD-L1:TRAIL to DLD-1 cells was analyzed by flow cytometry. **B)** Spheroid formation of DLD-1.PD-L1 or DLD-1 cells was assessed in the presence or absence of 100 ng/ml anti-PD-L1:TRAIL, anti- MCSP:TRAIL or anti-Ep-CAM:TRAIL. Cell viability was determined by MTS after 72 hours. **C)** Spheroid formation of NCI-H1975 cells was assessed in the presence or absence of 1 µg/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL. Cell viability was determined by MTS after 72 hours. **D)** DLD-1 cells were co-cultured with PBMCs at E:T ratio 5:1 in the presence of agonistic CD3 mAb (0.5 µg/ml) and an increasing dose of anti-PD-L1:TRAIL or anti-EpCAM:TRAIL. After 72 hours, IFN-γ levels in culture supernatant were determined by ELISA. **E)** PBMCs from CMV-positive or CMV-negative donors were treated with 500 ng/ml anti-EpCAM:TRAIL in the presence of CMV protein pp65 for 96 hours. IFN-γ levels in culture supernatant were determined by ELISA. **F)** A2058 cells were co-cultured with PBMCs at E:T ratio 5:1 and treated with 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL as indicated, in the presence of agonistic CD3 mAb (0.5 µg/ml). Where indicated, cells were co-treated with TRAIL-neutralizing antibody (1 ug/ml). After 48 hours,

IFN-γ levels in culture supernatant were determined by ELISA. **G)** A2058 cells were co-cultured with PBMCs at E:T ratio 5:1 in the presence or abscence of agonistic CD3 mAb (0.5 µg/ml).After 48 hours, PD-L1 expression on A2058 cells was measured by flow cytometry. **H)** As in G, PD-1 expression on PBMCs was measured by flow cytometry. Fold increase was calculated compared to medium control. **I)** A2058 cells were co-cultured with isolated $CD3^+$ cells at E:T ratio 5:1. Cells were co-treated with 500 ng/ml anti-PD-L1:TRAIL in the presence of CD3/CD28 beads. After 48 hours, loss of mitochondrial membrane potential (MMP) in T cells was measured by flow cytometry. **J)** As in F, HCT-116.wt or TRAIL resistantHCT-116.cFLIPs cells were co-cultured with PBMCs at E:T ratio 2.5:1 and loss of mitochondrial potential was assesed after 48 hours. **K)** M0, M1 or M2 macrophages were co-cultured with DLD-1 cells at E:T ratio 4:1 in the presence of 500 ng/ml anti-PD-L1:TRAIL with or without PD-L1 blocking mAb (10 µg/ml). After 18 hours, apoptosis was assessed by Annexin-V staining. Fold increase was calculatedcompared to medium control. All graphs represent mean ±SD. Statistical analysis was performed using Wilcoxon matched pairs test (E), unpaired two-sided Student t test (F) or ANOVA (K). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant)

cell line / patient sample	relative expression level	fold increase with IFN-γ
DLD-1	+	5
DLD-1.PD-L1	++++	2
A2058	+	1.5
HCT-116	+	2.5
SKMEL-28	++	1.5
MDA-MB-231	++++	nd
ES-2	++	nd
NCI-H1975	++	nd
Melanoma 1	+++	6
Melanoma 2	+++	1
Melanoma 3	++++	2
Melanoma 4	++	1.5
Melanoma 5	+	2
Melanoma 6	+++	2
Melanoma 7	+	2
Melanoma 8	+	3
Melanoma 9	+	1
Melanoma 10	+	4
Appendix Carcinoma	++++	2

Supplementary Table 1: PD-L1 expression levels of all cell lines and primary samples used in this study were determined by flow cytometry using PD-L1-APC antibody with appropriate isotype control. Relative expression levels were calculated by subtracting isotype control MFI from original MFI. (Relative expression index: MFI <20.000 = +, 20.000 <MFI> 50.000 = ++, 50.00 <MFI> 100.000 = +++, MFI >100.000 = ++++). Cells were also pre-treated with or with-out 20 ng/ml IFN-γ for 24 hours, after which PD-L1 expression was determined using flow cytometry. Fold increase was calculated compared to non-treated cells (nd=not determined).

cell line / patient sample	TRAILR1	TRAILR2
DLD-1	7.508	20.272
DLD-1.PD-L1	2.760	20.252
A2058	5.408	11.388
HCT-116	0	23.472
SKMEL-28	nd	67.733
Melanoma 1	50.984	47.653
Melanoma 2	11.678	501.374
Melanoma 3	117.701	10.992
Melanoma 4	109.621	180.582
Melanoma 5	64.969	191.333
Melanoma 6	76.436	49.606
Melanoma 7	356.732	71.917

Supplementary Table 2: TRAIL receptor expression levels of cell lines and primary samples used in this study. Tumor cells were incubated with 1 ug/ml TRAILR1 or TRAILR2 antibody for 1 hour at 4°C, washed twice with PBS (1000g, 5 min), stained with Goat-anti-Mouse conjugate for 30 minutes at 4°C, washed twice with PBS, after which TRAIL receptor expression was subsequently analyzed by flow cytometry. Relative expression levels were calculated by subtracting conjugate control MFI from original MFI (nd = not determined).

CHAPTER 5

A novel bispecific antibody for EGFR-directed PD-1/PD-L1 immune checkpoint inhibition

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Abstract

Purpose: PD-1/PD-L1-blocking antibodies can restore the antitumor activity of functionally-impaired antigen-experienced CD8+ T-cells and have provided significant clinical benefit in selected cancer patients with advanced stage disease. However, current PD-1/PD-L1-blocking antibodies lack intrinsic tumor selectivity and may indiscriminately reactivate all T-cells, including silenced autoreactive T-cells. The latter is evidenced by the frequent occurrence of severe autoimmune-related adverse events in patients receiving these antibodies. Here, we report on a novel bispecific antibody (bsAb), designated bsAb PD-L1xEGFR, which selectively directs PD-L1 blockade to EGFR-overexpressing cancer cells.

Experimental Design: We constructed bsAb PD-L1xEGFR (human IgG1) in which a PD-L1-blocking scFv antibody fragment is genetically fused to an EGFR-blocking scFv. EGFR-selective binding and blocking of PD-1/PD-L1 interaction by bsAb PD-L1xEGFR was assessed using EGFR-positive versus EGFR-negative cancer cell lines. The ability of bsAb PD-L1xEGFR to promote the antitumor activity of antigen-experienced CD8+ T-cells was assessed by co-culturing CMV-pp65 specific effector T-cells with HLA-matched target tumor cells ectopically expressing CMV-pp65 protein.

Results: Our pre-clinical data demonstrate that treatment with bsAb PD-L1xEGFR selectively directs PD-L1 blockade to EGFR-overexpressing cancer cells. In this process, bsAb PD-L1xEGFR augmented the capacity of T cells to proliferate, secrete IFN-γ and selectively kill EGFR-overexpressing target cells, while simultaneously blocking oncogenic EGFR signaling. Finally, EGFR-directed blockade of PD-L1 promoted the activity of antigen-experienced CMV-specific CD8+ T cells.

Conclusions: EGFR-directed PD-1/PD-L1 immune checkpoint inhibition can be achieved by applying bsAb PD-L1xEGFR. In this process, bsAb PD-L1xEGFR has multi-fold mutually reinforcing activities that promote CD8+ T-cells to selectively eliminate EGFR-overexpressing cancer cells. BsAb PD-L1xEGFR may be of clinical importance for enhancing selectivity, efficacy and safety of PD-1/PD-L1 checkpoint inhibition approaches in EGFR-overexpressing malignancies.

Introduction

Interaction between Programmed Death-1 receptor (PD-1) and PD ligand 1 (PD-L1) inhibits proliferation and cytokine production by antigen-experienced CD8+ T cells and serves to prevent collateral damage and autoimmunity.[1] Cancer cells can misuse this immune checkpoint in order to escape from elimination by anticancer CD8+ T cells.[2] Cancer cells may constitutively express PD-L1 due to aberrant oncogenic signals or upregulate PD-L1 in an adaptive response to IFN-γ released by anticancer CD8+ T cells in the tumor microenvironment.[2, 3] Expression of PD-L1 on cancer cells is associated with reduced survival and unfavorable prognosis in selected cancer types, including melanoma, lung cancer and renal cancer.[4-6]

Blocking the interaction between PD-L1 on tumor cells and PD-1 on effector T cells using antagonistic antibodies is a promising therapeutic approach and produced durable antitumor responses in multiple cancer types. Particularly, nivolumab and pembrolizumab yielded long-term remissions in advanced stage melanoma[7-9] and non-small-cell lung carcinoma (NSCLC)[10, 11]. However, PD-1 and PD-L1 are also broadly expressed on normal cells which may reduce the efficacy of PD-1/PD-L1 blocking antibodies. Consequently, treatment with PD-1/PD-L1 blocking antibodies can have severe autoimmune-related side effects in the skin, gastrointestinal tract, liver and lungs as observed for nivolumab.[7, 12]

To improve the clinical activity, various combinations of immune checkpoint-blocking antibodies have been evaluated, including combining the PD-1-blocking antibody nivolumab with the cytotoxic T lymphocyte antigen-4 (CTLA-4)-blocking antibody ipilimumab. This combination significantly enhanced response rates in melanoma patients, but is also associated with a higher incidence of toxicities than single antibody therapy.[12]

Recently, we proposed a novel approach to safely improve the efficacy of immune checkpoint blockade using a recombinant fusion protein, designated anti-PD-L1:TRAIL, in which a PD-L1-blocking scFv antibody fragment is fused to a soluble form of the tumor-selective pro-apoptotic death ligand TRAIL. *In vitro* evaluation demonstrated that anti-PD-L1:TRAIL not only enhanced the anticancer activity of T cells, but also selectively induced TRAIL-mediated apoptosis of PD-L1-positive cancer cells.[13]

Here, we describe a novel bispecific antibody (bsAb)-based approach that allows for more selectively directing PD-1/PD-L1 blockade to cancer cells. For this purpose, we produced recombinant bsAb PD-L1xEGFR that was designed to have both blocking activity for PD-L1 and high-affinity binding activity for the epidermal growth factor receptor (EGFR). EGFR is a well-established tumor target antigen that is overexpressed by various malignancies in which it correlates with poor prognosis.[14, 15] Aberrant EGFR signaling plays crucial roles in the pathogenesis of cancer by initiating the early stages of tumor development, sustaining tumor growth, promoting infiltration, and mediating resistance to therapy (reviewed in[16]). Several EGFR-targeted strategies that inhibit oncogenic EGFR signaling are FDA-approved (reviewed in[17]), including antibody cetuximab. Of note: cetuximab (IgG1) not only inhibits downstream signaling of EGFR[18] but also activates antibody-dependent cell-mediated cytotoxicity (ADCC)[19].

Taken together these notions prompted us to devise and pre-clinically evaluate a novel bispecific antibody-based approach to achieve EGFR-directed PD-1/PD-L1 immune checkpoint inhibition. To this end, we constructed recombinant bispecific antibody (bsAb) PD-L1xEGFR that comprises a PD-L1-blocking scFv antibody fragment fused in tandem to an EGFR-directed scFv and a human IgG1 Fc domain.

Materials and methods

Antibodies and reagents

Goat anti-human Ig-PE (Southern Biotech), anti-PD-L1-APC (clone 29E.2A3, BioLegend), anti-EGFR-FITC (clone 528, Santa Cruz Biotechnology), anti-CD137-PE (clone 4B4, eBioscience), anti-CD107a-APC (clone H4A3, BD Pharmingen), anti-IFN-γ-PerCP-Cyanine5.5 (clone 4S.B3, eBioscience), anti-CD3-PerCP-Cyanine5.5 (clone OKT-3, eBioscience), and anti-CD8-FITC, APC (clone HIT8a), anti-CD56-PE (clone B-A19), anti-CD14-FITC, PE (clone MEM-15), anti-CD25-FITC, APC (clone MEM-181), anti-HLA-DR-FITC, PE (clone MEM-12), mouse IgG1-FITC, PE, Mouse IgG2b-APC, Annexin-V-FITC (all from Immunotools). Recombinant human IFN-γ, TNF-α, PGE2, GM-CSF, IL-1β, IL-4, IL-6, IL12 and anti-CD3 mAb (clone UCHT-1) were purchased from Immunotools. PD-L1-blocking mAb was purchased from BPS Bioscience. Anti-EGFR mAb 425 was purchased from Merck. Cetuximab was obtained from the Department of Hospital Pharmacy, UMCG, The Netherlands. Secretion of cytokines by T-cells was measured using appropriate ELISA kits (IFN-γ from eBioscience and granzyme B from Mabtech) according to the manufacturer's recommendations.

Cell Lines and transfectants

Cell lines A431, FaDu, H292, OVCAR3, HT1080, DLD-1, LNCaP, A2058, A375m, A2780, CHO-K1 and Jurkat cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 or DMEM (Lonza), supplemented with 10% fetal calf serum (FCS, Thermo Scientific), CHO-K1 cells were cultured in GMEM (First Link) supplemented with 5% dialyzed FBS (Sigma Aldrich) at 37°C in a humidified 5% CO_2 atmosphere. Cell surface expression of PD-L1 and EGFR were analyzed by flow cytometry using anti-PD-L1-APC and anti-EGFR-FITC antibodies.

CHO.PD-L1 cells stably expressing human PD-L1 were generated by lipofection (Fugene-HD, Promega) with plasmid pCMV6-PD-L1 (Origene). A431.pp65 cells stably expressing CMV pp65 protein were generated by lipofection with plasmid pCMV6-pp65 (Origene). A2058-EpCAM cells stably expressing EpCAM-YFP were generated by lipofection with plasmid pEpCAM-YFP-N1 (kindly provided by Dr. Olivier Gires, Munich, Germany). Clones with stable expression of the indicated transgenes were selected using culture media supplemented with the appropriate antibiotic.

Construction of bsAb PD-L1xEGFR

Recombinant antibody fragments encoding scFvPD-L1 and scFvEGFR were generated by commercial gene synthesis service (Genscript) using published VH and VL sequence data of PD-L1-blocking antibody 3G10 and EGFR-directed antibody mAb 425, respectively. For

construction and production of bsAb PD-L1xEGFR we used eukaryotic expression plasmid pEE14-bsAb,[20] which contains 3 consecutive multiple cloning sites (MCS). MCS#1 and MCS#2 are interspersed by a 22 amino acid flexible linker derived from a CH1 domain. MCS#1, MCS#2 and MCS#3 were used for directional and in-frame insertion of DNA fragments encoding scFvPD-L1, scFvEGFR, and human IgG1, respectively, yielding plasmid pEE14-PD-L1xEGFR. Analogously, we constructed pEE14-PD-L1xMock by replacing scFvEGFR by scFv4-4-20 that is directed against fluorescein.

Eukaryotic production of bsAb PD-L1xEGFR

BsAb PD-L1xEGFR and bsAb PD-L1xMock were produced using the Expi293 expression system according to manufacturer's recommendations (ThermoFisher). Briefly, Expi293 production cells were transfected with plasmid pEE14-PD-L1xEGFR or pEE14-PD-L1xMock and cultured in suspension for 7 days on a shaker platform (125 rpm) at 37°C, 8% CO2. Next, conditioned culture supernatant was harvested and cleared by centrifugation (1,500g, 10 min), after which bsAb PD-L1xEGFR and bsAb PD-L1xMock were purified using an HiTrap protein A HP column connected to ÄKTA Start chromatography system (GE Healthcare Life Sciences).

Assessment of EGFR-specific binding by bsAb PD-L1xEGFR

EGFR-specific binding of bsAb PD-L1xEGFR was assessed by flow cytometry using EGFR-positive cancer cells (A431, FaDu, OVCAR3, HT1080, DLD1, LNCaP) versus EGFR-negative cancer cells (A2058, A375m, A2780, Jurkat). In short, cancer cells were incubated with bsAb PD-L1xEGFR (0.01-10 μg/ml, 45 min at 4°C), washed 3 times with PBS, and then incubated with a PE-conjugated anti-human mAb (45 min at 4°C).

Assessment of functional affinity of bsAb PD-L1xEGFR

Functional affinity (the accumulated strength of affinities for PD-L1 and EGFR) of bsAb PD-L1xEGFR was assessed using a competitive binding assay. In short, $PD\text{-}L1^+/EGFR^+$ A431 cells were pre-incubated (or not) with excess amounts of mAb 425 (50 μg/ml) for 15 min at 4°C, after which bsAb PD-L1xEGFR or bsAb PD-L1xMock was added in a concentration range from 0.01 to 50 μg/ml. After 1h, an APC-labeled PD-L1-blocking antibody (8 μg/ml) was added and allowed to compete for PD-L1 binding for 30 min. Competitive binding of anti-PD-L1-APC to A431 cells was quantified by flow cytometry.

Bioassay for PD-1/PD-L1 blockade

Blockade of PD-1/PD-L1 interaction was assessed using the PD-1/PD-L1 Blockade Bioassay (Promega). This assay uses two engineered cell lines; Jurkat-PD1-NFAT-luc T cells expressing both PD-1 and NFAT-inducible luciferase and CHO-PD-L1-CD3 cells expressing both PD-L1 and a membrane-linked agonistic anti-CD3 antibody. When co-cultured, PD-1/PD-L1 interaction between both cell types inhibits TCR signaling and NFAT-mediated luciferase activity in Jurkat-PD1-NFAT-luc indicator T cells. Addition of a PD-1/PD-L1 blocking antibody results in TCR signaling and subsequent NFAT-mediated luciferase activity in the Jurkat indicator T cells.

Bioassay for EGFR-directed PD-1/PD-L1 blockade by bsAb PD-L1xEGFR

The capacity of bsAb PD-L1xEGFR for EGFR-directed PD-1/PD-L1 blockade was assessed using a modified assay in which CHO-PD-L1-CD3 cells were replaced by A431 cells (EGFR^{pos}/EpCAM^{pos}/PD-L1^{pos}) pretreated with a suboptimal amount of bsAb BIS-1; an EpCAM-directed CD3-agonistic bispecific antibody[21]. The latter results in the decoration of A431 cells with BIS-1 and that endows them with capacity for anti-CD3-mediated TCR triggering of Jurkat-PD1-NFAT-luc indicator T cells. In short, Jurkat-PD1-NFAT-luc T cells were mixed with CHO-PD-L1-CD3 cells or BIS-1-pretreated A431 cells at a cell ratio of 5 to 1 and then cultured for 18h in the presence of bsAb PD-L1xEGFR, bsAb PD-L1xMock or appropriate control antibodies. Subsequently, Bio-Glo reagent was added after which bioluminescence was quantified using a Victor V3 multilabel plate reader (Perkin Elmer).

Assessment of T cell stimulation by bsAb PD-L1xEGFR

PBMCs were obtained from healthy volunteers after informed consent using standard density gradient centrifugation (Lymphoprep) and labeled with carboxyfluorescein succinimidyl ester (CFSE) (CellTracel Proliferation Kit, Invitrogen). CFSE-labeled PBMCs were cultured in a 96-wells plate at a density of 1x10^5 cells/well and stimulated with agonistic anti-CD3 mAb UCHT-1 (0.5 µg/ml). BsAb PD-L1xEGFR, bsAb PD-L1xMock or appropriate control antibodies were added to the wells (5 µg/ml) and T cell proliferation was measured at day 5 by analysis of CFSE dilution by flow cytometry.

Assessment of T cell activation by bsAb PD-L1xEGFR in MLR assay

The capacity of bsAb PD-L1xEGFR to promote activation of T cells was assessed in a mixed lymphocyte reaction (MLR). To this end, monocytes were isolated from PBMCs by adherence to culture flasks followed by culturing in X-VIVO-15 medium (Lonza), supplemented with 2% human serum (HS; PAA laboratories), IL-4 (500 U/ml) and GM-CSF (800 U/ml). After 3 days, MoDCs were matured by continuing culturing for an additional 24h in the presence of IL-1ß (5 µg/ml), IL-6 (15 µg/ml), TNF-α (20 µg/ml), and PGE2 (2.5 mg/ml), essentially as described before (Hobo, 2012). For the MLR, freshly isolated PBMCs were labeled with CFSE, resuspended in RPMI + 10% HS at 2x10^6 cells/ml and then stimulated with allogeneic MoDC in a final volume of 200µl/well using round-bottom 96-well plates (Corning Costar) at T cell to DC cell ratio of 10 to 1. Next, bsAb PD-L1xEGFR, bsAb PD-L1xMock, or appropriate control antibodies were added to the wells (5 µg/ml). After 5 days of co-culturing, spend culture medium was collected and assayed for cytokine secretion. Subsequently, induction of T cell proliferation was evaluated by CFSE dilution analysis using flow cytometry.

Assessment of inhibition of cancer cell proliferation by bsAb PD-L1xEGFR

Cancer cells were pre-cultured in 48-wells plates in RPMI-1640 (or DMEM) containing 10% FBS for 6h at a density of 8.000 cells/well, followed by addition of bsAb PD-L1xEGFR, bsAb PD-L1xMock or appropriate control antibodies (each 5 µg/ml). After 5 d, cancer cell proliferation was determined in a MTS-based colorimetric assay (CellTiter 96, Promega) using a Victor V3 multi-label plate counter (Perkin Elmer) at 490 nM.

Absorbance data for maximum cell death were obtained by control treatment with 70% ethanol for 15 min.

Assessment of promotion oncolytic activity of BIS-1-redirected T cells by bsAb PD-L1xEGFR

T cells were sorted from PBMC by MACS using the human Pan T cell isolation kit according to manufacturer's protocol (Miltenyi Biotec). Sorted T cells were incubated with bsAb BIS-1 (75 ng/ml) and then added to A431, FaDu or A2058.EpCAM cells in a 2 to 1 cell ratio, in the presence or absence of bsAb PD-L1xEGFR, bsAb PD-L1xMock, or appropriate control antibodies (5 µg/ml). At day 3, apoptosis induction in cancer cells (Annexin-V) and CD25 expression on T cells were evaluated by flow cytometry.

Assessment of activation of antigen-experienced T cells bsAb PD-L1xEGFR

We assessed whether bsAb PD-L1xEGFR could promote the cytotoxicity of CMV-specific CD8$^+$ T-cells towards HLA-matched EGFR$^+$ A431.pp65 versus wild type A431 target cells. To this end, A431.pp65 or A431 cells were incubated with bsAb PD-L1xEGFR, bsAb PD-L1xMock or appropriate control antibodies (5 µg/ml), washed to remove unbound antibody and then cultured in 48-wells plates at a density of 3x10^4 cells/well. Freshly isolated PBMCs from a CMV-positive donor were then mixed with cancer cells at cell ratio of 20 to 1. At day 4, the PBMCs were restimulated with A431 or A431.pp65 cells treated (or not) with the indicated antibodies. At day 7, 40% of PBMCs were removed, restimulated for 1h with fresh A431 or A431.pp65 cancer cells, followed by incubation for 16h in the presence of monensin (eBioscience) and anti-CD107a (BD Pharmigen). Subsequently, T-cells were stained with fluorescently-labeled anti-CD3 and anti-CD8 antibodies after which intracellular IFN-γ production was evaluated by flow cytometry using the FIX&PERM® kit (NordicMubi) and anti-IFN-γ antibody labeled with PerCp.Cy5.5 (eBioscience). At day 8, the remaining PBMCs were harvested and analyzed for CD3, CD8, HLA-DR, CD25 and CD137 expression by flow cytometry. The corresponding spend culture supernatants were assayed for cytokine production.

Assessment of NK cell-mediated ADCC by bsAb PD-L1xEGFR

NK cells were sorted from PBMCs using the MagnisortTM Negative Selection kit according to manufacturer's instructions (eBioscience). Sorted NK cells were stimulated for 24h with IL-12 (10 ng/ml) at a density of 1x10^6 cells/ml in RPMI-1640/10% FBS as described previously.[33] NK cells were washed with PBS and then mixed with cancer cells at indicated E to T ratio's and treated with bsAb PD-L1xEGFR, bsAb PD-L1xMock or appropriate control antibodies (5 µg/ml). After 18h of treatment, apoptosis was assessed by flow cytometry using Annexin-V staining.

Alternatively, LNCaP cells were treated with PBMCs at E to T ratio of 5 to 1 and then treated with bsAb PD-L1xEGFR or indicated control antibodies (5 µg/ml). After 48h, ADCC-mediated apoptosis induction in cancer cells was assessed by flow cytometry using Annexin-V staining procedure.

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by Bonferroni post-hoc test, as indicated using Prism software. P<0.05 was defined as a statistically significant difference. Where indicated * = P<0.05; ** = P<0.01; *** = P<0.001.

Results

Eukaryotic production of bsAb PD-L1xEGFR

BsAb PD-L1xEGFR was constructed as a so-called bispecific $(scFv)_4$-IgG1[22], a class of symmetric tetravalent bispecific human IgG1 molecules (Suppl. Fig. 1A), produced in human HEK293 cells and purified by protein A column chromatography.

BsAb PD-L1xEGFR selectively and simultaneously binds to PD-L1 and EGFR

In flow cytometric analysis, bsAb PD-L1xEGFR dose-dependently bound to CHO.PD-L1 cells and not to CHO cells (Fig. 1A). Similarly, bsAb PD-L1xEGFR showed potent binding to EGFR-expressing A431 cells, whereas bsAb PD-L1xMock only minimally bound to A431 cells (Fig. 1B). Binding levels of bsAb PD-L1xEGFR to a panel of $EGFR^+PD\text{-}L1^+$ cell lines closely correlated with respective expression levels of EGFR (Fig. 1C and Suppl. Fig. 1B). In contrast, relative low binding of bsAb PD-L1xEGFR was detected to $EGFR^-PD\text{-}L1^+$ cell lines (Fig. 1C). Furthermore, the binding of bsAb PD-L1xEGFR to A431 cells was strongly inhibited in the presence of excess amounts of parental anti-EGFR mAb 425, whereas presence of excess amounts of a PD-L1-blocking antibody only partly inhibited binding. Importantly, binding of bsAb PD-L1xEGFR to A431 cells was fully abrogated only in the combined presence of excess amounts of a clinically-used PD-L1-blocking antibody and mAb 425 (Fig. 1D), indicating that bsAb PD-L1xEGFR selectively and simultaneously binds to PD-L1 and EGFR.

Enhanced functional affinity of bsAb PD-L1xEGFR towards $PD\text{-}L1^+/EGFR^+$ cancer cells

Functional affinity (avidity) of bsAb PD-L1xEGFR was assessed in a competitive binding assay. The data indicated that bsAb PD-L1xEGFR strongly outperformed bsAb PD-L1x-Mock in preventing the binding of a conventional PD-L1-blocking antibody to $PD\text{-}L1^+/EGFR^+$ A431 cancer cells, with a calculated IC that was at least 100 times lower. The competitive binding capacity of bsAb PD-L1xEGFR for A431 cells was reduced to that of bsAb PD-L1xMock when incubation was performed in the presence of an excess amount of anti-EGFR mAb 425 (Fig. 1E). Together, this indicated that bsAb PD-L1xEGFR has strongly enhanced avidity towards $PD\text{-}L1^+/EGFR^+$ A431 cells compared to bsAb PD-L1x-Mock.

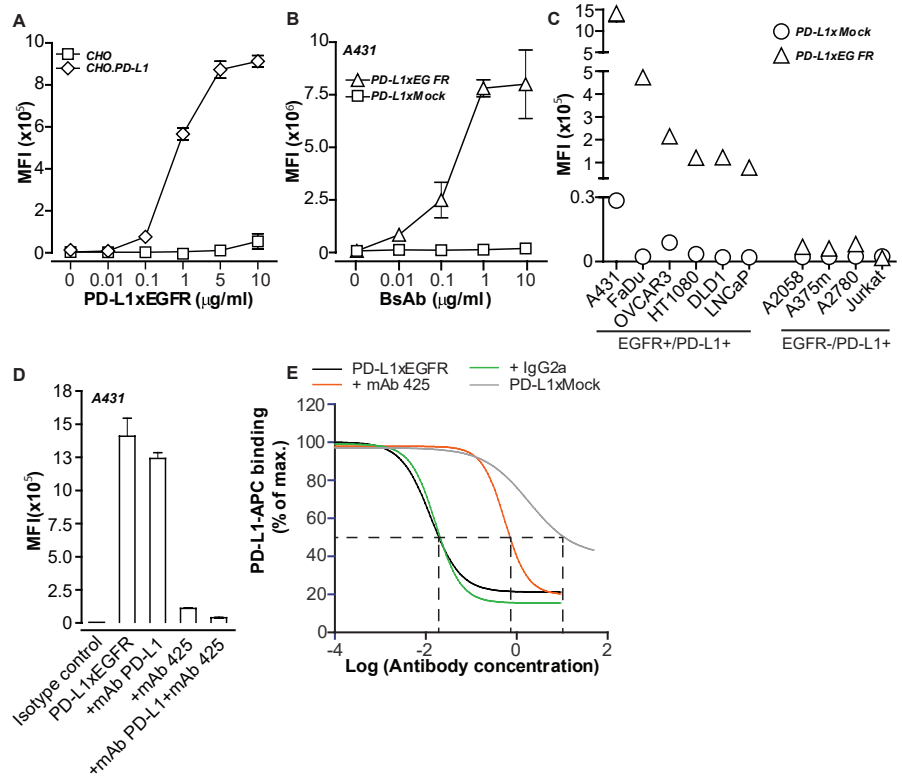


Figure 1: BsAb PD-L1xEGFR selectively and simultaneously binds to PD-L1 and EGFR. **A)** CHO.PD-L1 or parental CHO cells were incubated with an increasing dose of bsAb PD-L1xEGFR and binding was analyzed by flow cytometry. **B)** EGFR-positive A431 cells were incubated with an increasing dose of bsAb PD-L1xEGFR or PD-L1xMock and binding was analyzed by flow cytometry. **C)** Binding of bsAb PD-L1xEGFR or PD-L1xMock to $EGFR^+/PD\text{-}L1^+$ and $EGFR^-/PD\text{-}L1^+$ cell lines was analyzed by flow cytometry. **D)** Binding of bsAb PD-L1xEGFR (1 µg/ml) to A431 cells in the presence or absence of excess mAb PD-L1 and/or EGFR blocking mAb 425 was analyzed by flow cytometry. **E)** Binding of PD-L1-APC to A431 cells in the presence of an increasing dose (0.01-50 µg/ml) of bsAb PD-L1xEGFR or PD-L1xMock was analyzed by flow cytometry. Where indicated, A431 cells were pre-treated with excess EGFR-blocking mAb 425 (50 µg/ml) or isotype control IgG2a 15 min before addition of bsAb PD-L1xEGFR. All graphs represent mean ± SD.

BsAb PD-L1xEGFR and bsAb PD-L1xMock have comparable PD-1/PD-L1 blocking activity

The capacity of bsAb PD-L1xEGFR and bsAb PD-L1xMock to inhibit PD-1/PD-L1 interaction was evaluated using a commercially available PD-1/PD-L1 blockade bioassay. In this assay, both bsAb PD-L1xEGFR and bsAb PD-L1xMock showed similar capacity to dose-dependently release the PD-1/PD-L1 mediated block on TCR signaling in the Jurkat indicator T cells which resulted in prominent induction of luciferase activity (Fig. 2A). In the same assay, equimolar amounts of a clinically used high-affinity PD-L1-blocking antibody appeared to be more potent in blocking PD-1/PD-L1 interaction compared to bsAbs PD-L1xEGFR and PD-L1xMock, whereas an isotype-matched control antibody of irrelevant specificity did not block PD-1/PD-L1 interaction.

BsAb PD-L1xEGFR blocks PD1/PD-L1 interaction in an EGFR-directed manner

Next, we investigated whether EGFR-directed binding by bsAb PD-L1xEGFR also enhanced its capacity to block PD-1/PD-L1 interaction. To this end, we modified the standard PD-1/PD-L1 blockade bioassay by replacing CHO-PD-L1-CD3 cells by EGFR- and PD-L1-expressing A431 cells that were artificially equipped with anti-CD3 agonistic activity. Similar as observed in the standard PD-1/PD-L1 blockade bioassay, co-culturing of anti-CD3-equipped A431 cells with Jurkat indicator T cells resulted in minimal luciferase activity. In the modified assay, bsAb PD-L1xEGFR and a clinically used PD-L1 antibody showed comparable capacity for dose-dependent inhibition of PD-1/PD-L1 interaction as was evident from the comparable increase in luciferase activity (Fig. 2B). Importantly, PD-1/PD-L1 blocking activity of both bsAb PD-L1xMock and isotype-matched control antibody was not enhanced (Fig. 2B). Taken together, the data indicated that bsAb PD-L1xEGFR blocks PD1/PD-L1 interaction in an EGFR-directed manner.

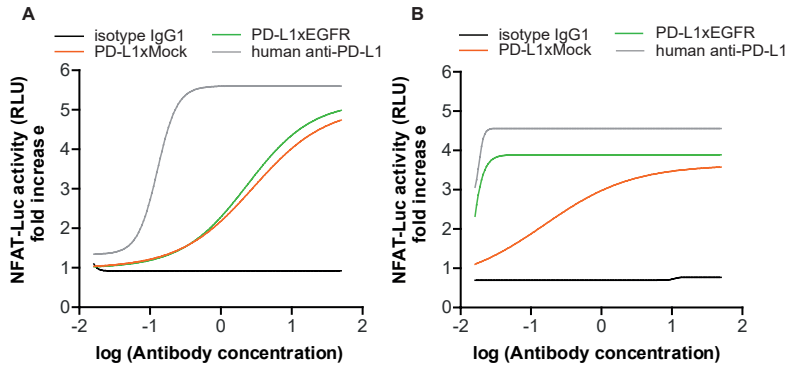


Figure 2: BsAb PD-L1xEGFR blocks the PD-1/PD-L1 interaction **A)** Blockade of the PD-1/PD-L1 interaction was analyzed using the commercially available PD-1/PD-L1 Blockade Bioassay (Promega). Briefly, mixed cultures of CHO-K1.PD-L1 cells and Jurkat.PD-1 cells were treated with an increasing dose (0.01-10 µg/ml) of bsAb PD-L1xEGFR, PD-L1xMock, human anti-PD-L1 mAb or isotype control. NFAT-RE-mediated luciferase activity was quantified using a plate reader and expressed as fold increase compared to medium control. **B)** Similar to A, mixed cultures of EGFR-positive A431 cells and Jurkat.PD-1 cells were treated with an increasing dose (0.01-10 µg/ml) of indicated antibodies in the presence of 75 µg/ml BIS-1.

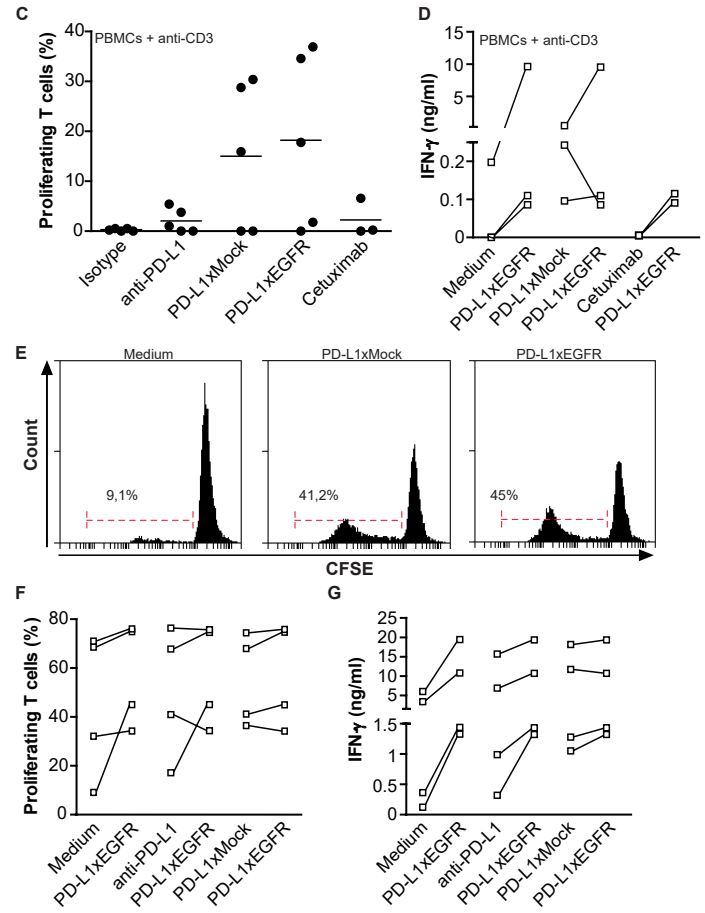


Figure 2 (continued): BsAb PD-L1xEGFR blocks the PD-1/PD-L1 interaction and promotes T cell activation. **C)** CFSE-labeled PBMCs were treated with agonistic CD3 mAb (0.5 µg/ml) in combination with bsAb PD-L1xEGFR, bsAb PD-L1xMock or cetuximab (5 µg/ml). After 5 days, cell proliferation was analyzed by flow cytometry. %proliferation in medium control was subtracted. **D)** IFN-γ levels in culture supernatant of C were determined by ELISA. **E)** Representative histograms of CFSE-labeled PBMCs co-treated with allogenic DCs and bsAb PD-L1xEGFR or bsAb PD-L1xMock. **F)** CFSE-labeled PBMCs were co-treated with allogenic DCs and bsAb PD-L1xEGFR, bsAb PD-L1xMock or anti-PD-L1. After 5 days, cell proliferation was analyzed by flow cytometry. **G)** IFN-γ levels in culture supernatant of F were determined by ELISA. All graphs represent mean ± SD.

BsAb PD-L1xEGFR and bsAb PD-L1xMock promote activity of T cells

Next, the capacity of bsAb PD-L1xEGFR and bsAb PD-L1xMock to activate suboptimal CD3-stimulated T cells was evaluated. The results indicated that bsAb PD-L1xEGFR and bsAb PD-L1xMock have comparable capacity to promote proliferation (Fig. 2C) and IFN-γ secretion (Fig. 2D) by suboptimal CD3-activated T cells. Similar treatment with agonistic anti-CD3 antibody alone or combined with anti-EGFR antibody cetuximab did not significantly enhance proliferation or IFN-γ secretion by T cells. Similarly, in a MLR of CFSE-labeled PBMCs and DCs, bsAb PD-L1xEGFR and bsAb PD-L1xMock showed comparable capacity to promote T cell proliferation (Fig. 2E and F) and IFN-γ production (Fig. 2G)

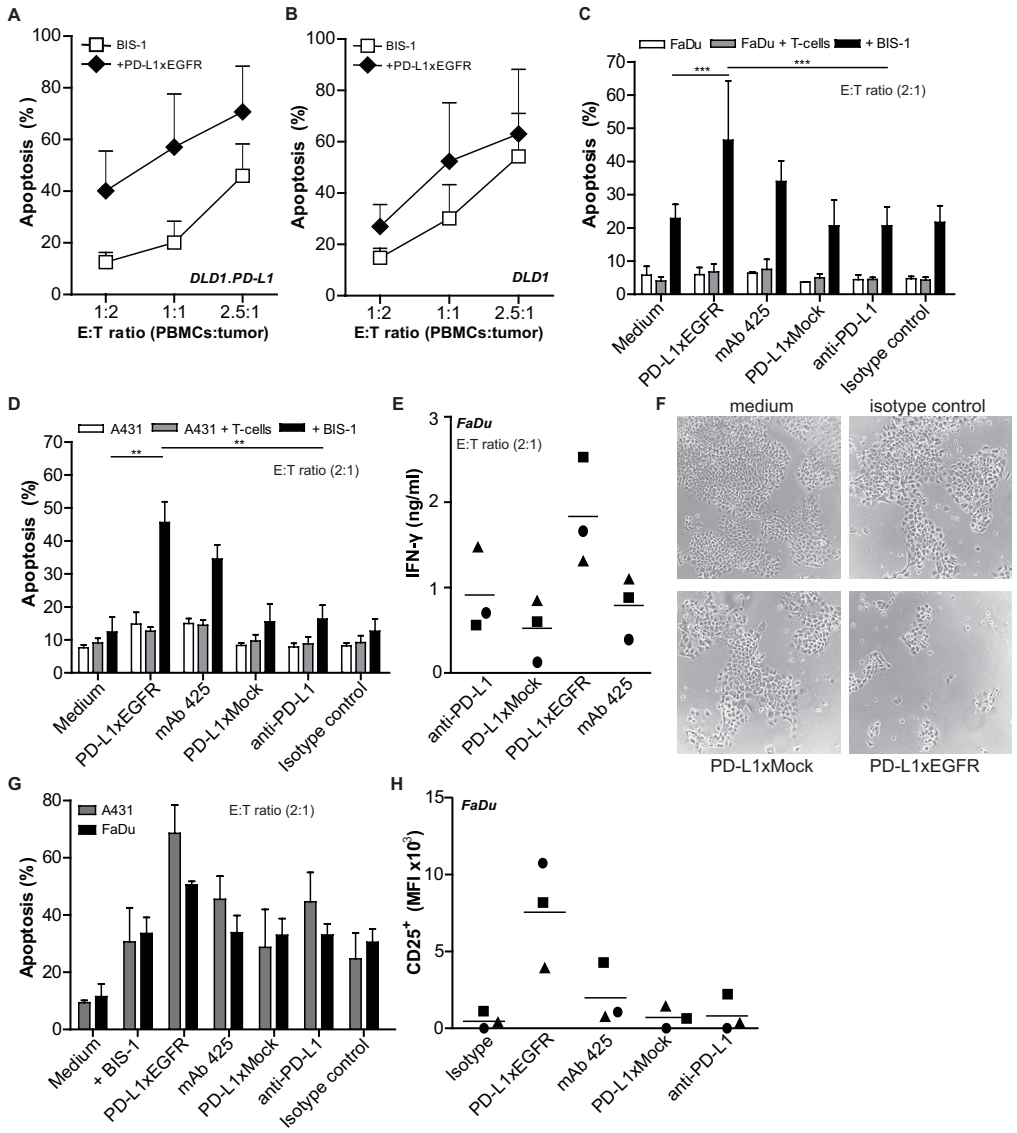
that outperformed a human anti-PD-L1 antibody. Taken together, both bsAb PD-L1xEGFR and bsAb PD-L1xMock promote proliferation and IFN-γ secretion by T cells that is most likely due to their capacity to block PD-1/PD-L1 interaction.

BsAb PD-L1xEGFR promotes cytotoxic activity of BIS-1-redirected T cells

To evaluate whether bsAb PD-L1xEGFR promoted cytotoxic activity, T cells were redirected to EpCAM expressing cancer cells using a suboptimal amount of BIS-1 (an EpCAM-directed CD3-agonistic bispecific antibody.[21] BIS-1-redirected T cells triggered apoptosis in EpCAM$^+$/EGFR$^+$ DLD-1 and DLD-1.PD-L1 colon cancer cells in an E to T ratio-dependent manner (Fig. 3A and B). However, apoptosis induction was significantly enhanced in DLD-1.PD-L1 cells, but not in DLD-1 cells, when the same experiment was performed in the continued presence of bsAb PD-L1xEGFR (Fig. 3A and B). Similarly, BIS-1-treated T cells induced moderate levels of apoptosis in EpCAM$^+$/EGFR$^+$ FaDu cells or A431 cells (~20% and ~15%, respectively; Fig. 3C and D) that was significantly enhanced when treatment was performed in the continued presence of bsAb PD-L1xEGFR (up to 60% in FaDu cells, up to 50% in A431 cells, respectively; Fig. 3C and D). BsAb PD-L1xEGFR-enhanced oncolytic activity of T cells was accompanied by a 2-fold increase in IFN-γ production (Fig. 3E) and significantly enhanced expression of activation marker CD25 (Suppl. Fig. 1C). Treatment of FaDu or A431 with bsAb PD-L1xEGFR alone showed minimal apoptosis induction (Fig. 3C and D), whereas treatment with mAb 425 alone resulted in moderate apoptosis induction up to 35%. The latter is likely the result of the capacity of mAb 425 to block oncogenic EGFR-signaling in cancer cells.

BsAb PD-L1xEGFR promotes cytotoxic activity of BIS-1-redirected T cells in an EGFR-directed manner

To evaluate whether bsAb PD-L1xEGFR promoted cytotoxic activity in an EGFR-directed manner, incubation with the indicated PD-L1-blocking agents was limited to only 1h, after which unbound antibodies were removed by washing. Under these conditions, treatment with bsAb PD-L1xEGFR almost completely eradicated the cancer cell monolayer while control antibodies had a minimal effect (Fig. 3F). Indeed, the data indicated that bsAb PD-L1xEGFR (and not bsAb PD-L1xEGFR or PD-L1 blocking mAb) promoted the pro-apoptotic activity BIS-1-redirected T cells towards EGFR-positive cancer cells (A431 and FaDu, 50% and 80% apoptosis, respectively; Fig. 3G). Importantly, neither bsAb PD-L1xEGFR nor bsAb PD-L1xEGFR promoted cytotoxic activity of T cells towards EGFR-negative A2058.EpCAM cells (Suppl. Fig. 1D). BsAb PD-L1xEGFR-enhanced oncolytic activity of T cells was accompanied by a 3-fold increase in CD25 expression (Fig. 3H). Taken together, bsAb PD-L1xEGFR appears to specifically enhance the anticancer activity of BIS-1-redirected T cells in an EGFR-directed manner.



C) A431 cells were pre-seeded 6h before T-cells were added at E:T ratio 2:1 in the presence of BIS-1 (75 µg/ml) and 5 µg/ml bsAb PD-L1xEGFR, anti-PD-L1, bsAb PD-L1xMock, mAb 425 or isotype control as indicated. After 72h, apoptosis was determined by flow cytometry using Annexin-V staining. **D)** FaDu cells were treated as described in D. **E)** IFN-γ levels in culture supernatant of C were determined by ELISA. IFN-γ levels for isotype control treatment were subtracted. **F)** Before pre-seeding, FaDu cells were loaded with bsAb PD-L1xEGFR or control antibodies as indicated. After 6h, T-cells were added at E:T ratio 2:1 in the presence of BIS-1 (75 ng/ml). After 72h, T cells were carefully washed away and microscopic images of the FaDu monolayer were taken. **G)** In mixed cultures with FaDu cells and A431 cells as described in F, apoptosis was determined by flow cytometry using Annexin-V staining. **H)** In mixed cultures with FaDu cells as described in F, expression of T cell activation marker CD25 was analyzed by flow cytometry. Mean fluorescence intensity (MFI) of BIS-1 treatment alone was subtracted. All graphs represent mean ± SD. Statistical analysis in C and D was performed using One-way ANOVA followed by a Bonferroni post-hoc test. (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 3: BsAb PD-L1xEGFR promotes cytotoxic activity of BIS-1-redirected T cells **A)** DLD1.PD-L1 or **B)** DLD1 cells were pre-seeded 24h before PBMCs were added at indicated effector to target (E:T) ratio's. Cells were co-treated in the presence of BIS-1 (100 ng/ml) with or without 5 µg/ml bsAb PD-L1xEGFR. After 72h, apoptosis was determined by flow cytometry using Annexin-V staining.

BsAb PD-L1xEGFR enhances cytotoxicity of antigen-experienced T cells

Next we assessed whether bsAb PD-L1xEGFR could promote the cytotoxicity of CMV-specific CD8⁺ T-cells towards HLA-matched EGFR⁺ A431.pp65 cells, that express CMV pp65 protein, versus wild type A431 target cells. CMV-specific CD8⁺ T cells showed enhanced cytotoxicity towards A431.pp65 (and not towards A431 cells) that were briefly pretreated with bsAb PD-L1xEGFR. Enhancement of the activity of CMV-specific T cells by bsAb PD-L1xEGFR was accompanied by an increased expression of activation markers CD25, HLA-DR, CD137 and CD107a compared to treatment with control antibodies (Fig. 4A to D). Of note, expression of CD137 is restricted to T cells recently activated through TCR-mediated signaling and as such identified specific activation of CMV-specific CD8⁺ T cells by bsAb PD-L1xEGFR towards A431.pp65 cells. Similarly, upregulation of degranulation marker CD107a indicated a concomitantly increased cytotoxic activity (Aktas 2009). These results were in line with the observed increase in secretion of IFN-γ and granzyme B by CD8⁺ T cells that were co-cultured with bsAb PD-L1xEGFR-pretreated A431.pp65 (Fig. 4E and F). Taken together, these results indicate that bsAb PD-L1xEGFR increases activity of antigen-experienced CD8⁺ T cells by blocking PD-1/PD-L1 in an EGFR-directed manner.

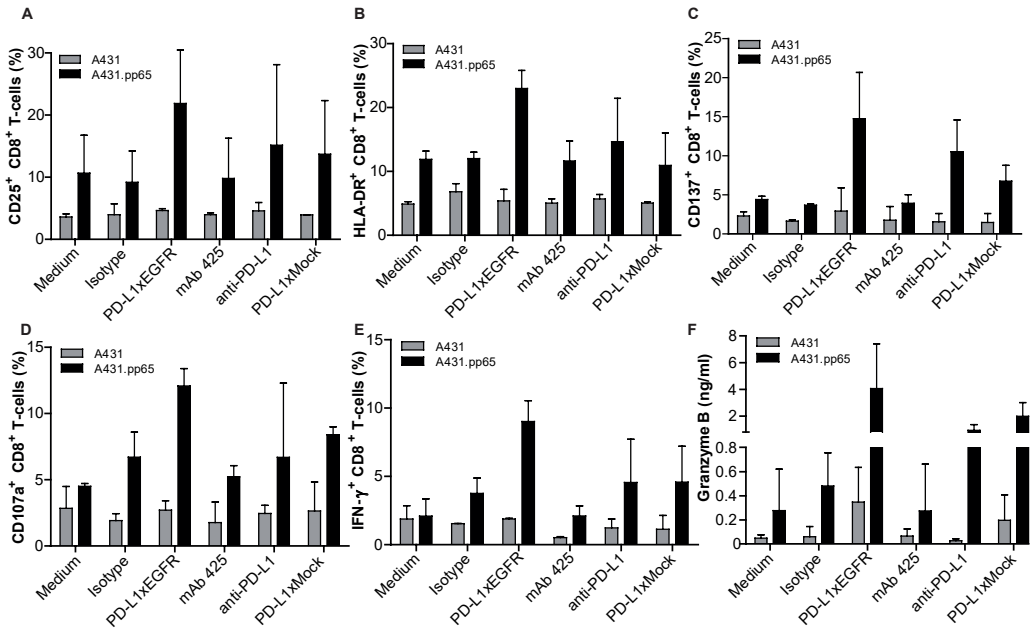


Figure 4: BsAb PD-L1xEGFR enhances cytotoxicity of antigen-experienced T cells **A)** A431 or A431.pp65 cells were incubated with bsAb PD-L1xEGFR or control antibodies as indicated and unbound antibody was washed away before pre-seeding. After 6h, T-cells from CMV-positive donors were added at effector to target (E:T) ratio 20:1. After 8 days, CD25 expression within CD8⁺ T cells was analyzed by flow cytometry. Additionally, **B)** HLA-DR, **C)** CD137, **D)** CD107a expression, and **E)** intracellular IFN-γ within CD8⁺ T cells was analyzed by flow cytometry. **F)** Granzyme B levels within culture supernatants of treatment conditions described in A were determined by ELISA. All graphs represent mean ± SD.

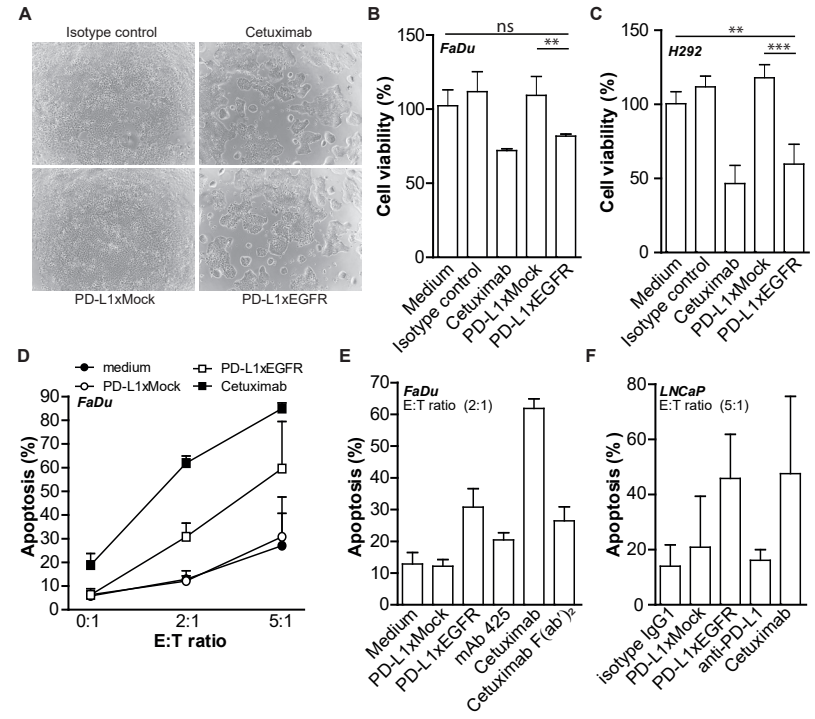


Figure 5: BsAb PD-L1xEGFR blocks oncogenic EGFR-signaling and induces NK-cell mediated ADCC **A)** Representative light microscopy images of FaDu cells after 120h treatment with 5 µg/ml bsAb PD-L1xEGFR, bsAb PD-L1xMock, cetuximab or isotype control as indicated. **B)** Cell viability of FaDu cells as treated in A was determined by MTS and expressed as percentage of medium control. **C)** Cell viability of H292 cells as treated in A was determined by MTS. **D)** FaDu cells were mixed with IL-12 pre-treated NK cells in indicated effector to target (E:T) ratio's in the presence of 5 µg/ml bsAb PD-L1xEGFR or control antibodies as indicated. After 24h, apoptosis was determined by flow cytometry using Annexin-V staining. **E)** FaDu cells were co-cultured with IL-12 pretreated NK cells at E:T ratio 2:1 as described in D. **F)** LNCaP cells were mixed with PBMCs in E:T ratio 5:1 in the presence of 5 µg/ml bsAb PD-L1xEGFR or control antibodies as indicated. After 48h, apoptosis was determined by flow cytometry using Annexin-V staining. All graphs represent mean ± SD. Statistical analysis in B and C was performed using One-way ANOVA followed by a Bonferroni post-hoc test. (*p < 0.05, **p < 0.01, ***p < 0.001, ns not significant).

BsAb PD-L1xEGFR blocks oncogenic EGFR-signaling

EGFR-blocking antibodies mAb 425 and cetuximab can inhibit oncogenic signaling by EGFR. Therefore, the capacity of bsAb PD-L1xEGFR to inhibit cancer cell proliferation of EGFR-expressing cancer cells was investigated. Indeed, both bsAb PD-L1xEGFR and cetuximab inhibited cell growth of FaDu and H292 cells, whereas bsAb PD-L1xMock and isotype control antibody had no effect (Fig. 5A to C). These results indicate that bsAb PD-L1xEGFR inhibits EGFR-mediated oncogenic signaling in cancer cells with an efficacy comparable to that of cetuximab.

BsAb PD-L1xEGFR induces NK cell-mediated ADCC

We investigated whether the human IgG1 domain of bsAb PD-L1xEGFR allowed for inducing NK cell-mediated ADCC towards EGFR-expressing cancer cells. Indeed, NK cell-mediated ADCC towards FaDu and LNCaP cancer cells was enhanced by bsAb PD-L1xEGFR or cetuximab, but not by bsAb PD-L1xMock or murine mAb 425 (Fig. 5D to F). Taken together, these data indicated that bsAb PD-L1xEGFR can induce NK cell-mediated ADCC towards EGFR-expressing cancer cells.

Discussion

PD-1/PD-L1-blocking antibodies can restore the anticancer activity of functionally-impaired antigen-experienced CD8$^+$ T cells and showed significant clinical efficacy in difficult-to-treat advanced stage malignant diseases.[8, 10] However, the benefits of current PD-1/PD-L1 checkpoint inhibition appear to be limited to only a selected group of cancer patients and is accompanied by the occurrence of sometimes severe and irreversible autoimmune-related adverse events.[7, 11, 23] This may be explained by the fact that current PD-1- and PD-L1-blocking antibodies lack intrinsic tumor-directed binding selectivity, whereas PD-1/PD-L1 checkpoint interactions are likely to be widespread in the human body and thus not restricted to the tumor microenvironment. This lack of tumor-selective binding reduces sufficient accretion of PD-1/PD-L1-blocking antibodies in the tumor microenvironment, particularly when local immune checkpoint expression is relatively low. Moreover, PD-1/PD-L1-blocking antibodies indiscriminately reactivate all T cells, including silenced yet potentially highly deleterious autoreactive T cells. In this respect, PD-L1-blocking antibodies generally show less severe side effects than PD-1-blocking antibodies, with e.g. 14% drug-related grade 3-4 adverse events reported for a PD-1-blocking antibody versus 9% for a PD-L1-blocking antibody.[7, 24] Moreover, no drug-related pneumonitis was observed in patients treated with the PD-L1-blocking antibody. In line with this, 3-5% grade 3 but no grade 4 adverse events or treatment-related deaths were observed in patients treated with PD-L1-blocking antibodies avelumab or MEDI4736.[25, 26] However, PD-L1 is constitutively expressed on various non-immune cells such as vascular endothelium, and endothelial and Kupffer cells in the liver.[1, 27] Moreover, PD-L1 expression is upregulated by many cell types in response to IFN-γ secretion during inflammatory responses.[2, 3] Therefore, we reasoned that generation of a PD-L1-blocking antibody format with enhanced tumor-selective binding capacity would have more favorable therapeutic characteristics. In this respect, bispecific antibodies may provide promising possibilities to enhance efficacy and selectivity of therapeutic immune checkpoint inhibition as they can be designed to simultaneously target cancer cells with high affinity and have PD-1/PD-L1 blocking activity.

Here, to the best of our knowledge, we report for the first time on a bispecific antibody (bsAb)-based approach that selectively directs PD-L1 blockade to EGFR-overexpressing cancer cells. EGFR is a well-established target for antibody-based therapy as it is overexpressed by various cancers in which it is involved in oncogenic proliferation and survival signaling. EGFR-specific antibodies, like cetuximab, are in clinical practice in oncology in which their anticancer action comes from their ability to inhibit EGFR-signaling and

induce ADCC.

We constructed bsAb PD-L1xEGFR in the so-called bispecific $(scFv)_4$-IgG1 format, a class of symmetric tetravalent bispecific IgGs in which each of the two arms of the molecule contains two scFv antibody fragments that are linked in tandem and possess different binding specificities.[22] The functional affinity, also known as avidity, of a tetravalent $bs(scFv)_4$-IgG1 is significantly enhanced compared to bivalent monospecific antibodies due to the combined effect of up to 4 individually participating binding affinities. Using various detailed preclinical assays we demonstrated that bsAb PD-L1xEGFR has multiple mutually reinforcing anticancer activities not available in any of the current conventional PD-L1-blocking antibodies. Our data demonstrated that bsAb PD-L1xEGFR (see Fig. 6): simultaneously binds to both PD-L1 and EGFR resulting in an enhanced avidity towards PD-L1$^+$/EGFR$^+$ cancer cells; blocked PD1/PDL-1 interaction, but less potently then a clinically used PD-L1-blocking antibody; blocked PD-1/PD-L1 interaction in an EGFR-directed manner with enhanced avidity; promoted anticancer activity of both BIS-redirected and antigen-experienced T cells in an EGFR-directed manner; blocked oncogenic EGFR-signaling in cancer cells and induced their elimination by NK cell-mediated ADCC.

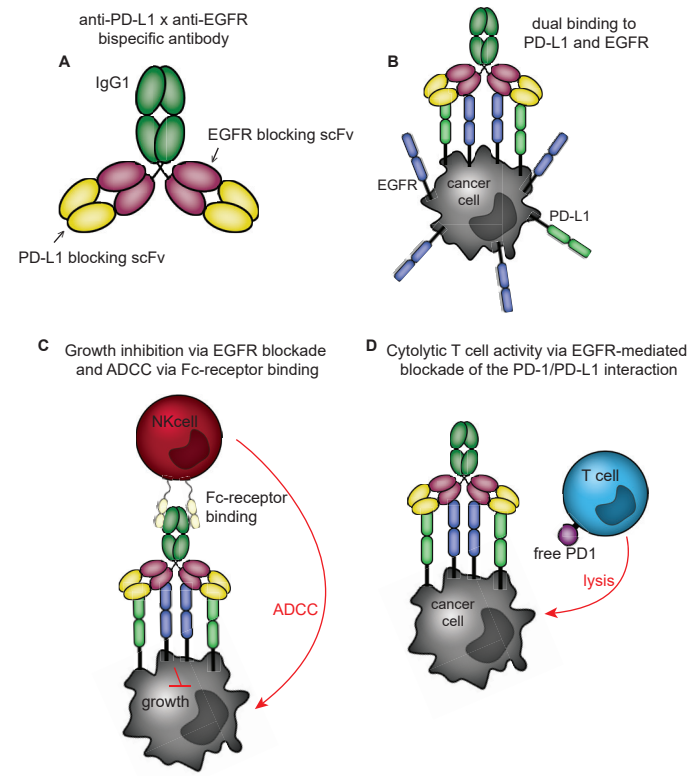


Figure 6: Proposed mechanism of action for bsAb PD-L1xEGFR. **A)** Tetravalent bsAb PD-L1xEGFR comprises EGFR-blocking and PD-L1-blocking antibody fragments (scFv) and a human IgG1 Fc domain. **B)** BsAb PD-L1xEGFR binds to both PD-L1 and EGFR on the cancer cell surface. **C)** BsAb PD-L1xEGFR blocks EGFR-mediated growth signaling and enhances NK-cell mediated antigen-dependent cellular phagocytosis (ADCC) via its IgG1 domain. **D)** BsAb PD-L1xEGFR enhances the antitumor reactivity of T cells towards EGFR$^+$ cells via EGFR-selective blockade of the PD-1/PD-L1 checkpoint.

We expect that these unique features of bsAb PD-L1xEGFR may prove to be of clinical importance for enhancing tumor selectivity, efficacy and safety of PD-1/PD-L1 checkpoint inhibition approaches in EGFR-overexpressing malignancies.

Of particular relevance is the fact that bsAb PD-L1xEGFR enhanced activation of antigen-experienced T-cells in an EGFR-directed manner. In particular, pre-treatment of CMV-pp65 transfected EGFR-positive cancer cells with bsAb PD-L1xEGFR, followed by removing unbound antibody, potently promoted the oncolytic activity of HLA-matched CMV-specific $CD8^+$ T cells, corroborated by an increased expression of CD137, CD107a and IFN-γ production. Previously, it was reported that PD-L1 blocking antibody avelumab has similar *in vitro* capacity to enhance activation of antigen-experienced T-cells directed against CMV, EBV, Flu or tetanus[28], yet obviously not in a tumor-directed manner.

Our data indicated that treatment of EGFR-expressing HNC and NSCLC cell lines with bsAb PD-L1xEGFR alone inhibited oncogenic EGFR-signaling with similar efficacy as EGFR-targeted antibody cetuximab, as was evident from a comparable reduction in cancer cell viability. These results indicated that bsAb PD-L1xEGFR has the combined capacity to block both PD-L1 and EGFR signaling. Recently, a link between EGFR-signaling and upregulation of PD-L1 expression was discovered in HNC and NSCLC.[29-32] Therefore, the simultaneous blockade of EGFR-mediated oncogenic signaling and PD-1/PD-L1 interaction by bsAb PD-L1xEGFR may be of therapeutic interest.

Furthermore, its fully functional human IgG1 domain endowed bsAb PD-L1xEGFR with the potential therapeutic capacity to promote NK cell-mediated ADCC in EGFR-expressing cancer cells. This is in line with recent reports stating that NK cell-mediated ADCC is induced by PD-L1 mAb avelumab (human IgG1)[33, 34] and as such enhanced its anticancer activity. Typically, PD-1/PD-L1-blocking antibodies are engineered to be of the human IgG4 isotype[11,24,35] or to contain an engineered IgG domain with reduced ADCC activity to avoid elimination of PD-1/PD-L1 expressing immune cells.[36, 37] Nevertheless, avelumab showed a toxicity profile comparable to ADCC-null PD-L1-blocking antibodies[25, 38, 39] with only low levels of avelumab-mediated lysis of PBMCs *in vitro*.[28, 33] Since bsAb PD-L1xEGFR selectively targets EGFR-positive cancer cells, we expect that IgG1-mediated ADCC will enhance the efficacy of bsAb PD-L1xEGFR with minor deleterious effects towards immune effector cells.

In conclusion, our results demonstrate that bsAb PD-L1xEGFR has multiple mutually reinforcing anticancer activities not available in any of the current conventional PD-L1-blocking antibodies. Clinical development of this novel approach appears warrented.

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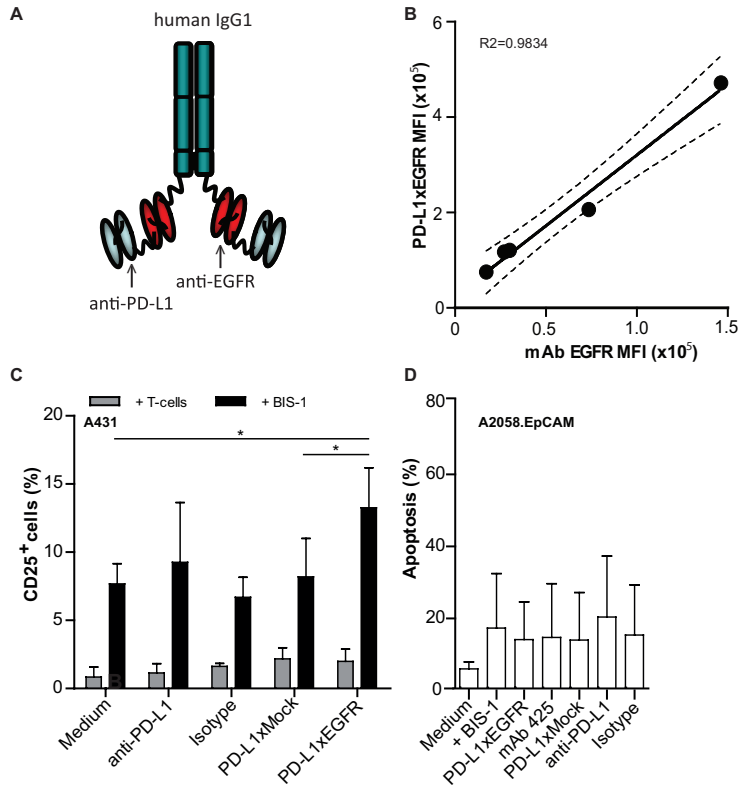

Supplementary Figure 1: A) BsAb PD-L1xEGFR was constructed as a so-called double-variable domain (DVD)-IgG1, a class of symmetric tetravalent bispecific IgGs, and contains EGFR-blocking and PD-L1-blocking antibody fragments (scFv) and a human IgG1 Fc domain. **B)** EGFR-expression and binding of 1 µg/ml bsAb PD-L1xEGFR to EGFR-positive cell lines was assessed by flow cytometry. **C)** A431 cells were pre-seeded 6 h before T-cells were added at effector to target (E:T) ratio 2:1 in the presence of BIS-1 (75 µg/ml) and 5 µg/ml bsAb PD-L1xEGFR, anti-PD-L1, bsAb PD-L1xMock, mAb 425 or isotype control as indicated. After 72 h, expression of T cell activation marker CD25 was analyzed by flow cytometry. **D)** A2058.EpCAM cells were loaded with 5 µg/ml bsAb PD-L1xEGFR, anti-PD-L1, bsAb PD-L1xMock, mAb 425 or isotype control as indicated and pre-seeded 6 h before T-cells were added at E:T ratio 2:1 in the presence of BIS-1 (75 µg/ml). After 72 h, apoptosis was determined by flow cytometry using Annexin-V staining. All graphs represent mean ± SD. Statistical analysis in C was performed using One-way ANOVA followed by a Bonferroni post-hoc test. (*p < 0.05, **p < 0.01, ***p < 0.001).

CHAPTER 6

Summary & Future perspectives

Summary

As outlined in **Chapter 2**, antibody-based cancer therapy has come of age with more than 20 antibodies currently approved and many more in clinical development.[1] However, many of the currently available antibodies are directed against antigens that are overexpressed rather than exclusively present on cancer cells. This lack of authentic tumor-selectivity may explain the observed limitations in clinical efficacy and the sometimes dose-limiting on-target/off-tumor side-effects. A large body of research is ongoing to leverage the specificity of antibodies towards improved cancer-selective therapy. Hereto, antibodies or antibody-derivatives can be equipped with cytotoxic or immunomodulatory domains, such as explored for the cytokine TRAIL in **Chapter 4**.

Alternatively, recombinant antibodies can be engineered to have specificity for two (or even more) tumor-associated target antigens, as explored in **Chapter 5**. The rapid advances in antibody engineering have increased the number of available molecular formats, providing tools to rationally design antibody-based drugs with multiple specificities and/or effector functions. Of note, suitable targets for antibody-based cancer immunotherapy are not limited to tumor antigens, as its efficacy may also be significantly enhanced by targeted blocking or activation of selected antigens on immune cells.

In **Chapter 3**, we describe a novel antibody-based approach to improve the efficacy of TRAIL-R2 agonistic antibodies. In brief, we constructed bispecific antibody (bsAb) MCSPxDR5, a bispecific tetravalent antibody that comprises a scFv antibody fragment derived from tigatuzumab, a clinically evaluated agonistic antibody targeting TRAIL-R2 (DR5), a high-affinity scFv antibody fragment targeting the melanoma-associated chondroitin sulfate proteoglycan (MCSP), and a human IgG1 Fc domain. BsAb MCSPxDR5 induced potent MCSP-restricted TRAIL-R2-mediated apoptosis in a panel of melanoma cell lines and primary patient-derived melanoma cells. BsAb MCSPxDR5 also inhibited colony formation of melanoma cells more efficiently than an agonistic anti-DR5 antibody, suggesting a synergistic effect of combined apoptosis induction via DR5 and simultaneous blockade of MSCP-mediated proliferative signaling by bsAb MCSPxDR5. Importantly, cross-linking of its Fc domain using either artificial cross-linker or Fc receptor positive immune effector cells significantly enhanced its anticancer activity. BsAb MCSPxDR5 also enhanced the anticancer efficacy of NK cells via Fc-mediated induction of antibody-dependent cellular cytotoxicity (ADCC). Taken together, bsAb MCSPxDR5 has promising MCSP- and DR5-restricted anticancer activity, which warrants further development for the treatment of melanoma and other MCSP-positive malignancies.

In **Chapter 4**, we describe a novel checkpoint inhibition strategy that combines reactivation of anticancer immunity via blockade of the PD-1/PD-L1 interaction with simultaneous induction of TRAIL-mediated apoptosis. Hereto, we constructed anti-PD-L1:TRAIL, a bi-functional TRAIL fusion protein that comprises a PD-L1 blocking scFv antibody fragment genetically fused to soluble TRAIL. Fusion protein anti-PD-L1:TRAIL selectively induced apoptosis in PD-L1-positive cancer cell lines and primary patient-derived melanoma cells. At the same time, anti-PD-L1:TRAIL enhanced anticancer activity and IFN-γ production of T cells via blockade of the PD-1/PD-L1 interaction. Since IFN-γ increases PD-L1 expression and sensitizes cancer cells to TRAIL-mediated apoptosis,

anti-PD-L1:TRAIL may trigger a feed-forward loop of increasing IFN-γ, increasing PD-L1 expression and increasing TRAIL sensitivity. In line with this, IFN-γ enhanced the efficacy of anti-PD-L1:TRAIL-mediated apoptosis in cell lines and in primary patient-derived melanoma cells. Additionally, anti-PD-L1:TRAIL was able to convert potentially immunosuppressive PD-L1-expressing immune cells into TRAIL-displaying effector cells that induce TRAIL-mediated apoptosis in cancer cells. In conclusion, fusion protein anti-PD-L1:TRAIL shows promising multi-fold and mutually reinforcing anticancer activity that may provide possibilities to enhance the efficacy of therapeutic PD-1/PD-L1 inhibition alone or in combinatorial strategies.

In **Chapter 5**, we describe a novel bispecific antibody-based strategy for tumor-selective blockade of PD-1/PD-L1 that may increase efficacy and safety of immune checkpoint inhibition. Hereto, we constructed recombinant bsAb PD-L1xEGFR that comprises a PD-L1-blocking scFv antibody fragment, an epidermal growth factor receptor (EGFR)-targeted scFv antibody fragment, and a human IgG1 Fc domain. BsAb PD-L1xEGFR was designed to selectively block the PD-1/PD-L1 interaction in an EGFR-directed fashion. BsAb PD-L1xEGFR inhibited the PD-1/PD-L1 interaction on EGFR-positive cells with similar efficacy as a conventional PD-L1-blocking antibody. Importantly, our data showed that treatment of EGFR and PD-L1-positive cancer cells with bsAb PD-L1xEGFR resulted in EGFR-directed blocking of PD-L1, which resulted in both enhanced anticancer activity and IFN-γ production of T cells. Additionally, bsAb PD-L1xEGFR enhanced NK cell-mediated ADCC towards cancer cells via its human IgG1 Fc domain and reduced the viability of EGFR-positive cancer cells by blocking oncogenic EGFR-signaling. Indeed, bsAb PD-L1xEGFR selectively enhanced the anticancer activity of T cells towards EGFR-positive cells and as such outperformed a PD-L1-blocking antibody that is in clinical use. In conclusion, the promising multi-fold EGFR-restricted anticancer activity of bsAb PD-L1xEGFR may provide possibilities to improve clinical efficacy and reduce side effects compared to conventional PD-1/PD-L1 antibodies.

Future perspectives

Antibody-based blockade of immune checkpoint molecules such as CTLA-4 and PD-1/PD-L1 has emerged as a promising strategy to improve anticancer immune responses.[2] In particular, antibodies that block the PD-1/PD-L1 checkpoint have triggered unprecedented curative anticancer immunity, most notably in advanced melanoma.[3, 4] However, since PD-1 and PD-L1 are broadly expressed on normal tissues, antibodies blocking the PD-1/PD-L1 interaction are not inherently tumor-selective. This can result in severe autoimmune-related side effects in the skin, gastrointestinal tract, liver and lungs as observed for PD-1 blocking antibodies nivolumab and lambrolizumab.[4-6] Additionally, despite their unprecedented response rates, only a subset of patients responds to single treatment with currently available PD-1 and PD-L1 blocking antibodies.[3, 4] Hence, there is a clear rationale to improve the efficacy of PD-1/PD-L1 blocking immunotherapy.

Based on our results in **Chapter 4-5**, we speculate that bsAb PD-L1xEGFR and fusion protein anti-PD-L1:TRAIL may be able to enhance therapeutic efficacy and reduce side effects of PD-1/PD-L1 blockade in cancer immunotherapy. Our *in vitro* results showed

that bsAb PD-L1xEGFR selectively delivers PD-L1 blockade to EGFR-positive target cells (**Chapter 5**). We expect that EGFR-directed blockade of the PD-1/PD-L1 checkpoint will result in less on-target/off-tumor side effects compared to currently available PD-1/PD-L1-blocking antibodies. Moreover, we showed that bsAb PD-L1xEGFR has additional anticancer activities, including blockade of pro-tumorigenic EGFR-signaling and induction of Fc-mediated ADCC. The multi-fold modes-of-action of bsAb PD-L1xEGFR may be responsible for its potent anticancer activity that appears to outperform a clinically used conventional PD-L1-blocking antibody.

Alternatively, the efficacy of PD-1/PD-L1 targeted therapy may be enhanced using fusion proteins that combine PD-1/PD-L1 blockade with a tumor-selective immune effector molecule such as TNF-related Apoptosis Inducing Ligand (TRAIL). In **Chapter 4**, we showed that TRAIL fusion protein anti-PD-L1:TRAIL efficiently combines reactivation of anticancer immunity via blockade of the PD-1/PD-L1 interaction with simultaneous induction of TRAIL-mediated apoptosis in cancer cells. The multi-fold and mutually reinforcing anticancer activities of anti-PD-L1:TRAIL appeared superior to the anticancer activity of conventional PD-L1-blocking antibodies. We therefore speculate that anti-PD-L1:TRAIL may be of use in PD-1/PD-L1-blocking immunotherapy for obtaining clinical efficacy at reduced dosages, thereby limiting side effects.

Reduction of the on-target/off-tumor side effects observed for current PD-1/PD-L1 blocking antibodies is particularly relevant in combinatorial regimes with other immunotherapeutic strategies, such as adoptive transfer of autologous tumor-infiltrating lymphocytes (TILs) or T cells that have been genetically engineered to express a chimeric antigen receptor (CAR), as discussed later.

Adoptive transfer of autologous TILs can induce durable MHC-restricted responses in patients with metastatic melanoma.[7] Such TILs are harvested after surgical resection, expanded and tested for appropriate anticancer activity and then re-infused into the same patient. However, such TILs are characterized by expression of PD-1,[8] implicating that their anticancer may be hampered by PD-1/PD-L1 interaction in the tumor microenvironment. This forms a rationale to combine adoptive transfer of autologous TILs with PD-1/PD-L1-blocking immunotherapy. In line with this, TILs from mice that were pretreated with a PD-L1-blocking antibody showed enhanced anticancer efficacy after adoptive transfer.[9] We speculate that combining adoptive transfer of TILs with tumor-selective blockade of PD-1/PD-L1 interaction (e.g. using a melanoma-directed PD-L1-blocking bsAb) may result in enhanced efficacy and possibly a more favorable safety profile compared to combinations with conventional PD-1/PD-L1-blocking antibodies.

CAR T cells are generated by transfection of peripheral blood T cells from a patient with a CAR construct that comprises an extracellular scFv domain, a transmembrane domain, and one or more intracellular co-stimulatory domains.[10] As a result, CAR T cells are redirected to cancer cells irrespective of their endogenous TCR specificity in a MHC-non-restricted manner. Upon binding to the tumor-associated target antigen, CAR T cells are engaged to proliferate and kill the targeted tumor cells. Thus, CAR T cells harness the capacity of both antibody-mediated target antigen recognition and selective delivery of

the full cytotoxic T cell armament. However, CAR T cells are typically directed against tumor-associated antigens that are overexpressed on cancer cells, but that may also be expressed at lower levels on normal tissues. Consequently, CAR T cell treatment often shows deleterious on-target/off-tumor activity towards normal cells that express these target antigens.[11] Moreover, in mouse models with established tumors, it was identified that several tumor-related factors may limit the clinical activity of CAR T cells,[12, 13] including PD-1/PD-L1 interaction in the tumor microenvironment. Of note, co-treatment of CAR T cells with conventional PD-1/PD-L1 blocking antibodies is likely to further enhance on-target/off-tumor side effects. Based on our results in **Chapter 4-5**, we speculate that combinatorial regimes with PD-L1xEGFR or anti-PD-L1:TRAIL and CAR T cells may be of use to enhance therapeutic efficacy while reducing side effects and as such may outperform combinations with conventional PD-1/PD-L1- blocking antibodies.

Of note, several strategies have been applied to reduce on-target/off-tumor side effects of CAR T cells. These include incorporation of 'ON-switches' for suicide genes that allow for controlled shut-down of CAR T cells[14, 15] and dual specificity CARs that redirect T cells to two different tumor antigens.[16, 17]

Recently, the use of so-called synthetic Notch (synNotch) receptors has been described. Using this approach, T cells can be engineered to only express the CAR in the tumor environment.[18] Binding of the synNotch receptor to its corresponding target antigen expressed by cancer cells induces cleavage of its intracellular domain, thereby releasing a transcription factor that initiates transcription of the CAR. Thus, such T cells only express the CAR after initial synNotch recognition at the tumor site. Other potential applications of the synNotch system included targeted therapeutic delivery of the apoptosis-inducing ligand TRAIL and release of therapeutic antibodies,[19] including αPD1, αCTLA4, and bispecific T-cell engagers (BiTE, see later). We hypothesize that the therapeutic potential and safety of this synNotch system can be enhanced by using tumor-directed scFv:TRAIL fusion proteins or tumor-directed PD-1/PD-L1 blocking bsAbs like PD-L1xEGFR.

In cancer immunotherapy, BiTEs represent an established antibody-based strategy to redirect T cells to attack cancer cells. In a BiTE, a CD3-targeted scFv antibody fragment is fused to a cancer-cell targeted scFv antibody fragment. Treatment with a given BiTE retargets and activates CD3-positive T cells to kill cancer cells that express the corresponding target antigen in a MHC-independent manner, irrespective of intrinsic TCR specificity. However, repeated challenging of BiTE-redirected T cells with fresh tumor cells resulted in a reduced killing capacity and upregulation of PD-1.[20] Correspondingly, blockade of the PD-1/PD-L1 interaction improved the *in vitro* efficacy of BiTEs targeted to CEA- and CD33-expressing cancer cells.[20, 21]

Of note, increased PD-L1 expression was observed in patients with acute lymphoblastic B-cell leukemia (B-ALL) that were resistant to treatment with blinatumomab, a CD19-targeted BiTE.[22] Hence, there is a clear rationale to combine blinatumomab treatment with blockade of the PD-1/PD-L1 interaction. We speculate that combinations of blinatumomab and tumor-selective blockade of the PD-1/PD-L1 interaction (e.g. using a leukemia-directed PD-L1 blocking bsAb) may help overcome the above-mentioned

therapy resistance and reduce side effects compared to combinations with conventional PD-1/PD-L1-blocking antibodies.

In **Chapter 4**, we present a novel PD-1/PD-L1-blockade approach that is based on the unique features of TRAIL fusion protein anti-PD-L1:TRAIL that efficiently combines reactivation of anticancer T-cells with induction of tumor-selective TRAIL-mediated apoptosis. Previously, we and others have demonstrated that scFv:TRAIL fusion proteins gain full pro-apoptotic activity only after tumor-selective binding via its tumor-directed antibody fragment.[23, 24] Furthermore, upon binding to target antigen-positive cancer cells, scFv:TRAIL fusion proteins may also trigger apoptosis in neighboring target antigen-negative cancer cells (Figure 1). This so-called bystander effect, can potentially reduce the risk of escape of target antigen-negative cancer cells from targeted therapy.[25]
An appealing feature of anti-PD-L1:TRAIL is that it can convert potentially immunosuppressive PD-L1-expressing immune cells into TRAIL-displaying effector cells able to induce TRAIL-mediated apoptosis in cancer cells. This is particularly relevant for various types of PD-L1 expressing myeloid-derived suppressor cells which are frequently present in the tumor microenvironment.[26, 27] The presence of such myeloid-derived suppressor cells is associated with poor prognosis in several cancer types.[28, 29] Although not investigated here, treatment with anti-PD-L1:TRAIL may inhibit PD-L1-expressing myeloid-derived suppressor cells while simultaneously inducing an anticancer bystander effect in neighboring cancer cells that express TRAIL-Receptors (Figure 1).

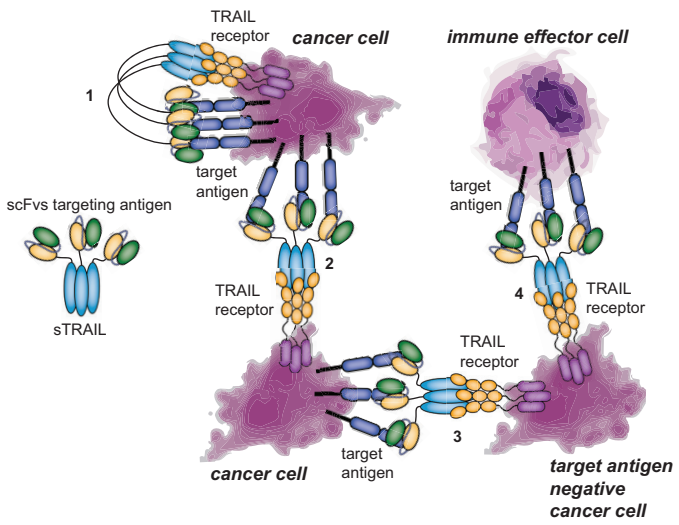

Figure 1: Schematic representation of the multiple modes-of-action of TRAIL fusion proteins. Upon binding of its scFv domain to the relevant target antigen, TRAIL fusion proteins activate TRAIL receptor-mediated apoptosis in the same cell **(1)** and in surrounding cancer cells **(2)**. Importantly, once bound to the target antigen, displayed TRAIL fusion proteins can also activate TRAIL receptors on antigen negative cancer cells **(3)** in a so-called bystander effect. Similarly, the bystander effect can be exploited by immune effector cells that express the target antigen **(4)**.

We have previously demonstrated that selective arming of immune effector cells such as T cells and granulocytes with an appropriate scFv:TRAIL fusion protein potently enhances their anticancer activity towards cancer cells with no or only minimal toxicity towards normal cells.[30, 31] Since there are multiple inhibitory interactions that regulate T cell responses it may be of interest to also engineer TRAIL fusion proteins that target and block checkpoint molecules expressed on T cells such as PD-1, CTLA-4, TIM-3 and LAG-3.

The anticancer efficacy of both recombinant soluble TRAIL and agonistic antibodies specific for TRAIL-R1 or TRAIL-R2 have been evaluated in early stage clinical trials in which favorable safety profiles were observed.[32] However, these first-generation TRAIL-R agonists had limited clinical efficacy, possibly due to intrinsic resistance to TRAIL and/or acquired resistance upon treatment with TRAIL-R agonists. Furthermore, TRAIL receptors are ubiquitously expressed throughout the human body,[33] limiting the accumulation of TRAIL-R agonists at the tumor site. Of note, unlike membrane-bound TRAIL, soluble TRAIL requires cross-linking to efficiently activate TRAIL-R2. Similarly, conventional TRAIL-R2 targeted antibodies appear to require additional cross-linking by Fc-receptor positive cells for effective induction of apoptosis.[24] However, ubiquitous maximal cross-linking and signaling through TRAIL-R2 may be associated with unwanted side effects. For example, nanobody TAS266, that did not require secondary cross-linking for efficient TRAIL-R-mediated apoptosis induction,[34] showed hepatotoxicity in a phase I clinical trial, leading to termination of the trial.[35]
In **Chapter 3**, we demonstrate that tumor-selective activation of TRAIL-R2 (DR5) can be achieved with a bispecific antibody directed against melanoma-associated antigen MCSP. BsAb MCSPxDR5 has high binding specificity for MCSP and potent TRAIL-R2-activating activity only towards MCSP-expressing cancer cells. We showed that Fc-mediated cross-linking could further enhance the anticancer activity of bsAb MCSPxDR5. Moreover, bsAb MSCPxDR5 additionally enhanced the anticancer efficacy of NK cells via Fc-mediated induction of ADCC. BsAb MCSPxDR5 showed minimal activity towards normal cells and Fc-mediated cross-linking did not enhance activity towards MCSP-negative cells. Thus, we expect enhanced anticancer activity of bsAb MCSPxDR5 compared to conventional TRAIL-R2 agonistic antibodies, possibly with a more a favorable safety profile. BsAb MCSPxDR5 may be of particular use for treatment of melanoma and other MCSP-positive malignancies.

To further enhance its anticancer activity, bsAb MCSPxDR5 may be incorporated into a combinatorial regime, for example with anticancer agents that upregulate TRAIL-Receptor expression, sensitize cancer cells to TRAIL-R-mediated apoptosis or overcome intrinsic TRAIL resistance mechanisms, such as chemotherapeutics and small molecule inhibitors. For example, RAF inhibitors could overcome TRAIL resistance in melanoma cells *in vitro*.[36] Furthermore, synergy between a TRAIL-R1 agonistic antibody and chemotherapeutic agents gemcitabine and cisplatin was observed in a Phase I clinical trial.[37] In line with this, TRAIL-R2 targeted bispecific antibody RG7386 showed synergy with chemotherapeutic agents doxorubicin and irinotecan in mouse models.[38] Indeed, we observed

synergy of bsAb MCSPxDR5 with several anticancer agents *in vitro*, including epigenetic drug valproic acid that has shown potent anticancer activity in pre-clinical studies.[39]

The therapeutic activity of antibodies is strongly influenced by the interaction of their Fc domain with the appropriate Fc receptors (FcR) on immune effector cells. Therefore, engineering of the IgG domain has been used to modify Fc-mediated effector mechanisms such as ADCC, ADCP and CDC. For example, interaction with inhibitory receptor FcγRIIb was found to be critical for agonistic antibodies that target TNFR family members such as TRAIL-R2 or co-stimulatory TNF family members such as CD40.[40, 41] In line with this, mutations in the IgG1 domain that caused a 200-fold increase in affinity for FcγRIIb enhanced the agonistic activity of a 4-1BB targeted antibody.[42] Incorporation of such IgG1 mutations into the Fc domain of bsAb MCSPxDR5 may be of interest to further enhance its anticancer activity.
In contrast, binding to FcγRIIb reduces the therapeutic efficacy of antibodies that directly target antigens on cancer cells as FcγRIIb is an important negative regulator of ADCC.[43] Therefore, such antibodies have been engineered to have a mutant IgG1 domain, which preferentially binds to activating FcγRs over FcγRIIb.[44] Interestingly, mutations in the IgG1 domain that enhanced the affinity to all FcγRs yielded up to a 100-fold increase in ADCC.[45] Therefore, incorporation of such IgG1 mutations into bsAb PD-L1xEGFR may be of interest too. Since bsAb PD-L1xEGFR showed EGFR-restricted anticancer activity, we expect that IgG1 engineering can enhance its tumor-selective activity without increasing off-target side effects.
Of note, current PD-1/PD-L1 blocking antibodies are typically engineered to have a human IgG4 isotype[3, 46, 47] or contain a "silenced" IgG1 domain[48, 49] to avoid elimination of PD-1/PD-L1 expressing immune cells by ADCC. However, recent reports showed that PD-L1-blocking antibody avelumab, that contains a fully functional human IgG1 domain, induced only low levels of ADCC-mediated lysis in PBMCs *in vitro*.[50, 51] Moreover, recent studies with avelumab in carcinoma patients showed promising response rates with a toxicity profile comparable to other PD-1/PD-L1-blocking antibodies.[52-54]

Despite recent advances, treatment of cancer is often not curative. New insights indicate that this may be attributable to a small population of therapy-resistant malignant cells with self-renewal capacity and the ability to generate large numbers of more differentiated cancer cells. These cancer-initiating cells are commonly referred to as cancer stem cells (CSCs). CSCs are regarded as the root of cancer origin and recurrence after seemingly successful therapy. Not surprisingly therefore, current and future cancer research is focused on ways to specifically eliminate CSCs.
In hemato-oncology, various CSC-associated surface antigens have been identified that may allow for CSC-selective therapy while sparing normal hematopoietic stem cells.[55] In this respect, it is worth mentioning that CSCs in selected cancers were found to be susceptible to TRAIL-mediated apoptosis,[56, 57] especially while simultaneously blocking pro-survival IL-4 signaling.[58] It may therefore be of value to construct a DR5-agonistic bispecific antibody with IL-4R-blocking capacity. More recently, it was reported that CSCs

in breast and colon cancer expressed elevated levels of PD-L1 compared to non-stem like cancer cells.[59] These results suggest that breast and colon cancer CSCs may be sensitive to PD-1/PD-L1 immunotherapy. In this respect, it appears of interest to develop bsAbs that allow for CSC-directed PD-1/PD-L1 blockade.

In conclusion, the novel antibody-based approaches described in this thesis appear to have promising anticancer activity that warrants further clinical development. Tumor-directed activation of pro-apoptotic TRAIL-Receptor signaling and/or tumor-directed blocking of the PD-1/PD-L1 checkpoint axis has the potential to enhance therapeutic efficacy and reduced side effects of current agents. BsAb PD-L1xEGFR, bsAb MCSPxDR5 and fusion protein anti-PD-L1:TRAIL each have promising multi-modal anticancer activities that may be further enhanced using rationally designed combinatorial therapeutic strategies.

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CHAPTER 7

Nederlandse samenvatting

Algemene introductie

In tegenstelling tot wat veel mensen denken is kanker geen moderne ziekte, maar is zij al bekend sinds de oudheid. Zo is er bijvoorbeeld bewijs voor tumoren aangetroffen in Egyptische mummies. Kanker komt tegenwoordig wel veel vaker voor dan in de oudheid. Alleen al in 2012 werden 14 miljoen nieuwe gevallen van kanker vastgesteld en overleden er wereldwijd 8 miljoen mensen aan kanker. De verwachting is dat het aantal nieuwe kankerpatiënten met 70% zal toenemen in de komende twee decennia. Kanker is dan ook een van de meest voorkomende doodsoorzaken wereldwijd.

Dankzij veel onderzoek is het begrip van kanker de laatste decennia flink toegenomen. Kort samengevat zijn kankercellen ontspoorde lichaamseigen cellen die bestaande biologische routes misbruiken om onder andere snel te groeien, zich oneindig te blijven vermenigvuldigen en aan het immuunsysteem te ontsnappen. Het immuunsysteem is continu bezig met het opsporen en elimineren van potentieel kwaadaardige cellen. Kankercellen zijn echter genetisch instabiel, waardoor ze nieuwe eigenschapen kunnen verwerven door een opeenstapeling van mutaties. Om tot de ziekte kanker uit te kunnen groeien, hebben kankercellen tijdens dit proces eigenschappen verworven die er uiteindelijk voor hebben gezorgd dat ze aan het immuunsysteem konden ontsnappen.

Dit proces wordt 'immunoediting' genoemd en is in drie fasen in te delen. In de eerste fase (eliminatie) is het immuunsysteem nog in staat om kankercellen te herkennen en te elimineren. In de tweede fase (equilibrium) is er een dynamisch evenwicht tussen kankercellen en het immuunsysteem waardoor tumorgroei wordt beperkt. In deze fase is er geen sprake van een detecteerbare tumor en er wordt dan ook gedacht deze evenwichtsfase soms decennialang onopgemerkt kan voortduren. Helaas ontstaat door de voortdurende selectiedruk uiteindelijk een kankercel (of meerdere typen kankercellen) die niet (meer) herkend worden door het immuunsysteem. Dit leidt tot de derde fase (escape) waarin kankercellen ontsnappen aan het immuunsysteem. In deze laatste fase kunnen kankercellen zich ongecontroleerd gaan delen waardoor grotere detecteerbare tumoren en uiteindelijk ook uitzaaiingen kunnen ontstaan.

Het is de laatste jaren steeds duidelijker geworden dat kankercellen niet alleen aan een antikanker immuunreactie kunnen ontsnappen, maar deze ook actief kunnen onderdrukken. Er zijn dan ook diverse strategieën ontwikkeld om dit tegen te gaan, ook wel immuuntherapie genoemd. Het uiteindelijke doel van immuuntherapie is om de antikanker immuunreactie weer effectief aan te zetten en patiënten te genezen. Vooral immuuntherapie die zich richt op het blokkeren van zogenaamde immuun 'checkpoint' interacties heeft veelbelovende resultaten behaald, voornamelijk in patiënten met vergevorderde huidkanker (melanoom).

Immuun 'checkpoint' moleculen komen echter niet alleen op kankercellen voor en algehele (re)activatie van immuuncellen kan daardoor tot heftige immuun-gerelateerde bijwerkingen leiden. Bovendien hebben veelgebruikte therapieën zoals radio- en chemotherapie veel bijwerkingen doordat ze zich richten op álle snelgroeiende cellen in het lichaam. Gelukkig hebben zowel het verbeterde begrip van kanker als doorbraken

in de biotechnologie ertoe geleid dat er steeds meer therapeutische opties beschikbaar zijn die zich selectief op de kankercellen richten, waaronder antilichaamtherapie. In dit proefschrift worden nieuwe antilichaam-gebaseerde moleculen beschreven die zijn ontworpen om de tumor-selectieve werking van immuuntherapie te verbeteren.

Antilichaam-gebaseerde kankertherapie

Antilichamen richten zich op zogenaamde tumor antigenen, moleculen die verhoogd tot expressie komen op de celmembraan van kankercellen en/of door mutaties veranderd zijn in vergelijking met normale cellen. Een antilichaam heeft een hoge affiniteit en specificiteit voor een bepaald tumor antigen, eigenschappen die het mogelijk maken om medicijnen te ontwikkelen die selectief kankercellen aanpakken. Er zijn diverse vormen van antilichaam-gebaseerde therapieën en in **Hoofdstuk 2** wordt een overzicht gegeven van recente ontwikkelingen op dit gebied.

Helaas blijken er vrijwel geen antigenen te bestaan die exclusief op kankercellen voorkomen. De meeste op dit moment beschikbare antilichamen richten zich daardoor op tumor antigenen die méér voorkomen op kankercellen ten opzichte van normale cellen. Dit betekent echter dat zulke antilichamen effect kunnen hebben op normale cellen waarop hetzelfde antigen ook voorkomt en dit kan leiden tot ongewenste bijwerkingen.

De tumorselectiviteit van antilichamen kan worden verbeterd door zogenaamde bispecifieke antilichamen te gebruiken die zich op twee tumor antigenen tegelijkertijd richten. Het idee hierachter is dat de kans dat twee tumor antigenen op gezonde cellen voorkomen veel kleiner is dan bij één tumor antigen. Antilichaamtherapie met bispecifieke antilichamen richt zich dus selectiever op kankercellen en zal daardoor minder bijwerkingen in gezonde cellen veroorzaken.

Een bispecifiek antilichaam kan zich echter ook richten op een combinatie van een tumor antigen op kankercellen en een target op immuuncellen. Er zijn op dit moment twee bispecifieke antilichamen op de markt die zich richten op een T-cel activerend signaalmolecuul (CD3) en een tumor antigen. Dit soort antilichamen activeert T-cellen alleen als ze in contact komen met kankercellen die het tumor antigen bevatten en leidt zo tot selectieve eliminatie van deze kankercellen. Deze strategie heeft voornamelijk in leukemiepatiënten veelbelovende resultaten behaald.

Antilichamen kunnen ook worden gebruikt om therapeutische ladingen af te leveren op plek van de tumor. Cytokines bijvoorbeeld, zijn belangrijke signaalmoleculen van het immuunsysteem die significante antikankeractiviteit kunnen hebben. Echter, doordat cytokines geen inherente tumor-selectieve eigenschappen hebben, is de dosis waarbij cytokines een therapeutisch effect hebben vaak zo hoog dat er heftige bijwerkingen optreden. Hierdoor zijn veel cytokines niet geschikt als medicijn. Door de therapeutische activiteit van cytokines te combineren met de tumor selectiviteit van antilichamen met behulp van zogenaamde antilichaam-cytokine fusie-eiwitten, kunnen cytokines selectief op de plaats van de tumor afgeleverd worden en kunnen heftige bijwerkingen worden voorkomen.

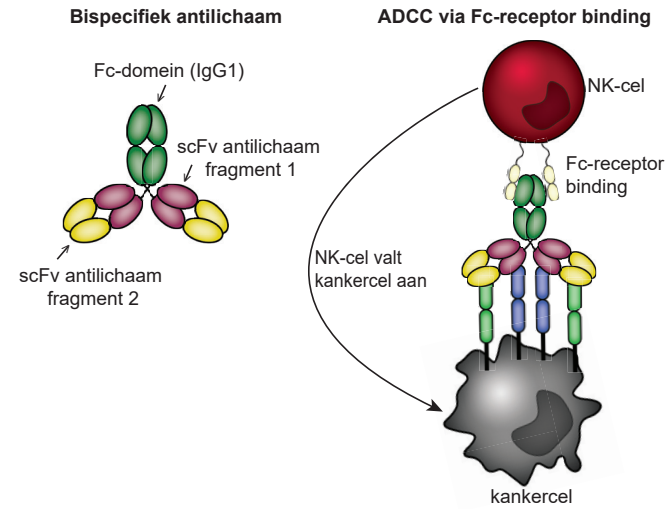
Selectieve eliminatie van kankercellen met TRAIL-Receptor gerichte therapie

Een interessant cytokine voor gerichte kankertherapie is TRAIL, een eiwit dat voorkomt op de celmembraan van bepaalde immuuncellen. TRAIL wordt door deze immuuncellen gebruikt om kankercellen te elimineren via zogenaamde geprogrammeerde celdood (apoptose). Dit natuurlijke proces stelt het lichaam in staat om overbodige of abnormale cellen snel en veilig op te ruimen. Het bijzondere van TRAIL is dat het selectief apoptose veroorzaakt in kwaadaardige cellen en geen effect heeft op normale cellen. Het exacte mechanisme hierachter is nog niet geheel opgehelderd, maar heeft onder andere te maken met een complex systeem van tenminste 5 verschillende TRAIL-Receptoren waar TRAIL aan kan binden, waarbij alleen efficiënte activatie van TRAIL-R1 en/of TRAIL-R2 tot geprogammeerde celdood leidt.

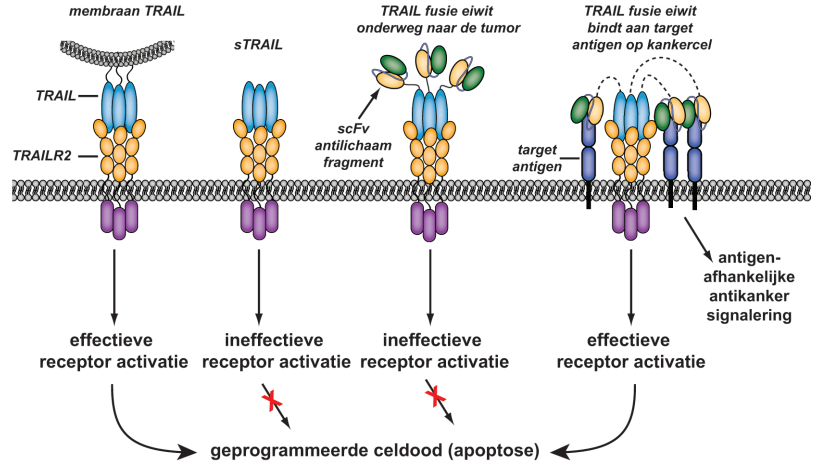
De antikankeractiviteit van zowel niet-membraan gebonden TRAIL (sTRAIL) als antilichamen die de TRAIL-R1 en TRAIL-R2 kunnen activeren is onderzocht in klinische studies, waaruit bleek dat deze middelen inderdaad weinig bijwerkingen veroorzaakten. Helaas bleek ook dat de antikankeractiviteit van zowel sTRAIL als TRAIL-Receptor antilichamen minimaal was. Zo wordt de effectiviteit van TRAIL-Receptor antilichamen onder meer beperkt doordat TRAIL-Receptoren in het hele lichaam voorkomen, waardoor het overgrote deel van het toegediende antilichaam niet op de plek van de tumor aankomt.

In tegenstelling tot TRAIL-R1 kan binding van sTRAIL aan TRAIL-R2 alleen celdood veroorzaken als er ook cross-linking van deze receptor optreedt. Dit geldt ook voor antilichamen gericht tegen TRAIL-R2. Maximale, niet tumor-selectieve cross-linking van TRAIL-R2 kan echter ongewenste bijwerkingen in de lever veroorzaken. In **Hoofdstuk 3** beschrijven we een nieuwe strategie om de effectiviteit van antilichamen gericht tegen TRAIL-Receptor 2 (ook wel DR5 genoemd) te verbeteren met behulp van bispecifieke antilichamen. Het door ons ontworpen bispecifieke antilichaam MCSPxDR5 combineert selectieve binding aan MCSP, een tumor antigen dat op bijna alle melanomen aanwezig is en onder andere de groei van melanoomcellen bevordert, met DR5 activatie. Binding van MCSPxDR5 aan MSCP zorgt voor effectieve cross-linking en leidt daardoor tot efficiëntere activatie van DR5.

Onze experimenten lieten zien dat MCSPxDR5 inderdaad selectieve celdood van MCSP-positieve melanoomcellen veroorzaakte en groei van melanoomcellen kon verhinderen. Verder is MCSPxDR5 in staat om de antikankeractiviteit van immuuncellen te verhogen door middel van zogenaamde ADCC (Figuur 1). Fc-receptor binding van immuuncellen aan MCSPxDR5 leidt echter ook tot efficiëntere activatie van DR5 en veroorzaakt zo meer celdood van MCSP-positieve melanoomcellen. De meervoudige antikankeractiviteit van MCSPxDR5 maakt dit bispecifieke antilichaam een veelbelovende kandidaat voor verdere doorontwikkeling voor klinische toepassing bij melanomen. Verder kan MCSPxDR5 waarschijnlijk ook van nut zijn voor de behandeling van andere kankersoorten, aangezien MCSP ook op andere moeilijk te behandelen kankers en op kankerstamcellen voorkomt.



Figuur 1: De in dit proefschrift beschreven bispecifieke antilichamen bestaan uit twee scFv antilichaam fragmenten en een IgG1 Fc-domein. Wanneer het bispecifieke antilichaam aan de target antigenen op de kankercel bindt, kan het Fc-domein worden herkend door Fc-receptoren die onder andere op NK-cellen voorkomen. Binding van de Fc-receptor aan het Fc-domein van het antilichaam activeert de NK-cel en initieert zogenaamde ADCC waarbij de NK-cel de kankercel aanvalt en uiteindelijk zal elimineren.

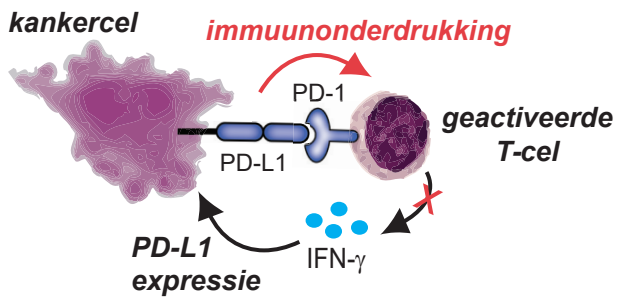


Figuur 2: In een TRAIL fusie-eiwit is een scFv antilichaamfragment genetisch gekoppeld aan sTRAIL. Op weg naar de tumor is een TRAIL fusie-eiwit niet in staat om de TRAIL-R2 te activeren. Echter, wanneer het scFv antilichaam fragment aan het target antigen op de kankercel bindt, lijkt het TRAIL fusie-eiwit op membraan TRAIL waardoor het wel in staat is om de TRAIL-R2 te activeren en apoptose te veroorzaken. Afhankelijk van het target antigen kan het binden van het scFv antilichaamfragment extra antikankeractiviteit hebben, zoals bijvoorbeeld het blokkeren van signalen die de groei van kankercellen bevorderen.

Versterking van de antikanker immuunreactie met TRAIL fusie-eiwitten

De effectiviteit van niet-membraan gebonden TRAIL (sTRAIL) kan worden verbeterd door gebruik te maken van antilichaam-cytokine fusie-eiwitten, waarin een antilichaamfragment gericht tegen een tumor antigen gekoppeld is aan sTRAIL. In tegenstelling tot TRAIL op de membraan van immuuncellen veroorzaakt binding van sTRAIL aan TRAIL-R2 geen receptoractivatie en dus geen apoptose (Figuur 2). In een TRAIL fusie-eiwit is sTRAIL dus relatief inactief. Pas na binding van het antilichaamfragment aan het tumor antigen op de kankercel zal TRAIL-R2 effectief geactiveerd worden (Figuur 2). Wanneer een tumor target antigen wordt gekozen dat een biologische functie heeft, kan binding van het antilichaamfragment een extra functie hebben die de antikankeractivietit van het TRAIL fusie-eiwit verhoogt, zoals bijvoorbeeld het blokkeren van signalen die de groei van kankercellen bevorderen.

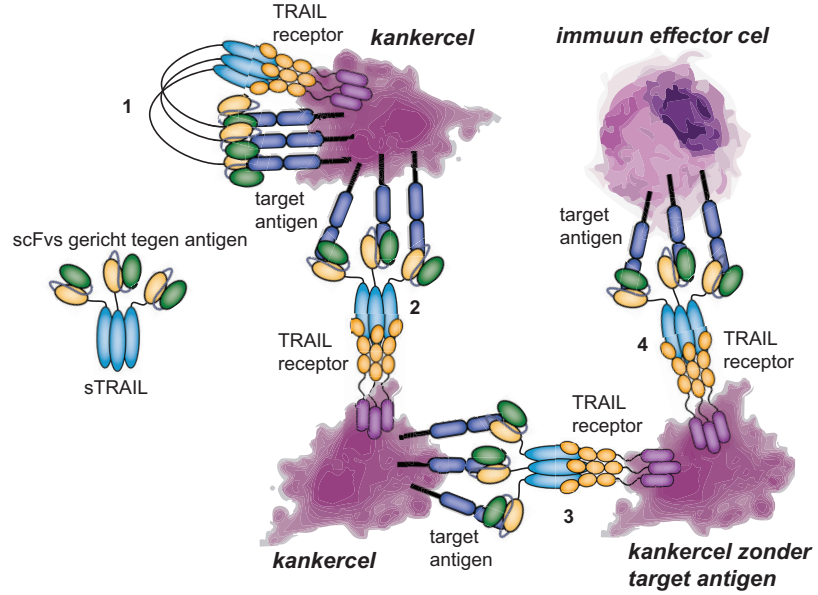
In dit proefschrift is onderzocht of TRAIL fusie-eiwitten ingezet kunnen worden om immuun-onderdrukkende signalen in het tumormilieu te blokkeren, zoals de interactie tussen PD-L1 en PD-1 (Figuur 3). Dit zogeheten immuun 'checkpoint' zorgt er normaal gesproken voor dat een immuunreactie op tijd wordt afgeremd om te voorkomen dat geactiveerde T-cellen gezonde cellen elimineren. Kankercellen misbruiken dit checkpoint echter om aan het immuunsysteem te ontsnappen door PD-L1 tot expressie te brengen. Er is bij diverse soorten kanker dan ook een relatie tussen expressie van PD-L1 op kankercellen en een slechte prognose. Antilichamen die de interactie tussen PD-L1 en PD-1 blokkeren kunnen de antikankeractiviteit van de onderdrukte T-cellen in het tumormilieu herstellen. Therapie met zulke antilichamen heeft in bepaalde typen kanker, met name huidkanker, indrukwekkende resultaten geboekt met zelfs genezing in een klein aantal patiënten. Er is echter ruimte voor verbetering aangezien de huidige PD-L1- en PD-1-blokkerende antilichamen slechts effect hebben in een minderheid van alle behandelde patiënten.



Figuur 3: Door mutaties of signalen in het tumormilieu (bijv. IFN-γ geproduceerd door geactiveerde T-cellen) brengen kankercellen PD-L1 tot expressie op hun celmembraan. De interactie tussen PD-L1 op kankercellen en de bijbehorende receptor (PD-1) op geactiveerde T-cellen onderdrukt de antikankeractiviteit van de T-cellen waardoor de kankercellen niet geëlimineerd worden.

In **Hoofdstuk 4** beschrijven we het door ons ontworpen TRAIL fusie-eiwit anti-PD-L1:TRAIL, dat behalve sTRAIL een PD-L1 blokkerend antilichaamfragment bevat. Uit onze experimenten bleek dat anti-PD-L1:TRAIL selectief celdood van PD-L1-positieve kankercellen veroorzaakte. Bovendien verbeterde anti-PD-L1:TRAIL de antikankeractiviteit van T-cellen door de interactie tussen PD-L1 en PD-1 te blokkeren. Dit zorgde *in vitro* voor verbeterde eliminatie van melanoomcellen door T-cellen afkomstig uit dezelfde patiënt.

Een interessante extra functie van anti-PD-L1:TRAIL is dat het PD-L1-positieve immuuncellen kan bewapenen met TRAIL. Dit is interessant omdat met name immuun-onderdrukkende immuuncellen in het tumormilieu vaak PD-L1-positief zijn. Anti-PD-L1:TRAIL kan zulke cellen bewapenen met TRAIL, waarna ze hiermee nabijgelegen kankercellen kunnen elimineren (Figuur 4). Daarnaast kan de immuun-onderdrukkende werking van zulke cellen worden verminderd via blokkering van de interactie tussen PD-L1 en PD-1. Bovendien kunnen TRAIL fusie-eiwitten via het zogenaamde 'bystander effect' celdood veroorzaken in nabijgelegen kankercellen waarop het target antigen niet voorkomt (Figuur 4). Kort samengevat heeft TRAIL fusie-eiwit anti-PD-L1:TRAIL veelbelovende meervoudige antikankeractiviteit die mogelijk de effectiviteit van PD-L1 blokkerende therapie zou kunnen verbeteren.



Figuur 4: Schematische weergave van de diverse werkingsmechanismen van TRAIL fusie-eiwitten: Binding van de scFvs aan het target antigen activeert TRAIL receptoren op dezelfde cel **(1)** of op een nabijgelegen kankercel **(2)**. Echter, wanneer een TRAIL fusie-eiwit aan het target antigen gebonden is, kan het ook celdood veroorzaken via het zogenaamde 'bystander effect'. Hierbij wordt celdood veroorzaakt in nabijgelegen kankercellen die geen target antigen maar wel TRAIL receptoren op hun celmembraan hebben **(3)**. Het 'bystander effect' kan ook plaatsvinden wanneer het target antigen zich op immuuncellen bevindt **(4)**.

Tumor-selectieve blokkering van de interactie tussen PD-L1 en PD-1

De huidige antilichamen die de interactie tussen PD-L1 en PD-1 blokkeren hebben geen tumor-selectieve werking, omdat PD-1 en PD-L1 ook op gezonde cellen voorkomen. Dit kan verklaren waarom therapie met dit soort antilichamen soms immuun-gerelateerde bijwerkingen kan veroorzaken. In **Hoofdstuk 5** beschrijven we een nieuwe antilichaam-gebaseerde therapeutische strategie die de tumorselectiviteit van PD-L1 en PD-1 blokkerende antilichamen zou kunnen verbeteren. Met behulp van bispecifieke antilichamen zou het mogelijk moeten zijn om de interactie tussen PD-L1 en PD-1 selectief op de plek van de tumor blokkeren, waardoor er waarschijnlijk minder bijwerkingen zullen optreden.

Het door ons ontworpen bispecifieke antilichaam PD-L1xEGFR bevat PD-L1- en EGFR-blokkerende antilichaamfragmenten. EGFR is een bekend tumor antigen dat vaak voorkomt op kankercellen en kankercelgroei kan bevorderen. Bovendien is recent ontdekt dat EGFR-mutaties kunnen leiden tot verhoogde PD-L1 expressie op kankercellen, wat associeert met een slechtere prognose in longkankerpatiënten. Antilichamen gericht tegen EGFR kunnen EGFR-signalering blokkeren en zo kankercelgroei verminderen. EGFR is daarom een interessant target voor tumor-selectieve PD-L1 blokkerende therapie met behulp van bispecifieke antilichamen.

Onze experimenten lieten inderdaad zien dat PD-L1xEGFR de interactie tussen PD-L1 en PD-1 met name op EGFR-positieve kankercellen blokkeerde. Dit leidde tot verhoogde antikankeractiviteit van T-cellen tegen EGFR-positieve kankercellen, terwijl de activiteit tegen EGFR-negatieve cellen niet veranderde. Uit onze experimenten bleek verder dat behandeling met PD-L1xEGFR de groei van kankercellen kon verminderen. Bovendien leidde behandeling met PD-L1xEGFR tot eliminatie van kankercellen via NK-cel afhankelijke ADCC (Figuur 2).

De meervoudige antikankeractiviteit van PD-L1xEGFR leidde tot efficiëntere eliminatie van EGFR-positieve kankercellen vergeleken met een PD-L1 antilichaam dat al in de kliniek wordt gebruikt. Kort samengevat heeft PD-L1xEGFR veelbelovende meervoudige antikankeractiviteit die de effectiviteit van PD-L1-blokkerende therapie zou kunnen verbeteren, terwijl het bijwerkingen zou kunnen verminderen.

Tot slot wordt in **Hoofdstuk 6** een korte samenvatting gegeven van de resultaten uit hoofdstuk 3 t/m 5 en worden perspectieven voor de verdere ontwikkeling van PD-L1- en TRAIL-Receptor-gerichte therapieën besproken. Kort samengevat vertonen de nieuwe antilichaam-gebaseerde moleculen die in dit proefschrift worden beschreven veelbelovende en veelzijdige antikankeractiviteit die doorontwikkeld zou kunnen worden voor klinische toepassing. Op basis van onze preklinische resultaten verwachten wij dat onze nieuwe moleculen de effectiviteit en tumor-selectiviteit van TRAIL-Receptor activerende en PD-L1 blokkerende therapie kunnen verbeteren en de bijwerkingen van PD-L1 gerichte immunotherapie kunnen verminderen door verhoogde tumor-selectiviteit en effectiviteit.

CHAPTER 8

Dankwoord

“Sometimes it’s the journey that teaches you a lot about your destination.” – Drake

Het zit erop. Dat is best wel een raar gevoel als je vier jaar intensief aan iets hebt gewerkt, het zal denk ik nog wel even duren voor het echt afgerond voelt. Terwijl ik dit typ, in Leiderdorp met een kat op schoot, bedenk ik mij dat het vooral een bijzondere periode was. Een promotie is sowieso een bijzondere en intense ervaring, maar wanneer je gedurende die periode ook dertig wordt en gaat trouwen, dan maak je wel wat mee zullen we maar zeggen. Vooral de laatste maanden van een promotietraject hebben wel iets van een eindeloze achtbaan of marathon. Dank aan alle collega's, vrienden en familieleden die ervoor hebben gezorgd dat ik de eindstreep heb gehaald.

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Djoke

Appendices

List of scientific publications

Curriculum Vitae

List of scientific publications

Hendriks D, Choi G, de Bruyn M, Wiersma VR, Bremer E. "Antibody-based cancer therapy: successful agents and novel approaches", International Review of Cell and Molecular Biology 2017; 331: 289-383.

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Curriculum vitae

Djoke Hendriks was born in Groningen, the Netherlands, on the 30th of July 1985. After graduation from secondary school in Franeker (HAVO) and Leeuwarden (VWO), she started studying chemistry at the University of Groningen in 2004. After obtaining her BSc degree in 2008, she continued with a master in Journalism. Although she really enjoyed writing, especially during her internship at the science section of national newspaper NRC Handelsblad, she decided to continue in science and started an additional master in Molecular Biology and Biotechnology in 2010. During her master, she became really interested in immunology and cancer research. This resulted in an internship at the Cancer Sciences Unit in Southampton in 2012, after which she decided to look for a cancer-related PhD position. Early 2013, she joined the laboratory for Translational Surgical Oncology at the University Medical Center Groningen. This thesis is the result of the KWF-funded PhD project she worked on.