CHAPTER 2

Antibody-based cancer therapy: successful agents and novel approaches

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Shortened version of:
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.
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 (Vaikus 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: IgG, IgD, IgE, IgA or IgM, which differ in abundance, 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) IgM 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 IgG, 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, Gul 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 Iggs, 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 a further important consideration for the design of therapeutic mAbs.
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 (Ofilioglu 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 Gaeta-no 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 Pescevitz 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 (TRAIL-R) 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 Liosis 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 FcyRIIIB (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 pretrained 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).

**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 pacitaxel and carboplatin improved progression free survival in chemotherapy-naive 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 signaling 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, with the 4-1BB antibody urelumab produced severe and fatal liver toxicity at the highest dose tested, leading to termination of a phase I clinical trial (reviewed in Bartkowski 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 naive and memory T cells, GITR expression is rapidly upregulated upon activation (Gurney et al. 1999, Zhan et al. 2004). Its ligand, GTRIL 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 Kne 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, 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 domain 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 camels 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 domains, which are derived from ‘heavy-chain-only antibodies’ expressed by camels and sharks. 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-mediated cross-linking and specifically recruit the activated T cells to the tumor by virtue of the tumor-targeting scFv.
**Chapter 2**

**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 FcR-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 Lympho mun, 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. 2012). A second bsAb of the same format, ertuxamomab, 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 (Klieve et al. 2006). However, clinical development of ertuxamomab 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 Huels 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 lyses 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 Leukemia (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 leukemia 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 Leukemia (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 (DART)" 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 leukemia 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 gpAA3 (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
As already mentioned above, the triplebody format can also be used to retarget NK cells (Rothe et al. 2015). A duobody exploits the same reaction environment (Schubert et al. 2012). This triplebody format 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 leukemia 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 developed to recruit γδ T cells as potent cytolytic effectors to malignant B-lineage target cells in vitro (Schiller 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 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 1 receptor (IGF-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-1 remaining on treatment longer. Further, IGF-IR and HER3 expression levels decreased upon treatment with MM-141 (Isakov et al. 2015). A more clinically advanced HER3 targeting bsAb 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 dulgotuzumab for EGFR and HER3 (Eigenbrot et al. 2013, Schaefer et al. 2011). Dulgotuzumab 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. 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. 2012).

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 synergize 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.

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 (Herwas-Stubbbs et al. 2011) and enhances cross-presentation of tumor-derived antigenic peptides in MHC-I by Dendritic Cells (DCs) (Gallicci et al. 1999, Ito et al. 2001). Further, IFN-α directly stimulates CD8 T cell expansion and acquisition of effector function (Herwas-Stubbbs 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. 2001). Further, IFN-α directly stimulates CD8 T cell expansion and acquisition of effector function (Hervas-Stubbs et al. 2011) and enhances cross-presentation of tumor-derived antigenic peptides in MHC-I by Dendritic Cells (DCs) (Gallicci et al. 1999, Ito et al. 2001). Further, IFN-α directly stimulates CD8 T cell expansion and acquisition of effector function (Herwas-Stubbbs 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).
and modest activity in a variety of human cancers (reviewed in Parker et al. 2016). Systemic treatment regime for IFN-α has proven difficult in view of its dose-limiting toxicity, which was 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 of 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 was designed to improve activity (Levin et al. 2012).

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 leukemia 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 I b study in advanced car-

fusin 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 Carcinomaembryonic Antigen (CEA) (Greiner et al. 1984), which may be of use for rational design of new type I IFN-containing immunocytokines.

Antibody-based cancer therapy: successful agents and novel approaches
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 (Mar-lind 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 immunocytokynes, where IL-2 was fused to the IgG light-chain, it was found that these cytokynes did not have improved tumor homing or recruitment of effector functions such as ADCC, but that efficacy of the immunocytokynes 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 antitumor 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-15Ra (Budagain 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 (Kasper 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-15Ra 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 4-1BB 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
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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 signalling (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 4-1BB (not included here), and TRAIL as discussed below.

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 TNFSF members can be processed into a soluble ligand comprising the extracellular domain. Membrane TNFSF ligands effectively trigger activation of cognate receptors (Figure 6A). However, soluble TNFSF ligands typically bind their cognate receptor(s), but are less effective in activation of downstream receptor signalling. It is crucial to understand the difference in activity profile of soluble vs. membrane TNFSF ligands. 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 4-1BB (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.

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).
**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 osteoprotegerin (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 (Giefers 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 (EH2D) 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 EH2D-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 ACPD 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 tumoridical 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 deacetylase 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.

**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 “armored” 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 release 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 (iCas9) 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 iCas9-expressing CAR T cells are currently being evalu-
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 \textit{in vivo} expansion of CD19 CAR T cells in patients. This phenotype could be selectively induced \textit{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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