Novel antibody-based drugs for PD-L1 and TRAIL-R targeted cancer immunotherapy
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CHAPTER 6

Summary & Future perspectives
Summary
As outlined in Chapter 2, antibody-based cancer therapy has come of age with more than 20 antibodies currently approved and many more in clinical development.1 However, many of the currently available antibodies are directed against antigens that are overexpressed rather than exclusively present on cancer cells. This lack of authentic tumor-selectivity may explain the observed limitations in clinical efficacy and the sometimes dose-limiting on-target/off-tumor side-effects. A large body of research is ongoing to leverage the specificity of antibodies towards improved cancer-selective therapy. Hereto, antibodies or antibody-derivatives can be equipped with cytotoxic or immunomodulatory domains, such as for the cytokine TRAIL in Chapter 4. Alternatively, recombinant antibodies can be engineered to have specificity for two (or even more) tumor-associated target antigens, as explored in Chapter 5. The rapid advances in antibody engineering have increased the number of available molecular formats, providing tools to rationally design antibody-based drugs with multiple specificities and/or effector functions. Of note, suitable targets for antibody-based cancer immunotherapy are not limited to tumor antigens, as its efficacy may also be significantly enhanced by targeted blocking or activation of selected antigens on immune cells.

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

In Chapter 4, we describe a novel checkpoint inhibition strategy that combines reactivation of antigenic immunity via blockade of the PD-1/PD-L1 interaction with simultaneous induction of TRAIL-mediated apoptosis. Hereto, we constructed anti-PD-L1:TRAIL, a bi-functional TRAIL fusion protein that comprises a PD-L1 blocking scFv antibody fragment genetically fused to soluble TRAIL. Fusion protein anti-PD-L1:TRAIL selectively induced apoptosis in PD-L1-positive cancer cell lines and primary patient-derived melanoma cells. At the same time, anti-PD-L1:TRAIL enhanced anticancer activity and IFN-γ production of T cells via blockade of the PD-1/PD-L1 interaction. Since IFN-γ increases PD-L1 expression and sensitizes cancer cells to TRAIL-mediated apoptosis, anti-PD-L1:TRAIL may trigger a feed-forward loop of increasing IFN-γ, increasing PD-L1 expression and increasing TRAIL sensitivity. In line with this, IFN-γ enhanced the efficacy of anti-PD-L1:TRAIL-mediated apoptosis in cell lines and in primary patient-derived melanoma cells. Additionally, anti-PD-L1:TRAIL was able to convert potentially immunosuppressive PD-L1-expressing immune cells into TRAIL-displaying effector cells that induce TRAIL-mediated apoptosis in cancer cells. In conclusion, fusion protein anti-PD-L1:TRAIL shows promising multi-fold and mutually reinforcing anticancer activity that may provide possibilities to enhance the efficacy of therapeutic PD-1/PD-L1 inhibition alone or in combinatorial strategies.

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

Future perspectives
Antibody-based blockade of immune checkpoint molecules such as CTLA-4 and PD-1/PD-L1 has emerged as a promising strategy to improve anticancer immune responses.2 In particular, antibodies that block the PD-1/PD-L1 checkpoint have triggered unprecedented-curable anticancer immunity, most notably in advanced melanoma.3-4 However, since PD-1 and PD-L1 are broadly expressed on normal tissues, antibodies blocking the PD-1/PD-L1 interaction are not inherently tumor-selective. This can result in severe autoimmune-related side effects in the skin, gastrointestinal tract, liver and lungs as observed for PD-1 blocking antibodies nivolumab and lambrolizumab.5-6 Additionally, despite their unprecedented response rates, only a subset of patients responds to single treatment with currently available PD-1 and PD-L1 blocking antibodies.3-5 Hence, there is a clear rationale to improve the efficacy of PD-1/PD-L1 blocking immunotherapy. Based on our results in Chapter 4-5, we speculate that bsAb PD-L1xEGFR and fusion protein anti-PD-L1:TRAIL may be able to enhance therapeutic efficacy and reduce side effects of PD-1/PD-L1 blockade in cancer immunotherapy. Our in vitro results showed
that bsAb PD-L1xEGFR selectively delivers PD-L1 blockade to EGFR-positive target cells (Chapter 5). We expect that EGFR-directed blockade of the PD-1/PD-L1 checkpoint will result in less on-target/off-tumor side effects compared to currently available PD-1/PD-L1-blocking antibodies. Moreover, we showed that bsAb PD-L1xEGFR has additional anticancer activities, including blockade of pro-tumorigenic EGFR-signaling and induction of Fc-mediated ADCC. The multi-fold modes-of-action of bsAb PD-L1xEGFR may be responsible for its potent anticancer activity that appears to outperform a clinically used conventional PD-L1-blocking antibody.

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

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

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

CAR T cells are generated by transfection of peripheral blood T cells from a patient with a CAR construct that comprises an extracellular scFv domain, a transmembrane domain, and one or more intracellular co-stimulatory domains.10 As a result, CAR T cells are redirected to cancer cells irrespective of their endogenous TCR specificity in a MHC-non-restricted manner. Upon binding to the tumor-associated target antigen, CAR T cells are engaged to proliferate and kill the targeted tumor cells. Thus, CAR T cells harness the capacity of both antibody-mediated target antigen recognition and selective delivery of the full cytotoxic T cell armament. However, CAR T cells are typically directed against tumor-associated antigens that are overexpressed on cancer cells, but that may also be expressed at lower levels on normal tissues. Consequently, CAR T cell treatment often shows deleterious on-target/off-tumor activity towards normal cells that express these target antigens.11 Moreover, in mouse models with established tumors, it was identified that several tumor-related factors may limit the clinical activity of CAR T cells,12, 13 including PD-1/PD-L1 interaction in the tumor microenvironment. Of note, co-treatment of CAR T cells with conventional PD-1/PD-L1 blocking antibodies is likely to further enhance on-target/off-tumor side effects. Based on our results in Chapter 4-5, we speculate that combinatorial regimes with PD-L1xEGFR or anti-PD-L1:TRAIL and CAR T cells may be of use to enhance therapeutic efficacy while reducing side effects and as such may outperform combinations with conventional PD-1/PD-L1-blocking antibodies.

Of note, several strategies have been applied to reduce on-target/off-tumor side effects of CAR T cells. These include incorporation of ‘ON-switches’ for suicide genes that allow for controlled shut-down of CAR T cells14, 15 and dual specificity CARs that redirect T cells to two different tumor antigens.16, 17 Recently, the use of so-called synthetic Notch (synNotch) receptors has been described. Using this approach, T cells can be engineered to only express the CAR in the tumor environment.18 Binding of the synNotch receptor to its corresponding target antigen expressed by cancer cells induces cleavage of its intracellular domain, thereby releasing a transcription factor that initiates transcription of the CAR. Thus, such T cells only express the CAR after initial synNotch recognition at the tumor site. Other potential applications of the synNotch system included targeted therapeutic delivery of the apoptosis-inducing ligand TRAIL and release of therapeutic antibodies,19 including αPD1, αCTLA4, and bispecific T-cell engagers (BiTE, see later). We hypothesize that the therapeutic potential and safety of this synNotch system can be enhanced by using tumor-directed scFvs:TRAIL fusion proteins or tumor-directed PD-1/PD-L1 blocking bsAbs like PD-L1xEGFR.

In cancer immunotherapy, BiTEs represent an established antibody-based strategy to redirect T cells to attack cancer cells. In a BiTE, a CD3-targeted scFv antibody fragment is fused to a cancer-cell targeted scFv antibody fragment. Treatment with a given BiTE retargets and activates CD3-positive T cells to kill cancer cells that express the corresponding target antigen in a MHC-independent manner, irrespective of intrinsic TCR specificity. However, repeated challenging of BiTE-redirected T cells with fresh tumor cells resulted in a reduced killing capacity and upregulation of PD-1.20 Correspondingly, blockade of the PD-1/PD-L1 interaction improved the in vitro efficacy of BiTEs targeted to CEA- and CD33-expressing cancer cells.21, 22 Of note, increased PD-L1 expression was observed in patients with acute lymphoblastic B-cell leukemia (B-ALL) that were resistant to treatment with blinatumomab, a CD19-targeted BiTE.23 Hence, there is a clear rationale to combine blinatumomab treatment with blockade of the PD-1/PD-L1 interaction. We speculate that combinations of blinatumomab and tumor-selective blockade of the PD-1/PD-L1 interaction (e.g. using a leukemia-directed PD-L1 blocking bsAb) may help overcome the above-mentioned
therapy resistance and reduce side effects compared to combinations with conventional PD-1/PD-L1-blocking antibodies.

In Chapter 4, we present a novel PD-1/PD-L1-blockade approach that is based on the unique features of TRAIL fusion protein anti-PD-L1:TRAIL that efficiently combines reactivation of anticancer T-cells with induction of tumor-selective TRAIL-mediated apoptosis. Previously, we and others have demonstrated that scFv:TRAIL fusion proteins gain full pro-apoptotic activity only after tumor-selective binding via its tumor-directed antibody fragment. Furthermore, upon binding to target antigen-positive cancer cells, scFv:TRAIL fusion proteins may also trigger apoptosis in neighboring target antigen-negative cancer cells (Figure 1). This so-called bystander effect, can potentially reduce the risk of escape of target antigen-negative cancer cells from targeted therapy.

An appealing feature of anti-PD-L1:TRAIL is that it can convert potentially immunosuppressive PD-L1-expressing immune cells into TRAIL-displaying effector cells able to induce TRAIL-mediated apoptosis in cancer cells. This is particularly relevant for various types of PD-L1 expressing myeloid-derived suppressor cells which are frequently present in the tumor microenvironment. The presence of such myeloid-derived suppressor cells is associated with poor prognosis in several cancer types. Although not investigated here, treatment with anti-PD-L1:TRAIL may inhibit PD-L1-expressing myeloid-derived suppressor cells while simultaneously inducing an anticancer bystander effect in neighboring cancer cells that express TRAIL-Receptors (Figure 1).

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

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

The anticancer efficacy of both recombinant soluble TRAIL and agonistic antibodies specific for TRAIL-R1 or TRAIL-R2 have been evaluated in early stage clinical trials in which favorable safety profiles were observed. However, these first-generation TRAIL-R agonists had limited clinical efficacy, possibly due to intrinsic resistance to TRAIL and/or acquired resistance upon treatment with TRAIL-R agonists. Furthermore, TRAIL receptors are ubiquitously expressed throughout the human body, limiting the accumulation of TRAIL-R agonists at the tumor site. Of note, unlike membrane-bound TRAIL, soluble TRAIL requires cross-linking to efficiently activate TRAIL-R2. Similarly, conventional TRAIL-R2 targeted antibodies appear to require additional cross-linking by Fc-receptor positive cells for effective induction of apoptosis. However, ubiquitous maximal cross-linking and signaling through TRAIL-R2 may be associated with unwanted side effects. For example, nanobody TAS266, that did not require secondary cross-linking for efficient TRAIL-R-mediated apoptosis induction, showed hepatotoxicity in a phase I clinical trial, leading to termination of the trial.

In Chapter 3, we demonstrate that tumor-selective activation of TRAIL-R2 (DR5) can be achieved with a bispecific antibody directed against melanoma-associated antigen MCSP. BsAb MCSPxDR5 has high binding specificity for MCSP and potent TRAIL-R2-activating activity only towards MCSP-expressing cancer cells. We showed that Fc-mediated cross-linking could further enhance the anticancer activity of bsAb MCSPxDR5. Moreover, bsAb MSCPxDR5 additionally enhanced the anticancer efficacy of NK cells via Fc-mediated induction of ADCC. BsAb MSCPxDR5 showed minimal activity towards normal cells and Fc-mediated cross-linking did not enhance activity towards MCSP-negative cells. Thus, we expect enhanced anticancer activity of bsAb MCSPxDR5 compared to conventional TRAIL-R2 agonistic antibodies, possibly with a more favorable safety profile. BsAb MCSPxDR5 may be of particular use for treatment of melanoma and other MCSP-positive malignancies.

To further enhance its anticancer activity, bsAb MCSPxDR5 may be incorporated into a combinatorial regime, for example with anticancer agents that upregulate TRAIL-Receptor expression, sensitize cancer cells to TRAIL-R-mediated apoptosis or overcome intrinsic TRAIL resistance mechanisms, such as chemotherapeutics and small molecule inhibitors. For example, RAF inhibitors could overcome TRAIL resistance in melanoma cells in vitro. Furthermore, synergy between a TRAIL-R1 agonistic antibody and chemotherapeutic agents gemcitabine and cisplatin was observed in a Phase I clinical trial. In line with this, TRAIL-R2 targeted bispecific antibody RG7386 showed synergy with chemotherapeutic agents doxorubicin and irinotecan in mouse models. Indeed, we observed...
synergy of bsAb MCSPxDR5 with several anticancer agents in vitro, including epigenetic drug valproic acid that has shown potent anticancer activity in pre-clinical studies.\textsuperscript{59}

The therapeutic activity of antibodies is strongly influenced by the interaction of their Fc domain with the appropriate Fc receptors (FcR) on immune effector cells. Therefore, engineering of the IgG domain has been used to modify Fc-mediated effector mechanisms such as ADCC, ADCP and CDC. For example, interaction with inhibitory receptor FcγRIIB was found to be critical for agonistic antibodies that target TNFR family members such as TRAIL-R2 or co-stimulatory TNF family members such as CD40.\textsuperscript{40, 41} In line with this, mutations in the IgG1 domain that caused a 200-fold increase in affinity for FcγRIIB enhanced the agonistic activity of a 4-1BB targeted antibody.\textsuperscript{42} Incorporation of such IgG1 mutations into the Fc domain of bsAb MCSPxDR5 may be of interest to further enhance its anticancer activity.

In contrast, binding to FcγRIIB reduces the therapeutic efficacy of antibodies that directly target antigens on cancer cells as FcγRIIB is an important negative regulator of ADCC.\textsuperscript{43} Therefore, such antibodies have been engineered to have a mutant IgG1 domain, which preferentially binds to activating FcRs over FcγRIIB.\textsuperscript{44} Interestingly, mutations in the IgG1 domain that enhanced the affinity to all FcRs yielded up to a 100-fold increase in ADCC.\textsuperscript{45} Therefore, incorporation of such IgG1 mutations into bsAb PD-L1xEGFR may be of interest too. Since bsAb PD-L1xEGFR showed EGFR-restricted anticancer activity, we expect that IgG1 engineering can enhance its tumor-selective activity without increasing off-target side effects.

Of note, current PD-1/PD-L1 blocking antibodies are typically engineered to have a human IgG4 isotype\textsuperscript{46, 47} or contain a “silenced” IgG1 domain\textsuperscript{48} to avoid elimination of man IgG4 isotype\textsuperscript{3}, 46, 47 or contain a “silenced” IgG1 domain\textsuperscript{48} to avoid elimination of PD-1/PD-L1 expressing immune cells by ADCC. However, recent reports showed that PD-L1-blocking antibody avelumab, that contains a fully functional human IgG1 domain, induced only low levels of ADCC-mediated lysis in PBMCs in vitro.\textsuperscript{50, 51} Moreover, recent studies with avelumab in carcinoma patients showed promising response rates with a toxicity profile comparable to other PD-1/PD-L1-blocking antibodies.\textsuperscript{52, 54}

Despite recent advances, treatment of cancer is often not curative. New insights indicate that this may be attributable to a small population of therapy-resistant malignant cells with self-renewal capacity and the ability to generate large numbers of more differentiated cancer cells. These cancer-initiating cells are commonly referred to as cancer stem cells (CSCs). CSCs are regarded as the root of cancer origin and recurrence after seemingly successful therapy. Not surprisingly therefore, current and future cancer research is focused on ways to specifically eliminate CSCs.

In hematopo-ology, various CSC-associated surface antigens have been identified that may allow for CSC-selective therapy while sparing normal hematopoietic stem cells.\textsuperscript{55} In this respect, it is worth mentioning that CSCs in selected cancers were found to be susceptible to TRAIL-mediated apoptosis,\textsuperscript{56, 57} especially while simultaneously blocking pro-survival IL-4 signaling.\textsuperscript{58} It may therefore be of value to construct a DR5-agonistic bspecific antibody with IL-4R-blocking capacity. More recently, it was reported that CSCs in breast and colon cancer expressed elevated levels of PD-L1 compared to non-stem-like cancer cells.\textsuperscript{59} These results suggest that breast and colon cancer CSCs may be sensitive to PD-1/PD-L1 immunotherapy. In this respect, it appears of interest to develop bsAbs that allow for CSC-directed PD-1/PD-L1 blockade.

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

\section*{References}

Chapter 6

Chapter 6


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