TNF receptor superfamily-based targeted cancer immunotherapy
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Chapter 1

Introduction to the thesis
General introduction

The Tumor Necrosis Factor (TNF) ligand and cognate TNF receptor superfamilies represent a pivotal immunoregulatory axis crucial to maintenance of immune homeostasis and the correct execution of immune responses. The TNF superfamily consists of 19 ligands that can interact with 29 cognate TNF receptors and regulates a host of cellular responses, including the induction of cell death in potentially dangerous and superfluous cells, a response governed by the sub-group of so-called Death Inducing Ligands and cognate Death Receptors. Further, TNF/TNFR signaling provides crucial co-stimulation at various stages of adaptive immune responses to ensure mounting of an effective adaptive immunity.

Most TNF ligands are expressed as type II transmembrane proteins, but can be proteolytically processed into soluble trimeric ligands. Although this soluble TNF ligand typically still binds to its receptor, the soluble ligand has a significantly reduced signaling activity compared to their transmembrane counterparts. It is thought that these soluble ligands may regulate or fine-tune the TNF/TNFR signaling outcome. Importantly, the activity of the soluble trimeric ligand can often be restored to 'membrane-like' activity by secondary cross-linking of this ligand.

This intrinsic regulatory feature of TNF/TNFR signaling makes this signaling axis an appealing target/effector for cancer immunotherapy. In chapter 2 we review the three TNFSF ligands (TRAIL, FasL and CD40L) and their cognate receptors that have been investigated in this thesis for use in cancer immunotherapy. Specifically, we briefly discuss their biological function, discuss the pre-clinical evidence for their utility in cancer therapy and detail challenges that were identified in early clinical trials using lead therapeutics. Further, we will detail the rationale for using antibody-based targeting, as being developed in our and other laboratories, to overcome the challenges of TRAIL/FasL/CD40L-based immunotherapy.

In chapter 3-5 we focused on developing new agonistic TRAIL-receptor targeting immunotherapeutics. In chapter 3, we developed a bispecific antibody-based strategy with the aim of augmenting the therapeutic activity of TRAIL-R2 or DR5-targeting antibodies. From literature, it is known that agonistic DR5 antibodies such as tigatuzumab require cross-linking by Fc receptors on myeloid effector cells to effectively induce apoptosis in cancer cells\(^1,2\). Moreover,
conventional agonistic DR5 antibodies have no tumor-selective binding activity in their own right, whereas TRAIL receptors are ubiquitously expressed on numerous normal tissues and cells\(^3\).

Consequently, there is large “target antigen sink effect” of normal healthy cells that can limit the therapeutic effect of DR5-targeting antibodies. Therefore, we generated recombinant dual-specificity antibody MCSPxDR5, comprising the epitope-binding domains of agonistic anti-DR5 antibody tigatuzumab and high-affinity anti-MCSP antibody 9.2.27 fused to the human IgG1. Our data demonstrates that MCSPxDR5 selectively binds to MCSP-positive melanoma cells and triggers direct DR5-mediated apoptosis in melanoma cells. Secondly, in the presence of Fc receptor positive immune cells, MCSPxDR5 triggers melanoma cell apoptosis through activation of ADCC.

In chapter 4 we build on our previous work with so-called scFv:TRAIL fusion proteins, in which an scFv antibody fragment is genetically fused to soluble TRAIL (sTRAIL). Antibody fragment binding to the targeted antigen converts the soluble and essentially inactive scFv:TRAIL fusion protein into a membrane-bound form of TRAIL that can effectively trigger apoptosis. Previously, we and other have shown that scFv:TRAIL fusion proteins have promising preclinical activity in both solid and hematological malignancies\(^4,5\). Here, we selected CD47 as a new target for scFv:TRAIL-based therapy. CD47 is a prominent immunomodulatory target on cancer cells that is often overexpressed in malignancies. Importantly, CD47 binds to SIRP\(\alpha\) on phagocytes, thereby inhibiting the immune cell-mediated removal of cancer cells. CD47 blocking antibodies have been shown to release the brake from phagocytes and trigger phagocytic elimination of CD47-overexpressing tumor cells\(^6\), with a CD47-blocking currently being evaluated in early clinical trials. Fusion protein anti-CD47:TRAIL combined the inhibition CD47-SIRP\(\alpha\) interaction, thereby potentiating phagocytic removal of cancer cells, with CD47-restricted induction of TRAIL-mediated apoptosis of cancer cells. Importantly, this dual function anti-tumor activity of antiCD47:TRAIL enhanced the efficacy of therapeutic anticancer antibodies, such as rituximab. Thus, antiCD47:TRAIL may be exploited as a novel approach to enhance the phagocytic and cytotoxic activity of current antibody-based cancer therapy.

In chapter 5 we report on a novel approach based on the multifunctional fusion protein “anti-PD-L1:TRAIL”. Fusion protein anti-PD-L1:TRAIL not only selectively trigger TRAIL-mediated cancer cell death in PD-L1-expressing malignant cells, but also reactivated functionally impaired T cells. The latter
was evidenced by increased T cell proliferation, IFN-γ secretion and enhanced killing capacity towards cancer cell lines and primary patient-derived cancer cells in mixed T cell/cancer cell culture experiments. Moreover, IFN-γ as induced by treatment with anti-PD-L1:TRAIL up-regulated PD-L1 expression on cancer cells and sensitized cancer cells to TRAIL-mediated apoptosis by anti-PD-L1:TRAIL. Of note, anti-PD-L1:TRAIL converted PD-L1-expressing monocytes, monocyte-derived dendritic cells and macrophages into TRAIL-displaying and pro-apoptotic effector cells that triggered apoptotic elimination of cancer cells. Thus, fusion protein anti-PD-L1:TRAIL has promising multiple and mutually reinforcing anticancer activities that may be exploited for therapeutic PD-L1/PD-1 checkpoint inhibition.

As detailed in chapter 2, various members of the TNFR/TNF superfamilies have important immunoregulatory activities that are of great interest for cancer immunotherapy. Unfortunately, both efficacy and safety issues have hampered the development of first-generation drugs targeting these families. Specifically, sFasL and sCD40L require hexamerization at a minimum in order to activate their cognate receptors. However, a ubiquitous cross-linking of agonistic receptors, using e.g. hexamerized soluble ligand or agonistic antibodies can trigger severe toxicity, as e.g. evidenced by dose-limiting liver toxicity of CD40 agonist antibodies in early clinical trials. In chapter 6 we set out to overcome these issues, by developing a pre-targeting strategy in which soluble forms of TRAIL, FasL and CD40L were genetically fused to a scFv antibody fragment specific not directly for a tumor antigen but for a fluorescent tag, fluorescein. These so-called scFvFITC:TNFSF fusion proteins are essentially inactive in solution and do not bind to cells. Only upon pre-targeting of cancer cells with FITC-labeled anti-cancer antibodies can a scFvFITC:TNFSF fusion protein bind and selectively gain agonist activity. Proof of concept for this approach was generated with in vitro studies using the pro-apoptotic ligands TRAIL and FasL and the co-stimulatory ligand CD40L. Our data indeed show that fusion proteins scFvFITC:sTRAIL and scFvFITC:sFasL induced potent target antigen-restricted apoptosis in cancer lines and in primary patient-derived cancer cells only when pretargeted with an appropriate FITC-conjugated antitumor antibody. In a similar setting, fusion protein scFvFITC:CD40L promoted tumor-directed maturation of immature monocyte-derived dendritic cells (iDCs). Using this versatile pre-targeting approach, toxicity towards normal cells that express the target may be controlled better and antigen escape of target antigen-negative cancer cells may be overcome by rational combinations of FITC-labeled...
anticancer antibodies. In summary, this novel approach provides a rapid and versatile way to delivery TNF family ligands to various cancer cells when pre-targeted with FITC-tagged antitumor mAbs or combinations thereof.

Finally, in **chapter 7 and 8** we provide a comprehensive summary of the results and conclusions as well as perspectives for further development of new TNFL/TNFR superfamily-based immunotherapeutics.

### References

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