Chapter 2

Therapeutic antibody-based fusion proteins for targeting members of the Tumor Necrosis factor superfamily in cancer

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Abstract

TNF/TNF receptor superfamily members play pivotal immunoregulatory roles in homeostasis and execution of immune responses. Selected ligand and receptor pairs of these superfamilies have been evaluated as possible suitable targets for cancer immunotherapy, including TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL receptors, Fas ligand/Fas and CD40 ligand/CD40.

This review highlights the rationale and current status of tumor-selective TNFL/TNFR-based fusion proteins for cancer therapy.

Introduction

In recent decades, antibody-based immunotherapy has been an intense area of research in oncology resulting in breakthroughs in the treatment of various forms of cancer. The first clinical success with antibodies was obtained with the FDA approval of Rituximab for the treatment of non-Hodgkin’s lymphoma (NHL) in 1997. Thereafter, various anticancer antibodies have entered the clinic, including trastuzumab (anti-HER-2/neu), cetuximab (anti-EGFR) and bevacuzimab (anti-VEGF).

More recently, antibody-based modulation of so-called immune checkpoints have revolutionized cancer immunotherapy. Immune checkpoints refer to a host of inhibitory pathways in the immune system that are paramount for maintaining self-tolerance and controlling duration and magnitude of immune responses in order to prevent collateral damage. Unfortunately, cancer cells can hijack one or more immune-checkpoint pathways, which allow them to evade from the immune system, in particular from tumor-directed T cells. Typically, immune checkpoints rely on the interaction between cell surface-expressed ligand–receptor pairs, which can be blocked (or modulated) by antibodies or recombinant soluble forms of the respective ligands or receptors.

The first immunomodulatory approach to enter clinical practice in oncology targets the CTLA-4 checkpoint molecule. CTLA4 is a dominant inhibitory immune checkpoint on T cells that plays a key role in central immune tolerance and homeostasis and is up-regulated on exhausted TILs. Specifically, CTLA-4 modulates the early phases of activation of naïve or memory T cells after TCR stimulation by cognate MHC-peptide complexes presented by APCs. In 2011, the CTLA-4 blocking antibody ipilimumab received FDA-approval for treatment of metastatic melanoma. More recently, unprecedented clinical efficacy was reported for antibodies that rejuvenated the anticancer activity of exhausted...
TILs by blocking the PD-1/PD-L1 axis. In early clinical trials the objective response rate of the PD-1 blocking antibody nivolumab reached 32%, with 4 complete responses and 34 partial responses metastatic melanoma patients. Nivolumab was first approved in 2014 for use in the second-line treatment of advance melanoma.

However, all recent clinical trials used conventional antibodies resulting in a generalized blockade immune checkpoints interaction. This indiscriminate blocking of immune checkpoints may explain the observed severe immune-mediated adverse reactions involving various organ systems, including enterocolitis, hepatitis, dermatitis, neuropathy, and endocrinopathy. The majority of these reactions appear during treatment while some may occur up to months after discontinuation.

Ideally, an anticancer agent acquires its therapeutic activity only after tumor cell surface binding and has no or only minimal adverse effects towards normal cells. In this respect, tumor-directed antibody-based fusion proteins containing a pro-apoptotic or co-stimulatory ligand of the TNF/TNF receptor superfamily appear promising for use in cancer immunotherapy.

TNF receptor superfamiliy is an important immunoregulatory family of cell surface proteins comprised of 29 members that interact with their corresponding cognate counterpart which are members of Tumor necrosis factor (TNF) superfamily (TNFSF). TNFSF members have diverse biological functions. TNFSF members such as nerve growth factor (NGF), CD40 ligand (CD40L), OX40 ligand (OX40L) and B cell activating factor (BAFF) can promote cell survival and trigger inflammatory signaling, whereas TNF, FAS ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) can induce (apoptotic) target cell death and are considered to be Death ligands that act via interaction of their corresponding death receptor (DR). Most prominent DRs are Fas and TRAIL-receptor 1 and 2 that are characterized by a hallmark intracellular death domain. The other subgroup of TNFRs consists of immune co-stimulatory receptors that typically trigger pro-inflammatory and pro-survival signal through TRAF molecules leading to e.g. activation of NFkB.

This mini review highlights and discusses recent insights in the biology and clinical applicability of TNFR superfamiliy members in oncology, with a particular focus on TRAIL-receptors, Fas and CD40.

**Death Receptor signaling**

The most interesting DRs for cancer therapy are those activated by the TNF-related apoptosis-inducing ligand (TRAIL), a member of TNFSF that is
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expressed on the surface of immune effector cells, such as NK cells and activated T-cells. TRAIL binds to a set of 5 different TRAIL receptors comprising two pro-apoptotic DRs, namely TRAIL-R1 (DR4) and TRAIL-R2 (DR5), two “decoy” receptors (TRAIL-R3 and TRAIL-R4) and the soluble receptor osteroprotegerin (OPG). These TRAILRs are expressed on most normal cells and on various tumor cells. Binding of TRAIL to DR4 or/and DR5 results in the induction of apoptosis via assembly of the death inducing signaling complex (DISC) and activation of caspase cascade. Of note, TRAIL treatment triggers prominent cell death in cancer cells, whereas normal cells are essentially resistant. In TRAIL-deficient mice, enhanced tumorigenesis and metastasis was detected, indicating TRAIL is involved in immune surveillance of cancer. Indeed, infusion of Dulanermin (sTRAIL) in patients was not associated with a maximum tolerable dose (MTD), indicating that sTRAIL is safe and tumor-specific. This sparked the development of an array of different DR4/5 agonists for cancer immunotherapy, including DR4 and DR5 agonistic antibodies.

Analogously, the trimeric receptor Fas (also known as CD95) is activated by binding of FasL, a type II transmembrane protein expressed on e.g. activated T cells. After binding FasL, Fas clusters and formation of the DISC is initiated. FasL can also bind to Decoy Receptor 3 (DcR3), a soluble receptor that lacks an intracellular DD and is thus considered a decoy or inhibitory receptor that regulates sensitivity to FasL. Correspondingly, DcR3 is up-regulated in various cancers. Agonistic antibodies that target Fas and recombinant forms of soluble FasL have shown anticancer activity in animal models. However, ubiquitous Fas activation resulted in severe liver toxicity, thus potentially hampering clinical applicability of Fas-targeting in cancer therapy.

Non-targeted activation of DRs for cancer therapy

TRAIL-receptor agonists have been at the forefront of DR-targeting agents, with TRAILR activation leading to selective cell death in cancer cells. Activation of TRAILRs for cancer therapy has been evaluated for both soluble forms of recombinant TRAIL (rhTRAIL) and agonistic TRAILR antibodies (Fig. 1). Recombinant sTRAIL has pronounced selective activity towards a variety of malignant cells, with treatment inducing apoptosis in over 40% of all tumor types, both malignancies of hematopoietic origin as well as solid tumors. In addition, treatment with TRAIL induced marked antitumor activity in various xenografted tumor models in mice. Furthermore, several agonistic anti-DR antibodies have been investigated and pre-clinically evaluated. For instance, AY4 is a novel anti-DR4 antibody that induces caspase-dependent apoptosis.
in several tumor types without cytotoxicity to normal hepatocytes. In vivo administration of AY4 significantly inhibited tumor growth of human NSCLC in athymic nude mice. Similarly, agonist anti-DR5 antibody TRA-8 has shown considerable antitumor efficacy towards colon cancer cell lines in vitro and in vivo. Of note, combined treatment of TRA-8 and CPT-11 increased antitumor efficacy against xenografts established from TRA-8-sensitive tumor cell lines, indicating a non-overlapping activity profile of ligand and agonistic antibody that may be exploited for combinatorial therapy.

Both recombinant human TRAIL (rhTRAIL) and agonistic anti-TRAIL-R antibodies have been tested in various early stage clinical trials. Importantly,
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treatment with a soluble homotrimeric TRAIL preparation (Dulanermin) was not associated with apparent toxicity in cancer patients in Phase I/II trials. Unfortunately, dulanermin treatment had disappointing clinical activity as a single agent treatment albeit with no apparent toxicity. However, the combinatorial synergistic activity of TRAIL with conventional anticancer drugs is reflected by a host of publications on preclinical studies both in vitro and in vivo. Thus, rational combination of TRAIL with synergizing chemotherapeutics and/or targeted drugs may yield more prominent clinical activity in human malignancies. In fact, dulanermin has also been tested in combination with chemotherapeutic drugs with encouraging trends towards antitumor efficacy. For example, a phase Ib clinical study of combination of dulanermin with paclitaxel and carboplatin (PC) and bevecizumab (PCB) induced 1 complete response and a high percentage of partial responses in patients with non-small-cell lung cancer (NSCLC) in the absence of dose-limiting toxicity.

However, a Phase II clinical study of combination of Dulanermin and paclitaxel and carboplatin (PC) and bevecizumab (PCB) showed no improvement of progression-free survival (PFS) and overall survival (OS) in patients with non-small-cell lung cancer. Importantly, there was a trend toward favorable PFS and OS with dulanermin treatment in NSCLC patients with high GalNT14 expression. Recently, a recombinant mutated form of human Apo2L/TRAIL, termed circularly permuted TRAIL (CPT), has been tested in a multicenter, open-label phase II trial for the treatment of multiple myeloma (MM) and other hematologic malignancies. Compared to Apo2L/TRAIL, CPT was reported to have better antitumor activity and stability in mice, rats and human, while sparing normal cells. In this study, the overall response (ORR) of 41 patients was 22%, including 2 patients with a complete response (CR), 3 patients with a near complete response (nCR), and 4 patients with a partial response (PR). In brief, CPT plus thalidomide was well tolerated with no occurrence of dose-limiting toxicities and demonstrated promising antitumor activity in patients with relapsed and/or refractory multiple myeloma.

At the same time various agonistic anti-TRAIL-R1 antibodies and agonistic anti-TRAIL-R2 antibodies have also been evaluated in clinical studies. For instance, the agonistic TRAIL-R1 antibody mapatumumab was evaluated either as monotherapy or in combination with chemotherapeutic drugs in patients with non-Hodgkin’s lymphoma, colorectal cancer or NSCLC. Mapatumumab proved to be well-tolerated, with no patients experiencing drug-related hepatic toxicity in early-stage clinical trials. In a phase Ib/2 study of mapatumumab in patients with relapsed/refractory NHL, patients with follicular lymphoma
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(FL) experienced clinical responses, including 2 CRs and 1 PR\textsuperscript{33}. However, limited clinical benefit for CRC patients was observed upon mono-treatment. A phase II trial of mapatumumab in patients with refractory colorectal cancer showed none of patients developed an objective tumor response\textsuperscript{35}.

In conclusion, disappointing clinical activity of single treatment of “first-generation” DRs-specific agonists was observed in patients. The reasons for the limited clinical benefit of these DR-specific agonists are likely multifold, but include the presence of intrinsic resistance to TRAILR signaling as reported for various tumor types. Furthermore, treatment with mapatumumab and chemotherapeutic drugs induced PRs in 19% of patients with advanced solid tumors, but no clinical benefit was found in patients with NSCLC\textsuperscript{33-36}. Targeting of TRAIL-R2 using agonistic antibodies, such as conatumumab, drozitumab, tigatuzumab and lexatumumab yielded more promising results. For instance, tigatuzumab combined with gemcitabine was well-tolerated and may be clinically active for the treatment of chemotherapy-naive patients with unresectable or metastatic pancreatic cancer\textsuperscript{37}. In this study, PFS rate of patients at 16 weeks was 52.5% and objective response rate (ORR) was 13.1%. Moreover, 46% of patients had stable disease and 23% patients had progressive disease (PD)\textsuperscript{37}.

Further, in an independent clinical study of conatumumab in combination with leucovorin, 5-fluorouracil, and irinotecan (FOLFIRI) chemotherapy was associated with a trend towards improved progression-free survival (PFS) with acceptable toxicity in patients with metastatic colorectal carcinoma\textsuperscript{45}. Tigatuzumab has been tested in Phase I and II clinical trials in patients with NSCLC, pancreatic cancer, metastatic colorectal carcinoma (mCRC)\textsuperscript{37, 39, 40}. A Phase I image and pharmacodynamics trial of tigatuzumab indicated clinical benefit of this antibody in the treatment of patient with mCRC\textsuperscript{39}.

Importantly, collectively all these “first-generation” TRAILR agonists do not have specificity for tumor cells and can bind to all normal cells that express TRAILRs. Therefore, their tumor accretion is likely to be suboptimal. Also these first generation TRAILR agonists do not optimally trigger activation of DR signaling, opening up ways of improving the efficacy of TRAILR-based agonist. This issue will be discussed later in this review.

The activation of apoptotic Fas signaling using sFasL and agonistic Fas antibodies demonstrated potent antitumor activity towards a host of tumor cell lines and primary patient-derived human tumor cells\textsuperscript{17} (Fig.2). However, systemic administration of Fas agonists in mice was associated with severe hepatotoxicity\textsuperscript{22}. In contrast, intra-peritoneal administration of sFasL induced
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*Fig. 2*.

Soluble homotrimeric FasL is essentially incapable of activating Fas-apoptotic signaling. However, hexamerized recombinant forms of sFasL have Fas-activating capacity analogous to membrane-expressed FasL. These findings suggested that localized application of Fas agonists might be feasible and could trigger efficient antitumor activity in the absence of systemic toxicity. In recent years, efforts have further focused on the design of more selective tumoricidal Fas agonists with improved safety profiles. In this respect, homogenous trimeric sFasL that does not contain higher order molecular aggregates is largely inactive and actually inhibits apoptotic activity of membrane-expressed FasL. However, secondary cross-linking of trimers of FasL to a hexameric state enables induction of Fas-mediated apoptosis in cancer cells. Based on these findings, an engineered hexameric FasL, termed mega FasL (MFL), was developed, which induced apoptosis in both established cell lines and primary cells from multiple myeloma (MM), acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and Burkitt’s lymphoma. Of note, MFL did not induce apoptosis in hematopoietic progenitor cells. MFL selectively induced antitumor activity in hematological cancer cells from lymphomas and leukemias, whereas it did not reduce the functional capacity of human hematopoietic stem cells in in vivo transplantation models.

Although many cancer models have shown sensitivity towards Fas agonist treatment in vitro and in vivo, resistance to FasL has also been reported.
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other cancer models\textsuperscript{47}. For instance, prostate cancer cells with an androgen-independent phenotype appear to up-regulate anti-apoptotic molecules like cFLIPL\textsuperscript{48}. In this respect, combination treatment with chemotherapeutics enhanced FasL-mediated apoptosis in both established cell lines and primary prostate cancer cells\textsuperscript{49}. Recently, combination of FasL and gemcitabine in pancreatic cancer cells was demonstrated to the simultaneous induce caspase-dependent and caspase-independent cell death, suggesting chemotherapeutics can sensitize cells towards necroptosis-type-mediated death\textsuperscript{50}.

**Targeted activation of DRs for cancer therapy**

As described above, “first-generation DRs-targeting agonists” showed limited therapeutic activity in clinical trials. In the past ten years, we and others have pioneered the development of improved TRAIL-based therapeutics exploiting scFv antibody fragment-targeted delivery of TRAIL to cancer cells. Such antibody fragment-mediated targeting of TRAIL selectively enhances the anti-tumor activity of TRAIL towards various types of cancer, including solid and hematologic malignancies. Briefly, genetic fusion of TRAIL to a scFv antibody fragment allows for the selective delivery of TRAIL to a pre-selected tumor-associated antigen at the tumor cell surface. The resulting high levels of tumor cell surface-bound TRAIL then efficiently activate apoptotic signaling via the agonistic TRAIL-receptors TRAIL-R1 and TRAIL-R2 in a mono- and/or bi/multicellular manner\textsuperscript{51-53}. Of note, non-targeted sTRAIL has no intrinsic tumor-selective binding activity and cannot effectively cross-link and activate TRAIL-R2, a receptor that is typically highly expressed on cancer cells and requires cross-linking to exert its full pro-apoptotic activity\textsuperscript{54}.

The selection of a particular tumor target antigen for this purpose can significantly contribute to the efficacy of scFv:sTRAIL-based therapeutics, as evidenced by our previous study in which sTRAIL was targeted using an EGFR-blocking antibody fragment derived from EGFR-blocking mAb 425. Treatment of EGFR-positive tumor cells with scFvEGFR:sTRAIL inhibited EGFR-mitogenic signaling, thereby sensitizing cells to apoptosis, while simultaneously inducing optimal TRAIL-apoptotic signaling via TRAILR1 and TRAILR2\textsuperscript{62}. Similarly, targeting of MCSP (Melanoma-Associated Chondroitin Proteoglycan Sulphate) expressed on melanoma cells, using fusion protein scFvMCSP:TRAIL, selectively induced TRAIL-mediated apoptosis and inhibited MCSP-dependent pro-metastatic signaling\textsuperscript{56}. In Table 1 an overview of TRAIL fusion proteins targeting cell surface antigens known to be highly expressed on various tumor cell types is presented.
In addition to direct tumor-targeting, the scFv targeting domain can also be used to selectively deliver TRAIL to the cell surface of immune effector cells, whereby these cells are equipped with an additional tumoricidal effector molecule. For instance, the T-cell targeting molecules anti-CD3:TRAIL and K12:TRAIL were designed to equip T cells with additional surface TRAIL and enhance the antitumor efficacy of T cells. In this study, both K12:TRAIL and anti-CD3:TRAIL selectively bound to the T-cell surface antigens CD3 and CD7, respectively, leading to cell surface accretion of TRAIL. Importantly, these two fusion proteins the tumoricidal activity of T cells was enhanced to over 500-fold toward cancer cell lines and primary patient-derived malignant cells in vitro. Furthermore, T-cell surface delivery of TRAIL strongly inhibited tumor growth and increased survival time of xenografted mice more than 6-fold. This approach may be used to augment the efficacy of conventional adoptive T-cell therapies.

In addition to T cell targeting, our group developed a TRAIL fusion protein designed to arm myeloid effector cells that express the C-type lectin-like molecule-1 (CLL-1). Treatment with fusion protein scFvCLL-1:TRAIL enhanced the antitumor leukocyte activity, as evaluated for granulocytes, and potentiated antibody-mediated cytotoxicity of clinically used therapeutic antibodies (e.g., rituximab, cetuximab). Along this line, our group produced a CD47-targeting/
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blocking TRAIL fusion protein. Fusion protein anti-CD47:TRAIL was designed to induce CD47-restricted apoptosis in cancer cells while simultaneously block CD47-SIRPα interaction\(^6\). Indeed, this protein did not only trigger CD47-restricted apoptosis in malignant B cell leukemia and primary B-cell malignancies, but also enhanced tumor cell phagocytosis. The later activity of anti-CD47:TRAIL enhance the pro-phagocytic activity of rituximab towards CD47+/CD20+ cancer cells\(^6\). This demonstrates that bi-functional TRAIL fusion proteins can have enhanced dual antitumor activity.

Various other forms of bifunctional TRAIL fusion proteins have been described. A nice example thereof is a CD40-directed TRAIL proteins, named G28:TRAIL. CD40 is a member of TNFSF that can activate dendritic cells and hereby trigger T cell-mediated antitumor activity. Treatment with G28:TRAIL induced selective apoptosis in CD40-expressing cancer cells. Importantly, binding of G28:TRAIL to CD40-positive cells not only led to activation of TRAIL receptor signaling but also activation of CD40-expressing dendritic cells\(^6\).

Similarly, another bifunctional fusion designated scFv:lαhCD70-TNC-TRAIL has been described that combines activation of CD27/CD70-mediated signaling with induction of cell death in CD70-expressing cancer cells. Expression of CD70 is restricted to B cells, dendritic cells and activated T cells. CD70 is the cognate ligand for CD27, also a member of TNFSF. Activation of CD27 is involved in T cell survival, activation and differentiation. High CD70 expression is observed on various cancers, including renal cell carcinoma, pancreatic carcinoma, ovarian and colon cancer\(^6\). Fusion protein was designed scFv:lαhCD70-TNC-TRAIL to induce TRAIL-mediated apoptosis in CD70-expressing cancer cells and simultaneously block immune inhibitory signaling of CD70/CD27 interaction. Taken together the studies detailed above have exploited the intrinsic activity profile of sTRAIL and improved its antitumor activity by selective targeting of the ‘inactive’ sTRAIL to tumor cells or immune cell effector cells. A similar approach used for agonistic DR antibodies, most those directed against DR5. As described above, DR5 only efficiently signals apoptosis upon sufficient cross-linking. Based on this feature, we have engineered a recombinant bi-specific tetravalent antibody, designated MCSPxDR5, which has both high binding affinity for the melanoma-associated antigen MCSP and potent agonistic activity towards DR5\(^6\). The mode of action of MCSPxDR5 involves high affinity binding to tumor cell surface-expressed MCSP with concomitant localized enhanced cross-linking of DR5. BsAb MCSPxDR5 showed potent MCSP-directed pro-apoptotic activity towards MCSP-positive melanoma cells, with essentially no or minimal toxicity towards normal cells. Antitumor activity by MCSPxDR5 was enhanced by cross-
linking of its IgG domain by either by artificial crosslinker or Fc receptors on myeloid immune effector cells\textsuperscript{63}. This concept was also confirmed by RG7386, a tetravalent FAP-TRAIL-R2 antibody\textsuperscript{64}. Both in vitro and in vivo studies showed that RG7386 triggers apoptosis in cancer cells. The antitumor efficacy of RG7386 was FAP-dependent and FcR crosslinking-independent and proved to be superior to conventional anti-DR5 antibodies. Combined treatment of irinotecan or doxorubicin with RG7386 led to substantial tumor regression in patient-derived xenograft models.

In analogy to sTRAIL, essentially sFasL lacks apoptotic activity, but this activity can be readily reactivated by secondary crosslinking of sFasL into hexamers. Therefore, sFasL appears an interesting candidate as an effector molecule in antibody-targeted approach for cancer therapy. Antibody fragment-targeted sFasL, using an scFv:sFasL fusion protein format, ensures that en route sFasL remains inactive, but is converted to its fully active membrane-like form upon antibody-mediated binding to the tumor cell surface. Feasibility of this concept was first demonstrated using fusion protein sc40-FasL, which selectively targeted the fibroblast activation protein (FAP). FAP-targeted delivery of sFasL resulted in selective Fas-mediated apoptosis in FAP-expressing tumor cells in vitro\textsuperscript{65}. Importantly, the intravenous application of sc40-FasL in mice showed no signs of systemic toxicity and prevented growth of xenotransplanted FAP-positive, but not FAP-negative, tumor cells. Analogously, we explored targeted delivery of sFasL to the T-cell leukemia-associated antigen CD7 as well as the B cell leukemia antigen CD20 using fusion protein scFvRIT:FasL (anti-CD20:FasL). We demonstrated that scFvRIT:FasL induced target antigen-restricted apoptotic elimination of cancer cells. Specifically, treatment of acute lymphoblastic leukemia (T-ALL) cell lines and patient-derived T-ALL, peripheral T-cell lymphoma (PTCL), and CD7-positive acute myeloid leukemia (AML) cells with scFvCD7:FasL showed potent CD7-restricted induction of apoptosis. Of note, antitumor activity by scFvCD7:FasL in T-cell leukemia cells was enhanced by various small molecule inhibitors, including farnesyl transferase inhibitor L-744832 and the proteasome inhibitor bortezomib (valcde). Of note, long-term treatment of CD7-positive resting lymphocytes with scFvCD7:sFasL did not induce apoptosis, although activated CD7+ T cells were partly sensitive to apoptosis induction\textsuperscript{66}. The latter finding was further evaluated in studies on rheumatoid arthritis, in which scFvCD7:FasL was used to eliminate activated autoreactive T-cells\textsuperscript{66}.

The CD20-targeted fusion protein scFvRIT:sFasL comprises an scFv derived from clinically used anti-CD20 antibody rituximab. Rituximab has been reported
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to trigger CD20 cross-linking and apoptotic elimination of malignant B cells. In line with this, scFvRit:sFasL potently induced apoptosis in CD20-positive malignant cells, which was superior compared to co-treatment of rituximab and FasL. These data suggest a unique cooperative effect of simultaneous CD20 cross-linking and Fas-activation. This phenomenon is probably due to the fact that crosslinking of CD20 target molecule induces apoptosis in selected CD20-positive B-cell malignancies. In fact, it has been reported that rituximab induced apoptosis in patients with B-cell malignancies partly relies on crosslinking of surface CD20 on targeted cells.

B7 molecules are highly expressed on B-cell lymphoma cells and regarded as an interesting target antigen for treatment of B-cell NHL. CTLA-4 is the natural counterpart of B7. Therefore, fusion protein CTLA-4-FasL was designed to induce potent apoptotic activity in B7+/Fas+ B-cell lymphoma cell lines. Indeed, CTLA4-FasL bound to B7+ B-cell malignancies and induced apoptosis in vitro or in vivo. Importantly, other examples of tumor-selective sFasL fusion proteins are c49scFv- FasLext and L6scFv-FasLext, which target TAG-72 and TAL-6, respectively. Of note, fusion protein c49scFv-FasLext induced highly effective in vitro killing of TAG-72-positive Jurkat-Ras tumor cells with a 3000-fold greater cytotoxicity as compared to soluble FasL.

CD40L biology

CD40L is mainly expressed on T cells and binds to CD40 on antigen-presenting cells, such as dendritic cells (DCs). One of the main functions of the CD40L/CD40 system is to activate and “license” or “condition” DCs to prime cytotoxic CD8+ T-cell responses. CD40L is expressed on e.g. antigen-specific CD4+ T helper cells and, upon helper T cell binding to DCs, CD40L cross-links and activates CD40 on DCs. Following CD40L activation, CD40L-CD40 interaction triggers a multi-pronged response, including activation and survival of DCs, up-regulation of MHC class I and co-stimulatory molecules such as CD80/CD86 as well as induction of cytokines such as IL-12 and IL-6. Thus, CD40L initiates a pro-survival signal in DC and promotes antigen presentation by CD40-expressing DC. In brief, CD40L-licensed DCs present peptide-MHC-class I complexes in the context of appropriate co-stimulatory molecules to CD8-positive CTL precursors. Of note, CD40 signaling on DC with agonistic anti-CD40 antibodies or CD40L can mimic this cellular effect and trigger direct activation of specific CD8-positive CTL responses. In addition to the biological function of CD40/CD40L on immune effector cells, CD40 is also aberrantly expressed on many lymphoid malignancies, with CD40 expression detected on...
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e.g. Hodgkin lymphoma (HL), NHL, acute myeloid leukimas (AML), and multiple myeloma (MM)\textsuperscript{76}. Moreover, CD40 is expression on some solid malignancies, such as bladder, renal, pancreatic, prostate, colon and lung cancer \textsuperscript{69, 70}. Based on these findings, CD40 has been regarded as a bona fide tumor target for certain types of cancer\textsuperscript{78, 79}. Taken together, some anti-CD40 agonists were designed to induce direct apoptosis in lymphocytic malignancies, while other CD40-targeting agonists were designed to attribute to activation of APC and subsequently augment T-cell-mediated antitumor responses.

**CD40L/CD40-based approaches for cancer therapy**

From the above, it is clear that CD40 ligation can on the hand be used to trigger activation of anti-tumor immunity and on the other hand to trigger direct cytotoxicity towards CD40-expressing malignancies. Immunotherapy targeting CD40/CD40L is focused on both sides and has been tested in pre-clinical or clinical studies using soluble recombinant CD40 ligand (CD40L) and CD40-targeting antibodies. When CD40L is expressed on CD4\textsuperscript{+}T cells it can effectively multimerize and activate APCs. Reversely, trimeric soluble CD40L is not effective in inducing CD40-signaling. However, higher order oligomerization, e.g. using a 4-trimer form of CD40L, restores full co-stimulatory signaling by CD40L for B cells\textsuperscript{80}. Along with these findings, soluble recombinant CD40L (rCD40L) as an antitumor therapeutic has been tested in pre-clinical studies. Two major observations evidenced that rCD40L could be used to target cancer cells, 1) rCD40L triggers immune stimulation observed after crosslinking of surface CD40, including augmentation of antigen presentation by DCs, monocytes and B cells\textsuperscript{81, 82}. 2) rCD40L induces cytotoxicity against CD40-expressing tumors. Pre-clinical studies showed that treatment with rCD40L upregulate antigen presentation and subsequently stimulate autologous CTL responses in B cell malignancies\textsuperscript{82, 83}. A phase I trial using recombinant CD40L showed that daily administration of rCD40L for 5 days resulted in PR in 2 of 32 patients with advanced cancers, probably due to both of enhancement of immune stimulation and direct cytotoxicity against CD40-expressing tumors\textsuperscript{84}. Furthermore, a human CD40L fused to an isoleucine zipper trimerization domain (CD40L-IZ) displayed a significant increase in CD40-induced B cell proliferation and activation. These findings demonstrate that CD40-targeting can enhance T-cell immunity and T cell-mediated antitumor activity in pre-clinical studies\textsuperscript{85}.

In addition to rCD40L, CD40-targeting antibodies also categorized into two types of antibodies that can either activate CD40-mediated immune responses or induce direct cytotoxicity in CD40+ cancers. Agonistic anti-CD40 antibodies
activate DCs via cross-linking of surface CD40, whereupon priming of CD8+ T cells is enhanced, leading to development of anticancer T cell immunity. The first anti-CD40 antibody tested in human is CP-870893, a fully human IgG2 mAb, which triggers CD40-mediated stimulation of immune responses but not due to direct cytotoxic effects on cancer cells, e.g. ADCC or CDC\textsuperscript{86}. CP-870893 was well-tolerated in combination with gemcitabine and induced partial responses (PR) in 15-20% of patients with pancreatic ductal adenocarcinoma (PDA)\textsuperscript{87}. Recently, CP-870893 has been tested in combination with anti-CTLA-4 blocking antibody tremelimumab in patients with multiple myeloma (MM). MM patients received CP-870893 every 3 weeks and tremelimumab every 3 months for up to 1 year. Amongst 22 patients, tumors disappeared in 2 and diminished in 4. The ORR by combined treatment is 27.3%, higher than that achieved with either single treatment of tremelimumab or ipilimumab\textsuperscript{88}. Co-administration of anti-CD40 antibody and polyI:CLC significantly enhanced CD4+ and CD8+ T cell responses in lung, compared either to adjuvant given alone\textsuperscript{96}. In contrast, no objective clinical responses have been observed upon combination of CP-870893 with carboplatin and paclitaxel. These findings indicate that treatment of tumor cells with agonistic anti-CD40 antibodies can activate antitumor immune responses and subsequently leads to induction of tumor regression.

In addition, CD40 is not only expressed on APCs but also on various cancers\textsuperscript{90}, such as B-cell lymphoma\textsuperscript{91}. To this end, another approach is to use of CD40-targeting agonists to selectively target surface CD40, resulting in direct apoptosis or inhibition of proliferation in CD40-expressing cancer cells. It has been found that recombinant human CD40L (rhCD40L) induced significant antitumor activity in SCID mice xenografted with ovarian tumor cells\textsuperscript{92}. Similarly, rhCD40L improved survival of several-combined SCID mice implanted with mouse-human lymphoma models\textsuperscript{91}. Furthermore, CD40-targeting antibodies can mediate ADCC or induce direct CD40-mediated signaling that leads to apoptosis. Some anti-CD40 antibodies, such as dacetuzumab and lucatumumab, were investigated and evaluated for killing CD40-positive cancers. To evaluate the rate and duration of objective responses and safety of single-agent dacetuzumab, a phase II study was undertaken in patients with relapsed diffuse large B-cell lymphoma (DLBCL)\textsuperscript{93}. In this study, overall response rate was 9% and disease control (CR+PR+SD) was 37%, indicating single-agent dacetuzumab has modest activity and manageable toxicity in patients with relapsed DLBCL\textsuperscript{100}. Recently, a phase IA/II study of another anti-CD40 antibody lucatumumab was designed to determine the maximum tolerated dose (MTD) and activity in patients with relapsed/refractory lymphoma. In this study, 74 patients with NHL and 37
with HL were enrolled and responses were observed in various subtypes of lymphoma. Specifically, the OR by computed tomography among patients with follicular lymphoma (FL) and marginal zone lymphoma of mucosa-associated lymphatic tissue (MZL/MALT) was 33.3% and 42.9%, respectively, suggesting modest activity induced by lucatumumab in relapsed/refractory patients with advanced lymphoma. Of note, these antibodies do not prevent CD40-CD40L interaction like blocking anti-CD40 antibodies, but behave as a partial agonist. Recently, a phase I clinical trial of a chimeric IgG1 anti-CD40 antibody ChiLo7/4 was performed in patients with a range of CD40-expressing solid tumors and DLBCL who are resistant to conventional therapy. MTD of ChiLo7/4 was 200mgx4 with dose-limiting toxicity of liver transaminase at 240 mg/kg. ChiLo7/4 can activate B and NK cells at doses that can be administrated safely. In this study, patients with SD had a median time to progression of 6 months and overall median survival for all patients was 11 months. In general, CD40-targeting antibody-based approaches appear promising in cancer therapy, especially in combination with other anticancer therapeutics.

Tumor-selective CD40L/CD40-based approach for cancer therapy

Unfortunately, there are indications from pre-clinical studies and early stage clinical studies for dose-limiting toxicity of CD40L towards normal hepatocytes. In fact, treatment of mice with anti-CD40 agonists was associated with inflammatory side effects. To solve this problem, tumor-selective CD40L/CD40-based approaches have being developed. To enhance tumor-selective delivery of CD40L, a 4 trimer form of CD40L was fused to scFvgp100 in order to target gp100-positive melanoma tumor antigen and to simultaneously promote DC maturation. Fusion protein SPD-gp100-CD40L activated dendritic cells by aggregating CD40 trimers on the DC membrane surface and, upon DNA vaccination with SPD-gp-CD40L plasmid together with plasmids encoding IL-12 and GM-CSF, significantly inhibited tumor growth in mice.

To improve antitumor activity and reduce systemic toxicity of soluble CD40L, antibody-based CD40L fusion proteins were designed and tested in the pre-clinical studies. In particular, fusion proteins anti-EpCAM:CD40L and anti-CD20:CD40L were designed to selectively bind to EpCAM-positive carcinoma cells and CD20-positive B cell leukemic cells, respectively, whereupon target antigen-restricted activation of CD40 induced DC maturation. In brief, anti-EpCAM:CD40L selectively delivered CD40L to the carcinoma marker EpCAM on carcinoma cells and subsequently induced paracrine maturation of DCs over 20-fold more than a non-targeted scFv:CD40L fusion protein. Analogously,
fusion proteins targeting TNFSF

Fusion proteins targeting TNFSF

fusion protein anti-CD20:CD40L promoted paracrine maturation of DCs in the presence of CD20-expressing B cell malignancies. Importantly, anti-CD20:CD40L also induced granulocyte-mediated phagocytosis in B-cell malignancies and triggered CD20-mediated apoptosis in certain B cells. In addition to tumor-selective delivery of CD40L, fusion protein scFv:G28-TRAIL selectively targets CD40 by virtue of its scFv domain and was found to not only enhance TRAIL-mediated apoptosis in cancer cells but also in activation of CD40. In conclusion, tumor-selective CD40L-based approaches may help pave the way for CD40L-based cancer immunotherapy due to target cell-localized anti-tumor and DC maturation activity.

Conclusions

As discussed in this review, both pro-apoptotic and co-stimulatory TNFR ligand/TNF receptor pairs are of clear interest for use in cancer immunotherapy and can be targeted both by recombinant soluble TNFR ligands and agonistic TNFR antibodies. A primary challenge to address is to obtain the required specificity and tumor-localized TNFR-signaling without off-target effects. To this end, antibody-based TNFR-targeting fusion proteins appear promising candidates. Important features of this approach include the selective accretion to the surface of targeted tumor cells and subsequent localization/activation of TNFR ligands to full membrane-like antitumor activity.

Of note, clinical trials evaluating single treatment with TRAIL-receptor agonists and CD40 agonists met with limited clinical benefit, but combination therapy with chemotherapeutics did have beneficial clinical activity. In line with this, several therapeutics synergize with TRAIL and/or CD40 in preclinical studies. Therefore, design of rational combinatory strategies that provide maximal synergistic antitumor efficacy and minimal side effect becomes current trend in the clinic. Currently, there are several clinical trials that combine targeted therapy and immunotherapy in patients with melanoma, NSCLC or RCC. In this respect, combination approach of immune checkpoint-targeted anti-CTLA-4 antibody with CD40-targeting vaccine was investigated in mice implanted with melanoma. This combination approach caused significant regression of melanoma in vivo compared to control groups with single treatment, while other observations included prolonged CD8+ T cell responses and increased survival. Of note, some essential considerations for these combinations include selection of optimal combination, reduction of treatment-related toxicity and standard efficacy assessment. One particular sample is triple combination treatment of primary fibrosarcomas with anti-DR5 antibody,
Fusion proteins targeting TNFSF

anti-CD40 antibody and anti-CD137 antibody\textsuperscript{101}. This triple-antibody approach enhanced tumor-specific CD8+ T cell responses against tumor cells. However, the limitation of this approach is that it is efficient only in TRAIL-sensitive cancers. Therefore, another combination approach may include the use of for instance HDAC inhibitors to possibly address this limitation\textsuperscript{102}. Thus, research in the upcoming years should focus not only on further identification of optimal tumor targets for such strategies, but also on synergistic combinatorial treatment strategies using standard chemo/radiotherapy and new targeted drugs. In conclusion, tumor-cell localized activation of pro-apoptotic and co-stimulatory TNFR superfamily signaling has yielded promising pre-clinical data that warrants further (pre)clinical evaluation.
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