REVIEW ARTICLE

Targeting Co-Stimulatory Receptors of the TNF Superfamily for Cancer Immunotherapy

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Abstract

The clinical approval of immune checkpoint inhibitors is an important advancement in the field of cancer immunotherapy. However, the percentage of beneficiaries is still limited and it is becoming clear that combination therapies are required to further enhance the treatment efficacy. The potential of strategies targeting the immunoregulatory network by "hitting the gas pedal" as opposed to "blocking the brakes" is being recognized and intensively investigated. Hence, next to immune checkpoint inhibitors, agonists of co-stimulatory receptors of the tumor necrosis factor superfamily (TNF-SF) are emerging as promising options to expand the immunomodulatory toolbox. In this review the development of different categories of recombinant antibody and ligand-based agonists of 4-1BB, OX40, and GITR is summarized and discussed in the context of the challenges presented by the structural and mechanistical features of the TNFR-SF. An overview of current formats, trends, and clinical studies is provided.

Key Points

Targeting the co-stimulatory receptors 4-1BB, OX40, and GITR of the TNF superfamily holds potential for cancer immunotherapy.

Current developments of agonists focus on effective receptor clustering, site-specific activity, and reduced toxicity.

A variety of mono- and bispecific antibodies as well as antibody-ligand fusion proteins has been generated, which are now being evaluated in clinical trials.

1 Introduction

Interfering with the regulatory network of the immune system holds great potential for cancer immunotherapy. This has been impressively demonstrated by the successful clinical development of many immune checkpoint inhibitors that act by enhancing an antitumor immune response blocking coinhibitory receptors (e.g. CTLA-4, PD-1). However, treatment responses are still limited to a small percentage of patients [1]. Thus, current efforts focus on also exploring the opposite regulatory approach, i.e. enhancing an antitumor immune response by activating co-stimulatory receptors. Members of the tumor necrosis factor receptor superfamily (TNFR-SF), in particular 4-1BB, OX40, and GITR, have emerged here as promising targets [2]. However, the translation of the concept has been challenged by their particular structural and mechanistic features. Their influence and impact on the development of therapeutic reagents are discussed in this review.

2 Costimulatory Receptors of the Tumor Necrosis Factor Superfamily (TNF-SF)

4-1BB (CD137/TNFRSF9), OX40 (CD134/TNFRSF4), and GITR (CD357, TNFRSF18) are amongst the most intensively investigated co-stimulatory members of the TNFR-SF for cancer therapy so far. They are mainly expressed on activated T cells and NK cells, enhancing the processes of proliferation, differentiation, survival, and effector functions (for reviews, see [3, 4]). Accordingly, treatment effects of 4-1BB agonists in several preclinical mouse models were demonstrated to impact and depend on CD8+ T cells and NK cells [5, 6]. Importantly,

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upregulation of 4-1BB on antigen-primed T cells in the tumor allowed to identify and target tumor-specific T cells on site [7]. The expression of 4-1BB was shown to be enhanced by the hypoxic conditions of the tumor microenvironment [8]. Treatment with 4-1BB agonists induced expansion of tumor infiltrating CD8+ T cells [9, 10], prevention of activation-induced cell death (AICD) [11], and restoration of exhausted tumor infiltrating lymphocyte (TIL) function [12]. Furthermore, intratumoral persistence [13], reversion of anergy [14], and an increase in effector memory CD8+ T cells [15] was reported. In addition, the expression of 4-1BB on tumor microvessels was shown to be involved in enhancing the recruitment of activated T cells [16]. Early co-stimulatory studies indicated a more prominent effect of 4-1BB on CD8+ T cells and OX40 on CD4+ T cells, respectively [17, 18]. Indeed, OX40 signaling was shown to enhance the cooperation between CD4+ T cells and CD8+ T cells for antitumor activity [19], and both subpopulations were shown to participate in agonist-mediated tumor regression in preclinical mouse models [20]. Also, enhanced infiltration and function of tumor-specific CD8+ T cells and the generation of tumor-specific memory was reported [21, 22]. In the case of GITR, co-stimulation by agonistic antibodies was shown to promote antitumor response by enhancing both CD8+ and CD4+ effector T-cell activity and in particular reducing the number and activity of tumor-infiltrating Tregs [23, 24]. Although GITR, OX40, and 4-1BB appear to have the potential to drive the proliferation of Tregs, they also seem to be implicated in antagonizing Treg generation and Treg-mediated suppression (for review, see [25, 26]). Thus, the co-stimulatory impact on Tregs and the implication for cancer treatment still remain unclear. Importantly, agonists of 4-1BB, OX40, and GITR have shown great potential for combination therapies, for example, with each other, immune checkpoint inhibitors, and conventional strategies (for review, see [26, 27]).

2.1 Mechanism of Activation

In general, co-stimulatory TNFRs are expressed on immune cells and interact with their respective ligands expressed on antigen presenting cells (APC). Consequently, under physiological conditions, co-stimulation takes place in a local manner via cell-cell interaction. From a structural point of view, TNF-SF receptors are characterized by repeats of a cysteine-rich domain (CRD) in their ectodomain that can promote diverse degree of receptor self-assembly. Thus, prior to their activation the receptors present in monomeric, dimeric or trimeric state. TNF-SF ligands on the other side are characterized by an external TNF homology domain (THD) that usually leads to stable homotrimeric ligand assemblies [28]. X-ray crystal structures showed that receptor-ligand binding takes place in a symmetric ligand trimer-receptor trimer configuration, involving the typical THD and CRD

domains [29]. According to the prevalent two-step model, further clustering of this trimeric receptor-ligand complex is required to achieve efficient signaling pathway activation [30]. This step is supported by the given alignment, restricted mobility, and high local density of the ligand in its transmembrane form. In fact, many TNF-SF ligands can bind in soluble form with high affinity to their receptors, but fail to activate them efficiently, unless additional oligomerization is induced. Most of the co-stimulatory members of the TNFR-SF, including 4-1BB, OX40, and GITR, fall into this category. Thus, the induction of receptor clustering is considered essential for the efficacy of agonistic reagents. This insight has ultimately guided the development of agonists, leading to diverse antibody and ligand-based formats (Fig. 1), many of them now entering clinical trials (Tables 1, 2 and 3).

2.2 Agonistic Monospecific Antibodies

In principle, the bivalency of a classical monoclonal IgG antibody entails the potential for cross-linking and agonistic cluster induction, whereupon the position of the epitope rather than high affinity is critical [31, 32]. However, it is actually the Fc region that plays a dominant role in modulating this process. It was shown that FcγR-mediated cell surface binding of the targeted antibody can become crucial for the efficacy of co-stimulatory TNFR-SF clustering and activation [33]. Unfortunately, this makes the approach dependent on the presence of FcγR-expressing immune cells and prone to unreliable factors like FcγR expression levels and competition with serum IgG. Furthermore, isotype-dependent binding to particular FcγR types impacts the therapeutic outcome. For instance, in preclinical studies with 4-1BB agonistic antibodies of different isotypes it was shown, that binding to inhibitory FcγRIIB was required for anti-tumor efficacy, while binding to the activating FcγRIII reduced tumor effects due to T cell depletion by antibody-dependent cellular cytotoxicity (ADCC). However, an isotype with low activating/inhibitory FcγR binding ratio (reduced ADCC) was only combinable with a weak intrinsic agonist. In combination with a strong intrinsic agonist liver toxicity was observed [34]. Thus, intrinsic cross-linking capacity, isotype and availability and distribution of FcγR types determine not only the treatment efficacy, but also the side effect profile. Consequently, the development of monospecific antibodies has been challenged by these factors.

By now several 4-1BB-directed agonistic monoclonal antibodies have entered clinical trials (Table 1). Initial leading molecules were Urelumab (BMS-663513) [35] and Utolimumab (PF-05082566) [36]. Urelumab is a non-ligand-blocking fully human IgG4 antibody with a hinge mutation (S228P) for improved stability that showed clinical activity, but also dose-limiting hepatotoxicity (doses ≥ 1mg/kg)
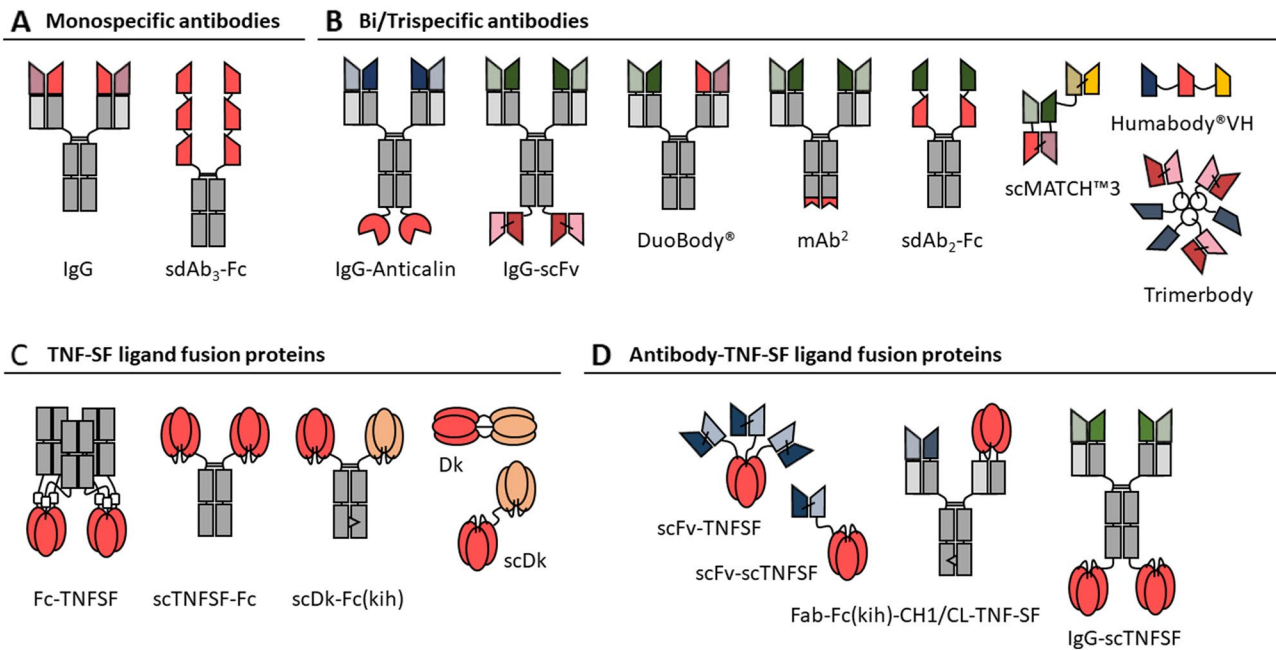
Fig. 1 Schematic overview of agonists for co-stimulatory tumor necrosis factor superfamily (TNF-SF) receptors. (**A**) monospecific antibodies, (**B**) bispecific antibodies, (**C**) TNF-SF ligand fusion proteins, (**D**) antibody-TNF-SF ligand fusion proteins. Target specificity: red/orange, costimulatory receptor; blue, TAA; green, PD-1/PD-L1; yellow, human serum albumin. *sdAb* single-domain antibody, *TNF-SF* extracellular domain of costimulatory TNF super family ligand, *kih* knob-into-hole, *Dk* duokine, *sc* single-chain

[37, 38]. Utolimumab on the other hand is a ligand-blocking humanized IgG2 antibody that has shown a favorable safety profile, but was less effective relative to Urelumab [31, 39]. Structural analysis revealed that the epitope position of Urelumab in comparison to Utolimumab enabled stronger 4-1BB cross-linking through the bivalent binding of the IgG, enhancing its intrinsic agonistic strength. In addition, both isotypes presented reduced ADCC capacity and enabled FcγRIIB-mediated cross-linking [31], whereupon affinity for FcγRIIB is 10-fold higher for IgG4 than for IgG2 [40]. Looking into optimizing the cross-linking balance of agonistic strength and FcγR affinity led to the development of LVGN6051 that combines weak intrinsic 4-1BB agonism, i.e. FcγR cross-linking requirement, with engineered FcγRIIB selectivity. Preclinical mouse studies showed effective antitumor activity without signs of concomitant liver toxicity [34]. Mutations in isotypes of immunostimulatory antibodies are reviewed in detail by Boulard et al. [41]. Most clinical studies with co-stimulatory agonists include the evaluation of combinatory treatments with immune checkpoint inhibitors (Table 1).

Further developments focus on enhancing the intrinsic agonistic efficacy. Here, a consistent strategy to improve the cross-linking property of an antibody is the generation of recombinant antibody formats with increased multivalency. This included fusing small binding units, e.g. three OX40-directed single-domain antibodies (sdAb), in a row to an Fc part, leading to hexavalent antibodies with enhanced avidity and therefore cross-linking capacity (ES102/INBRX-106). Also, a tetravalent hinge-stabilized IgG4 molecule targeting GITR (ASP1951), has been reported [42]. Both formats are currently listed in clinical trials (Table 1). Moreover, in preclinical studies the design of tetravalent and in addition biepitopic antibodies was shown to retrieve robust OX40 agonists, independent of extrinsic crosslinking [43].

Other approaches address in particular the reduction of immune-related adverse events. Thus, to avoid systemic toxicity, local treatment and local activation of monoclonal antibodies is being investigated. Local treatment by low-dose intratumoral injections with a 4-1BB agonistic antibody in mice was shown to result in antitumor effects without liver inflammation [8]. A clinical phase I/II study with intratumoral urelumab treatment in combination with systemic applied nivolumab in patients with solid tumors has been announced (NCT03792724). Local activation is the strategy of the Probody-approach with a 4-1BB agonist antibody prodrug. Here, a peptide fused via a protease-cleavable linker to the N-terminus of the light chain, masks the antigen-binding site in solution. Once arrived at the tumor microenvironment (TME) the peptide is cleaved by tumor-associated proteases, enabling co-stimulatory receptor binding, i.e. agonistic activity at the tumor site. Thus, in syngeneic mouse models the antitumor efficacy of the original antibody was preserved while liver inflammation

Table 1 Tumor necrosis factor super family (TNF-SF) agonistic monospecific antibodies in clinical studies (www.clinicaltrials.gov)

Target	Name	Format	Tumor type	Combination	Phase	Status	Clinical ID	Information by
4-1BB	Urelumab (BMS-663513)	huIgG4	Melanoma	–	II	Completed (10/2009)	NCT00612664	Bristol-Myers Squibb
			Urothelial carcinoma/bladder cancer	Nivolumab (αPD-1)	II	Recruiting	NCT02845323	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins
	Utomilumab (PF-05082566)	huIgG2	Adv. solid tumors	Pembrolizumab (αPD-1)	I	Completed (02/217)	NCT02179918	Pfizer
			Solid tumor/B-cell lymphoma	Rituximab (αCD20)	I	Completed (02/2019)	NCT01307267	Pfizer
			Her2-positive breast cancer	Trastuzumab (αHer-2)/Trastuzumab Emtansine (αHer2-ADC)	I	Active	NCT03364348	George W. Sledge Jr., Stanford University
			Adv. cancers	Avelumab (αPD-L1)/CMP-001 (TLR9 agonist)/PF-04518600 (αOX40)	II	Active	NCT02554812	Pfizer
	YH004	huIgG1	Cancer	–	I	Recruiting	NCT05040932	Eucure (Beijing) Biopharma Co., Ltd.
	ATOR-1017	huIgG4	Solid tumor	–	I	Recruiting	NCT04144842	Alligator Bioscience AB
	EU101	n.i.a.	Solid tumor	–	I/II	Recruiting	NCT04903873	Eutilex
	ADG106	huIgG4	Metastatic NSCLC	Nivolumab (αPD-1)	I/II	Recruiting	NCT05236608	National University Hospital, Singapore
	AGEN2372	huIgG4	Adv. solid tumor	AGEN1181 (αCTLA-4)	I	Recruiting	NCT04121676	Agenus Inc.
	LVGN6051	huIgG (huFcγRIIB selective)	Soft tissue sarcoma	Anlotinib	I/II	Not yet recruiting	NCT05301764	Lyvgen Biopharma Holdings Limited

Table 1 (continued)

Target	Name	Format	Tumor type	Combination	Phase	Status	Clinical ID	Information by
OX40	MOXR0916	huIgG1	Adv. or metastatic solid tumors	Atezolizumab (αPD-L1)		Completed (11/2019)	NCT02410512	Genentech, Inc.
	PF-04518600	huIgG2	Adv. or metastatic carcinoma	PF-05082566 (α4-1BB)	I	Completed (11/2020)	NCT02315066	Pfizer
			Adv. malignancies	Avelumab (αPD-L1)/Utomilumab (α4-1BB)/radiation therapy	I/II	Active	NCT03217747	M.D. Anderson Cancer Center
			Adv. malignancies	Avelumab (αPD-L1)/Utomilumab(α4-1BB)/CMP-001 (TLR9 agonist)/PD 0360324 (Anti-M-CSF)	II	Active	NCT02554812	Pfizer
			Recurrent/refractory acute myeloid leukemia	Avelumab (αPD-L1)/Azacitidine	I/II	Active	NCT03390296	M.D. Anderson Cancer Center
			Follicular Lymphoma	Rituximab (αCD20)/Utomilumab (α4-1BB)	I	Active	NCT03636503	Caron A. Jacobson, Dana-Farber Cancer Institute
			Triple negative breast cancer	Avelumab (αPD-L1)	II	Recruiting	NCT03971409	Hope Rugo, MD, University of California, San Francisco
			Metastatic kidney cancer	Axitinib	II	Recruiting	NCT03092856	University of Southern California
	MEDI6469	mIgG1	Metastatic breast cancer	–	I	Completed (08/2018)	NCT01862900	Providence Health & Services
			Head and neck cancer	–	I	Active	NCT02274155	Providence Health & Services
	MEDI0562	huIgG	Adv. solid tumors	–	I	Completed (01/2018)	NCT02318394	MedImmune LLC
			Ovarian cancer	Durvalumab (αPD-1), Tremelilumab (αCTLA-4), MEDI 9447 (αCD73)	II	Completed (09/2021)	NCT03267589	Nordic Society of Gynaecological Oncology - Clinical Trials Unit
			Adv. solid tumors	Durvalumab (αPD-1)/Tremelimumab (αCTLA-4)	I	Completed (08/2019)	NCT02705482	MedImmune LLC
			Head and neck squamous cell carcinoma or melanoma	–	I	Active	NCT03336606	Providence Health & Services
	BMS-986178	huIgG1	Adv. solid tumors	Nivolumab (αPD-1)/Ipilimumab (αCTLA-4)	I/II	Completed (11/2020)	NCT02737475	Bristol-Myers Squibb
			Adv. or metastatic solid tumors	SD-101 (TLR9 agonist)	I	Active	NCT03831295	Ronald Levy, Stanford University
			Low-grade B-cell non-Hodgkin lymphomas	Radiation/SD-101 (TLR9 agonist)	I	Active	NCT03410901	Ronald Levy, Stanford University

Table 1 (continued)

Target	Name	Format	Tumor type	Combination	Phase	Status	Clinical ID	Information by
	INCAGN01949	huIgG1	Adv. or metastatic solid tumors	–	I/II	Completed (03/2019)	NCT02923349	Incyte Corporation
			Adv. malignancies	Nivolumab (αPD-1)/Ipilimumab (αCTLA-4)	I/II	Completed (09/2019)	NCT03241173	Incyte Corporation
			Pancreatic cancer and others	CMP-001 (TLR9 agonist)	I/II	Recruiting	NCT04387071	University of Southern California
	IBI101	huIgG1	Adv. solid tumors	Sintilimab (αPD-1)	I	Active	NCT03758001	Innovent Biologics (Suzhou) Co. Ltd.
	HFB301001	huIgG1	Adv. solid tumors	–	I	Recruiting	NCT05229601	HiFiBiO Therapeutics
	ES102/INBRX-106	sdAb$_3$Fc	Adv. solid tumors	–	I	Recruiting	NCT04730843	Elpiscience Biopharma. Ltd.
			Adv. solid tumors	Toripalimab (αPD-1)	I	Recruiting	NCT04991506	Elpiscience Biopharma. Ltd.
			Adv. or metastatic solid tumors	Pembrolizumab (αPD-1)	I	Recruiting	NCT04198766	Inhibrx, Inc.
	BGB-A445	n.i.a.	Adv. solid tumors	Tislelizumab (αPD-1)	I	Recruiting	NCT04215978	BeiGene
	BAT6026	n.i.a.	Adv. solid tumors	-	I	Recruiting	NCT05105971	Bio-Thera Solutions
			Adv. solid tumors	BAT1308 (αPD-1)	I	Not yet recruiting	NCT05109650	Bio-Thera Solutions
GITR	MK-4166	huIgG1	Solid tumors	Pembrolizumab (αPD-1)	I	Completed (07/2019)	NCT02132754	Merck Sharp & Dohme LLC
	TRX518	huIgG1	Melanoma/solid tumors	–	I	Completed (09/2018)	NCT01239134	Leap Therapeutics, Inc.
	GWN323	IgG1	Solid tumors/lymphomas	PDR001 (αPD-1)	I	Completed (03/2020)	NCT02740270	Novartis
	INCAGN01876	huIgG1	Adv. or metastatic solid tumors	–	I/II	Completed (12/2019)	NCT02697591	Incyte Corporation
			Adv. or metastatic malignancies	Ipilimumab (αCTLA-4)/Nivolumab (αPD-1)	I/II	Completed (11/2021)	NCT03126110	Incyte Corporation
			Glioblastoma	INCMGA00012 (αPD-1)/Stereotactic Radiosurgery	II	Active	NCT04225039	University of Pennsylvania
	ASP1951	tetravalent huIgG4	Adv. solid tumors	Pembrolizumab (αPD-1)	I	Active	NCT03799003	Astellas Pharma Inc
	REGN6569	n.i.a.	Squamous cell carcinoma of head and neck	Cemiplimab (αPD-1)	I	Recruiting	NCT04465487	Regeneron Pharmaceuticals
	BMS-986156	huIgG1	Metastatic lung/chest/liver tumors	Ipilimumab (αCTLA-4)/Nivolumab (αPD-1)/radiation	I/II	Recruiting	NCT04021043	M.D. Anderson Cancer Center

n.i.a. no information available, *adv.* advanced

Table 2 Tumor necrosis factor super family (TNF-SF) agonistic bi- and trispecific antibodies in clinical studies (www.clinicaltrials.gov)

Targets	Name	Format	Tumor type	Combination	Phase	Status	Clinical ID	Information by
Her2 x 4-1BB	PRS-343	(αHer2) IgG4mut-(α4-1BB) Anticalin	Her2-positive adv. or metastatic solid tumors	–	I	Completed (10/2021)	NCT03330561	Pieris Pharmaceuticals, Inc.
			Adv. or metastatic Her2-positive solid tumors	Atezolizumab (αPD-L1)	I	Active	NCT03650348	Pieris Pharmaceuticals, Inc.
			Her2-positive gastric cancer	Ramucirumab (αVEGFR2)/Paclitaxel/Tucatinib	II	Active	NCT05190445	Pieris Pharmaceuticals, Inc.
PD-L1 x 4-1BB	PRS-344/ S095012	(αPD-L1) IgG4mut-(α4-1BB) Anticalin	Solid tumors	–	I/II	Recruiting	NCT05159388	Pieris Pharmaceuticals, Inc.
	GEN1046	DuoBody®	Solid tumors	–	I	Recruiting	NCT04937153	Genmab
			Metastatic NSCLC	Pembrolizumab(αPD-1)	II	Recruiting	NCT05117242	Genmab
	ABL503	(αPD-L1) IgG1mut-(α4-1BB) scFv	Adv. solid tumors	–	I	Recruiting	NCT04762641	ABL Bio, Inc.
	INBRX-105	(αPD-L1) sdAb-(α4-1BB) sdAb-Fcmut	Solid tumors	Pembrolizumab (αPD-1)	I	Recruiting	NCT03809624	Inhibrx, Inc.
	FS222	mAb²	Advanced cancers	–	I	Recruiting	NCT04740424	F-star Therapeutics Limited
OX40 x 4-1BB	FS120	mAb²	Adv./ metastatic cancer	–	I	Recruiting	NCT04648202	F-star Therapeutics Limited
PD-L1 x OX40	EMB-09	FIT-Ig®	Adv. solid tumors	–	I	Not yet recruiting	NCT05263180	Shanghai EpimAb Biotherapeutics Co., Ltd.
4-1BB x PD-L1 x HSA	NM21-1480	scMATCH™3	Adv. solid tumors/ NSCLC	–	I/II	Recruiting	NCT04442126	Numab Therapeutics AG
PSMA x 4-1BB x HSA	CB307	Humabody®	Adv. and/or metastatic PSMA-positive solid tumors	–	I	Recruiting	NCT04839991	Crescendo Biologics Ltd.

Adv advanced, *NSCLC* non-small cell lung cancer

was reduced [44]. Next to the efforts to improve monospecific antibodies, the introduction of bispecific antibodies is on the rise in the field.

2.3 Bispecific Antibodies

Bispecific antibodies targeting a co-stimulatory receptor and a tumor-associated antigen (TAA) have the potential to localize the agonistic activity at the tumor site. Binding to the co-stimulatory receptor on the immune cell is in general not sufficient for an effective activation, but tethering the antibody by its TAA-specificity to the tumor cell surface, i.e., adopting a transmembrane-like form, enables the dynamic of efficient receptor clustering and therefore target-dependent activation. Thus, the strategy seeks for high local co-stimulatory efficacy and reduced peripheric

Table 3 Agonistic tumor necrosis factor super family (TNF-SF) ligand-fusion proteins in clinical studies (www.clinicaltrials.gov)

Targets	Name	Format	Tumor type	Combination	Phase	Status	Clinical ID	Information by
OX40	MEDI6383	Fcγ4(S228P)-TD-OX40L	Adv. solid tumors	+/− Durvalumab (αPD-L1)	I	Completed (07/2017)	NCT02221960	MedImmune LLC
GITR	MEDI1873	Fcγ1-TD-GITRL(N161D)	Adv. solid tumors	–	I	Completed (12/2018)	NCT02583165	MedImmune LLC
FAP x 4-1BB	RO7122290	(αFAP) Fab-Fcγ1mut-CH1/CL-4-1BBL	Metastatic colo-rectal cancer	Cibisatamab (αCEAxCD3) / Obinutuzumab (αCD20)	I/II	Recruiting	NCT04826003	Hoffmann-La Roche
CD19 x 4-1BB	RO7227166	(αCD19) Fab-Fcγ1mut-CH1/CL-4-1BBL	Lymphoma, non-Hodgkin	Obinutuzumab (αCD20)/ Glofitamab (αCD20xCD3)/ Tozilizumab (αIL-6R)	I	Recruiting	NCT04077723	Hoffmann-La Roche
PDL-1 x OX40	SL-279252	PD1-Fcγ4-OX40L	Adv. solid tumors or lymphomas	–	I	Recruiting	NCT03894618	Shattuck Labs, Inc.

TD trimerization domain, *adv.* advanced

toxicity. Furthermore, the tumor-directed antibody unit can also contribute to diversify the mode of action, for example, by blocking a target receptor (TAA). Most advanced developments include a variety of monovalent/bivalent bispecific antibodies with a silenced Fc part or a human serum albumin (HSA) binding unit (Fig.1B, Table 2). PRS-343 was the first bispecific 4-1BB agonist to enter clinical trials. It is a Her-2 specific IgG4 variant of trastuzumab, fused at the C-terminus to a 4-1BB-directed, non-ligand-blocking, anticalin molecule. The Fc region is engineered (S228P, F234A, L235A) to avoid half-antibody exchange and FcγR-binding (i.e., excluding ADCC and targeting-independent cross-linking), without interfering with FcRn-binding (i.e., prolonged plasma half-life). It was shown that the co-stimulatory activity of PRS-343 was related to the Her-2 expression levels in vitro and induced localized immune effects and antitumor efficacy in preclinical in vivo studies [45]. The first clinical phase I trial as monotherapy was recently completed (NCT03330561). PRS-343 was well tolerated and showed clinical benefit, associated with increased CD8+T cell numbers and proliferation index [46]. A second phase I trial of PRS-343 in combination with atezolizumab (NCT03650348) and a phase II trial of PRS-343 in combination with ramucirumab and paclitaxel or tucatinib (NCT05190445) is ongoing. Another important target on the rise is PD-L1. The ligand forms part of the PD-L1/PD-1 checkpoint inhibitor axis and is overexpressed in many solid tumors [47]. Bispecific antibodies targeting PD-L1 and a co-stimulatory receptor seem a particular promising strategy, because combination of localized checkpoint inhibition and co-stimulation is expected to synergize in enhancing T-cell and NK-cell function, increasing treatment response

rate and durability. Bispecific antibodies in development seek to translate this concept mostly by targeting PD-L1 and 4-1BB. 4-1BB is prominently expressed on PD-1 high positive CD8+ TILs, and PD-1 blockade can further upregulate the 4-1BB expression [48], thus supporting a combined action. Formats entering clinical trials include IgGs fused at the C-terminus to an Anticalin [46] or scFv [49], DuoBody® [50], mAb² [51], and sdAbs fused to either a Fc region or an HSA-specific sdAb [52] (Fig.1B, Table 2). Preclinical studies confirmed blocking of checkpoint inhibition and targeting-dependent co-stimulatory activity. Furthermore, bispecific antibodies were able to outperform the combination of respective monoclonal antibodies in vitro and showed superior antitumor effects in comparison with the treatment with immune checkpoint inhibitor only in divers tumor mouse models [46, 49–51]. Mechanistic studies with NM21-1480, a monovalent trispecific antibody (single-chain of three λcap™-stabilized Fvs) targeting PD-L1, 4-1BB, and HSA, respectively, addressed the issues of target density, epitope position and antibody affinities. Targeting-mediated co-stimulation was demonstrated at a broad range of PD-L1 expression levels, whereupon the co-stimulatory strength correlated with the target density. For this antibody format, 4-1BB clustering resulted more effectively from binding a membrane distal epitope than a proximal one. Furthermore, increasing the affinity to PD-L1 significantly over 4-1BB converged the dosing for maximal dual activity [53]. Hence, target density, epitope position, and affinity need to be concerted adequately to deliver the strategy. PD-L1x4-1BB bispecifics were in general well tolerated in toxicity studies in cynomolgus monkeys without signs of liver inflammation [49–51, 53]. First results of a phase I

trial of the PD-L1x4-1BB DuoBody GEN1046 in heavily pretreated patients with advanced refractory solid tumors (NCT03917381) showed a manageable safety profile and disease control in 65.6% of the patients mostly in the form of stable disease [50].

Other developments focus on the combination of small antibody or antibody-like units without including an Fc-part. In the trimer body format, a 4-1BB-directed scFv is connected to an EGFR-directed V_{HH} by a linker with the murine collagen XVIII homotrimerization domain. Consequently, the molecule assembled into a homotrimer with the binding units presented in a hexagonal conformation. Targeting-enhanced co-stimulation was confirmed in vitro and antitumor effects demonstrated in CDX and PDX humanized mouse models [54]. No signs of systemic or liver toxicity were observed for respective surrogates in corresponding syngeneic mouse models [55, 56]. The principle of targeting-mediated co-stimulation is also pursued by bispecific antibody-mimetics composed of designed ankyrin repeat proteins (DARPins). MP0310, a bispecific DARPin® drug candidate directed against the fibroblast activation protein (FAP) and 4-1BB, is currently being evaluated in a clinical phase I trial in patients with advanced solid tumors (NCT04049903).

2.4 Costimulatory TNF-SF Ligands

Naturally, co-stimulatory receptors of the TNFR-SF can also be activated by recombinant forms of their respective ligands (Fig. 1C, Table 3). The basic functional unit is usually a self-assembling, non-covalently linked homotrimer of the extracellular domain (ECD) of the ligand, which requires further oligomerization to induce effective receptor clustering. This can be facilitated for example by fusing the ECD of the ligand to an Fc region and enforcing ligand trimerization by introducing an isoleucine zipper coiled coil domain in the linker. Thus, a Fc-mediated covalently linked hexameric ligand form was generated that showed co-stimulatory properties for Fc-GITRL and Fc-OX40L in preclinical in vitro and in vivo studies [57, 58]. Similar to the situation observed with agonistic monoclonal antibodies, cross-linking via Fc/FcγR interactions were shown to play an important role in the activity of these molecules. Both fusion proteins entered clinical phase I studies with patients with advanced solid tumors (NCT02221960, NCT02583165) (Table 3). Fc-GITRL (MEDI1873) was reported to show an overall acceptable safety profile and prolonged stable disease in some patients. However, the lack of tumor response discouraged the company from further clinical development [59].

Other developments include the generation of recombinant ligands in the single-chain format, i.e. connecting three ECDs with short linkers, thus enforcing intramolecular trimerization rather than intermolecular trimerization.

Concomitant fusion to the N-terminus of a silenced Fcγ1 region retrieves a covalently linked homodimer with a hexavalent ligand configuration. scGITRL-Fc showed co-stimulatory activity and antitumor effects that were independent of FcγR-mediated cross-linking [60]. This property was also confirmed for scCD40L-Fc and scCD27L-Fc [61, 62].

Another approach conceives the generation of Duokines (Dk), i.e. fusion proteins composed of two different co-stimulatory TNF-SF ligands (e.g., combinations of 4-1BBL, OX40L, CD27L, and CD40L). Here, the respective ECDs are connected by a 15-20 amino acid linker, leading to a bifunctional homotrimer formation. Alternatively, ligand units in the single-chain format are fused, generating scDuokines (scDk) [63]. Receptor clustering is here facilitated by simultaneous receptor binding in cis or trans. Thus, dual-targeting translates into combined co-stimulatory activity. Following the same principle, further developments to increase the plasma half-life included scDk-Fc fusion proteins utilizing a silenced, heterodimeric (knob-into-hole) Fc design [64]. Both formats, scDk and scDk-Fc, showed similar co-stimulatory properties and the potential to enhance the antitumor effect of a T-cell bispecific antibody (TAA × CD3) in a syngeneic tumor mouse model [63, 64].

2.5 Antibody-Fusion Proteins with Co-Stimulatory TNF-SF Ligands

Antibody-fusion proteins composed of a tumor-directed antibody and the ECD of a co-stimulatory TNF-SF ligand constitute another approach to achieve tumor-localized co-stimulation. Antibody-mediated binding to a tumor-associated antigen leads to the cell surface presentation of the co-stimulatory ligand, mimicking its physiological active membrane-bound form. Targeting-dependent activity was demonstrated for antibody fusion proteins with different TNF-SF members (e.g., 4-1BBL, OX40L, GITRL, LIGHT), target specificities (e.g., FAP, EGFR, Endoglin, EDA, CD19), and formats [65–70] (Fig. 1D, Table 3). To translate this concept, initially scFv-TNF-SF were created by fusing a scFv antibody to the N-terminus of the TNF-SF ligand (ECD). Due to the trimerization property of the ligand, homotrimeric molecules with three antibody units and a trimeric ligand unit were generated [65–68]. Advanced design introduced the ligand in the single-chain format, creating a monomeric scFv-scTNF-SF variant with only one antibody unit and one trimeric ligand unit, showing improved activity and stability. Importantly, targeting a ligand trimer to the cell surface was shown to be sufficient for the induction of an effective receptor stimulation in vitro and to enhance antitumor effects in mice [71]. Furthermore, the single-chain design of the TNF-SF ligand enabled single-site fusion of the ligand trimer and consequently the incorporation into fusion protein formats of higher complexity [72]. Currently, the most

advanced and in clinical studies is an IgG-like format composed of a FAP or CD19-directed Fab fragment, a heterodimeric Fcγ1 region, and a 4-1BBL trimer (RO7122290/RO7227166). The ligand trimer assembles from two ECDs fused as single-chain to the CL(RK) domain connected to the CH2-CH3 of the Fc region and a single ECD fused to a CH1(EE) domain forming a complementary light chain-like arm. The heterodimeric (knob-into-hole) Fc region is modified to inhibit FcγR binding without interfering with FcRn binding. Thus, tumor target-dependent, but FcγR-cross-linking independent, co-stimulatory activity was combined with a prolonged serum half-life [70]. Preclinical studies in xenograft-humanized mouse models showed FAP- and CD19-directed antibody-4-1BBL fusion proteins to increase the accumulation and activation of intratumoral CD8+ T cell and enhance the antitumor effects of T-cell bispecific CEA × CD3 and CD20 × CD3 antibodies, respectively. No accumulation of immune cells in the liver was observed [70]. Clinical phase I studies with patients with metastatic colorectal cancer (NCT04826003) and non-Hodgkin lymphoma (NCT04077723) have been initiated (Table 3).

Recently, a format for the blockade of PD-1 checkpoint inhibition in combination with GITR agonism has been proposed. The corresponding antibody-fusion protein is composed of an anti-PD-1 IgG1 antibody fused at the C-terminus of the silenced Fc to scGITRL. Taking advantage of the co-expression and cross-regulation of PD-1 and GITR on activated T cells, PD-1 targeting-mediated GITR-clustering in cis was shown to induce effective tumor growth inhibition in diverse syngeneic, genetically engineered, and xenograft-humanized mouse tumor models [73]. Instead of using an antibody, targeting and blocking of PD-L1 can also be achieved by introducing the ECD of PD-1. In the design of PD-1-Fc-OX40L, the ECD of PD-1 and OX40L were fused to the N- and C-terminus of a silenced Fc region, respectively. Indeed, the stimulatory activity on activated T cells and the antitumor responses in mice appeared to be superior to the treatment effect obtained by the combination of corresponding monoclonal antibodies [74]. Currently, a clinical phase I study is recruiting participants (NCT03894618).

In the current treatment strategies co-stimulatory agonists and immune checkpoint inhibitors are usually combined simultaneously, either in the form of a single molecule or as co-applied separate molecules. Preclinical studies in mice showed for the combination of an OX40 agonist and a PD-1 checkpoint inhibitor that the sequential administration and the order of application were crucial to improve the antitumor efficacy and obtain effects superior to the concurrent combination therapy [75]. Thus, accounting the dynamic of a natural immune response, exploring the potential of different timing should be of interest to further improve dosing and treatment efficacy of co-stimulatory agonists in combinatory approaches. Considering their mode of action as

enhancer molecules, their therapeutic efficacy will always be intrinsically dependent on the presence of a natural underlying or an artificially induced antitumor immune response. Thus, in order to tune the antitumor immune response adequately and minimize immune-related adverse events, their application will have to be carefully adjusted for each particular combination strategy.

3 Conclusions

Agonists of co-stimulatory TNF-SF receptors are required to induce effective receptor clustering. The application of conventional monoclonal antibodies has been shown to be complicated by the dependence on FcγR-mediated cross-linking. Thus, current drug developments focus mainly on enhancing the cross-linking capacity of antibodies and ligands in an FcγR-independent manner. Next to the generation of multivalent antibody and oligomeric ligand molecules, the design of bispecific antibodies and bifunctional antibody-ligand fusion proteins driving receptor complex clustering by cell-cell interactions is emerging as a promising option to enhance and localize the co-stimulatory activity in the tumor. Site-directed activity in combination with immune checkpoint inhibition is expected to further increase the therapeutic efficacy. Currently, multiple co-stimulatory TNF-SF agonists have entered clinical trials. In the near future upcoming results of toxicity and treatment efficacy will define the potential of the optimized formats and concepts. It will be interesting to see which candidates will come out on top and take the lead.

Declarations

Conflicts of interest/competing interests D.M. is named inventor on a patent application covering the Duokine and scDuokine technology.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and material No datasets were generated for this article. References are indicated.

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