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Harnessing co-stimulatory TNF receptors for cancer immunotherapy: Current approaches and future opportunities

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Abstract. Co-stimulatory tumor necrosis factor receptors (TNFRs) can sculpt the responsiveness of T cells recognizing tumor-associated antigens. For this reason, agonist antibodies targeting CD137, CD357, CD134 and CD27 have received considerable attention for their therapeutic utility in enhancing anti-tumor immune responses, particularly in combination with other immuno-modulatory antibodies targeting co-inhibitory pathways in T cells. The design of therapeutic antibodies that optimally engage and activate co-stimulatory TNFRs presents an important challenge of how to promote effective anti-tumor immunity while avoiding serious immune-related adverse events. Here we review our current understanding of the expression, signaling and structural features of CD137, CD357, CD134 and CD27, and how this may inform the design of pharmacologically active immuno-modulatory antibodies targeting these receptors. This includes the integration of our emerging knowledge of the role of Fcγ receptors (FcγRs) in facilitating antibody-mediated receptor clustering and forward signaling, as well as promoting immune effector cell-mediated activities. Finally, we bring our current preclinical and clinical knowledge of co-stimulatory TNFR antibodies into the context of opportunities for next generation molecules with improved pharmacologic properties.

Keywords: TNFR, TNFRSF, human, antibodies, immunotherapy, oncology, cancer, clinical trials, 4-1BB, CD137, GITR, CD357, OX40, CD134, CD27, FcγR, TRAF, co-stimulation

1. Introduction

In humans, 19 ligands of the tumor necrosis factor (TNF) superfamily have been described to interact with 29 members of the TNF receptor (TNFR) superfamily [2]. TNFR superfamily members coordinate diverse aspects of the innate and adaptive immune response. As a result, a number of therapeutic antibodies have sought to either attenuate or enhance TNFR signaling in the context of tissue inflammation, autoimmunity and cancer [15,208]. Immunomodulatory antibodies that engage T cell-expressed antigens can be broadly classified based on their primary mechanism of action: 1) antagonist antibodies that block the interaction between receptor and cognate ligand(s), and 2) agonist antibodies which induce or facilitate receptor-mediated forward signaling. The preclinical testing of agonist antibodies targeting co-stimulatory TNFRs has convincingly shown their ability to influence the magnitude, quality and durability of tumor-specific $CD4^+$ and $CD8^+$ T cell immune responses, as well as the potential to overcome immune suppression mediated by regulatory T (Treg) cell populations [208].

Here we discuss evidence supporting the utility of antibodies targeting CD137 (also known as 4-1BB or TNFRSF9), CD357 (also known as GITR or TNFRSF18), CD134 (also known as OX40 or TNFRSF4) and CD27 (also known as TNFRSF7) to enhance anti-tumor immune responses, as well as efforts to combine these agents with antibodies targeting the co-inhibitory T cell pathways involving cytotoxic T-lymphocyte-associated protein 4 (CTLA-4 or CD152) and programmed cell death protein 1 (PD-1 or CD279) (Ta-

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ble 1) [21]. We consider the anti-tumor potential of antibodies targeting CD137, CD357, CD134 and CD27 expressed on conventional T cell and Treg cells, while acknowledging the preclinical evidence that supports a broader function for these receptors in coordinating innate and adaptive immune cell responses. An extended discussion on the impact that TNFR superfamily members have on immune cell regulation is provided elsewhere [183,198]. Importantly, we survey the available structural information for these TNFR and ligand pairs, and the implications this provides for defining a threshold for receptor signaling. Finally, we discuss the additional role of Fcγ receptors (FcγRs) in promoting antibody-mediated receptor forward signaling, and how these combined features might be harnessed to generate next generation therapeutic antibodies targeting co-stimulatory TNFRs.

2. Co-stimulatory TNFR superfamily members utilize TRAF proteins to coordinate diverse signaling outcomes in T cells

The TNF superfamily of ligands acts through cognate receptors to control a range of biological outcomes, including key aspects of immune regulation [101]. Death receptors are TNFR superfamily members that possess a cytoplasmic death domain (DD) and function *via* recruitment of TNFR-associated DD (TRADD) or Fas-associated DD (FADD) adapter proteins to coordinate a variety of cellular processes including apoptosis, necrosis, as well as homeostatic and pro-inflammatory processes [198]. A second class of co-stimulatory TNFR superfamily members, which lack a defined DD, instead contain binding sites for TNFR-associated factors (TRAFs) in their cytoplasmic domain, referred to as TIMs (TRAF interacting motifs) [186]. Biochemical and structural analyses identified the major ((P/S/A/T)x(Q/E)E) and minor (PxQxxD) consensus motifs present in TNFRs that are important for TRAF 1, 2, 3 and 5 binding (where x defines any amino acid) [210]. Consistent with its unique receptor-binding specificity, TRAF6 recognizes a distinct consensus motif (PxExx(Aromatic/Acidic residue)) [209,210].

TRAF proteins are major signal transduction components in T cells and coordinate pathways that impact proliferation, survival, differentiation, cytokine and chemokine production, migration, metabolic changes, as well as responses to cellular stress [71,161,205]. TRAFs share certain structural characteristics and

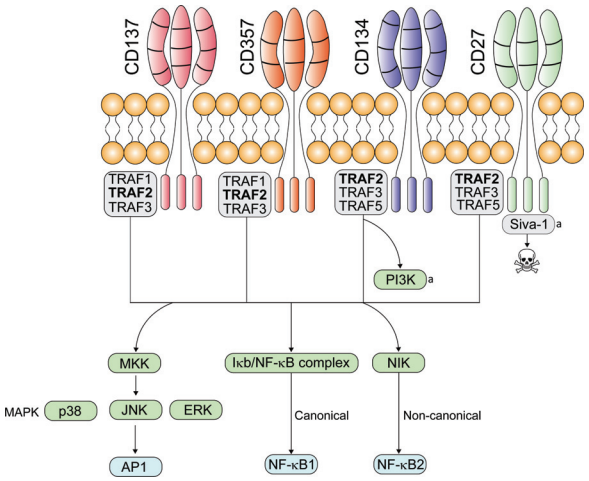

Fig. 1. Schematic representation of TRAF recruitment and subsequent activation of downstream signaling pathways following CD137, CD357, CD134 and CD27 activation. (A) TRAF recruitment is mediated by one or more TRAF interacting motifs in the cytoplasmic tails of TNFR superfamily members. TNFR-associated TRAFs provide ubiquitin-ligase activity and scaffolding for additional signaling components, facilitating the activation of numerous signal transduction pathways important for T cell activity such as canonical and non-canonical NF-κB, p38 MAPK, JNK, AP1 and ERK. [a]Alternative signaling adaptors, such as the pro-apoptotic protein Siva-1 and the multifaceted enzyme PI3K, have also been reported to interact with certain TNFR superfamily members [129, 160]. PI3K, phosphatidylinositol 3-kinase; NIK, NF-κB-inducing kinase; MKK, MAP kinase kinase (s).

function as apical signaling adapters in CD137, CD357, CD134 and CD27 signaling complexes. TRAFs collectively function to orchestrate the activation of diverse intracellular signaling cascades including canonical and non-canonical NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), p38 MAPK (mitogen-activated protein kinase), JNK (c-Jun N-terminal kinase), AP-1 (activator protein 1) and ERK (extracellular signal-regulated kinase) pathways (Fig. 1) [161,205]. A detailed review on individual TRAF adapter proteins and their role in cell signaling pathways is covered elsewhere [205].

A summary of the reported TRAF interactions with human CD137, CD357, CD134 and CD27 is provided (Table 2). Notably, the pro-apoptotic protein Siva-1, which plays a role in apoptotic signaling, is also described to bind to the intracellular domain (ICD) of human CD27 [129]. Siva-1 can also interact with the RING finger domain of TRAF2, which is essential for its E3 ubiquitin ligase activity and its ability to activate NF-κB [68]. Despite these features, the role of Siva-1 in modulating primary human T cell function remains undefined. The potential bifurcation of signaling networks downstream of co-stimulatory TNFRs is

Table 1
Antibodies targeting CD137, CD357, CD134 and CD27 under clinical investigation

Antibody	Target	Human isotype	Status	Company
Utomilumab	CD137	IgG2	Phase 3	Pfizer
Urelumab	CD137	IgG4	Phase 1/2	Bristol-Myers Squibb
TRX518	CD357	IgG1-N297A	Phase 1	Leap Therapeutics
MK-4166	CD357	IgG1	Phase 1	Merck, USA
MK-1248	CD357	IgG*	Phase 1	Merck, USA
INCAGN1876	CD357	IgG1	Phase 1/2	Incyte/Agenus
GWN323	CD357	IgG1	Phase 1/1b	Novartis
BMS-986156	CD357	IgG2	Phase 1/2	Bristol-Myers Squibb
MOXR0916	CD134	IgG1	Phase 1	Roche/Genentech
MEDI0562	CD134	IgG1	Phase 1	MedImmune/AstraZeneca
PF-04518600	CD134	IgG2	Phase 1	Pfizer
GSK3174998	CD134	IgG1	Phase 1	GlaxoSmithKline
BMS-986178	CD134	IgG2	Phase 1/2	Bristol-Myers Squibb
INCAGN1949	CD134	IgG1	Phase 1/2	Incyte/Agenus
Varlilumab	CD27	IgG1	Phase 1/2	Celldex Therapeutics

* IgG Fc details not provided at time of writing.

Table 2
Signaling adapter proteins of human CD137, CD357, CD134 and CD27

Target	Apical signaling components	Reference
CD137	TRAF1, TRAF2, TRAF3	[6,81]
CD357	TRAF1, TRAF2, TRAF3	[73,92]
CD134	TRAF2, TRAF3, TRAF5	[6,73,85]
CD27	TRAF2, TRAF3, TRAF5, Siva-1	[5,129,164,207]

further exemplified by the recruitment of E3 ubiquitin protein ligases cellular inhibitor of apoptosis (cIAP)1 and cIAP2 by TRAF proteins [217]. Cellular IAPs can regulate canonical and non-canonical NF-κB pathways *via* their ability to mediate the post-translational addition of lysine (Lys)-48- or Lys-63-linked ubiquitin chains to target proteins [192]. Ubiquitin is an 8 kDa polypeptide that when covalently linked to target proteins can alter their half-life, localization or function [181]. Lys-48 polyubiquitin chains lead to proteasome-mediated target degradation, resulting in attenuated signaling, while Lys-63-linked polyubiquitin chains generally provide a scaffold to promote further complex assembly and downstream signaling cascades [181,192]. For example, TRAF2 conjugated with Lys-63-linked polyubiquitin chains can recruit transforming-growth-factor-β-activated kinase-1 (TAK1)/TAK1-binding protein (TAB) 2/TAB3 complex and the IκB kinase (IKK) complex, leading to IKK activation (referred to as the canonical NF-κB pathway) [158]. By contrast, TRAF2 can complex with TRAF3 to bridge NF-κB-inducing kinase (NIK) with cIAPs, leading to Lys-48 polyubiquitination and degradation of NIK, attenuating the non-canonical NF-κB pathway [181,214].

TRAF2 is considered the prototypical TRAF family member and has been implicated in nucleating the signaling complexes of CD137, CD357, CD134 and CD27 in T cells [161,186]. Consistent with this, TRAF2-deficient T cells, or T cells expressing a dominant-negative version of TRAF2 that lacks the N-terminal RING finger domain, exhibit profound defects in survival, cytotoxic activity, cytokine production and memory cell reactivation following co-stimulatory TNFR activation [22,130,177]. TRAF2-mediated activation of canonical NF-κB appears to be a well-conserved downstream signaling outcome of CD137, CD357, CD134, and CD27 activation [5,81,85,92]. TRAF5 is closely related to TRAF2 in both structure and function, including overlap in the ability to induce NF-κB activation [169]. Like TRAF2, TRAF5 is reported to play an important role in coordinating downstream signaling for several co-stimulatory TNFRs including CD357, CD134 and CD27 [49,114,162]. For example, T cells from TRAF5-deficient mice exhibit an impaired proliferative response to T cell receptor (TCR) activation in the context of CD27 co-stimulation [114]. Moreover, TRAF5-deficient mice exhibit altered $CD4^+$ T cell polarization when immunized with a vaccine together with an agonistic antibody to CD134 [162].

TRAF1 recruitment to certain co-stimulatory TNFRs has been proposed to regulate both positive and negative signaling outcomes. On one hand, CD137-stimulated $CD8^+$ T cells from TRAF1-deficient mice show reduced cell survival, which was found to be associated with impaired ERK activation and increased pro-apoptotic B cell lymphoma protein-2 (Bcl-2)-like protein 11 (or Bim) accumulation [142]. By contrast, TRAF1 was reported to negatively regulate NF-κB activation downstream of co-stimulatory TNFRs, such

as CD40 [55]. This apparent divergence in signaling outcome was attributed to the ability of TRAF1 to form heterotrimeric interactions with TRAF2, thereby modifying the affinity of TRAF2 for cIAPs [217]. Finally, TRAF6 has also been ascribed a role in T cell TNFR pathway activation, including CD134, whereby TRAF6 signaling can mediate the conversion of conventional $CD4^+$ T cell into IL-9 producing cells *via* the non-canonical (NIK-mediated) NF-κB signaling [204].

In addition to the role of TRAFs in coordinating downstream TNFR signaling, these adapters also serve as critical conduits for TCR and cytokine receptor pathways, providing an additional layer of complexity in coordinating signals within the immune synapse. Indeed, impairment of key signaling events induced by TCR and CD28 stimulation have been described in TRAF3-deficient T cells, including reduced Zeta-chain-associated protein kinase 70 (Zap70) activation [206]. Further, TCR signaling utilizes TRAF2 and TRAF6 signal downstream of the CARMA1-BCL10-MALT1 (CBM) complex to the IKK complex through TAK1, which activates both NF-κB and AP-1 [167]. Therefore, TRAF proteins provide significant scope for signaling crosstalk between the TCR and co-stimulatory TNFR pathways in T cells. In addition, CD134 has been demonstrated to directly modify the TCR signaling complex by recruiting PI3K/PKB (PKB, protein kinase B) to detergent-insoluble membrane lipid microdomains (DIM) [160]. CD134-TRAF2-dependent recruitment of PI3K/PKB helps support TCR signaling by providing these components for TCR-induced T cell activation. Notably, CD134 has also been reported to bypass TCR signaling and activate NF-κB using TRAF2 to recruit receptor-interacting protein (RIP) kinases and the CBM complex as signaling intermediaries [163]. In contrast to TCR and co-stimulatory TNFR signaling, several TRAFs have been shown to play a negative regulatory role in T cell-associated cytokine receptor signaling. For instance, TRAF3 can suppress interleukin (IL)-2 signaling in T cells by facilitating the recruitment of T cell protein tyrosine phosphatase (TCPTP) to the IL-2 receptor complex [212]. TRAF3-mediated recruitment of TCPTP results in dephosphorylation of the Janus kinases, Jak1 and 3, which are critical mediators of IL-2-induced signaling in T cells. TRAF6 has also been shown to inhibit IL-2 signaling by associating with the cytoplasmic tail of IL-2 receptor β (IL-2Rβ), preventing Jak1 binding and downstream pathway activation [111]. Taken together, these observations highlight the importance of TRAF-mediated signaling in T cells following CD137, CD357, CD134 and CD27 activation, but also raise questions of how coordinated signaling *via* these shared adapters converge given the overlap in TRAF usage between receptors expressed within the immune synapse.

3. Regulation of conventional and regulatory T cell responses by co-stimulatory TNFRs

In humans, quiescent peripheral blood T cells express little or no CD137, CD357 and CD134, with the exception of distinct subsets of Treg and effector memory T cells [186]. Transcriptional control of these receptors has been linked with TCR activation [172,191,215]. In this dynamic environment, functionally diverse co-stimulatory and co-inhibitory molecules are expressed in an overlapping, spatiotemporal fashion. For instance, the expression level of CD357 and CD134 has been shown to be associated directly with TCR signal strength, as assessed by a correlation with Nur77 expression, an immediate early gene upregulated following TCR triggering [104]. CD27 is distinct, and is more broadly expressed by quiescent conventional $CD4^+$ and $CD8^+$ T cells [77]. However, like CD134, CD137 and CD357, CD27 is rapidly upregulated following TCR activation, consistent with its role in contributing to T cell priming and memory cell differentiation events [75].

Regulatory T cells play an indispensable role in controlling immune responses to pathogens and maintaining immunological self-tolerance and immune homeostasis [171]. Co-stimulatory TNFRs, together with co-inhibitory receptors, are essential in the development, maintenance and function of Treg cells [13]. A feature consistent in mice and humans is that relative to the expression level on activated conventional T cells, activated Treg cells display the highest surface levels of CD137, CD357 and CD134 [20,58,104,106,182]. While this finding also holds true for CD27 in humans, the expression differential for CD27 between activated conventional T cells and Treg cells in mice is less clear [45,104]. Despite this observation, as well as the widespread use of these TNFRs as activation markers to define the Treg lineage, signaling events downstream of CD137, CD357, CD134 and CD27 in Treg *versus* conventional T cells remain controversial. For example, a number of TNFRs are reported to promote the expansion and suppressive function of activated Treg cells [33,47,141,175]. Conversely,

there is evidence that TNFR-mediated Treg cell expansion is minimal compared to the extent of proliferation induced in conventional T cell populations, and that signaling by certain co-stimulatory TNFRs may actually attenuate the suppressive function of Treg cells [30,83,88,128,154,174,180]. Further complicating the interpretation of TNFR signaling outcomes in distinct T cell populations is the finding that TNFRs can decrease the susceptibility of conventional T cells to suppression by activated Treg cells [10,133, 165,170]. Indeed, conventional T cells activated *via* the TCR and CD134, prior to the addition of Treg cells, have been shown to be protected from Treg cell-mediated suppression *in vitro* [170,179]. Similarly, conventional T cells stimulated *via* CD137 show enhanced IL-2 production, which is able to overcome Treg cell-mediated suppression [10]. To delineate the contribution of CD357 signaling in Treg cells *versus* conventional T cells, CD357-deficient Treg cells were adoptively transferred into an inflammatory disease setting [47]. In this model, the ability of CD357 to modulate regulatory *versus* effector T cell responsiveness was complex and context dependent. A similar conclusion was reached for CD134 signaling in Treg *versus* conventional T cell compartments, which appeared highly dependent on the cytokine milieu, a feature also consistent with other TNFRs [50,141]. Forkhead box P3 (Foxp3) is indispensable for the development and maintenance of the suppressive phenotype of Treg cells [54]. It has been proposed that certain Treg cell populations retain plasticity, and therefore might downregulate Foxp3 expression to generate a so-called 'exTreg' state *in vivo* [143]. Related to this, several reports have claimed that CD357 and CD134 signaling might promote Foxp3 destabilization in Treg cells, although this mechanism remains controversial [20,47,145,180].

As discussed, CD137, CD357, CD134 and CD27 recruit TRAF proteins as apical adapters in their respective signaling complexes (Table 2). Therefore, precisely how these co-stimulatory TNFR signaling complexes might coordinate distinct functional outcomes in conventional T *versus* Treg cells remains to be elucidated. To understand this difference, parallels may be inferred from the distinct downstream transcriptional profiles in conventional T *versus* Treg cells following TCR and CD28 stimulation [185]. How transcriptional outcomes might diverge in different T cell subsets following CD137, CD357, CD134 and CD27 activation may include: differential TRAF expression, recruitment of other lineage-specific signalosome components, differences in ubiquitin-mediated signaling pathways, unique transcription factor expression, epigenetic modifications or a combination of these features. A comparison of CD137, CD357, CD134 or CD27 signalosome components between conventional T and Treg cells remains to be thoroughly characterized, although TRAF3 and TRAF6 are described as critical components for Treg cell-intrinsic development and function [26,155]. Non-TRAF signaling components, such as protein kinase C-θ (PKC-θ) recruitment, may also alter the response of Treg cells to TNFR co-stimulation [213]. For example, reduced Treg cell suppressive function downstream of CD357 is associated with the selective activation of the JNK pathway *versus* ERK or p38 MAPK [83]. An attractive hypothesis is that the distinct epigenetic characteristics of Treg cells confer unique transcriptional profiles downstream of co-stimulatory TNFR activation, provided that apical signaling events may be conserved between conventional T and Treg cells [182]. Taken together, the finding that co-stimulatory TNFR signaling might elicit distinct functional outcomes in T cell populations has important implications for antibodies targeting these receptors and their utility to promote effector T cell responses while overcoming Treg cell-mediated suppression in the tumor.

4. Structure-based implications for antibodies targeting T cell co-stimulatory TNFRs

TNFRs exist within the cell membrane at an equilibrium between monomeric, dimeric and trimeric states, with the potential to form higher order complexes [183]. This receptor pre-assembly dynamic is defined by the extracellular, transmembrane and intracellular domain sequences and associated tertiary structure, with a canonical view that downstream signaling is generally facilitated by ligand-induced receptor oligomerization [24,147,203]. TNFRs share certain organizational features, whereby the ligand-receptor stoichiometry can define an initial signaling threshold [28,35,201,203]. In general, progressive receptor organization not only increases ligand avidity, but also regulates signaling duration, magnitude and even bifurcation of survival *versus* cell death pathways [125,176]. The characterization of an extracellular domain in some TNFRs, commonly called the pre-ligand assembly domain (PLAD) as well as transmembrane cholesterol binding domains (CBD), provide additional mechanisms that may define a recep-

Table 3
Presence or absence of CBD/CRAC or CBD/CRAC-like motifs in the transmembrane domains of CD137, CD357, CD134, CD27 and their cognate ligands

Target	UniProt #	*CBD/CRAC or CBD/CRAC-like* sequence
CD137	Q07011	ECD-IISFFLALTSTALLFLLFFLTLRFSVVKRGRKKLLY
CD137L	P41273	C*RVLPWAL*VAGLLLLLLLAAACAVFLACPWAVSGAR-ECD
CD357	Q9Y5U5	ECD-LGWLTVVLLAVAACVLLLTSAQLGLHIW
CD357L	Q9UNG2	RSSW*KLWLFCSIV*MLLFLCSFSWLIFIFLQLETAK-ECD
CD134	P43489	ECD-VAAILGLGLVLGLLGP*LAILLALYLLR*R
CD252	P23510	NKLLLVASVIQGLGLLLCFTYICLHFSALQVSHRYPRI-ECD
CD27	P26842	ECD-SLCSSDFIRILVIFSGMFLVFT*LAGALFLHQRR*KYRS
CD70	P32970	R*RRPYGCVL*RAALVPLVAGLVICLVVCIQRFAQAQQQL-ECD

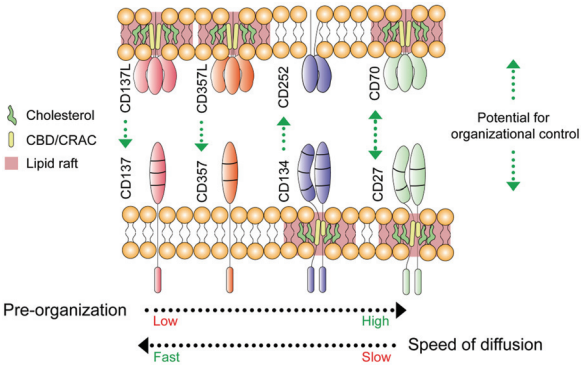

Fig. 2. TNFR lipid raft partitioning and degree of ligand- or receptor pre-organization. Based on the presence of CBD/CRAC motifs, CD137, CD357, CD134, CD27 or their cognate ligands may exhibit differential organizational states, cellular localization and rates of lateral membrane diffusion prior to ligand-receptor interactions. In some cases, receptors may mediate ligand organization or vice versa depending on pre-organization.

tor signaling threshold by regulating pre-organization or diffusion within the membrane (Fig. 2) [25]. For example, PLADs may support ligand-independent receptor pre-organization while CBD or cholesterol recognition amino acid consensus motifs (CRAC) might help to partition co-stimulatory TNFRs into lipid raft fractions and facilitate the formation of signaling competent synapses between cells [51]. Here we discuss the available structural information on CD137, CD357, CD134 and CD27 and their respective ligands, including their ability to segregate into micro-clusters and lipid rafts and how this relates to ligand (or antibody)-mediated signaling [12,27,28,35,201] (Fig. 2).

How CD137 interacts with its ligand (CD137L also known as TNFSF9) remains unclear, and has been largely inferred from the structure of its ligand, as well as mutagenesis studies of the ligand-receptor interface [201,211]. CD137L exhibits a 3-fold symmetric assembly, resembling a three-bladed propeller, and is not predicted to undergo further conformational change upon receptor engagement. The pre-organized ligand structure may serve to increase the spacing between co-engaged monomeric receptors, resulting in an increased distance between CD137 intracellular domains as a mechanism to set a threshold for pathway activation. Like CD137, the organizational state of CD357 has been extrapolated from structural analysis of its ligand [28]. Human CD357L (also known as TNFSF18) forms homotrimeric structures with a large degree of separation between protomers. This results in a flattened structure with reduced receptor contact points and a significant level of monomer-trimer fluctuation reported in solution. Consequently, the interaction between human CD357L and its receptor is relatively weak, as compared with other TNFR superfamily members [28]. Unlike their ligands, CD137 and CD357 lack CBD/CRAC motifs, suggesting minimal lipid raft partitioning and diffuse membrane organization in the steady state (Table 3). This observation suggests that CD137L and CD357L may be needed to co-engage and facilitate oligomerization of their receptors to achieve an active signaling complex (Fig. 2). By contrast, CD134 contains a CBD/CRAC motif which may enable a higher degree of receptor pre-organization within the membrane (Table 3) [35]. Lipid raft-mediated migration and organization of CD134 has been shown to be critical in achieving optimal signaling, as cholesterol depletion or the inhibition of cholesterol synthesis abolishes CD134-induced IL-2 production in T cells [160]. Consistent with this model, the ligand for CD134 (CD252, also known TNFSF4) lacks a CBD, supporting the hypothesis that CD134 may facilitate ligand organization (Table 3 and Fig. 2) [37]. However, despite potential lipid raft partitioning and the formation of assembled microclusters, further CD134 clustering to facilitate higher order oligomerization is required for downstream signaling [112].

There is a paucity of structural information available for CD27 or its ligand, CD70 (also known as TNFSF7). Sequence homology with other TNF ligands

supports a trimeric organizational state of CD70, while CD27 is predicted to exist as a disulfide-linked homodimer [184]. In order to maintain a 1:1 receptor-to-ligand ratio, which is observed for other TNFR superfamily members, the existence of CD27 as a dimer suggests an asymmetrical receptor-ligand interface *viz* $(CD27_2)_3$-$(CD70_3)_2$ [184]. Regardless of its structural organization, ligand oligomerization was required to activate CD27 [203]. Despite CD27 and CD70 containing CBD/CRAC motifs that may favor lipid raft localization, further studies are needed to understand how this receptor-ligand pair coordinates signaling threshold in immune cells (Table 3 and Fig. 2).

5. Anti-tumor activity of agonist antibodies targeting CD137, CD357, CD134 and CD27

The events leading to the transition from a naïve T cell to its activated state is termed T cell priming [199]. T cell activation requires two distinct signals: an antigen specific signal arising from TCR binding to major histocompatibility (MHC)-peptide complexes displayed on APCs, as well as a co-stimulatory signal provided by B7 family ligands (CD80 or CD86) expressed on APCs that binds to CD28 [199]. The "*tide*" signal model has been proposed to explain how co-stimulatory and co-inhibitory molecules cooperatively function within the APC:T cell interface to shape T cell responses [219]. A limited number of co-stimulatory receptors are expressed on naïve T cells that are able to synergize with TCR signaling to initiate priming, suggesting that this early activation phase is tightly regulated.

Antibodies designed to engage and mediate CD137, CD357, CD134 and CD27 forward signaling are under investigation for their therapeutic utility in cancer patients (Table 1). The limited information on the structural features of these receptors as well as their cognate ligands, provides challenges on how therapeutic antibodies optimally engage these receptors to achieve a signaling threshold that modulates an immunological outcome, while avoiding the potential immune-related adverse events associated with superagonists [80]. In order to recapitulate the level of clustering mediated by ligand co-engagement, bivalent antibodies targeting co-stimulatory TNFRs typically require the co-engagement with FcγRs expressed on accessory cells in order to facilitate receptor oligomerization and downstream pathway activation [86,122,200]. Related to this mechanism, different IgG subclasses have varying affinities and specificities for distinct FcγRs [118]. FcγRs are characterized by two distinct functional classes: activating and inhibitory. Activating receptors contain an intracellular immunoreceptor tyrosine-based activation motif (ITAM) which enables cells expressing this class of FcγRs to mediate various effector functions, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and/or antibody-dependent cell-mediated phagocytosis (ADCP) of antibody-labeled target cells. FcγRIIB is an inhibitory FcγR and contains a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM), which negatively regulates downstream ITAM-mediated cellular responses, including those mediated by activating FcγRs [118]. In preclinical models, FcγRs have been demonstrated to mediate cross-linking of co-stimulatory TNFR antibodies to potentiate receptor forward signaling [95, 194,200]. Both activating and inhibitory FcγRs can mediate antibody cross-linking *in vitro*; however, several studies have shown that FcγRIIB is particularly efficient *in vivo* in mediating this cross-linking function [86,122,200]. Below we discuss the available preclinical information involving the use of surrogate antibodies that support the immunomodulatory potential of antibodies targeting CD137, CD357, CD134 and CD27 (Table 4). In addition, we dissect mechanisms associated with the co-engagement with FcγRs and bring this discussion into the context of their therapeutic application, either alone or in combination with other immunomodulatory antibodies.

5.1. CD137

The ability of CD137 agonist antibodies to enhance T cell responsiveness was originally demonstrated *in vitro* using purified populations of $CD4^+$ and $CD8^+$ T cells [41,156]. In these studies, CD137 co-stimulation was described to preferentially promote $CD8^+$ T cell proliferation, although later studies found that CD137 signaling also improved $CD4^+$ T cell function [38]. Indeed, two recent studies reported that CD137 signaling could convert conventional $CD4^+$ T cells into cytotoxic effector T cells, suggesting they may contribute directly to the elimination of tumor cells [4,38]. The administration of CD137 agonist antibodies has been shown to mediate potent single agent activity in tumor-bearing mice, in some instances leading to the eradication of pre-established tumors [107,113,188,195].

In addition to monotherapeutic potential, the ability of anti-CD137 agonist antibodies to combine with other immunomodulatory antibodies has been described,

Table 4
Antibodies targeting CD137, CD357, CD134 and CD27 in preclinical models

Target	Clone	Isotype	Reference
CD137	2A	Rat IgG2a	[79,90,113,188,195]
CD137	LOB12.3	Rat IgG1	[39,69,187]
CD137	3E1	Rat IgG2a	[156]
CD137	22B6	Rat IgG2a	[156]
CD137	3H3	Rat IgG2a	[96,156]
CD137	1D8	Rat IgG2a	[156,188]
CD357	DTA-1	Rat IgG2b	[19,36,89,96,102,109,131,145]
CD357	DTA-1	Mouse IgG2a; IgG2a N297A	[19]
CD357	G3c	Rat IgM	[119]
CD134	OX86	Rat IgG1	[18,20,62,110,135]
CD134	OX86	Mouse IgG2a; IgG2a N297A	[20]
CD27	RM27-3E5	Rat IgG2a	[3,138,144]
CD27	RM27-3C1	Rat IgG2b	[144]
CD27	RM27-1F3	Rat IgG1	[144]
CD27	AT124-1	Rat IgG*	[18,56,74,138]

* IgG Fc details not provided.

including antibodies targeting the CTLA-4 and PD-1 pathways [21]. For example, the combination of anti-CD137 and -CTLA-4 antibodies was shown to enhance anti-tumor activity in mouse tumor models, as compared to either agent alone [39,90]. In this instance, tumor efficacy was attributed to differential effect on T cell subsets, with CD137 co-stimulation enhancing $CD8^+$ T cell activity, and CTLA-4 blockade improving the $CD4^+$ T cell response. The administration of antibodies targeting either CD137 or CTLA-4 has been associated with certain autoimmune manifestations [90, 127]. Paradoxically, the combination of anti-CTLA-4 and CD137 antibodies alleviated autoimmunity, while enhancing anti-tumor efficacy in a colon carcinoma model [90]. The reduction in immune-related pathologies correlated with enhanced suppressive activity of splenic Treg cells. This finding appears somewhat contradictory given the role of Treg cell-mediated immune suppression within the tumor microenvironment [124]. While the explanation for this observation remains unclear, an attractive possibility is that the anti-CTLA-4 antibody mediated the selective depletion of intratumoral populations of CTLA-4-expressing Treg cells *via* the co-engagement of activating FcγR on effector cells within the tumor [11]. The depletion of Treg cells within the tumor would remove this suppressive barrier, while maintaining Treg cell function outside the tumor microenvironment, thereby mitigating systemic immune-related adverse events [11,148]. Consistent with the cooperative anti-tumor efficacy of CD137 co-stimulation combined with intratumoral Treg cell depletion, systemic Treg cell depletion was found to increase the anti-tumor activity of an anti-CD137 antibody monotherapy [79]. Anti-CD137 agonist antibodies combine effectively with anti-PD-1 antibodies, resulting in increased tumor-specific T cell effector function and improved anti-tumor efficacy [69,187]. In contrast to the differential impact on $CD4^+$ *versus* $CD8^+$ T cells proposed for anti-CTLA-4 and -CD134 antibodies, the combination of PD-1 blockade together with CD137 activation was suggested to predominately potentiate $CD8^+$ T cell cytotoxic responses [187]. Finally, anti-CD137 antibodies are reported to cooperate with other immunomodulatory antibodies, including antibodies targeting CD134 and T cell immunoglobulin and mucin domain containing-3 (TIM-3), which further exemplifies their versatility as a therapeutic modality in cancer [65,69,132].

Anti-CD137 antibodies that bind in close proximity to the ligand interface (ligand competitive) have been shown to be more potent at inducing receptor forward signaling *in vitro* [41,156]. Consistent with this observation, other TNFR agonist antibodies shown to mediate downstream signaling *via* a bivalent interaction alone are reported to bind to the receptor within or near the ligand binding site, likely mimicking properties of ligand co-engagement and promoting receptor clustering [1,137]. However, in most instances, antibody Fc cross-linking has been demonstrated to enhance the agonist activity of antibodies by increasing receptor clustering, superior to that of a bivalent interaction alone [200]. Consistent with this notion, a non-ligand competitive anti-mouse CD137 antibody (rat IgG2a) showed weak agonist potential *in vitro*; however, *in vivo* it was effective at enhancing $CD8^+$ T cell proliferation [156]. This finding supports that FcγR-mediated cross-linking of certain antibodies targeting CD137 may potentiate further receptor forward signaling [86,122].

5.2. CD357

Lymphoid and myeloid immune cells in mice broadly express CD357 under steady state conditions, while cell surface expression of CD357 is low or undetectable on the majority of human peripheral blood immune cell populations, with the exception of subpopulations of Treg and transitional memory T cells [140]. In mice and humans, CD357 is rapidly upregulated by Treg and conventional T cells following activation [84,154]. In mouse preclinical models, antibodies targeting CD357 were initially proposed to enhance anti-tumor immunity by inducing receptor forward signaling in antigen-specific T cells, while inhibiting Treg cell suppressive activity [89,152]. Interestingly, binding of CD357 with a bivalent antibody has been found to be sufficient for potent activation of T cell NF-κB signaling and co-stimulation, *in vitro* [84]. This finding may relate to the proposed dimeric form of murine CD357, which differs from the predicted trimeric human receptor [27,218]. These findings suggest that additional antibody clustering may not be required to promote receptor signaling, a result supported by *in vitro* experiments showing that cross-linking an anti-CD357 antibody did not further enhance T cell co-stimulation. However, in mouse syngeneic tumor models optimal anti-tumor activity of an anti-CD357 antibody required co-engagement of FcγRs; a result confirmed using mice deficient for activating FcγRs as well as an aglycosylated antibody variant, which eliminated FcγR binding [19,87]. Consistent with the dependence on activating FcγRs expressed by tumor-infiltrating effector cells, anti-CD357 antibody selectively and rapidly depleted intratumoral Treg cells. An alternative hypothesis proposed to explain the loss of Foxp3-expressing intratumoral Treg cells in response to CD357-specific antibody treatment is the destabilization of Treg cells to an 'exTreg' state [145]. However, given that the same anti-CD357 antibody (clone DTA-1) was used in both studies and the anti-tumor efficacy was dependent on the co-engagement of activating FcγRs, the relative contribution of Treg cell destabilization therefore remains unclear [19,145]. In support of this notion, a second anti-CD357 antibody, which lacked the ability to mediate the depletion of intratumoral Treg cells, failed to elicit similar anti-tumor activity [119]. Taken together, the finding that certain anti-mouse CD357 antibodies required an IgG isotype capable of interacting with activating FcγRs to mediate anti-tumor efficacy may have implications for development of antibodies targeting the human receptor. In addition to monotherapeutic activity, CD357-specific antibodies have shown compelling combination activity with anti-PD-1 and -CTLA-4 antibodies in preclinical mouse tumor models [89,102,109,131].

5.3. CD134

In preclinical tumor models, CD134 co-stimulation has been shown to increase $CD4^+$ T cell proliferation, survival and memory responses, and in some instances promote differentiation into cytotoxic $CD4^+$ T cell effectors [62,132]. However, a role for CD134 stimulation in promoting tumor-specific $CD8^+$ T cell responses has also been defined [9]. Agonistic antibodies targeting CD134 on activated T cells were proposed to enhance the cytotoxic effector function of effector T cells, while counteracting the immunosuppressive effects of Treg cells. It was initially hypothesized that anti-CD134 antibodies enhanced the responsiveness of tumor-specific T cells exclusively by mediating CD134 forward signaling through receptor clustering [82,190]. However, a secondary mechanism of action, similar to that described for a surrogate anti-CD357 antibodies, was the contribution of the anti-CD134 antibody Fc region to anti-tumor activity [20]. Indeed, a single intravenous dose of an anti-CD134 antibody resulted in selective elimination of Treg cells within the tumor microenvironment, which correlated with anti-tumor efficacy. In the absence of FcγR co-engagement, either using activating FcγR-deficient mice or an aglycosylated anti-CD134 antibody variant, only weak anti-tumor activity was observed. These observations suggest that both antibody-mediated receptor forward signaling and Treg depletion may be required for optimal therapeutic activity [20]. The FcγR-mediated depletion of intratumoral Treg cells expressing CD134 was consistent with the involvement of the immune effector cell mechanisms, such as ADCC and/or ADCP. These pre-clinical findings therefore highlight at least two potential anti-tumor mechanisms of action for a human anti-CD134 agonist antibody in a clinical setting. First, effective CD134 forward signaling may promote enhanced conventional T cell responsiveness to tumor antigens, and/or reduce the suppressive activity of intratumoral Treg cells. Secondly, that optimal anti-tumor efficacy may require antibody co-engagement of activating FcγRs on tumor-associated effector cells to selectively eliminate immunosuppressive Treg cells within the tumor [19,20]. Finally, a number of preclinical studies have highlighted the potential to combine antibodies targeting CD134 together

with other immuno-modulatory antibodies, including molecules targeting the CTLA-4, PD-1 and CD137 pathways [70,97,132,135]. An attractive rationale for the combination of an anti-CD134 and -CTLA-4 antibody is the potential that CD134 agonists might cooperate to promote tumor-specific T cell responses, while also promoting the expansion on non-tumor associated Treg cells in other compartments, including promoting the homeostasis of intestinal Foxp3-expressing Treg cells [66].

5.4. CD27

CD27 has been shown to play an important cooperative role with CD28 in enhancing the proliferation and survival of activated T cells [76]. Consistent with this, anti-CD27 antibodies in preclinical tumor models have been shown to increase the cytotoxic potential of tumor-specific $CD8^+$ T cells, resulting in anti-tumor efficacy [138,144]. Although the expression of CD27 on human Treg cells may support an antibody capable of Treg cell depletion, the level of constitutive CD27 expression on human and mouse naïve T cells may preclude exploiting this mechanism [45,64,94]. One study revealed that treatment of tumor-bearing mice with a non-depleting anti-CD27 agonist antibody was more effective at eliciting anti-tumor immune responses compared to an anti-CD27 antibody capable of effectively engaging activating FcγR [144]. This would suggest that, unlike CD357 and CD134, activating FcγR co-engagement is not required for the anti-tumor activity of CD27-specific antibodies. However, in non-human primate and humanized mouse models, an anti-human CD27 antibody (human IgG1), capable of binding to activating FcγRs and depleting cells with high CD27 expression, promoted antigen-specific T cell responses, leaving the possibility that FcγR co-engagement contributed to either receptor clustering and/or Treg cell depletion [74,178]. Importantly, there was no evidence for the depletion of CD27-expressing naïve or activated conventional T cell populations [74]. These findings may relate to discrepancies in CD27-expressing T cell subsets in mice and humans, whereby CD27 expression on human Treg cells is higher than on conventional human T cells, a feature not conserved in mice [45,104]. Therefore, although the optimal Fc backbone for anti-CD27 agonist antibodies remains controversial in rodents, studies utilizing a human IgG1 anti-CD27 antibody suggest that activating FcγR binding might contribute to anti-tumor activity.

Like other TNFR agonist antibodies, the combination of a surrogate CD27-specific agonist antibodies targeting the PD-1:PD-L1 pathway was shown to enhance $CD8^+$ T cell proliferation, function and promote tumor eradication [3,18]. Interestingly, while $CD8^+$ T cell tumor infiltration was improved, the combination of CD27 activation and CTLA-4 blockade failed to enhance the anti-tumor efficacy of either monotherapy [3].

6. Clinical experience with antibodies targeting CD137, CD357, CD134 and CD27

Given the compelling preclinical evidence to support the monotherapeutic and combination potential of therapeutic antibodies targeting CD137, CD357, CD134 and CD27, a number of clinical studies are now underway to evaluate antibodies targeting the human receptors to treat cancer patients (Table 1). However, a number of limitations hinder extrapolating mouse preclinical anti-tumor activity into patient populations [99,100]. In addition to the issue of translational relevance of mouse models to humans, there is the consideration of differences between human and mouse receptor expression profiles and receptor-ligand structural organization. For example, CD357 is predicted to form a more typical trimer-based TNFR signaling complex in humans, and its expression restricted almost exclusively to recently activated human T cells [120]. By contrast, mouse CD357 is predicted to exist in a dimeric signaling state, and is broadly expressed by a variety of immune cell populations, including quiescent conventional T and Treg cell populations [140]. Another consideration is the discrepancies in FcγR and IgG biology between mice and humans [16]. Importantly, human IgG1 therapeutic antibodies have the potential to induce multiple FcγR-mediated functions upon co-engagement with cell expressed target antigens. This includes antibody-mediated receptor forward signaling *via* FcγR-mediated clustering, as well as the potential to deplete cells expressing high levels of target antigen [122]. In humans, the ability to co-engage activating FcγRs, in particular FcγRIIIA, FcγRIIA and FcγRI, is a feature of the IgG1 and IgG3 Fc isotype subclasses, while the IgG2 Fc isotype can engage the activating receptor, FcγRIIA [17,103,116]. By comparison, the human IgG4 Fc isotype binds primarily to the activating FcγR, FcγRI, and the ITIM-containing inhibitory FcγR, FcγRIIB, with minimal binding to FcγRIIA or FcγRIIIA [16,17].

6.1. CD137

Two anti-CD137 antibodies are under clinical investigation for their utility to treat cancer: utomilumab (a human IgG2 isotype) and urelumab (a human IgG4 isotype). Both antibodies co-operate effectively with TCR activation to enhance primary human T cell proliferation and cytokine responses *in vitro* [8,53]. However, these antibodies have unique binding epitopes on CD137, as well as distinctions in their IgG isotype that would impact their ability to co-engage FcγRs *in vivo*. For example, utomilumab binds to a site on CD137 that competes with ligand association and therefore is likely to block ligand-mediated reverse signaling [53]. This may be an important feature, given the various immune functions associated with CD137L reverse (or retrograde) signaling into APCs, including pro-inflammatory IL-12 production [72]. By contrast, urelumab is reported to not compete with ligand binding, and therefore during T cell priming would preserve CD137L reverse signaling. Interestingly, utomilumab has a manageable adverse event profile across a broad dose range in patients (up to 5 mg/kg), while urelumab has been associated with dose-limiting toxicities, in particular hepatotoxicity at doses above 1 mg/kg [150]. Notably, hepatocellular pathologies are also associated with repeat administration of an anti-mouse CD137 antibody, although the binding characteristics and ligand blocking properties of this antibody remain to be described [44]. Importantly, when administered at lower dose levels, urelumab was found to have a manageable safety profile, with evidence of pharmacologic activity in patients [150]. As previously discussed, the pre-organizational state of CD137-CD137L supports that a bivalent antibody interaction alone may not elicit maximal downstream signaling, and that antibody cross-linking may be required to enhance receptor clustering. In the case for urelumab, receptor clustering may be mediated *via* the ability of an IgG4 isotype to co-engage FcγRIIB. Conversely, utomilumab (IgG2) is predicted to retain only minimal interactions with FcγRs, predominantly the activating receptor, FcγRIIA [17]. However, urelumab was not observed to require cross-linking to potently stimulate primary human T cells *in vitro*, which may relate to a binding epitope on CD137 that facilitates substantial FcγR-independent clustering [150]. Consistent with preclinical experience in mouse tumor models, utomilumab and urelumab are currently being tested in combination with anti-PD-1 antagonist antibodies pembrolizumab (NCT02179918) and nivolumab (NCT02253992). In support of preclinical data showing enhanced anti-tumor activity with anti-CD137 and -CD134 antibody combinations, utomilumab is under evaluation in patients with anti-CD134 (PF-04518600, NCT02315066), as well as the triple combination of anti-CD134 and -CD137 antibodies with an anti-PD-L1 antibody (avelumab, NCT02554812) [93]. Surprisingly, given the preclinical rationale that anti-CD137 antibodies might effectively combine with CTLA-4 blockade, not only improving tumor efficacy but also tolerability, the only clinical study to evaluate cooperativity was withdrawn (NCT00803374).

6.2. CD357

There is limited clinical experience with CD357, with a number of antibodies only recently having entered dose escalation studies. Consistent with the preclinical rationale to support at least two mechanisms of action, INCAGN1876, MK-4166 and GWN323 molecules contain a human IgG1 Fc region, potentially taking advantage of both FcγR-mediated receptor clustering and intratumoral Treg cell depletion [86,115]. The only clinical experience reported to date is with TRX518, a CD357-specific antibody with an aglycosylated Fc region [91]. This Fc-engineered antibody is not predicted to significantly co-engage activating FcγRs, and is therefore not anticipated to be an optimal Fc for receptor clustering or intratumoral Treg cell depletion [19,153]. Two other anti-CD357 antibodies in clinical trials are BMS-986156 (a human IgG2) and MK-1248, for which the human IgG backbone has not been disclosed. Notably, MK-4166 (NCT02132754), BMS-986156 (NCT02598960) and GWN323 (NCT02740270) are also under evaluation in combination with antibodies targeting the PD-1:PD-L1 pathway.

6.3. CD134

With the exception of a mouse antibody targeting human CD134 (a murine IgG1), there has been only limited clinical experience with anti-CD134 antibodies [189]. In this early study, patients were administered up to 2 mg/kg of the mouse anti-human antibody (clone 9B12) with some evidence of a pharmacodynamic response, albeit with the limitation that this antibody could not be dosed repetitively due to the formation of human anti-mouse antibodies (HAMA). Human IgG antibodies MOXR0916, MEDI0562, GSK3174998 and INCAGN1949 are now in clinical development.

All four antibodies were generated with a human IgG1 Fc backbone, and therefore retain the potential to promote FcγR-mediated receptor clustering and intratumoral Treg cell depletion [60,61,105]. Two other anti-CD134 antibodies are also in clinical trials, BMS-986178 and PF-04518600, which have human IgG2 Fc regions [17]. Consistent with preclinical findings, anti-CD134 antibodies are also under clinical evaluation in combination with anti-CTLA-4, -PDL-1 or -PD-1 antibodies. For example, MOXR0916 has entered into clinical trials in combination with an anti-PD-L1 antibody (atezolizumab, NCT02410512), while GSK3174998 will be evaluated with PD-1 blockade (pembrolizumab, NCT02528357). BMS-986178 has entered into a dose escalation study with nivolumab or ipilimumab (NCT02737475). MEDI0562 is currently being tested in combination with either anti-CTLA-4 (tremelimumab) or anti-PD-L1 antibodies (durvalumab, NCT02705482).

6.4. CD27

Finally, varlilumab is a human anti-CD27 antibody (human IgG1) that is reported to activate CD27 on conventional T cells, while retaining the potential to deplete CD27 expressing intratumoral Treg cells [74,178]. Varlilumab is currently the only anti-CD27 antibody in the clinic, and is under evaluation alone and in combination with PD-L1 (atezolizumab, NCT02543645) or PD-1 (nivolumab, NCT02335918) antibodies. Finally, the combination of varlilumab with CTLA-4 blockade (ipilimumab, NCT02413827) is also being tested.

7. Potential next generation opportunities for therapeutic antibodies targeting CD137, CD357, CD134 and CD27

Epitope, specificity and affinity are three common parameters routinely considered during monoclonal antibody discovery [23]. In addition, a further opportunity to modify the efficacy of therapeutic antibodies is to engineer their Fc regions to have altered affinities for activating or inhibitory FcγRs [118]. This may be achieved by the more conventional selection of an IgG isotype, commonly human IgG1, IgG2 or IgG4, each of which has its own intrinsic FcγR binding profile [117]. Alternatively, antibody Fc functionality can be tuned by mutagenesis or altering the glycan groups attached to the asparagine residue (N297A) in the Fc region [23]. Here we discuss emerging areas of consideration in design of next generation antibodies for CD137, CD357, CD134 or CD27, with the potential to improve both their therapeutic efficacy and potential tolerability in patients.

7.1. Antibody design features: i) optimizing antibody affinity to improve agonistic dose response

Unlike antibodies designed to block or neutralize target antigens, where high affinity is typically a desired and sought after property. The relationship between affinity and potency of agonistic antibodies targeting co-stimulatory TNFRs is more complex, and can be considered an optimizable feature to fine-tune receptor signaling. For example, a panel of agonist antibodies targeting the TNFR superfamily member CD95 were shown to have an inverse correlation between higher affinity and reduced receptor forward signaling [29]. The authors concluded that antibodies with a faster off-rate (k_{off} (s^{-1})) might favor an avidity driven bivalent antibody interaction with the target antigen, thereby favoring trimer complexing and oligomeric formation upon cross-linking through the Fc region. This contrasts with slow off-rate antibodies, where increasing monovalent binding *via* a single Fab arm at higher concentrations would reduce receptor clustering and result in an inverted U-shaped dose response curve [146]. A second mechanistic possibility is when slow off-rate antibodies that maintain an increasing dose-response relationship may engage autoregulatory feedback loops, such as those involved in attenuating the NFκB response [48,136]. Whether these observations can be generalized and translated to antibodies targeting CD137, CD357, CD134 and CD27 remains to be systematically explored, but may provide an opportunity to further optimize antibody-mediated receptor signaling in immune cells.

7.2. Antibody design features: ii) optimizing binding epitope to promote receptor forward signaling, while preserving ligand-mediated reverse signaling

The concept of reverse, or retrograde, signaling was coined to describe the potential for ligand members of the TNF superfamily to mediate downstream signaling by virtue of their ability to function as counter receptors [46,168]. Indeed, there is compelling evidence for bidirectional signaling for the ligands of CD134, CD137, CD357 and CD27 expressed at the interface

of T cell priming on activated APCs [7,37,67,98,168]. The potential that reverse ligand signaling might function to condition APCs is not routinely considered in the development of therapeutic antibodies targeting these receptors, and the benefit or liability of this signaling may need to be considered on a case-by-case basis. For example, a number of studies suggest that CD252 and CD137L reverse signaling may improve the quality of APC-mediated T cell priming, including the production of pro-inflammatory cytokines that enhance T cell priming and polarization to effector cells [52,72]. As discussed, the anti-CD137 agonist antibody urelumab does not inhibit the binding of CD137L to CD137, and may therefore permit ligand-mediated retrograde signaling in APCs. By contrast, utomilumab, targeting the same receptor, is a ligand blocking antibody [53]. How these contrasting features might translate into efficacy in patients remains to be explored. In particular, whether apparent differences in tolerability might be ascribed to the ligand blocking or non-blocking feature of these two antibodies remains unclear [149]. A second example of beneficial ligand co-operative binding has been demonstrated with an antibody targeting the TNFR superfamily member CD262 (DR5, TRAIL-R2). In this example, the anti-CD262 antibody, conatumumab, has been shown to co-operate with ligand binding (Apo2L (TRAIL, CD253)) and enhance receptor forward signaling, leading to superior anti-tumor activity [63,173]. Finally, further adding to the complexity of ligand-mediated reverse signaling, CD357L is reported to promote tolerogenic functions in APCs *via* upregulation of indoleamine-pyrrole 2,3-dioxygenase (IDO), an enzyme that catalyzes the degradation of tryptophan, leading to T suppression [67]. Therefore, therapeutic antibodies that activate CD357 while preventing ligand reverse signaling may be more therapeutically active.

7.3. Antibody Fc engineering: i) enhanced receptor forward signaling via FcγRIIB

Skewing the binding of the antibody Fc region toward inhibitory or activating FcγRs can profoundly alter the function of antibodies *in vivo*, as demonstrated in preclinical animal models and in cancer patients [34, 59,200]. Modifying the affinity toward certain FcγR can be achieved by selection of different human IgG isotypes, which have intrinsic differences in their respective FcγR binding profiles [118]. In addition, mutations within the Fc region have been identified that can dramatically alter the activating or inhibitory FcγR binding affinities [153]. For example, FcγRIIB is recognized as an important mediator of TNFR agonist antibody cross-linking *in vivo* [194,200]. In order to exploit the properties of FcγRIIB-mediated antibody cross-linking, engineering the Fc region to increase the binding affinity to FcγRIIB is considered to be a viable approach. Indeed, specific Fc mutations have been previously defined that increase the binding affinity of antibodies for FcγRIIB, including the S267E/L328F and P238D/E233D/G237D/H268D/P271G/A330R Fc variants [31,32,108]. With an increased binding affinity for FcγRIIB and associated cross-linking potential, the agonistic potential for antibodies targeting various TNFR superfamily members can be significantly enhanced [108,166]. However, the clinical application of Fc-modified antibodies with the potential for enhanced FcγRIIB-mediated cross-linking has been relatively limited, with the exception of an anti-CD19 antibody with an Fc region containing the S267E/L328F mutation [31]. One potential caveat with this approach is that FcγRIIB is expressed by B cells and sinusoidal endothelial cells within the liver, which may have a profound impact on the biodistribution and half-life of antibodies with enhanced FcγRIIB affinity [196]. In addition, while the S267E/L328F IgG1 Fc variant has been described to have an increased binding affinity to FcγRIIB (up to 400-fold), it is also reported to increase binding affinity to the activating receptor, FcγRIIA [159]. In particular, the naturally occurring FcγRIIA variant harboring an R131 (arginine) *versus* H131 (histidine) polymorphism. Enhanced antibody binding to complexed Fc γRIIA has also been associated with increased platelet activation and aggregation, suggesting the risk of thromboembolism might be increased with the S267E/L328F variant [108]. The identification of additional mutations that more selectively engage FcγRIIB may have important therapeutic applications, particularly for their application to next generation co-stimulatory TNFR agonists with enhanced forward signaling potential [108].

7.4. Antibody Fc engineering: ii) enhanced effector cell activity via activating FcγRs

In addition to strategies to improve the agonist potential of co-stimulatory TNFR antibodies *via* FcγRIIB clustering, other Fc mutations have been identified that enhance activating FcγR binding and subsequent elimination of target cells, such as intratumoral Treg cells [153]. Consistent with this approach, surrogate antibodies targeting mouse CD357 and CD134 showed

superior anti-tumor activity when the antibody Fc region effectively co-engaged activating FcγRs [19,20, 151,157]. In humans, FcγRIIA and FcγRIIIA are primarily associated with effector cell-mediated ADCC and ADCP [116]. Many Fc-engineered therapeutic antibodies with improved FcγRIIIA binding have been evaluated in clinical studies, including the S239D/I332E, L235V/F243L/R292P/Y300L/P396L, P247I/A339Q and S298A/E333A/K334A IgG1 mutants, as well as glycan modified (afucosylated) IgG1 Fc variants [14,57, 78,121,123,202]. In addition to modifying the therapeutic molecule, an interesting alternative approach to skew antibody binding to activating FcγRs is the co-administration of agents targeting FcγRIIB that neutralize its binding to other IgG antibodies [134, 139,197]. Finally, while human IgG1 antibodies show a preference to engage activating FcγRs, in particular FcγRIIIA, they also retain an interaction with FcγRIIB [17]. Therefore, human IgG1 antibodies targeting co-stimulatory TNFRs might provide both effective receptor clustering (forward signaling) while retaining the ability to engage activating FcγRs within the tumor microenvironment [19,200].

7.5. Antibody Fc engineering: iii) FcγR- independent receptor forward signaling

Several alternative antibody formats have been described that might circumvent the need for FcγR-mediated antibody clustering. For example, a novel tetrameric nanobody agonist specific for CD262 demonstrated superior ability to induce receptor forward signaling, as compared with cross-linked bivalent antibodies [126]. In addition to this approach, point mutations within the Fc region of IgG antibodies have also been shown to promote the ability of IgG antibodies to form hexamers upon target engagement [42,43]. Therefore, strategies that promote FcγR-independent oligomerization of antibodies may promote superior receptor clustering over a bivalent interaction alone. However, it was recently demonstrated that the agonism induced by an anti-CD134 antibody hexabody was still inferior to an Fc variant with mutations that increased FcγRIIB binding [216]. Further, forward signaling mediated by the anti-CD134 hexameric antibody was further enhanced by FcγRIIB cross-linking. Finally, interchain disulfide bonds within the hinge region of a naturally occurring IgG isotype, IgG2, was found to stabilize the overall antibody structure and impart FcγR-independent agonist activity of co-stimulatory TNFR antibodies [193]. However, the ability of this IgG2 scaffold to promote substantial FcγR-independent receptor clustering *in vivo* remains controversial, with evidence that both CD40- and CD134-specific antibodies still required FcγR co-engagement for optimal forward signaling [40,216].

8. Summary

The design of human antibodies that can optimally engage and activate CD137, CD357, CD134 and CD27 on immune cells presents an exciting opportunity to harness the immunomodulatory potential of these signaling pathways for cancer therapy. This therapeutic strategy is exemplified by the significant amount of preclinical experience evaluating the biology of these co-stimulatory TNFR receptors in T cell and non-T cell compartments, which has been complemented by the evaluation of surrogate antibodies in preclinical tumor models, both as single agents and in combination with antibodies targeting the clinically validated PD-1 and CTLA-4 pathways. Indeed, a number of early-stage human clinical trials are now in progress to evaluate a range of functionally disparate antibodies targeting CD137, CD357, CD134 and CD27 (Table 1). Historically, agonist antibody properties including IgG isotype, epitope and affinity may not have been mechanistically interrogated. Rather, development candidate selections often relied on *in vitro* cell-based assays and extrapolation to pharmacologic activity in patients. It is now appreciated that the pharmacologic modulation of antibodies targeting co-stimulatory TNFRs can be fine-tuned through several parameters, therefore presenting a significant opportunity for a class of next generation molecules with improved pharmacologic and tolerability properties. For example, antibody epitope specificity and affinity may dictate whether receptor pharmacologic modulation of the TNFR pathway can be maintained across a broad dose range in patients. Secondly, whether preserving ligand-mediated reverse ligand signaling is a desirable feature that may augment the T cell co-stimulatory properties of APCs needs to be considered. Finally, IgG engineering approaches that integrate our knowledge of the role of FcγRs in facilitating antibody-mediated receptor clustering and forward signaling, as well as promoting immune effector cell-mediated activities, show important promise in the development of next generation antibodies.

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