The promise and challenges of immune agonist antibody development in cancer

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Abstract | Immune cell functions are regulated by co-inhibitory and co-stimulatory receptors. The first two generations of cancer immunotherapy agents consist primarily of antagonist antibodies that block negative immune checkpoints, such as programmed cell death protein 1 (PD1) and cytotoxic T lymphocyte protein 4 (CTLA4). Looking ahead, there is substantial promise in targeting co-stimulatory receptors with agonist antibodies, and a growing number of these agents are making their way through various stages of development. This Review discusses the key considerations and potential pitfalls of immune agonist antibody design and development, their differentiating features from antagonist antibodies and the landscape of agonist antibodies in clinical development for cancer treatment.

Immune cell function is tightly regulated by co-stimulatory receptors, which are activated in response to exposure to foreign antigens, and co-inhibitory receptors, which dampen signalling to avoid excessive immune activation, tissue damage and autoimmunity. Collectively, such immune checkpoints are critical to control immune cell activity and can be regulated through blocking or activating antibodies. Many cancer cells hijack immune checkpoint pathways to prevent clearance by the host immune response[1]. Antagonist antibodies targeting immune checkpoint co-inhibitory receptors can reverse immune resistance of some tumours, and a number of these agents have been approved for the treatment of cancer. However, co-stimulatory pathways are equally important in driving productive anticancer immunity[2,3], with strong genetic evidence supporting their role in mediating anticancer immune responses[4–7]. The role of co-stimulatory receptors in influencing cancer immune surveillance is more nuanced than that of co-inhibitory receptors. For example, the timing and duration with which co-stimulatory receptors are induced following activation of T cell receptors (TCRs), as well as the context of co-inhibitory receptor expression on cells, may dictate whether co-stimulatory receptor activation produces a functional response. This is well illustrated through the design of second and third generations of engineered chimeric antigen receptor (CAR)-targeted T cells, which require intracellular co-stimulatory domains to achieve substantial clinical responses[8,9].

The B7 family consists of structurally related, cell surface protein ligands, which bind to the CD28 family of receptors on lymphocytes and regulate immune responses via co-stimulatory and co-inhibitory signals. The two main co-inhibitory receptors of the B7–CD28 family, cytotoxic T lymphocyte protein 4 (CTLA4) and programmed cell death protein 1 (PD1), are the targets of the first wave of successful cancer immunotherapy drugs, with multiple agents now approved or in the late stages of clinical development[10]. CD28, the namesake of the family, is the prototypic co-stimulatory receptor and a critical mediator of T cell signalling following TCR activation[11]. Upon binding to the ligands B7-1 (also known as CD80) and B7-2 (also known as CD86), CD28 activates downstream signals that drive T cell function, proliferation and survival[11]. Similarly, inducible T cell co-stimulator (ICOS) is another co-stimulatory receptor important for the function and survival of activated and memory T cells[12–14] in response to ICOS ligand (ICOSLG). Early attempts to develop potent agonists of CD28 were met with unacceptable clinical toxic effects[15]; however, the field has made substantial advances in the past decade. Therefore, both CD28 (REFS [16–18]) and ICOS[19,20] are targets in the development of therapeutic agonist agents for the treatment of cancer (TABLE 1).

The tumour necrosis factor (TNF) receptor superfamily (TNFRSF or TNFR) is a large and functionally diverse class of receptors with related structures capable of mediating a range of immune and non-immune cell functions[21,22]. Of the 29 receptors that are known to

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Table 1 | **Summary of co-stimulatory agonist antibodies in clinical development**

Target	Molecule	Antibody isotype	Company	Stage
CD27	Varlilumab (CDX-1127)	IgG1	Celldex	Phase I/II
CD40	CDX-1140	IgG2	Celldex	Phase I
	SEA-CD40	Non-fucosylated IgG1	Seattle Genetics	Phase I
	RO7009789	IgG2	Roche	Phase I/II
	JNJ-64457107 (ADC1013)	IgG1	Janssen	Phase I
	APX-005M	IgG1	Apexigen	Phase I
	Chi Lob 7/4	Mouse/human chimaera IgG1	BioNTech RNA Pharmaceuticals GmbH, University of Southampton	Phase I
GITR	TRX-518	Aglycosyl IgG1	Leap Therapeutics	Phase I
	MK-4166	IgG1	Merck & Co.	Phase I
	MK-1248	IgG4	Merck & Co.	Phase I
	GWN-323	IgG1	Novartis	Phase I
	INCAGN01876	IgG1	Incyte	Phase I/II
	BMS-986156	IgG1	Bristol-Myers Squibb	Phase I/II
	AMG-228	IgG1	Amgen	Phase I
OX40	Tavolimab (MEDI0562)	IgG1	AstraZeneca	Phase I
	PF-04518600	IgG2	Pfizer	Phase II
	BMS-986178	IgG1	Bristol-Myers Squibb	Phase II
	MOXR-0916	IgG1	Roche	Discontinued; phase at termination: phase II clinical
	GSK-3174998	IgG1	GlaxoSmithKline	Phase I
	INCAGN01949	IgG1	Incyte	Phase II
4-1BB	Utomilumab (PF-05082566)	IgG2	Pfizer	Phase II
	Urelumab (BMS-663513)	IgG4	Bristol-Myers Squibb	Phase II
ICOS	GSK-3359609	IgG4	GlaxoSmithKline	Phase I
	JTX-2011	IgG1	Jounce Therapeutics	Phase I
CD28	Theralizumab (TAB-08)	IgG4	TheraMAB	Phase I/II

GITR, glucocorticoid-induced tumour necrosis factor receptor-related protein; ICOS, inducible T cell co-stimulator; IgG, immunoglobulin G.

belong to this family, 6 receptors have been characterized and validated to date to have a primary role as immune co-stimulators (TNFRSF5 (also known as CD40), TNFRSF4 (also known as OX40), TNFRSF9 (also known as 4-1BB), TNFRSF7 (also known as CD27), TNFRSF18 (also known as glucocorticoid-induced TNFR-related protein, GITR) and TNFRSF8 (also known as CD30)) (FIG. 1). Other family members have demonstrated immune co-stimulatory potential (such as TNFRSF25 (also known as death receptor 3, DR3), TNF receptor 1 (TNFR1; also known as TNFRSF1A), TNFR2 (also known as TNFRSF1B), lymphotoxin-β receptor (LTβR) and TNFRSF14 (also known as herpesvirus entry mediator, HVEM)). However, this co-stimulatory mechanism of action is not fully understood, as it can be complicated by additional functional activities such as cell death induction via intracellular death domains (in the case of DR3 and TNFR1), or, in the case of HVEM, TNFR2 and LTβR, substantial crossreactivity with other TNF ligands and receptors[23]. These co-stimulatory receptors can be expressed on a number of immune cell types,

including T cells, B cells and natural killer (NK) cells as well as antigen-presenting cells (APCs), and have been shown to drive immune cell function, proliferation and survival[2]. The death receptors are an additional subclass within the TNFRSF that can also mediate immune activation in some contexts; however, their primary function is to activate cell death through intracellular death domains[24,25]. Although studies of these receptors have provided substantial mechanistic insight into agonists targeting the TNF co-stimulatory receptor class, agonist antibodies targeting death receptors are beyond the scope of this Review.

Here, we discuss the current landscape of agonist antibodies that target immune co-stimulatory receptors in cancer, differentiating them from antagonist antibodies and highlight the therapeutic promise and development challenges associated with this class of agents. We focus on the mechanistic basis of how therapeutic agonist antibodies interact with their target receptors to explain the unique challenges in the design and development of these immune agonist therapies. We hope to
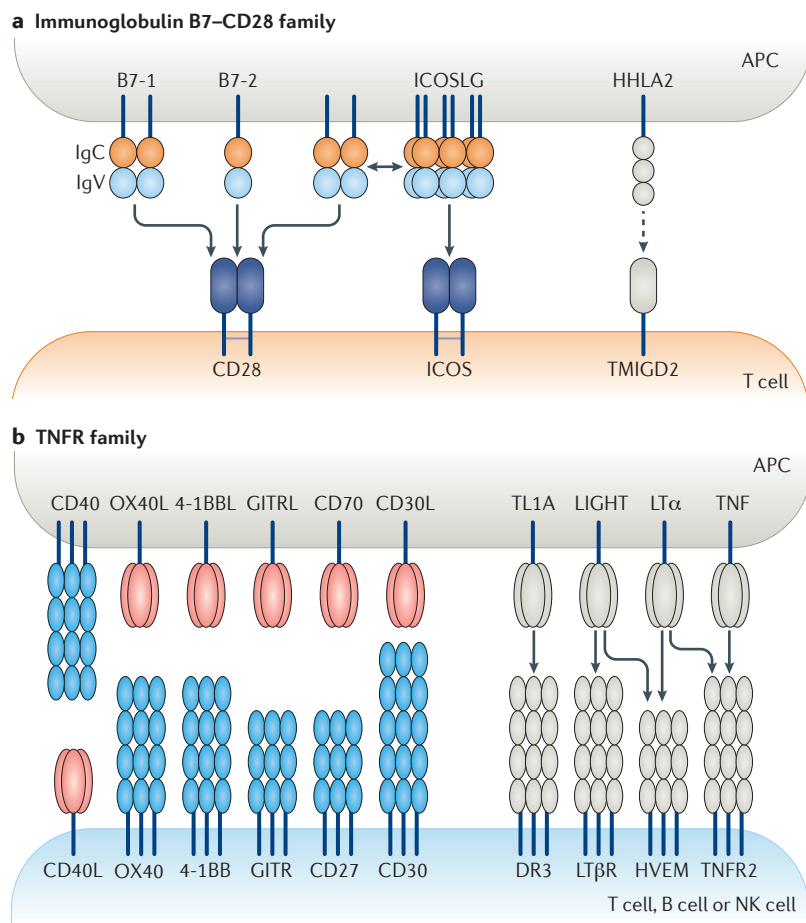
Figure 1 | **The B7–CD28 and TNFR families of co-stimulatory receptors. a** | CD28 and inducible T cell co-stimulator (ICOS) have a single extracellular immunoglobulin variable-like (IgV) domain coupled to an intracellular tail that mediates intracellular signalling[14,200]. Each receptor is expressed as a glycosylated, disulfide-linked homodimer at the cell surface[201,202]. The predominant ligands for CD28, B7-1 and B7-2 each contain an IgV domain and an immunoglobulin constant-like (IgC) domain[203]. B7-1 binds to CD28 with a fivefold to tenfold higher affinity than B7-2 and is expressed as a mixed population of monomers and non-covalent dimers, whereas B7-2 is exclusively monomeric[204–207]. The difference in oligomerization potential of B7-1 versus B7-2 has an effect in their interaction with CD28 (REF. [205]). Neither B7-1 nor B7-2 bind or activate ICOS[19,208,209], but ICOS ligand (ICOSLG) can bind weakly to both CD28 and cytotoxic T lymphocyte antigen 4 (CTLA4)[210,211]. Like B7-1, ICOSLG favours a non-covalent dimeric and higher-order oligomeric form on the cell membrane[44]. Transmembrane and immunoglobulin domain-containing protein 2 (TMIGD2) is an additional co-stimulatory receptor in the B7–CD28 family that has been reported to bind to HERV-H LTR-associated protein 2 (HHLA2). Dotted lines indicate limited data in the literature supporting these interactions. **b** | Tumour necrosis factor receptors (TNFRs) are transmembrane proteins with intracellular signalling domains varying between 36 (OX40) and 188 amino acids in length (CD30). Some receptors in the family, including the co-stimulatory class, can also be present in soluble form owing to proteolytic cleavage of the extracellular domain (CD40, CD30, CD27 and 4-1BB)[212,213]. The hallmark of TNFRs is the extracellular cysteine-rich domains (CRDs) that are repeated in an elongated arrangement to form the ligand-binding interface[21]. The co-stimulatory class contains anywhere between three and five CRDs. Co-stimulatory TNF ligands are non-covalent, trimeric transmembrane proteins with extracellular TNF homology domains (protomers), which are responsible for receptor binding[46]. Grey receptor–ligand pairs indicate receptors for which clinical stage agonists targeting the receptor have not been reported. 4-1BBL, 4-1BB ligand; APC, antigen-presenting cell; CD30L, CD30 ligand (also known as TNFSF8); CD40L, CD40 ligand; DR3, death receptor 3; GITR, glucocorticoid-induced TNFR-related protein; GITRL, GITR ligand; HVEM, herpesvirus entry mediator; LTα, lymphotoxin-α; LTβR, lymphotoxin-β receptor; NK, natural killer; OX40L, OX40 ligand; TL1A, TNF ligand-related molecule 1 (also known as TNFSF15).

demonstrate that this class of agents requires antibody design features and development approaches that are different from those used for first-generation immune checkpoint antagonists. Finally, we provide a summary of the clinical contexts and combination approaches in which these agents are currently being developed for the treatment of cancer.

Activating a receptor with an antibody

A desired and defining feature of an agonist antibody is the ability to bind and activate the target receptor in a way that mimics the activity of the native ligand. The propensity of an antibody to act as an agonist can be influenced by many factors that include the binding epitope, valency, degree of receptor occupancy and the interaction of the antibody crystallizable fragment (Fc) domain with Fcγ receptors (FcγRs). Unlike antagonists — for which the general rule is to design an antibody with the highest strength of binding (affinity) possible to interact competitively with the ligand-binding domain of the receptor — there are no hard-and-fast rules that can easily predict the potential of an antibody to act as an agonist. For this reason, historically, the process of identifying and selecting agonist antibodies has largely been an empirical exercise driven by functional characterization of the antibody using cell-based and in vivo model systems[26–29]. However, a detailed understanding of the specific stoichiometric and binding properties of the native ligand–receptor complex can help inform the design of an antibody with optimal agonist function against a specific receptor. The receptor structure as well as the ligand-binding and signalling-engagement mechanisms tend to be generally conserved within receptor families but are quite unique between families[30]. As such, the mechanisms of co-stimulatory receptor binding and activation for the two main classes of cancer immune agonists (B7–CD28 and TNFR) should be considered independently.

Receptor–ligand engagement and signalling

B7–CD28 family. The propensity of B7 ligands, particularly B7-1 and ICOSLG, to configure as homodimers on the cell surface favours the bivalent association of a single ligand dimer with two separate dimers of CD28 or ICOS at the cell–cell interface of an immune synapse[31–34] (FIG. 1). This suggests a model in which the avidity effect of dimeric B7-1 ligand bridging with adjacent CD28 or ICOS receptor dimers helps to stabilize the ligand–receptor interaction, leading to receptor supercluster formation and subsequently inducing more effective co-stimulatory receptor signalling. Additionally, the sequestration of CD28 and ICOS receptor within immunological synapses following T cell activation may further assist in receptor supercluster formation and productive co-stimulatory receptor signalling in response to B7 ligand binding owing to the increased proximity to neighbouring receptors[35–37]. Crystallographic and cryo-electron microscopy studies of the complexes formed by CD28 and agonist antibodies have identified a more compact conformation[38]. These compact CD28–agonist antibody

complexes are consistent with a 'kinetic segregation model' in which the phosphatase CD45 (also known as PTPRC) is displaced from the immune synapse, leading to more effective signalling through CD28 (REF. [39]). The bivalent association of B7 ligands with CD28 and ICOS suggests that an agonist antibody against these receptors must mimic this bridging activity to achieve optimal receptor supercluster formation and activation.

CD28 and ICOS are non-redundant in their effect on T cell proliferation, function and survival[40]. Although, following ligand binding, both CD28 and ICOS can activate phosphoinositide 3-kinase (PI3K) signalling and nuclear factor of activated T cells (NFAT)-responsive genes, CD28 is unique in its ability to also significantly induce both c-Jun N-terminal kinase (JNK; also known as MAPK) and nuclear factor-κB (NF-κB) signalling[11,41]. CD28 and ICOS recruit regulatory subunits of PI3K to the membrane proximal YXXM motif of their intracellular domains[41]. However, the ICOS YMFM motif has been shown to induce stronger PI3K signalling than has the CD28 YMNM motif[42]; PI3K subsequently drives phosphorylation and activation of AKT and activation of NFAT. Additionally, CD28 contains membrane-distal proline-rich motifs that ICOS does not have, and these motifs can interact with SH3 domain-containing proteins such as growth factor receptor-bound protein 2 (GRB2) and LCK, which can mediate signalling through NF-κB and JNK and/or the adaptor protein 1 (AP1) complex[11,41].

TNFR family. Despite the wide range of biological functions performed by the TNFR family, the structure of both the receptors and the ligands as well as the mechanisms of binding and signalling engagement are generally well conserved between the family members. However, the aforementioned group of six TNF co-stimulatory ligands is the most divergent of the TNF superfamily ligands in terms of sequence and tertiary structure (FIG. 1). Compared with the canonical bell-shaped trimer of most other family members, TNF co-stimulatory ligands can assume unique tertiary confirmations[43–47]. Although some TNF ligands are produced as soluble proteins or cleaved by proteases, which release them to act as soluble ligands, none of the ligands against the six immune co-stimulatory TNFRs are believed to act in a soluble form[46,48]. A number of mechanisms have been proposed for how TNF ligands can activate co-stimulatory receptor signalling. One mechanism proposes that a trimeric TNF ligand coordinates binding of three monomeric TNFRs into a 3:3 configuration[49]. However, some TNFRs exist predominantly in a preformed trimeric configuration even in the absence of ligand[50–52]. In this scenario, TNF ligand binding may result in a conformational change to the preformed trimeric receptor, leading to intracellular domain colocalization and signal transduction[50,51]. A third model, which seems to be more in line with functional observations, is that the TNF ligand trimer bridges multiple preformed receptor-trimer complexes, leading to receptor superclusters[30,50] or that preformed ligand superclusters can bind and mediate receptor superclustering[53]. Multiple lines of evidence

suggest that receptor superclustering is an important factor in mediating proficient signalling induction of TNF co-stimulatory receptors. One example is that the agonist potential of some soluble TNF ligands is dramatically reduced in soluble form 'trimer-only configurations' compared with membrane-bound forms[54]; however, the inactive soluble ligand forms can gain activity by forming high-order oligomers (with more than three monomers) that are able to induce signalling[55]. Similar evidence has been demonstrated with GITR ligand (GITRL; also known as TNFSF18) and CD40 ligand (CD40L) — which can potentially exist in dimeric (GITRL), trimeric and higher-order oligomers — in which increased agonist activity is strongly correlated with the higher-order oligomeric ligand forms[53,56,57].

The structure–function relationship of the native TNF ligand–receptor interaction predicts that an agonist antibody that targets a co-stimulatory TNFR must be capable of mediating the higher-order oligomerization (superclustering) of co-stimulatory receptors that is required to induce productive signalling. Therefore, much like antibodies targeting the B7–CD28 family, multivalent binding and the potential to induce receptor superclustering are key requisites of optimal agonist activity.

Unlike CD28 and ICOS, which predominantly signal through the NFAT transcription factor, co-stimulatory TNFRs predominantly drive NF-κB signalling[21,58]. Co-stimulatory TNFRs activate intracellular signalling through differential interaction with six known TNFR-associated factor (TRAF) family members, each with different expression patterns and different upstream and downstream mediators[59,60]. TRAF2 is the most widely expressed and is believed to bind to all six of the co-stimulatory TNFRs covered in this Review[59]. The other TRAFs bind to the different co-stimulatory TNFRs with different preferences, resulting in overlapping but distinct signalling patterns. TRAFs predominantly mediate NF-κB signalling through activation of the inhibitor of NF-κB (IκB) kinase (IKK). Phosphorylation and degradation of IκB leads to translocation of NF-κB to the nucleus to activate transcription. Certain TRAFs can also mediate JNK signalling via activation of either mitogen-activated protein kinase kinase kinase 7 (MAP3K7), as is the case with TRAF6, or via activation of other upstream MAP3Ks, which in turn can activate JNK signalling[60].

Binding affinity, epitope and valency
Important biophysical criteria that dictate antagonist antibody activity — such as the receptor-binding epitope and affinity — can also be important in the design of agonist agents. However, the general rules that drive the structure–function relationship of antagonist antibody design (high affinity and ligand competition) do not hold true for agonists[61]. The role of epitope and affinity in controlling agonist antibody activity has been mostly studied in the TNFR family, particularly in agonist antibodies that target TNF death receptors[62–66]. However, lessons learned from agonists targeting death receptors can be applied to agonist antibodies targeting

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Valency
The number of antigen binding sites that an individual antibody can engage.

Fcγ receptors
(FcγRs). Receptors expressed on the surface of cells that can bind specifically to the crystallizable fragment (Fc) region of an antibody.

Immune synapse
The interface between an antigen-presenting cell (such as a dendritic cell) and a T cell or natural killer cell.

Receptor supercluster
A large grouping of receptors on the cell membrane typically occurring in lipid rafts or the immune synapse.

TNF co-stimulatory receptors as well. How these rules influence agonists in the B7–CD28 class is less clear but can be inferred to be similar on the basis of the general principle of driving receptor supercluster formation to induce agonist function, which is true of both classes of receptors. A study by Chodorge et al. used phage display to identify a potent agonist antibody targeting TNFRSF6 (also known as FAS)[62]. The agonist antibody was then affinity-matured to select four additional variants with a range of affinities for FAS, which were tested for their

agonist potential. Surprisingly, a strong negative correlation between FAS affinity and agonist activity was observed, with the highest affinity antibodies having lost nearly all agonist activity[62]. However, with the addition of protein A crosslink, the agonist activity increased to a similar level for all antibodies in the panel, eliminating the effect of affinity on agonist function. This led to the proposal of a model in which antibody binding affinity can be inversely correlated with agonist function. This model is supported by previous reports that show that panels of antibodies targeting FAS can bind to the same or similar epitopes but can result in different biological effects, with some acting as agonists and others as antagonists. Although the epitope was not predictive of function, relative binding affinity did seem to inversely correlate with agonist activity against FAS[64,67]. These reports, while proving the importance of affinity for achieving agonist function, also clearly demonstrate that the binding epitope alone does not dictate agonist function. Agonist antibodies have generally shown partial or considerable overlap with the ligand-binding epitope of their target receptor[62,68,69]. However, there are also examples of agonist antibodies that have almost no overlap with ligand-binding domains and that can bind their receptor in completely different conformations than the native ligand[27,70]. Bjorck et al. showed that multiple antibodies targeting different epitopes on CD40 were able to cause agonist signalling activation[71]. In fact, two antibodies synergized CD40 signalling when used together but not when used in combination with CD40L[71]. In these instances, an agonist antibody may differ in the mode of receptor activation compared with the native ligand. Examples of alternative agonist modes of action may include antibodies that preferentially bind to and stabilize preformed receptor oligomers (FIG. 2a). Therefore, the receptor-binding epitope of an agonist antibody can be an important factor responsible for signalling activity; however, for individual receptors, there is likely to be more than just one binding epitope capable of achieving agonist activity. For this reason, the receptor-binding epitope, in isolation, is not predictive of the agonist potential of an antibody.

The ability of an antibody to induce receptor supercluster formation is critical for co-stimulatory agonist function. Therefore, the bivalency of native immunoglobulin G (IgG) is a key biophysical characteristic that must be preserved. The bridging of multiple receptors via the two antigen-binding fragments (Fab) can result in monomer–monomer interactions or higher-order (greater than two) preformed clusters[72,73], which differs from antagonist antibody design, in which monovalent binding antibodies or antibody fragments can act as potent antagonists[74]. This difference has important implications for bispecific antibody design. New generations of engineered IgG-like bispecific antibodies have emerged in which a single IgG can bind two distinct receptors via two Fab arms with distinct antigen specificities[75,76]. It is clear that this format has the potential to allow for simultaneous blockade of two axes (antagonist–antagonist pairings), with each antagonist Fab forming a monovalent binding interaction with its
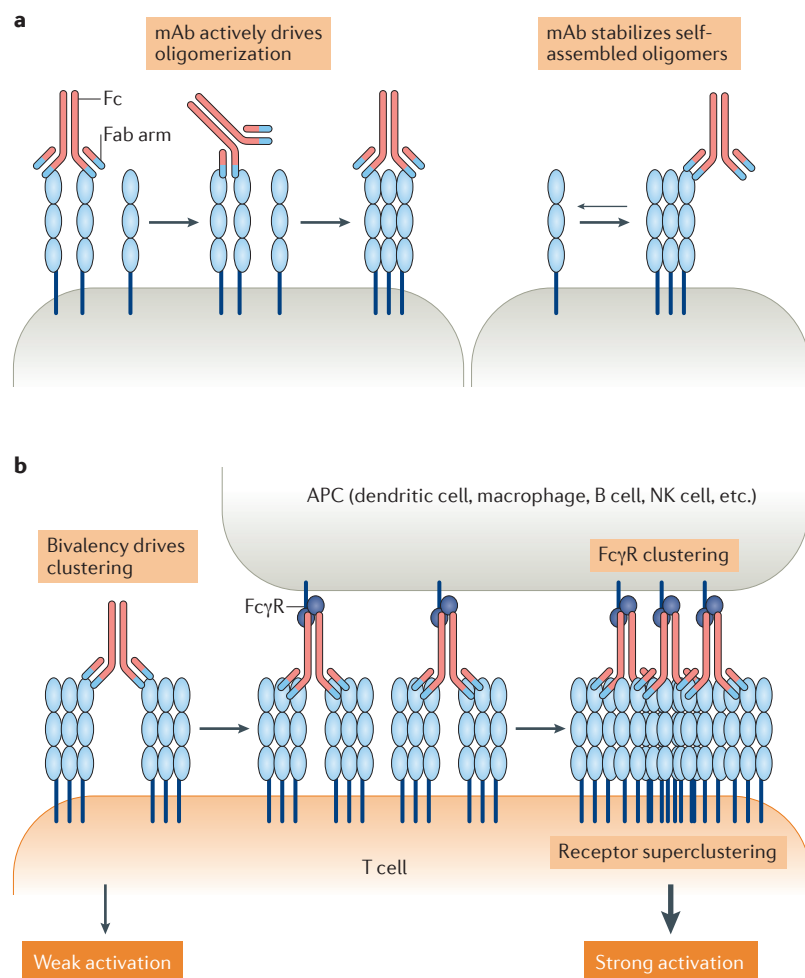

Figure 2 | **Mechanisms of receptor supercluster formation with co-stimulatory agonists. a** | Antibody binding affinity can be inversely correlated with agonist function. In this model of agonist antibody activation, partial dissociation of antibodies allows the antigen-binding fragment (Fab) arms of a single antibody to interact with more than two receptors in a dynamic fashion, resulting in recruitment of multiple receptor monomers into a receptor oligomer where signalling activation can be triggered (left-hand side). In an alternative model of co-stimulatory receptor agonistic activation, an antibody preferentially binds to and stabilizes preformed receptor oligomers, shifting the equilibrium away from the monomeric form and towards oligomeric receptor clusters (shown here as tumour necrosis factor receptor (TNFR) trimers, but this can also be true of B7–CD28 dimers) (right-hand side). **b** | Crystallizable fragment (Fc)-γ receptors (FcγRs) expressed on antigen-presenting cells (APCs) act as a scaffold to crosslink agonist antibody bound to co-stimulatory receptor, leading to receptor supercluster formation and increased agonist signalling. Interactions occur in *trans* between FcγR-expressing APCs and lymphocytes, which express co-stimulatory receptors[87]. mAb, monoclonal antibody; NK, natural killer.

respective receptor target. What is less clear is whether agonist–antagonist or agonist–agonist pairings are also achievable in this format. Of particular interest is whether antibody-induced heterodimerization of different co-stimulatory receptors can induce similar or synergistic activation compared with homodimerization of individual co-stimulatory receptors. There are examples of some TNFR family members that are able to signal as mixed oligomers[77].

The importance of multivalency for co-stimulatory receptor activation has also been demonstrated through the generation of agonistic aptamers against 4-1BB and OX40 (REFS [78,79]), as well as soluble recombinant forms of co-stimulatory TNF ligands, including CD40L, 4-1BB ligand (4-1BBL; also known as TNFSF9) and OX40 ligand (OX40L; also known as TNFSF4), in which higher-order oligomer ligand fusions or crosslinked trimers induce significantly greater TNFR activation than native soluble trimeric forms or monomeric forms of the ligands[55,80,81].

Interaction with the FcγR domain

Although the target-binding properties of Fab domains in an agonist antibody are critical, the interaction between the antibody Fc domain and FcγRs can also dictate agonist potential. FcγR binding is now understood as a requisite for optimal co-stimulatory agonist antibody function[82]. The interaction between human antibodies and FcγRs is highly complex and nuanced. Humans have six known FcγRs, five activating (high affinity immunoglobulin-γ FcR I (FcγRI), low affinity immunoglobulin-γ FcR IIa (FcγRIIA), low affinity immunoglobulin-γ FcR IIc (FcγRIIC), low affinity immunoglobulin-γ FcR IIIa (FcγRIIIA) and low affinity immunoglobulin-γ FcR IIIb (FcγRIIIB)) and one inhibitory (FcγRIIB), all of which can bind to the four human IgGs (IgG1, IgG2, IgG3 and IgG4) with varying specificities and affinities[83]. The complexity is further increased by the fact that FcγRs are differentially expressed on different human cell types, particularly on immune cells[84]. A number of reports have demonstrated the requirement of FcγRIIB for optimal agonist activation of co-stimulatory receptors such as CD28, CD40, OX40 and 4-1BB[85–89], as well as death receptors DR4 (also known as TNFRSF10A), DR5 (TNFRSF10B) and FAS[90]. It is unlikely that agonist function is mediated by intracellular signalling through FcγRIIB. Instead, agonist activity seems to be mediated by the scaffolding and anchoring activity of the receptor or through increased receptor crosslinking potential following antibody binding in *trans* to the target cell and FcγR-expressing cell populations[87] (FIG. 2b). Activating FcγRs can also induce IgG crosslinking and agonist function[91]. However, in ex vivo immune cell assays and in vivo models, the increased potential for FcγRIIB to mediate crosslinking is believed to be due to the high relative expression and availability of FcγRIIB-expressing cell types (primarily B cells)[91,92]. Induction of antibody-dependent cellular cytotoxicity (ADCC) following engagement with activating FcγRs further complicates the interpretation of whether these receptors can mediate crosslinking and agonist activation[90,93].

Interestingly, human IgG2 antibodies against a number of immune co-stimulatory receptors (CD40, 4-1BB and CD28) seem to be capable of inducing agonist activation in an FcγR-independent manner[92,94]. The proposed explanation for this unique activity is that human IgG2 can adopt dynamic conformations as a result of disulfide rearrangements in the hinge region. It is the more rigid confirmation IgG2b, where the Fab arms are disulfide linked to the hinge region, that results in FcγR-independent agonist function[92,94]. In the case of CP-870,893 (a fully human anti-CD40 IgG2 agonist antibody now developed by Roche as RO7009789), Fc crosslinking was not required, but rather the CD40 epitope recognized by the antibody was an important determinant of potency[95]. It will be important to further understand the functional relevance of the FcγR-independent agonist activity of human IgG2 antibodies, as a number of these agents are in development against co-stimulatory receptors in cancer (TABLE 1). Whether the human IgG2 isotype obviates the need for binding interactions with FcγR-expressing cells in humans and what implications this has on antitumour activity as well as toxicity could have implications for future agonist antibody development. Other strategies to improve the agonist potential of antibodies include antibody engineering approaches to increase the affinity of native IgG for FcγRIIB[89,96] because the affinity of native IgGs for FcγRIIB is relatively low[97]. FcγRIIB-enhanced antibodies have shown improved agonist potential relative to native IgG in some studies[88,89,98].

Receptor occupancy

The relationship between function and the degree of receptor binding is another key variable that must be considered with respect to agonist antibody development. The rules governing this relationship for agonists are again in contrast with classic antibodies that have been developed for the treatment of cancer, particularly receptor antagonists (such as PD1, CTLA4 or vascular endothelial growth factor (VEGF)) or antibodies that induce target cell killing through ADCC or complement-dependent cytotoxicity (CDC) mechanisms (such as CD20, ERBB2 (also known as HER2) and EGFR)[99]. These antagonists and ADCC and CDC antibodies generally act in a sigmoidal dose–response fashion, in which the functional activity peaks near 100% receptor occupancy (binding saturation) and plateaus at concentrations above those needed for binding saturation. This classic dose–response relationship predicts that the dose level at which full blockade of the receptor or function is achieved determines the highest activity in humans and is not exceeded through higher doses[100–102]. However, there is direct evidence to suggest that the same sigmoidal dose–response relationship does not apply to agonist antibodies targeting co-stimulatory receptors. Instead, some co-stimulatory agonists have a bell-shaped dose–response. For example, in studies testing human T cell activation and cytokine secretion following treatment with TGN1412 (an agonist antibody targeting CD28), immune activation was found to occur in a bell-shaped profile. In the in vitro assay system used, peak immune activation occurred at 5 μg/ml, whereas at

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Protein A crosslink
The simultaneous binding of multiple immunoglobulin G (IgG) molecules by a single protein A molecule, leading to IgG clustering or crosslinking.

Receptor crosslinking
The active process of receptor clustering that occurs when bivalent antigen-binding fragment (Fab) domains of a single antibody bind to multiple receptors; the process can be mediated by the interaction of the crystallizable fragment (Fc) domain with Fcγ receptors.

Sigmoidal dose–response
A dose–response relationship in which the logarithm of the drug concentration plotted on the x axis results in an activity–response curve with a sigmoidal shape, wherein drug activity increases exponentially at the middle concentrations and plateaus at high concentrations.

Bell-shaped dose–response
A dose–response relationship in which the logarithm of the drug concentration plotted on the x axis results in an activity–response curve with a bell or umbrella shape, wherein drug activity is maximal at middle concentrations and decreases as concentration increases further.

concentrations ≥100 µg/ml, nearly all activity was lost[103]. A separate study demonstrated that at the concentration levels at which TGN1412-induced immune activation was greatest (5–10 µg/ml), the percentage of receptor occupancy for TGN1412 binding to CD28+ T cells was in the range of 50–80%, whereas concentrations ≥100 µg/ml resulted in binding saturation[104]. A similar study testing a panel of murine CD28 agonist antibodies also showed a bell-shaped dose–response profile for the antibodies tested[105]. Bell-shaped dose–response profiles have also been observed for other agonist antibodies; however, the same receptor occupancy–function relationship has not been closely studied[68,94,106].

Figure 3 | **Optimal stoichiometry for receptor supercluster formation.** In theory, to achieve maximal receptor supercluster formation, a molar equivalence of both antibody and receptor would be desirable to facilitate uninterrupted bridging between antibody and receptor. In a situation in which the receptor abundance far exceeds the antibody concentration, there would be inadequate bridging owing to lack of antibody, leading to isolated receptor–antibody complexes with a 2:1 stoichiometry (top panel). In the opposite situation, antibody concentration far exceeds receptor, which would also result in suboptimal antibody–receptor bridging, leading to isolated complexes with a 1:2 stoichiometry (bottom panel). However, equimolar amounts of both antibody and receptor would lead to optimal, uninterrupted receptor bridging and supercluster formation (middle panel). Mechanistically, this model fits well with the known structure– function relationship of immune co-stimulatory receptors[30]. The example shown in this figure highlights the B7–CD28 family receptors; however, the same principle applies to the tumour necrosis factor receptor (TNFR) family as well. Additional studies are needed to better characterize the binding–function relationship for the class of co-stimulatory immune agonist antibodies, as these data are likely to have important consequence on the dose–response relationship of these agents in patients.

Many instances of bell-shaped dose–response profiles have been reported for other drug classes[107]. With respect to immune co-stimulatory agonist antibodies, there are a few mechanisms that may explain the potential for a bell-shaped dose–response with these agents. One possibility is that immune overstimulation with high concentrations of antibody leads to immune exhaustion, signalling downregulation or activation-induced cell death. This phenomenon has been well described with T cells in response to antigen stimulation[108]. However, exhaustion has most often been characterized as a consequence of chronic antigen exposure, in which the duration of T cell activation is what drives the exhaustion phenotype. There is less evidence to support the concept that strong activation over a short interval can lead to the same effect. Likewise, the acute time points measured in the in vitro assay formats in which bell-shaped dose–responses have been demonstrated suggest that high concentrations fail to ever induce sufficient levels of T cell activation[103]. Another potential mechanism relates to the stoichiometric binding properties between agonist antibody and receptor that lead to the greatest potential for receptor oligomerization and function (FIG. 3).
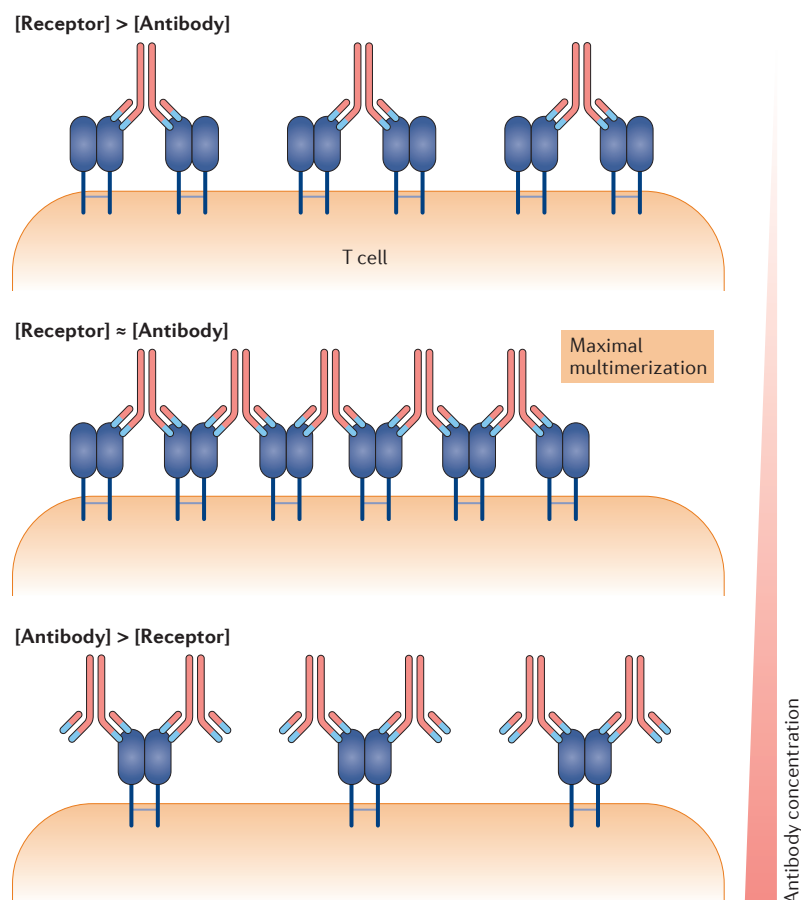
Balancing effector–agonist functions

Because FcγR engagement is a key property required for optimal activity of co-stimulatory agonist antibodies, IgG isotype selection is critical for the design of these agents. Nearly all of the co-stimulatory agonist antibodies in development are IgG isotypes, which are capable of FcγR engagement (TABLE 1). Of the human IgG isotypes, which are commonly used as therapeutic antibodies (IgG1, IgG2 and IgG4), IgG1 is the strongest binder to FcγRs, particularly the activating FcγRs[97,109]. This property makes it the most effective IgG isotype at inducing ADCC or CDC and thus makes it the isotype of choice for antibodies designed to induce targeted cell killing[110]. IgG2 and IgG4 have significantly less potential to bind FcγR (in particular FcγRIII, the primary activating receptor responsible for ADCC in humans) than IgG1 (REF. [111]). Both IgG2 and IgG4 can bind to human FcγRII, and IgG4 can bind to FcγRI, with lower binding affinity than IgG1 (REFS [97,112]). The human system of FcγRs and IgG is somewhat more complex than the murine system, wherein mice have three main activating FcγRs (FcγRI, FcγRIII and low affinity immunoglobulin-γ Fc region receptor IV (FcγRIV)) as well as one inhibitory receptor (FcγRIIB), as in humans[113]. There are three murine IgG isotypes (mIgG1, mIgG2a and mIgG2b), in contrast to four in humans, with mIgG2a being the most homologous to human IgG1 in terms of its affinity for activating FcγRs and potential for inducing ADCC[113]. Therefore, murine studies using mIgG2a can be considered an approximation of the expected activity of an IgG1 isotype in humans[114,115].

Interpretation of studies with murine-targeted versions of co-stimulatory agonist antibodies (murine surrogates) in mouse models of cancer can be largely influenced by the respective isotype of the antibody used. In some instances, the antitumour activity observed with these agents is directly related to the respective isotype used.

For example, the antitumour responses observed in mice using OX40 and GITR-targeted murine surrogate agonist antibodies depended on the use of the mIgG2a variant of the agonist antibodies[116–119]. This was due to the ability of mIgG2a to engage with activating FcγRs and deplete regulatory T (T$_{reg}$) cells by an ADCC mechanism, as antitumour activity was strongly impaired in mice lacking activating FcγRs[116,117,118,119]. The authors demonstrated that the unique ability of these agonist antibodies to preferentially deplete T$_{reg}$ cells in these mouse models is due to the high expression of OX40 and GITR on intratumoural populations of T$_{reg}$ cells. These studies suggest that the therapeutic effect of human OX40 and GITR antibodies, many of which are IgG1 (TABLE 1), is related to the ability of these agents to deplete intratumoural populations of T$_{reg}$ cells by ADCC.

This mechanism may also be considered for other co-stimulatory agonist antibodies that are IgG1 and leads to a hypothetical model in which some co-stimulatory agonist antibodies may function by a dual-mechanism: agonist activation of some immune cell populations in addition to immune cell depletion via ADCC of other immune cells, particularly T$_{reg}$ cells (FIG. 4). Both OX40 and GITR agonist antibodies can reverse T$_{reg}$ cell suppressive function via direct co-stimulatory receptor activation in this cell population[28,119–122]. T$_{reg}$ cell inhibition by OX40 and GITR agonist antibodies, whether through targeted inhibition or ADCC depletion, is expected to be beneficial in cancer treatment.

A key question for the development of immune co-stimulatory agonists in patients with cancer is whether the same window of intratumoural T$_{reg}$ cell
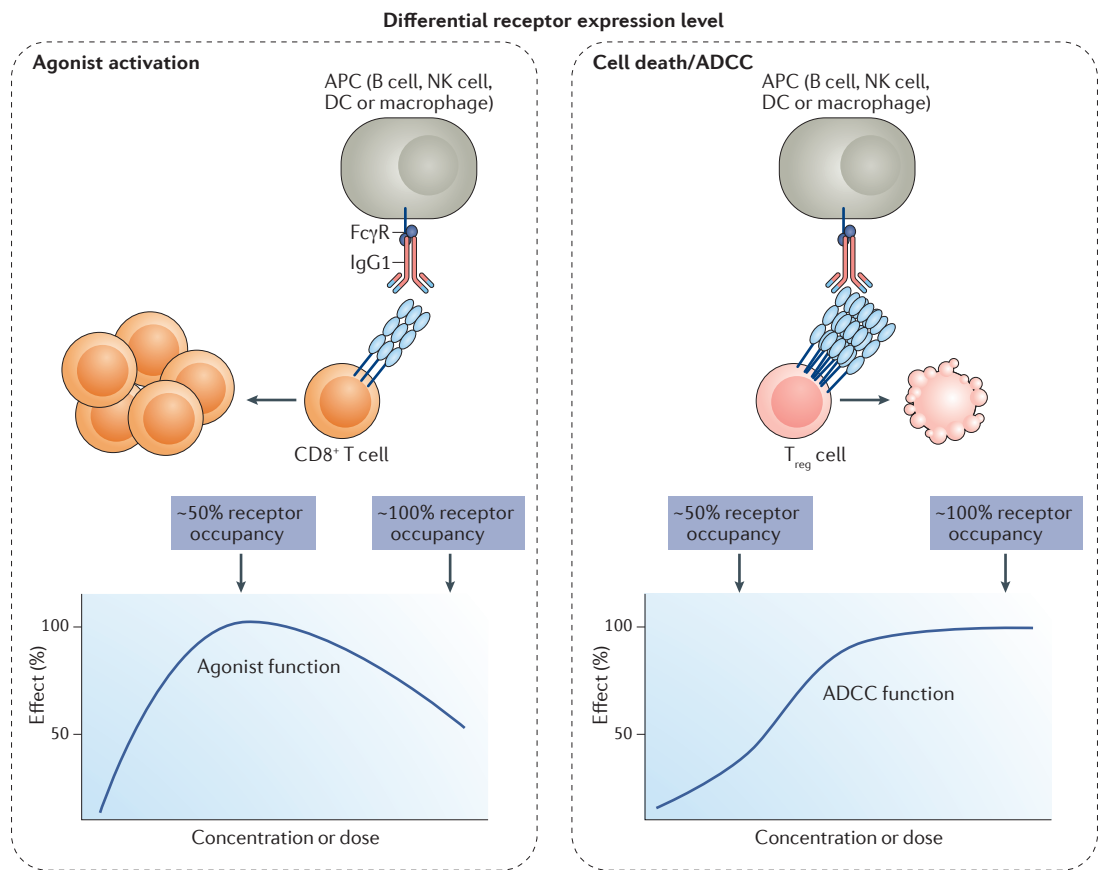

Figure 4 | **Model for potential dual activities of some immune agonist antibodies.** Some co-stimulatory agonist antibodies may function by a dual mechanism, including agonist activation of some immune cell populations in addition to immune cell depletion of other cells, particularly regulatory T (T$_{reg}$) cells, via antibody-dependent cellular cytotoxicity (ADCC). A hypothesis for how these mechanisms can coincide is that the expression density of co-stimulatory receptors on immune cell subsets is different. The ability of an antibody to induce ADCC can be positively correlated with the expression levels of the target receptor[214]. Therefore, for receptors with widely varying expression levels across immune cell subsets, a scenario can be envisioned in which cells that express high levels of the co-stimulatory receptor are killed by ADCC (right panel), whereas cells that express low levels are activated by the agonist (left panel). This remains a hypothesis for which more data are needed, particularly for human applications. Another interesting caveat when considering these dual mechanisms is the different dose–response profile for each function. The agonist mechanism may act via a bell-shaped dose–response, but the ADCC mechanism may act via a sigmoidal dose–response, which may require greater levels of drug to achieve optimal activation. Whether the two mechanisms can occur at the same dose and/or receptor occupancy of agonist remains to be determined. APC, antigen-presenting cell; DC, dendritic cell; FcγR, crystallizable fragment-γ receptor; IgG1, immunoglobulin G1; NK, natural killer.

overexpression observed in murine tumour models is also observed in human tumours. Some recent studies that characterized immune co-stimulatory receptor expression in human tumours suggest that high intratumoural T_{reg} cell expression exists in some contexts for some receptors but is not a feature common to all co-stimulatory receptors[123–126]. For this reason, close consideration will be required when determining the relative effect of immune cell activation versus immune cell depletion via ADCC in cancer patients, particularly with co-stimulatory agonists that are IgG1. As the immune context may vary widely between patients even with the same type of cancer, patient selection based on immune profiles may become critical for the effective use of agonist antibodies.

Current agonist antibodies

As of April 2018, there are 25 different immune agonist antibodies in clinical development for cancer indications (TABLE 1). These agents are being tested in 48 ongoing combination trials (TABLE 2). The agonist antibodies covered here are directed against seven different targets (CD27, CD40, OX40, 4-1BB, GITR, ICOS and CD28). An additional six antibodies are also in development against CD30; however, all of these agents are being developed with the primary mechanism of directed cell killing against CD30-expressing cancer cells[125]. Recombinant ligand therapies targeting the respective co-stimulatory receptors are also briefly covered below. Remaining challenges exist with this class of therapies, such as complexities of exposure, immunogenicity, stability and patient immune profiling. Clinical investigations are largely still early, and additional data are required to determine the full potential of these therapies.

CD27

CDX-1127 (varlilumab) is the only agonist antibody targeting CD27 currently in clinical development for the treatment of cancer. It was initially described in 2012 as an antibody that could elicit direct antitumour effects against leukaemia and lymphoma T and B cells that express CD27 (REF. [126]). As varlilumab is a human IgG1, the antitumour activity is proposed to be a result of ADCC directed killing of T and B cells[126]. Subsequent studies demonstrated that, in addition to the direct killing of CD27-expressing cancer cells, varlilumab also induces antitumour immunity as a result of agonist activation of CD27+ non-tumorigenic T cells[127]. A phase I dose escalation study was performed in patients with advanced B cell lymphoma. Some clinical activity was observed in this study, including a patient with stage IV Hodgkin lymphoma who achieved a complete response. However, it was noted that, of the patients analysed, this patient had the highest level of expression of CD27 on tumour cells, making it difficult to determine whether clinical activity was a result of agonist activation of antitumour immune cells or through a tumour cell-directed ADCC mechanism[128]. Varlilumab has also been studied in solid tumours, in which evidence of clinical and biological activity has been observed, including

immune changes consistent with CD27 activation. In particular, varlilumab has demonstrated potent depletion of T_{reg} cells, which is believed to be due to high levels of CD27 expression on this population of cells, making them a target of ADCC[129,130]. Varlilumab is in continued phase I/II development against selected solid tumour types as well as haematological malignancies (TABLE 1). Additionally, several ongoing studies are testing varlilumab in combination with immune-directed agents (anti-PD1, anti-PDL1 and tumour vaccines) as well as in combination with agents directed against tumour cells and the tumour microenvironment (including anti-VEGF and a transmembrane glycoprotein NMB (gpNMB) antibody–drug conjugate) (TABLE 2).

CD40

Agonist antibodies against CD40 were among the first agents to demonstrate the therapeutic potential of targeting an immune co-stimulatory receptor in cancer[131–133]. Clinical testing of the first CD40 agonist antibodies in cancer patients began nearly 10 years ago, and a meaningful amount of clinical data have been generated since then[134–137]. There are currently six anti-CD40 antibodies undergoing active clinical testing (TABLE 1). Development paths and design of CD40 antibodies have diverged around the two main mechanisms of CD40 targeting. Some antibodies such as selicrelumab (RO-7009789, previously known as CP-870893) have been designed as human IgG2 isotypes, thus promoting strong agonist activation while avoiding CD40+ cell depletion via ADCC and CDC (TABLE 1). CD40 is unique among the TNFR co-stimulatory targets in that its expression is predominantly found on APCs such as dendritic cells, B cells and macrophages and is not significantly found on T cells[138]. Therefore, the predominant agonist mechanism of CD40 is inducing antigen presentation, as well as APC proliferation and function[136]. However, CD40 is also expressed on some B cell malignancies and solid tumours, and therefore some CD40 antibodies designed as human IgG1 isotypes can mediate direct tumour cell killing via CDC and ADCC (TABLE 1). In line with this directed tumour cell kill mechanism, SEA-CD40 has been designed as an afucosylated IgG1 monoclonal antibody (mAb), which improves the ADCC potential of the antibody[139]. Some reports have also shown that agonist activation of CD40 receptor on tumour cells can mediate apoptosis independent of ADCC, suggesting that direct tumour cell killing is also possible with pure CD40 agonists such as CP-870,893 (REFS [140,141]). The class of CD40 agonist antibodies has demonstrated some positive signals of clinical activity across multiple studies in patients with advanced-stage cancer. There have been some dose-limiting toxic effects observed, as well as instances of moderate, transient cytokine release syndrome (CRS) in some patients[142]. Nevertheless, to date, its activity either as single agent or in combination with chemotherapy has not justified late-phase development[137]. The emphasis of current CD40 agonist antibody clinical development is on its combination with other immunomodulatory therapies such as anti-CTLA4, anti-PD1, anti-PDL1 and anti-macrophage colony-stimulating

Table 2 | **Clinical combination studies with co-stimulatory agonist antibodies in cancer**

Combination agent	Combination	Indication	Sponsor	Status	ClinicalTrials. gov identifier
CD27					
PD1	Varlilumab and nivolumab	Relapsed or refractory aggressive BCL	National Cancer Institute	Recruiting	NCT03038672
Vaccine	Varlilumab, IMA950 vaccine and poly-ICLC	Neoadjuvant treatment of LGG	University of California San Francisco	Recruiting	NCT02924038
CD20	Varlilumab and rituximab	BCL	University Hospital Southampton	Recruiting	NCT03307746
PDL1	Varlilumab and atezolizumab	Multiple solid tumours	Celldex	Terminated	NCT02543645
VEGF	Varlilumab and sunitinib	Metastatic clear-cell RCC	Celldex	Terminated	NCT02386111
PD1	Varlilumab and nivolumab	Advanced-stage refractory solid tumours	Celldex	Active	NCT02335918
gpNMB ADC	Varlilumab and glembatumumab vedotin	Advanced-stage melanoma	Celldex	Recruiting	NCT02302339
CD40					
CTLA4	CP-870,893 and tremelimumab	Metastatic melanoma	University of Pennsylvania	Active, not recruiting	NCT01103635
CSF1R	Selicrelumab and emactuzumab	Advanced-stage solid tumours	Roche	Recruiting	NCT02760797
VEGF and ANG2	Selicrelumab and vanucizumab	Metastatic solid tumours	Roche	Recruiting	NCT02665416
PDL1	Selicrelumab and atezolizumab	Locally advanced and metastatic solid tumours	Roche	Recruiting	NCT02304393
Chemotherapy	Selicrelumab, nab-paclitaxel and gemcitabine	Neo-adjuvant and adjuvant treatment of pancreatic carcinoma	University of Pennsylvania	Recruiting	NCT02588443
PD1	APX005M and pembrolizumab	Metastatic melanoma	MD Anderson Cancer Center	Recruiting	NCT02706353
	APX005M and nivolumab	NSCLC or metastatic melanoma	Apexigen	Recruiting	NCT03123783
GITR					
PD1	MK-4166 and pembrolizumab	Advanced-stage solid tumours	Merck & Co.	Recruiting	NCT02132754
	MK-1248 and pembrolizumab	Advanced-stage solid tumours	Merck & Co.	Recruiting	NCT02553499
	GWN-323 and PDR-001	Advanced-stage malignancies and lymphomas	Novartis	Recruiting	NCT02740270
	BMS-986156 and nivolumab	Advanced-stage solid tumours	Bristol-Myers Squibb	Recruiting	NCT02598960
PD1 or IDO	INCAGN01876 and pembrolizumab (anti-PD1) or INCAGN01876 and epacadostat (anti-IDO)	Advanced-stage solid tumours	Incyte	Recruiting	NCT03277352
PD1 or CTLA4	INCAGN01876 and nivolumab or INCAGN01876 and ipilimumab	Advanced-stage solid tumours	Incyte	Recruiting	NCT03126110
OX40					
CTLA4 and PDL1	MEDI0562 and durvalumab or MEDI0562 and tremelimumab	Advanced-stage solid tumours	MedImmune	Recruiting	NCT02705482
4-1BB	PF-04518600 and utomilumab	Advanced or metastatic carcinoma	Pfizer	Recruiting	NCT02315066
PDL1	PF-04518600 and avelumab	Selected tumour types	Pfizer	Recruiting	NCT02554812
4-1BB and PDL1	Utomilumab, PF-04518600 and avelumab (triple combo)	Selected tumour types	Pfizer	Recruiting	NCT02554812
Multi-kinase inhibitor	PF-04518600 and axitinib	RCC	Pfizer	Recruiting	NCT03092856
PD1	BMS-986156 and nivolumab	Advanced-stage solid tumours	Bristol-Myers Squibb	Recruiting	NCT02598960
PD1 and CTLA4	INCAGN01949 and nivolumab, INCAGN01949 and ipilimumab	Advanced malignancies	Incyte	Recruiting	NCT03241173
PDL1	MOXR0916 and atezolizumab	Locally advanced or metastatic solid tumours	Genentech	Active, not recruiting	NCT02410512

Table 2 (cont.) | **Clinical combination studies with co-stimulatory agonist antibodies in cancer**

Combination agent	Combination	Indication	Sponsor	Status	ClinicalTrials. gov identifier
OX40 (cont.)					
PDL1 and VEGF	MOXR0916, atezolizumab and bevacizumab (triple combo)	Locally advanced or metastatic solid tumours	Genentech	Active, not recruiting	NCT02410512
PDL1	MOXR0916 and atezolizumab	Locally advanced or metastatic urothelial carcinoma	Genentech	Active, not recruiting	NCT03029832
PD1	GSK-3174998 and pembrolizumab	Advanced-stage solid tumours	GlaxoSmithKline	Recruiting	NCT02528357
TLR4	GSK-3174998 and GSK-1795091	Advanced-stage solid tumours	GlaxoSmithKline	Recruiting	NCT03447314
TLR9 and RT	BMS986178, SD-101 and RT	Low-grade B cell NHL	Stanford University	Recruiting	NCT03410901
4-1BB					
CCR4	Utomilumab and mogamulizumab	Advanced-stage solid tumours	Pfizer	Recruiting	NCT02444793
HER2	Utomilumab and trastuzumab or trastuzumab emtansine	HER2+ breast carcinoma	Stanford University	Recruiting	NCT03364348
PD1	Utomilumab and pembrolizumab	Solid tumours	Pfizer	Completed	NCT02179918
CD20	Utomilumab and rituximab	CD20+ NHL	Pfizer	Recruiting	NCT01307267
OX40	PF-04518600 and utomilumab	Advanced or metastatic carcinoma	Pfizer	Recruiting	NCT02315066
PDL1	Utomilumab and avelumab	Selected tumour types	Pfizer	Recruiting	NCT02554812
OX40 and PDL1	Utomilumab, PF-04518600 and avelumab	Selected tumour types	Pfizer	Recruiting	NCT02554812
PDL1 and CD20	Utomilumab, avelumab and rituximab (triple combo)	Relapsed or refractory DLBCL	Pfizer	Recruiting	NCT02951156
PDL1 and azacitidine	Utomilumab, avelumab and azacitidine (triple combo)	Relapsed or refractory DLBCL	Pfizer	Recruiting	NCT02951156
LAG3 and PD1	Urelumab, BMS986016 and nivolumab	Recurrent GBM	Sidney Kimmel Comprehensive Cancer Center	Recruiting	NCT02658981
Chemotherapy	BMS663513 and chemotherapy	Advanced solid malignancies	Bristol-Myers Squibb	Terminated	NCT00351325
Chemotherapy and RT	BMS-663513 and chemoradiation	NSCLC	Bristol-Myers Squibb	Terminated	NCT00461110
CD20	BMS663513 and rituximab	B cell NHL	Bristol-Myers Squibb	Completed	NCT01775631
EGFR	Urelumab and cetuximab	Advanced or metastatic CRC or advanced or metastatic HNSCC	Bristol-Myers Squibb	Active, not recruiting	NCT02110082
SLAMF7	Urelumab and elotuzumab	Multiple myeloma	Bristol-Myers Squibb	Active, not recruiting	NCT02252263
PD1	Urelumab and nivolumab	Solid tumours and B cell NHL	Bristol-Myers Squibb	Recruiting	NCT02253992
	Neoadjuvant urelumab and nivolumab	Cisplatin-ineligible MIBC	Sidney Kimmel Comprehensive Cancer Center	Recruiting	NCT02845323
	Urelumab and nivolumab	Recurrent GBM	Sidney Kimmel Comprehensive Cancer Center	Recruiting	NCT02658981
PD1 and cell therapy	Urelumab, nivolumab and adoptive cell therapy	Metastatic melanoma	H. Lee Moffitt Cancer Center and Research Institute	Recruiting	NCT02652455
ICOS					
PD1	GSK-3359609 and pembrolizumab	Selected solid tumours	GlaxoSmithKline	Recruiting	NCT02723955
	JTX-2011 and nivolumab	Advanced-stage solid tumours	Jounce Therapeutics	Recruiting	NCT02904226

ANG2, angiopoietin 2; BCL, B cell lymphoma; CCR4, CC-chemokine receptor 4; CRC, colorectal cancer; CSF1R, macrophage colony-stimulating factor 1 receptor; CTLA4, cytotoxic T lymphocyte protein 4; DLBCL, diffuse large B cell lymphoma; EGFR, epidermal growth factor receptor; GBM, glioblastoma; GITR, glucocorticoid-induced tumour necrosis factor receptor-related protein; gpNMB ADC, glycoprotein NMB antibody–drug conjugate; HER2, human epidermal growth factor receptor 2; HNSCC, head and neck squamous cell carcinoma; ICOS, inducible T cell co-stimulator; IDO, indoleamine 2,3-dioxygenase; LAG3, lymphocyte activation gene 3 protein; LGG, low-grade glioma; MIBC, muscle-invasive bladder cancer; NHL, non-Hodgkin lymphoma; NSCLC, non-small-cell lung cancer; PD1, programmed cell death protein 1; PDL1, programmed cell death 1 ligand 1; RCC, renal cell carcinoma; RT, radiotherapy; SLAMF7, SLAM family member 7; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor.

factor 1 receptor (CSF1R) (TABLE 2). Although limited data have been reported to date, it is likely that, should CD40 agonists succeed, it will be through these types of rational immune targeted combinations.

OX40

A total of six OX40 agonist antibodies have entered clinical trials in the past 3 years (TABLE 1), in large part owing to the excitement around the promising preclinical activity observed with surrogate OX40 agonist antibodies in mouse tumour models[143]. Starting with a first report in 2000 (REF. [29]), the OX86 rat IgG1 clone has been the predominant OX40 agonist antibody used for the majority of studies in mouse tumour models in the literature[144]. The promising immune-stimulatory and antitumour activity of OX86 in these early studies led to the development of a murine monoclonal anti-human OX40 agonist antibody (9B12) by the Providence Cancer Center and AgonOx. This murine antibody, which later became MEDI0562, entered human clinical trials in 2006 (REF. [145]). Of the 30 patients who were treated in this trial, no tumour responses were observed; however, promising immunomodulatory activity was evident, including increases in T cell activation and proliferation[146]. Many nonclinical studies have subsequently demonstrated the antitumour potential of agonist activation of OX40 in mouse models in combination with a multitude of other agents, including immune checkpoint antibodies, cancer vaccines, targeted inhibitors and others[143]. The more recent evidence that antitumour activity in mice is driven predominantly via the FcγR-dependent depletion of T_{reg} cells by an ADCC mechanism[117] points to the potential importance of this mechanism in patients treated with OX40 agonist antibodies. Interestingly, five of the six OX40 agonist antibodies in clinical development are human IgG1 isotype and hence capable of ADCC depletion of OX40+ cells, including T_{reg} cells. The emerging clinical data have shown relatively little evidence of T_{reg} cell depletion by OX40 compared with CD27 agonist antibodies (REFS [131,132,138,139]). The role of OX40-mediated cell depletion is further confounded by recent nonclinical data that showed that anti-PD1 blockade inhibits the antitumour potential of an OX40 agonist in some mouse models when used in combination with vaccine antigen stimulation[147]. One possibility for the antagonistic activity of the OX40 and PD1 combination observed in this study is that PD1 blockade may increase OX40 expression on CD4+ effector and on CD8+ cytotoxic T cell populations, therefore making these cells susceptible to OX40 depletion as well. In the MMTV-PyMT breast cancer model, concurrent PD1 blockade significantly attenuated the therapeutic effect of an agonistic anti-OX40 antibody (OX86 clone), whereas sequential therapy initiated with anti-OX40 and followed by PD1 blockade significantly enhanced tumour growth inhibition and survival outcomes compared with anti-OX40 monotherapy[148]. This phenomenon must be carefully tracked in human trials as well, as at least eight trials combining an OX40 agonist with a PD1 or PDL1 antibody are currently under way (TABLE 2). A deeper mechanistic understanding of the activity of OX40 agonist antibodies in human tumours will help inform future rational combination studies and may also point to the importance of drug sequencing to achieve optimal therapeutic benefit. Other OX40 agonist combinations currently under way include the combination of PF-04518600 with a 4-1BB agonist (utomilumab, also known as PF-05082566) as well as the combination of MEDI0562 (tavolimab) with the CTLA4 blocker tremelimumab (TABLE 2). Preclinical evidence also supports the combination of an OX40 agonist together with an ICOS agonist; however, clinical investigation of this particular combination has not been done[149].

GITR

Like with OX40, much of the therapeutic rationale for the agonist activation of GITR has come from a single anti-mouse agonist antibody developed in the early 2000s (REF. [28]). DTA-1 is a rat IgG2b anti-mouse GITR agonist antibody that has antitumour activity in a wide range of syngeneic mouse models[150] via co-stimulation of CD4+ and CD8+ T cells[151,152] and via inhibition of the immune-suppressive activity of intratumoural T_{reg} cells. It can also deplete intratumour T_{reg} cells through an FcγR-dependent mechanism, possibly owing to the high differential expression of GITR on intratumoural T_{reg} cells compared with other T cells in the tumour microenvironment[116,153]. Whether GITR agonist activity in mice will be translatable to humans remains an open question[69]. However, an anti-human GITR antibody (MK-4166) — which binds to a similar epitope on human GITR as DTA-1 does on murine GITR — has shown both immune-stimulatory and T_{reg} cell-suppressive activity in human T cells[153,154]. Interestingly, the complementarity-determining regions from this antibody have also been cloned as humanized IgG4 (MK-1248), which has significantly reduced FcγR-mediated effector function[154]. Both MK-4166 and MK-1248 are now in phase I clinical studies in cancer patients (TABLE 1). Four other GITR agonist antibodies undergoing human clinical evaluation are native human IgG1, which confers them with an ADCC effector function that TRX518 — an aglycosylated human IgG1 — lacks (TABLE 1). Therefore, determining whether FcγR-mediated effector depletion of GITR+ cells offers a therapeutic advantage or liability is of substantial importance to the field. There are a number of GITR agonists currently being tested in combination with immune checkpoint antibodies against PD1 and CTLA4, as well as a study testing the combination of the anti-GITR agonist antibody INCAGN01876 and the IDO antagonist epacadostat (TABLE 2). Mechanistic insights into the actual profile of immunological activity of GITR agonists in the microenvironment of human tumours will be a key outcome of ongoing clinical studies. This knowledge will inform the design of future rational drug combination and sequencing approaches.

4-1BB

Among the members of the class of immune co-stimulatory receptor targets, some of the most compelling data generated to date support the therapeutic

rationale for agonist activation of 4-1BB. Multiple lines of evidence support this conclusion, including preclinical combination studies in mouse tumour models, clinical data with 4-1BB agonist antibodies in cancer patients and the use of the intracellular signalling domain of 4-1BB in the design and successful clinical use of CAR autologous T cell therapies. The recent US Food and Drug Administration approval of the first CAR therapy (CTL019) from Novartis contains an intracellular signalling domain of 4-1BB in the CD19-targeted CAR construct[155]. Significant clinical activity and durable responses were observed with this class of therapies — termed second-generation CARs — only after the addition of the 4-1BB co-stimulatory domain to the CD3ζ domain found in first-generation CARs[156]. This observation highlights the importance of 4-1BB co-stimulation for optimal T cell activation and antitumour response. Preclinical data in mouse models have also shown the potential antitumour activity of 4-1BB agonist antibodies — through co-stimulation of T cell immunity, as well as through activation and enhancement of NK cell function — both alone and in combination with other agents[157–165]. Clinically, there are currently two 4-1BB agonist antibodies being tested for cancer treatment: BMS-663513 (urelumab) and utomilumab (TABLE 1). Urelumab was the first 4-1BB agonist antibody to enter human clinical trials in 2005 (REF. 166). It is a fully human IgG4 with a point mutation (S228P) to stabilize the hinge region. Interestingly, urelumab does not compete with the 4-1BB ligand for binding, a feature that makes it distinct from utomilumab, which is a ligand blocker[68]. In 2008, two ongoing clinical studies assessing urelumab as monotherapy were terminated owing to the occurrence of hepatotoxicity, which was later deemed to be related to the administration of urelumab at doses of 1 mg/kg and above through mechanisms still unknown[70]. Subsequently, in 2012 a new monotherapy dose escalation study was initiated[167] to evaluate urelumab monotherapy at doses below 1 mg/kg (REF. 168). Some signs of liver toxicity have also been observed in preclinical studies of 4-1BB agonists in mice and suggest that the cause is related to increased CD8^{+} T cell accumulation and activation in the liver following treatment[169–171]. Urelumab has demonstrated some signals of disease stabilization as well as immune activation. A number of ongoing combination studies for urelumab take advantage of the NK cell-enhancing activity of the antibody by combining it with ADCC antibodies that target HER2, EGFR, SLAM family member 7 (SLAMF7) and CD20 (TABLE 2). Additionally, urelumab is also being tested in a number of studies in combination with the PD1-blocking antibody nivolumab in multiple cancer settings (TABLE 2). The second clinical stage 4-1BB agonist antibody, utomilumab, is a fully human IgG2 that was developed by Pfizer and to date has shown an excellent nonclinical safety profile in monkeys and mice[68], as well as having shown no dose-limiting toxic effects in humans at doses up to 10 mg/kg. Signs of promising clinical activity have been observed in patients with advanced-stage solid tumours, in whom several tumour responses were observed with utomilumab monotherapy. Additionally,

a recent report of 26 patients who received utomilumab in combination with pembrolizumab (NCT02179918) (TABLE 2) showed that 26% of patients with different advanced-stage cancers had confirmed complete or partial responses[172]. The promising antitumour activity observed with this study supports further investigation of the 4-1BB agonist combinations with PD1 and PDL1-blocking antibodies.

CD28

There is a single CD28 agonist antibody in clinical development, TAB-08 (theralizumab), which was formally known as TGN-1412 (TABLE 1). However, much can be learned about the immune-activating potential and perils of immune co-stimulatory agonist antibody development from this one agent. In 2006, six healthy volunteers that had received a single dose (0.1 mg/kg infused at a rate of 2 mg/min) of TAB-08 experienced CRS[15]. All patients became critically ill with immune-related multi-organ failure. It was subsequently determined that effector memory T cells in the tissues of patients were the source of cytokines responsible for CRS following TGN1412 treatment[173,174]. Further mechanistic studies in human T cell cultures determined that the severe CRS observed in this study was a result of the high dose of TAB-08 that was tested[175]. With careful consideration, human testing of TAB-08 was re-initiated in 2011 (REF. 176) starting at doses 1,000-fold lower than that tested in 2006. TAB-08 was well tolerated in all 30 healthy volunteers treated at the reduced dose levels[177]. Subsequent investigation of TAB-08 in patients with cancer started in 2016 (TABLE 1) and must determine whether a therapeutic window can be established that could allow for induction of antitumour immune activation in the absence of severe systemic CRS.

ICOS

Agonist antibodies targeting ICOS are the newest entrants into clinical development, and as such, they have the least amount of clinical data. The rationale for targeting ICOS stems from biomarker data obtained as part of the ipilimumab (anti-CTLA4) clinical development programme, in which upregulation of ICOS on T cells correlated with increased probability of response or prolonged survival[178–180]. This mechanistic link makes CTLA4-blocking antibodies strong candidates for combination with an ICOS agonist, and preclinical evidence supports this combination[180–182]. There are also signalling similarities between ICOS, CD28 and CTLA4 and, to a lesser extent, PD1 (REF. 183). Recombinant murine ICOS ligand has shown antitumour activity in mouse models[181,184,185]. The two ICOS agonist antibodies in clinical development, GSK-3359609 and JTX-2011, both started clinical trials in 2016 (TABLE 1) and are currently being tested both as monotherapies as well as in combination with PD1-blocking antibodies (TABLE 2). GSK-3359609 is a humanized IgG4 antibody, whereas JTX-2011 is a humanized IgG1 antibody. The difference in isotype between the two agents may result in a unique mechanistic profile for each of the respective antibodies. The engineered IgG4 format of

GSK-3359609 is designed to act as a true agonist to stimulate T cells without the cell-depleting function. The ADCC effector-enabled IgG1 isotype of JTX-2011 may induce the FcγR-mediated depletion of ICOS⁺ T cells. Many tumour-infiltrating T_{reg} cells are ICOS⁺, which suggests that depletion of these cells will have a beneficial therapeutic effect in some clinical settings[185–187]. However, expression of ICOS is also high on CD4⁺ helper and cytotoxic CD8⁺ T cells in some tumours[178–180]. Therefore, careful consideration must be paid to whether an effector-enabled ICOS agonist mAb may also result in FcγR-mediated ADCC depletion of ICOS⁺ effector T cell populations. Clinical biomarker data from treated tumours that demonstrate the immunomodulatory impact of each of these mechanistic approaches will be necessary to understand the full therapeutic potential of ICOS agonist antibodies and will aid in better selecting patient populations that are most likely to respond to each approach as well as in designing rational drug combinations.

Novel targets and alternative formats

There are a number of additional agonist antibodies and proteins in preclinical development against several co-stimulatory receptor targets including DR3, HVEM, transmembrane and immunoglobulin domain-containing protein 2 (TMIGD2) (FIG. 1) and NKG2D. Limited characterization of these agents has been reported and, in many cases, additional studies supporting the co-stimulatory function of these receptors are required. Additionally, therapeutic agents with alternative formats have been developed against co-stimulatory receptors, including bispecific antibodies and recombinant ligands.

Bispecific antibodies and TNFR agonists

Co-stimulatory agonistic antibodies are being extensively evaluated in early clinical development in combination with checkpoint blockade and standard-of-care therapies in both haematological malignancies and solid tumours (TABLE 2). A potential limitation in the clinical application of TNFR co-stimulation is the induction of systemic side effects associated with autoimmunity. To address these concerns, early investments are being made in the use of antibodies against tumour-associated antigens (TAAs) to direct TNFR agonists to the tumour microenvironment. AbbVie has initiated a phase I clinical trial of a tumour-directed anti-CD40 bispecific antibody (ABBV-428)[188]. Agonist activation of CD40 receptor can activate and mature APCs, leading to the efficient priming, activation, proliferation and memory development of antigen-specific CD8⁺ T lymphocytes[189,190]. Moreover, agonist activation of CD40 receptor with the use of stabilized CD40L trimers has emerged as a vaccine adjuvant strategy[191]; however, systemic activation of the immune system manifests dose-limiting side effects that are being addressed through targeted delivery strategies. Targeted delivery of the ABBV-428 bispecific antibody construct to the tumour is designed to increase therapeutic efficacy while minimizing the toxic effects associated with systemic activation of CD40

receptor through tumour-localized APC activation and T cell priming. Pieris Pharmaceuticals is developing a portfolio of bispecific antibodies that induce 4-1BB clustering and activation with the use of TAA-specific antibodies. For example, the bispecific antibody (PRS-342), which targets the oncofetal protein glypican 3 (GPC3) and 4-1BB, is in preclinical development for potential application in cancer indications in which 4-1BB⁺ tumour-infiltrating lymphocytes have been identified, such as hepatocellular carcinoma, Merkel cell carcinoma and melanoma. In a related strategy, Pieris produced a HER2-directed bispecific antibody targeting 4-1BB with an agonistic 4-1BB-specific anticalin protein (PRS-343), which is in phase I clinical trials. Antibody-mediated cell surface immobilization or oligomerization of stabilized TNFR ligand trimers (CD27 ligand (CD27L; also known as CD70), CD40L, 4-1BBL and GITRL) can activate localized cognate receptor signalling with high efficiency[81]. In the case of 4-1BBL, forced hexamerization with the use of an Ig Fc domain or cell surface immobilization using a fusion protein containing a single-chain variable region fragment (scFv) and 4-1BBL (scFv–4-1BBL) significantly increases receptor activation[81]. Consistent with these observations, preferential binding of the Pieris bispecific antibodies to the TAAs, GPC3 or HER2 promotes clustering (crosslinking) of 4-1BB and subsequent activation of tumour-specific 4-1BB⁺ T cells in the tumour microenvironment.

Likewise, preclinical studies are examining the use of anti-TAA antibodies to deliver stabilized TNF ligand trimers to the tumour. LIGHT (also known as TNFSF14), is a potent regulator of antitumour immunity through the engagement of two cellular receptors, LTβR and HVEM. The intratumoural introduction of LIGHT promotes chemokine production, the expression of adhesion molecules and the development of tertiary lymphoid structures that promote the infiltration and activation of naive lymphocytes; however, the systemic administration of LIGHT has limited therapeutic efficacy[192,193]. By constructing a fusion protein combining anti-EGFR and a human mutant form of LIGHT that can also bind to the mouse receptor (hmLIGHT), Tang and colleagues were successful in delivering a LIGHT trimer to EGFR-expressing tumours, resulting in profound antitumour efficacy and the reversal of PDL1 resistance[194]. Treatment with the anti-EGFR–hmLIGHT bispecific construct significantly elevated production of chemokines and cytokines from effector T helper 1 (T_H1) cells (such as interferon-γ (IFNγ), TNF and IL-12) in a LTβR-dependent manner[194]. These studies establish the preclinical proof of concept that tumour-directed TNFR agonists have the potential to yield robust antitumour immunity.

Hexavalent TNFR agonists

In an effort to improve current strategies to activate TNFR with agonists, a number of groups are building stabilized trimers of TNFR ligands in hexavalent configurations to maximize the efficiency of receptor engagement, clustering and activation on target cells (FIG. 5). Murine GITRL fusion protein (mGITRL-FP) agonists

promote antitumour immunity and tumour growth inhibition in syngeneic mouse models by inducing the expansion of antigen-specific CD8+ T cells and durable CD8+ T cell memory development with concomitant reductions in the intratumoural T_{reg} cell content[195]. Combination of mGITRL-FP with a murine OX40L fusion protein (mOX40L-FP) or checkpoint blockade (PD1, PDL1 or CTLA4) increased antitumour immunity over single agent therapy in the CT26 colon carcinoma model[196]. These findings provide preclinical support for the further exploration of GITRL combination strategies with OX40 agonists and PD1 and PDL1 antagonists. To extend this strategy into the clinic, MedImmune has developed a hexameric human GITR agonist, MEDI1873, comprising an IgG1 Fc domain and stabilized trimers of the human GITRL extracellular domain[197]. In non-human primates, MEDI1873 dose-dependently improved antigen-specific humoral and T cell proliferative responses[197]. These studies support a role for the use of a hexameric GITRL agonist in promoting antitumour immunity in early clinical development. MedImmune initiated a phase I clinical trial to evaluate the safety and efficacy of MEDI1873 in patients with advanced-stage solid tumours[198]. Similarly, OncoMed announced the initiation of a phase I clinical trial of its trimeric GITRL–Fc fusion protein (OMP-336B11) in September 2017 (REF. [199]).

Investing in a broader commitment to the use of TNFR ligands as receptor agonists, Apogenix has developed a hexavalent TNF superfamily receptor agonists (HERA) platform targeting CD40, CD27, HVEM, OX40, GITR, 4-1BB and TNF-related apoptosis-inducing ligand (TRAIL; also known as TNFSF10). The HERA agonists are formed by a single polypeptide chain comprising three repetitive TNFR binding domains (scT-NF-RBD) connected by peptide linkers that trimerize when folded as a protein. Fusing a human IgG1 Fc domain to the carboxy terminus of each scTNF-RBD creates a homodimeric molecule with hexavalent receptor engagement. The hexavalent architecture of the HERA platform is designed to promote TNFR clustering and downstream signalling independent of Fc-mediated crosslinking. Apogenix is collaborating with AbbVie to develop TRAIL receptor 1 (TRAILR1) and/or TRAILR2 agonists on the HERA platform — such as APG880 (ABBV-621) — which are designed to induce immunogenic tumour cell death. Additionally, Apogenix reports that its HERA-GITRL is stable in vivo in CD-1 mice and promotes lymphocyte activation, proliferation and production of effector cytokines (TNF and IFNγ) in functional assays using human CD4+ lymphocytes from healthy donors. This preclinical pipeline of molecules represents a novel and still developing technology for potent TNFR agonists.

Conclusions

Agonist antibodies targeting immune co-stimulatory receptors have been tested clinically in cancer now for well over 10 years. Yet none have been approved to date, nor have any begun phase III randomized trials. This speaks to the complexity of agonist antibody design and
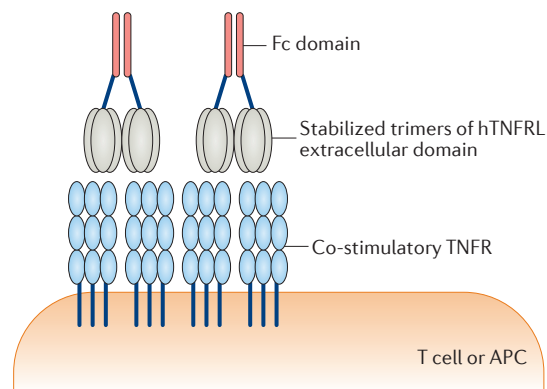

Figure 5 | **Hexavalent TNFR agonist.** An emerging alternative to antibody-mediated receptor activation is the development of crystallizable fragment (Fc) fusion constructs comprising stabilized trimers of tumour necrosis factor receptor (TNFR) ligands (TNFRL). The fusion of a human Fc domain to the carboxy terminus of each single-chain TNFR binding domain (scTNFR-BD) produces a homodimeric molecule with hexavalent receptor engagement. The hexavalent architecture is designed to promote efficient TNFR clustering and activation on target cells independent of Fc-mediated crosslinking. Hexavalent TNFR agonists are in preclinical discovery and phase I clinical trials. APC, antigen-presenting cell.

development. The challenges of discovering and characterizing agonist antibodies inherent in the clinical trial design needed to develop these agents make them unique compared with the methods used for the development of other antibody therapies in cancer. There are no sets of biophysical properties or profiles that can reproducibly predict that an antibody will act as an agonist. Only through full functional characterization can one be confident that an antibody demonstrates agonist properties, and even then, secondary mechanisms, such as ADCC, can complicate the interpretation of their effects.

Knowledge of what antibody properties make an effective agonist has expanded greatly in the past few years. In particular, the realization that the IgG isotype and Fc domain properties are as important as the Fab domain for making an effective immune agonist antibody has aided greatly in the design of better agonists. Additionally, preclinical experiments have demonstrated the promise of immune agonist antibodies in the treatment of cancer, particularly when used in combination with other immune-activating approaches. As a result, there has been a recent explosion of activity in this space, with the majority of the 25 different antibodies that are currently in development having entered clinical trials within the past 3 years. Many of the most promising co-stimulatory receptor targets now have six or seven competing antibody programmes currently undergoing clinical evaluation. The race is on to identify the most effective dose, regimen and combination approach for each of these agents. Traditional understandings, gained largely through the development of antibody antagonists, about the optimal target saturation, dose frequency

and duration required for maximal therapeutic effect, likely do not apply to most antibodies in the immune agonist class. Therefore, new clinical designs must be instituted, new rules must be established and alternative approaches must be evaluated to determine how best to use these therapies. It will be of particular importance to immune profile T cell subsets and receptor expression in patients considered for agonist therapy to understand the effects of these agents in different subpopulations and guide therapy accordingly. It remains possible that some agonist therapies can be highly active in patients with one immune profile but have no activity in patients with another profile even if both share the same cancer diagnosis.

It is clear that immune co-stimulatory receptors are an essential component of antitumour immune cell function. Effectively activating these receptors may have important therapeutic benefits and contribute to the treatment repertoire in immuno-oncology. The key to success will likely be in determining in what treatment settings and patient populations and in combination with which agents will agonist antibodies targeting these receptors provide the most therapeutic benefit. In this regard, some of the next-generation approaches covered here that have been designed to activate immune co-stimulatory receptors through carefully engineered monoclonal antibodies, recombinant ligands and bispecific antibodies may help unlock the full therapeutic potential of this class of targets.

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