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TNFRSF agonists: Mode of action and therapeutic opportunities

Edited by
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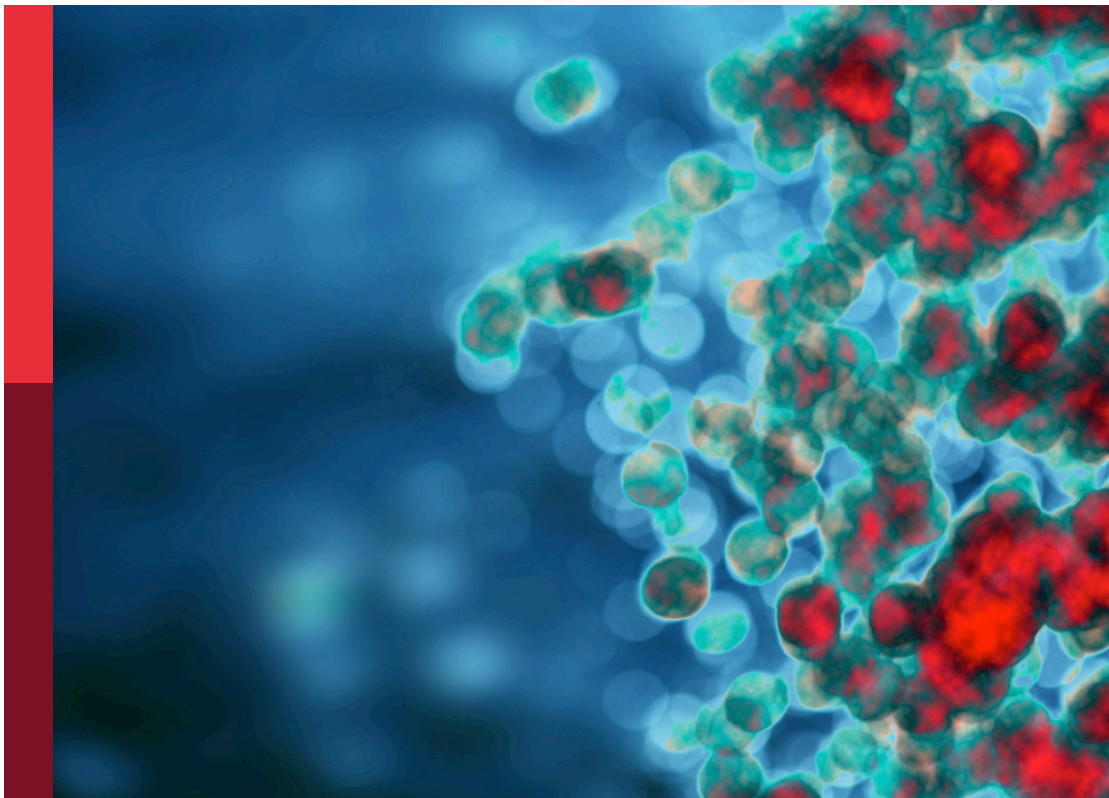

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TNFRSF agonists: Mode of action and therapeutic opportunities

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Editorial: TNFRSF agonists: mode of action and therapeutic opportunities

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Editorial on the Research Topic
TNFRSF agonists: mode of action and therapeutic opportunities

The receptors of the tumor necrosis factor (TNF) receptor superfamily (TNFRSF) fulfill crucial and manifold immunomodulatory functions and are involved in maintenance of tissue homeostasis and development. TNFRSF receptors (TNFRs) exhibit specific, though partially overlapping expression profiles, and elicit functional effects on the vast majority of immune cells at different stages of an immune response. TNFRs also crucially contribute to the communication between immune cells and immune cells with other cell types. The repertoire of immunomodulatory functions of TNFRs spans both adaptive as well as innate immunity. For example, TNFRs co-stimulate T-cells, engage antigen presenting cells (APCs) and regulate B-cell maturation. On the other hand, they contribute to tumor surveillance and are also involved in tissue regeneration and development.

In line with the diverse functions of TNFRs, inhibition as well as activation of these receptors (or their ligands) has considerable potential for the treatment of a variety of immune pathologies, cancers, and infectious diseases. Agents targeting TNFR1 and TNFR2, RANK and BAFF-R pathways have long been approved for the therapy of autoimmune diseases (TNF blockers) (1), osteoporosis and giant cell tumors of bone and bone metastases of solid tumors (anti-RANKL antibody) (2) and systemic lupus erythematosus (anti-BAFF, TACI-Fc) (3, 4). For more than the past two decades there have also been tremendous preclinical and clinical efforts to bring TNFR agonists into the clinic, especially as cancer therapeutics. However, to date these efforts have resulted in only a single niche approval of recombinant TNF for the treatment of soft tissue sarcomas in isolate limb perfusion (5). The lack of translational success of TNFR agonists can be largely attributed to the challenging design of potent agonists due to the special mode of TNFR activation, and dose limiting toxicities arising from systemic TNFR activation. The later can be further exacerbated by FcγR binding in the case of TNFR-specific agonistic antibodies. Therapeutic targeting of TNFRs with agonists remains in a nascent stage. However, the improved molecular understanding of TNFR activation achieved in recent years prompted new concepts guiding the design of TNFR agonists with potent and/or

conditional agonism. Ongoing and forthcoming clinical trials will be essential to validate the efficacy and safety of these novel agonists.

This Research Topic, compiling 7 review/perspective articles and 4 original studies, provides an overview of the molecular mode of action, as well as the clinical development and possible applications of TNFR agonists. The reviews deliver a comprehensive summary of our current understanding of the mechanisms of TNFR activation, and further outline how these learnings have shaped and changed the development of TNFR agonists in recent years, spotlighting 4-1BB agonists as an example. The original articles build on this premise and, with additional examples of several therapeutically relevant TNFRs, illustrate how current knowledge of TNFR agonism can guide the rational design of novel TNFR-activating therapeutics.

Vanamee and Faustman review the current state of our understanding of TNFR signaling mechanisms and present a uniform model of TNFR activation that can accommodate all members of the TNFRSF. The model highlights the importance of pre-formed hexagonal honeycomb-like TNFR clusters in instructing the recruitment of a likewise hexagonal assembled honeycomb lattice of downstream components to trigger the intracellular signaling pathways culminating in the induction of cell death or activation of the NFκB pathway upon engagement of the death domain-containing and TRAF-interacting receptors of the TNFRSF, respectively. Based on this model, Faustman and Vanamee furthermore discuss the possible underlying mechanism of action of antagonistic and agonistic anti-TNFR antibodies, with a goal to aid the development of better therapeutics.

Fromm et al. continue on the realm of oligomerization being the decisive prerequisite for TNFR activation and further discuss the ability of different types of synthetic agonists to facilitate higher-order clustering of TNFRs. The focus of their review is to synthesize how the available human clinical data reflect the underlying mechanisms of synthetic agonist compounds that have been evaluated in the clinic. Fromm et al. present examples of frequently observed 'bell-shaped' dose response effects in patients and highlight the variables to consider in selecting an optimal dose for TNFR agonists and common themes across different TNFR agonist modalities that should be considered in advancing future agents to the clinic.

In their review, Dadas et al. advance the discussion by describing the biomedical rationale, efficacy, and limitations of the currently available agents delivering co-stimulatory TNFR agonism for cancer immunotherapy. They propose considerations for the development of next generation immunostimulatory agents to overcome challenges in translating pre-clinical successes into the clinic. Dadas et al. provide an overview of the co-stimulatory TNFR targeting agents in clinical trials and list examples of preclinical and clinical studies of targeting T cell co-stimulatory TNFRs 4-1BB, CD27, GITR, OX40 and TNFR2 and the APC stimulatory CD40 receptor. Building on a summary of the recent approaches in targeting these TNFRs, they supplement with a discussion of potential modifications to achieve curative clinical immune responses while avoiding toxicity.

Apart from addressing common features of TNFR activation and the development of TNFR agonists in a rather general way, the next three reviews by Salek-Ardakani et al., Liu and Luo, and Chen et al. focus specifically on 4-1BB and TNFR2 to comprehensively cover therapeutic targeting of these two receptors.

Salek-Ardakani et al. begin by describing the structure of 4-1BB and the mechanisms of action of 4-1BBL and antibody-based agonists, including structural superposition of several agonist modalities targeting 4-1BB. The authors then summarize some of the major clinical efforts agonizing 4-1BB to date in immuno-oncology. In particularly, they outline the knowledge gained from the early studies with the two prominent 4-1BB therapeutics urelumab and utomilumab, provide a perspective on strategies that are being attempted to generate greater specificity in targeting and biological activity, and highlight opportunities in other clinical arenas such as viral vaccines and autoimmunity that have yet to be pursued.

In a related review on 4-1BB, Liu and Luo present 4-1BB biology in the context of anti-4-1BB agonist drug discovery. Comparing anti-4-1BB antibodies urelumab, utomilumab, and ADG106, Liu and Luo discuss in detail the relevance of the binding epitope and ligand-blocking properties in inducing 4-1BB clustering and signaling activation, the role of FcγR binding and antibody isotype for agonistic activity and regulatory T cell depletion, and the preferential reduced affinity and higher dissociation rate for agonism. They delve into strategies for conditional activation of 4-1BB to improve the therapeutic index by localizing the agonistic activity, and further describe the vast potential of combinatorial approaches either as multi-specific antibodies, in combination with cancer vaccines or T cell engaging antibodies.

Chen et al. provide a perspective on the therapeutic potential of TNFR2 agonists and their use to stimulate immunosuppressive regulatory T cells (T_{reg} cells) and myeloid derived immunosuppressive cells (MDSCs) for the treatment of autoimmune diseases, and also their potential use as co-stimulators of cytotoxic T lymphocytes (CTLs) and activators of NK cells in tumor therapy. An overview of TNFR2 agonists is intervened with most relevant factors that may determine their therapeutic outcome. The intricate bi-phasic effects of TNF-TNFR2 signaling, dual role of TNFR2 on T_{reg} cells and effector CD8 and NK cells and tissue-specificity of responses are discussed to explain the complicated nature of TNFR2 agonist responses.

In their article, Vredevoogd and Peeper indirectly address the development of TNFR agonists, by providing examples of proof-of-concept analyses of mechanisms of TNF resistance. By exploiting heterogeneity of DepMap dependency database, Vredevoogd and Peeper present an analytical approach for discovery of novel immune sensitivity modifiers. Comparing gene perturbation effects between cell lines that are positive vs negative for expression of TNF and TRAIL signaling intermediates, respectively, they validate previously described data and identify novel immune sensitivity modifiers. They further probe the fidelity of such an approach by comparing the drug sensitivity profile of these specific tumor cell lines in the DepMap.

The series of the above 7 review/perspective articles included in this Research Topic, is accompanied by 4 original research manuscripts, complementing the discussion on critical aspects of

TNFRSF structure-function and receptor clustering with pre-clinical research data. The research papers focus on the requirement and role of FcγR binding in anti-tumor immunity and provide possible alternatives to achieve desired TNFRs agonism.

Melo et al. created a novel immune co-stimulatory CD27xEGFR IgG1 bispecific antibody lacking effector function and present its *in vitro* characterization in their research article. They describe selective and simultaneous binding of this tetravalent CD27xEGFR bsAb to both targets (CD27 and EGFR), T cell co-stimulation in co-cultures with a range of $EGFR^+$ cell lines, and anti-cancer activity - both by co-stimulation of T cells at the sites of EGFR expression as well as by directly blocking EGFR on cancer cells. Melo et al. argue the unique features of CD27xEGFR and offer a compelling rationale for its further exploration in preclinical and clinical settings as a promising immunotherapeutic agent for $EGFR^+$ tumors.

Dadas et al. performed pre-clinical side-by-side comparisons of soluble variants of CD70 (either trimeric (t) or dimer-of-trimers (dt)) to an agonist anti-CD27 antibody. Whereas tCD70 failed to co-stimulate $CD8^+$ T cells, both dtCD70-Fc and an agonist anti-CD27 antibody could enhance T cell proliferation *in vitro*. When evaluating the dependence on FcγR binding, the activity of anti-CD27 antibody and dtCD70-Fc in FcγR-deficient mice remained active. Nevertheless, although a substantial part of the stimulatory activity of dtCD70-Fc was retained in the absence of FcγR interaction, FcγR binding of dtCD70-Fc was required for maximal induction of a $CD8^+$ T cell response *in vivo*. Their data reveal that TNFSF ligands can be generated with a tunable activity profile and suggest that this class of immune agonists could have broad applications in immunotherapy.

In their comprehensive *in vitro* assessment of the individual contribution of different human FcγR classes on the agonistic activity of antibodies targeting 4-1BB (urelumab and utomilumab) and CD27 (varlilumab), Leitner et al. used a T cell reporter system to show that urelumab could induce 4-1BB signaling without FcγR cross-linking, but also that the presence of the FcγRs CD32a/b and CD64 augmented intrinsic agonistic activity of this antibody. However, utomilumab and varlilumab exerted agonistic function only when crosslinked (utomilumab via CD32A/B and varlilumab via any FcγR). In addition, they analyzed the capacity of these TNFR agonistic antibodies to augment PBMC activation. While the 4-1BB agonists induced T cell activation comparably well as a CD3 antibody alone, the capability of the CD27 agonist varlilumab to augment T cell responses in primary human PBMCs was counteracted by its FcγR-mediated cytotoxic effects. The data by Leitner et al. highlight the importance to account for the FcγR-mediated effects, such as ADCC and AICD, which critically impact the activity of antibody-based co-stimulatory TNFR agonists.

Zaitseva et al. generated various oligovalent variants of the fibroblast growth factor (FGF)-inducible 14 (Fn14)-specific antibody 18D1 and compared their agonism with that of soluble and membrane TWEAK (sTWEAK and memTWEAK), the natural ligands of Fn14 engaging different patterns of Fn14-associated signaling pathways. In their research article, Zaitseva et al. present that the number and type of the Fn14 binding domains within an oligovalent 18D1 construct determine whether sTWEAK- or memTWEAK-like activity is mimicked, and hypothesize that qualitatively different TNFR agonism with preference for specific TNFR-associated signaling pathways can be achieved by modifying the antibody design. Further, using one of their intrinsically agonistic 18D1 variants, Zaitseva et al. provide evidence that Fn14 activation *per se* can elicit anti-tumoral response and argue that apart from blocking Fn14 pro-tumoral activity or targeting it as a tumor associated antigen (6), inducing Fn14 agonism may serve as an alternative targeting approach for tumor therapy.

In conclusion, this Research Topic provides comprehensive overview of the TNFRs, focusing on the mechanisms of their activation, and their potential as therapeutic targets. Combining the data from pre-clinial research with the findings from clinical studies, the articles address the imminent challenges of TNFR activation related to clustering-enabled signal transduction and necessity for conditional agonism to avoid systemic activation.

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NO: Conceptualization, Writing – original draft, Writing – review & editing. AZ: Writing – review & editing. HW: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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Delivering co-stimulatory tumor necrosis factor receptor agonism for cancer immunotherapy: past, current and future perspectives

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The tumor necrosis factor superfamily (TNFSF) and their receptors (TNFRSF) are important regulators of the immune system, mediating proliferation, survival, differentiation, and function of immune cells. As a result, their targeting for immunotherapy is attractive, although to date, under-exploited. In this review we discuss the importance of co-stimulatory members of the TNFRSF in optimal immune response generation, the rationale behind targeting these receptors for immunotherapy, the success of targeting them in pre-clinical studies and the challenges in translating this success into the clinic. The efficacy and limitations of the currently available agents are discussed alongside the development of next generation immunostimulatory agents designed to overcome current issues, and capitalize on this receptor class to deliver potent, durable and safe drugs for patients.

KEYWORDS

TNFR, agonism, co-stimulation, cancer, immunotherapy

Introduction

Members of the tumor necrosis factor superfamily (TNFSF) and their receptors (TNFRSF) are important regulators of the immune system. Interaction between these ligands and receptors can mediate proliferation, survival, differentiation, and function of immune cells (1, 2). There are 19 TNFSF ligands and 29 TNFRSF receptors, representing a large and diverse family.

The TNFSF ligands are type II proteins which are characterized by the presence of a C-terminal TNF homology domain (THD) responsible for ligand trimerization and receptor binding (3). In comparison, the TNFRSF receptors have between one to six cysteine rich domains (CRD) in their extracellular region (Figure 1) that are involved in ligand binding and receptor auto-association (5).

TNFRs can be sub-divided into three groups according to functional and structural differences; death domain (DD) containing receptors, decoy receptors and TNF receptor associated factor (TRAF) binding receptors (Figure 1). The DD is an 80 amino acid domain present in the cytoplasmic tail of the DD containing receptors. Although the DD containing receptors mainly initiate cell death signaling, they can also mediate other outcomes, such as NF-κB signaling (1, 5). The decoy receptors lack signal initiation capacity and consist of glycosylphosphatidylinositol (GPI) tethered receptors, soluble receptors and receptors possessing a non-functioning DD (5). Finally, TRAF binding receptors possess TRAF-interacting motifs (TIF) in their cytoplasmic tail that is responsible for recruiting TRAFs to mediate downstream signaling upon receptor activation.

Following expression on the cell surface, several members of the TNFRSF can self-associate into dimers or multimers prior to ligand binding. Although some members can be found as covalently linked dimers (e.g. CD27 (6)), self-association for others is mainly driven by the pre-ligand assembly domain (PLAD), largely covering the N-terminal CRD1 (7, 8), and glucocorticoid-induced TNFR related protein (GITR) is an exception as dimerization of this TNFR is driven by interactions within CRD3 (9). Formation of receptor dimers or trimers for several members of the TNFRSF before ligand binding has been shown to be crucial for their interaction with ligand. Deletion of the PLAD domain in TNFR1 and TNFR2, significantly reduced TNFα binding to both receptors (7, 10). Although the ligand binding domain is located in CRD2/3, the reduced binding suggested that ligand-independent multimerization, driven by the PLAD domain, is important for ligand binding.

The TNFSF ligands can be found in soluble or membrane bound forms. Although one group of TNFRSF members (category I) can be activated by soluble ligand trimers, others (category II) require interaction with the membrane bound ligand to be fully activated (5). For example, soluble TNFα binds with higher affinity to TNFR1 than TNFR2 and primarily activates TNFR1 signaling, whereas TNFR2 is mainly activated by membrane bound ligand (11, 12). Although CD40 and GITR are both activated by trimeric ligands, activation is further enhanced with higher valency ligands or cross-linking of the trimeric molecules, presumably through induction of higher-order clustering (9, 13, 14). In contrast, CD27 and 4-1BB show minimal activation and require higher-order clustering (13). As described above activation of TNFRSF members can lead to multiple cellular outputs including proliferation, survival and differentiation, several of which may be therapeutically beneficial.

Rationale behind targeting TNFRSF

In addition to T-cell receptor (TCR) interaction with peptide-MHC (major histocompatibility complex), T cells require co-stimulatory signaling to be fully activated and generate an optimal response (15). Co-stimulatory TNFRSF members

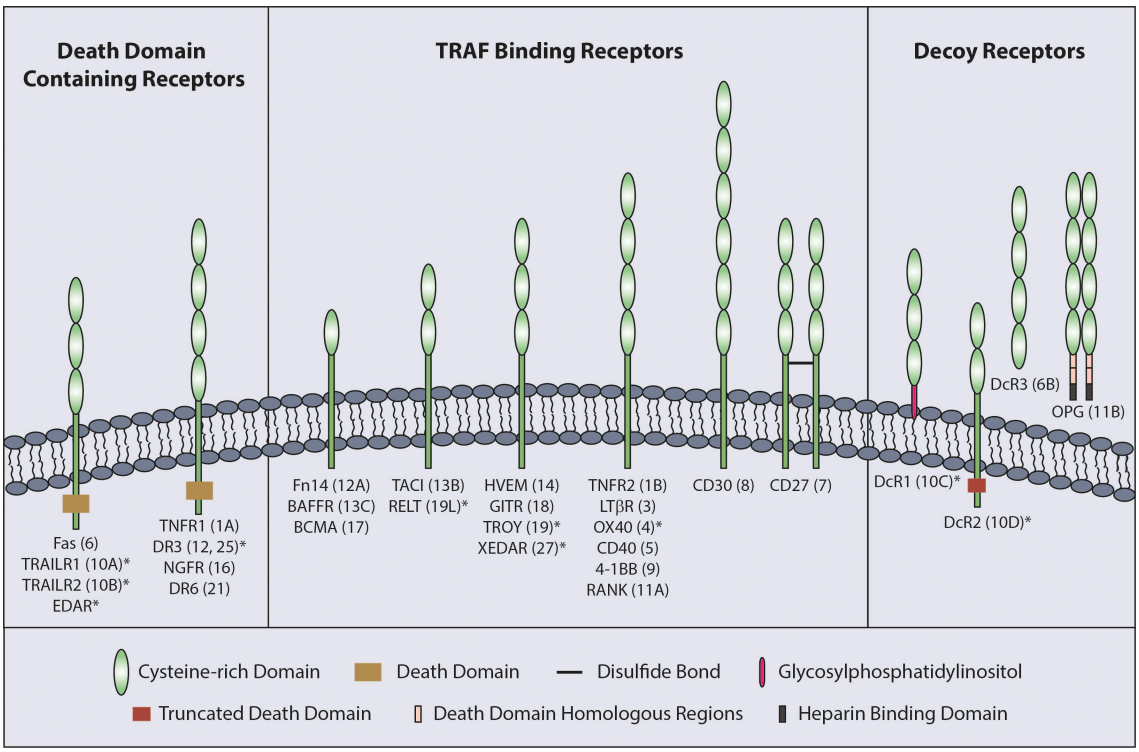

FIGURE 1
Classification of TNFRSF. The TNFRSF can be classified into three sub-families. All twenty-nine members of the family, grouped into three sub-families, are indicated with the number of CRDs on their extracellular region and TNFRSF number in brackets. CRD domains are defined by Uniprot with the exception of RELT which was published as having two CRDs (4). * indicates the receptors with a truncated CRD domain.

expressed on T cells include CD27, OX40, 4-1BB, TNFR2 and GITR. Co-stimulatory receptors on antigen presenting cells (APCs) are also important, with molecules such as CD40 playing a critical role in licensing and activation of dendritic cells (DCs) and B lymphocytes during an immune response (16), to elicit appropriate humoral and cellular adaptive immunity. DCs can be excluded from the tumor microenvironment and multiple immunosuppressive mechanisms can suppress their maturation and full activation, preventing effective T-cell responses (17–19). The DCs up-regulate multiple TNFSF ligands after maturation which are required for the optimal co-stimulation of T cells. Thus, targeting the TNFRSF members to provide co-stimulation is an attractive approach to elicit effective T-cell responses.

The majority of the T-cell co-stimulatory receptors are only upregulated and appreciably expressed after TCR activation, e.g. 4-1BB expression on adoptively transferred T cells is detected 12 to 24 hrs after stimulation (20) whereas others, most notably CD27, are constitutively expressed on T cells (2). Once expressed, the various TNFR are available for engagement by their ligands, which themselves also possess specific kinetics of expression (21). Although the downstream signaling pathways of the co-stimulatory TNFRSF members are not identical, signals are mainly initiated after TRAF recruitment to their cytoplasmic tails which leads to NF-κB and JNK pathway activation (22).

Stimulation of these co-stimulatory receptors contributes to enhanced effector function but also survival of the T cells. For instance, CD27 stimulation through engagement of its ligand CD70 leads to expression of cytokines such as IFN-γ, Interleukin-12 (IL-12), IL-5, IL-4 and IL-2 (2, 23), alongside the complementary cytokine receptors including IL-12R and IL-2R. Similar to CD27, stimulation of OX40 leads to upregulation of cytokines and cytokine receptors such as IL-12R and IL-2R on T cells, supporting their activation (24, 25). GITR stimulation also promotes the expression of IFN-γ, IL-2 and IL-2R (26) and is required for optimal $CD8^+$ effector T-cell generation as absence of GITR on $CD8^+$ T cells significantly reduces their expansion following an influenza infection (27). CD27 engagement can alter cellular metabolism to support the rapid expansion of T cells after activation. Here, the expression of the serine threonine kinase Pim-1 is upregulated to facilitate increased aerobic glycolysis and protein translation during proliferation (28–30).

TNFR signaling also supports survival of activated T cells. CD27 increases expression of the anti-apoptotic protein Bcl-XL in T cells, reduces the level of FasL on $CD4^+$ T cells and reduces $CD8^+$ T-cell sensitivity to FasL-stimulated apoptosis (29, 31). Similarly, anti-apoptotic proteins such as Bcl-XL and Bcl-2 are upregulated following OX40 stimulation (32), Bcl-XL and Bfl-1 are upregulated by 4-1BB (33) and Bcl-XL is upregulated after GITR engagement (27).

CD27 signaling induces $CD8^+$ T-cell differentiation into cytotoxic T lymphocytes (CTL) and $CD4^+$ T-cell differentiation into Th1 cells (28). Increased cytotoxic capacity of CTLs is supported by mechanisms such as upregulated of IL-2, important for their survival, and IFN-γ, which is further upregulated by IL-2 signaling. Increased cytotoxic capacity and effector functions of $CD8^+$ T cells has also been shown after 4-1BB stimulation (34). Similar activities are evident on APCs, where CD40 signaling is critical for their ability to induce effective $CD8^+$ T-cell responses. Stimulation of CD40 on DCs is important for their maturation and ability to present antigens to T cells. Activation of CD40 also leads to production of pro-inflammatory cytokines such as IL-12, IL-6 and IL-1β (35). Moreover, CD40 stimulates expression of co-stimulatory ligands such as CD80 and CD86, that interact with the receptors on T cells (e.g. CD28) for further activation.

In addition to the effects during naïve T-cell priming, co-stimulatory receptors of the TNFRSF contribute to the generation of the memory T-cell pool. CD27 signaling during the initial activation phase of $CD8^+$ T cells is required for the development of memory $CD8^+$ T-cell subsets and efficient expansion during the secondary response. Stimulation of CD27 during the initial response leads to IL-7Rα expression on effector $CD8^+$ T cells, which in turn increases the frequency of memory precursor cells (36, 37). Similarly, 4-1BB and OX40 signaling are required for the generation of robust memory T-cell pools (38, 39). Stimulation of antigen specific $CD8^+$ T cells with a 4-1BB agonist during priming leads to the generation of a strong memory $CD8^+$ T-cell pool, resulting in a high secondary response (40). OX40 signaling is also important for T-cell memory. Although the primary expansion of $CD8^+$ T cells was not impaired in OX40L-/- mice following influenza infection, there were defects in the secondary response of the virus specific $CD8^+$ T cells (41). GITR has also been shown to be important for the secondary expansion of memory $CD8^+$ T cells as *in vitro* generated WT or GITR-/- memory cells showed significantly different expansion capacity in an influenza infection recall response (27).

Additionally, the crucial role of co-stimulatory members of the TNFRSF in generating immune surveillance is evidenced by the development of various pathologies in individuals with TNFR deficiencies/mutations. For example, deficiency of CD27 or CD70 can lead to development of Epstein-Barr virus (EBV)-related immunodeficiency and lymphoproliferative disorders including B-cell malignancies (42, 43). Characterization of the immune response of an individual with CD27 deficiency who had hypogammaglobulinemia and persistent symptomatic EBV viremia revealed impaired IL-2 production in their $CD8^+$ T cells which are the primary immune cells responsible for clearing EBV infections. IL-2 is critical for $CD8^+$ T-cell function and impaired IL-2 production contributes to defective immune responses (42, 44, 45). 4-1BB deficiency can also lead to EBV driven complications and individuals can have persistent EBV viremia and EBV-related lymphoproliferation. $CD8^+$ T cells from 4-1BB deficient individuals showed reduced proliferative and cytotoxic capacity (46). Deficiency in functional OX40 can lead to Kaposi sarcoma development in individuals with human herpes virus 8 infection (47). Similarly, CD40 or CD40 ligand deficiency can lead to immunodeficiency due to impaired APC function, which subsequently leads to impaired T-cell responses (48, 49), alongside an absence of germinal center-mediated somatic hypermutation and class switching in the humoral response known as hyper-IgM syndrome (50, 51). Dysregulation of the TNFRSF co-stimulatory receptor signaling and associated diseases identified to date are illustrated in Table 1. Further, the importance of co-stimulatory TNFRSF members in functional immune response generation is also supported in multiple constitutive

TABLE 1 TNFRSF co-stimulatory receptor dysregulation and disease development.

Receptor	Defect	Associated disease	Reference
CD27	Absent or reduced receptor expression	EBV related immunodeficiency B-cell malignancies	(42, 52–54)
4-1BB	Absent receptor expression	EBV-related lymphoproliferation	(46)
OX40	Reduced receptor expression and defective ligand binding	Kaposi sarcoma after human herpes virus 8 infection	(47)
CD40	Defective receptor expression or defective ligand binding	Impaired T-cell responses Hyper-IgM syndrome	(48, 55, 56)
TNFR2	Gene polymorphisms (Effects on the receptor not yet characterized)	Autoimmune diseases Hepatitis B virus related liver disease	(57, 58) (59)
BAFFR	Loss of function mutation Gain of function mutation	Common variable immunodeficiency Non-Hodgkin lymphoma Autoimmunity	(60, 61) (62) (63)
TACI	Defective receptor expression, defective ligand binding or defective signaling	Common variable immunodeficiency IgA deficiency	(64–66)
HVEM	Absent or reduced receptor expression	B-cell malignancies	(67–69)
RELT	Loss of function mutations or mutations predicted to reduce protein stability	Amelogenesis Imperfecta	(70, 71)

These co-stimulatory receptors have been reported to contribute to a clinical condition as a consequence of defects in their normal expression, function or ligand binding.

and conditional TNFRSF knock out (-/-) models. For example, 4-1BBL deficiency in mice leads to impaired $CD8^+$ T-cell responses against viral infections and predisposes the mice to B-cell lymphoma development (72–74). Similarly, CD27-/- mice have defects in the generation and accumulation of effector T cells at the site of infection following influenza infection, with the memory T-cell pool impaired (75, 76).

As the importance of co-stimulatory TNFRSF members in the development of a functional immune response has become clear, many of these receptors have subsequently been targeted to modulate the immune response in the context of immunotherapy. In this review we have restricted ourselves to discussing findings mainly in the field of cancer immunotherapy. Moreover, as various definitions of agonism exist, here we have defined agonism as activating the target receptor either *via* Fc gamma receptor (FcγR) dependent or independent mechanisms.

Therapeutic targeting of the TNFRSF

Agonistic targeting of the co-stimulatory members of the TNFRSF has shown to be effective in pre-clinical tumor models. Targeting 4-1BB in tumor models representing liver cancer, floor of mouth squamous cell cancer, colorectal cancer and lymphoma, using monoclonal antibodies (mAb) or recombinant 4-1BBL has generated robust anti-tumor responses (77–80). Buchan et al. demonstrated that two different mechanisms can contribute to a robust anti-tumor response induced by anti-4-1BB antibodies in certain models and contexts; 1) stimulating the effector T cells and 2) depleting T regulatory (Treg) cells. Additionally, depleting Tregs first and then agonizing the effector T cells induced better responses than only depleting the Tregs or agonizing the effector T cells (79). Similar to 4-1BB, targeting OX40 or GITR has been shown to stimulate robust anti-tumor responses in several pre-clinical tumor models, through a similar mechanism of action i.e. agonizing effector T cells or depleting Tregs (81–83). Treatment of solid tumors in a pre-clinical study with an agonistic anti-GITR mAb, increased the infiltration and activity of effector $CD4^+$ and $CD8^+$ T cells (84). In another study with the same agonistic mAb targeting a different solid tumor model however, the effect was mainly through depletion of intra-tumoral Tregs and slight increase in the infiltration of CD8s which resulted in a significantly improved $CD8^+$ to Treg ratio (85). Additionally, the $CD8^+$ T cells exhibited a more activated phenotype. These results indicate that anti-GITR mAbs can also act through different mechanisms and the dominant mechanism of action can vary depending on the tumor model. Moreover, it has been shown for OX40 and GITR targeting that the differential level of expression on effector T cells vs Tregs can lead to preferential depletion of Tregs as a consequence of higher levels of receptor expressed on them, enhancing immunotherapy (86–88).

Targeting CD27 has also been shown to induce significant anti-tumor responses in several pre-clinical models. Agonistic anti-CD27 antibody was efficacious in murine lymphoma models such as BCL_1 and A31 (89). In a study where DCs in CD27-/- mice were manipulated to exhibit constitutive expression of CD70, an ovalbumin (OVA) expressing melanoma model (B16-OVA) was rejected following OVA specific (OT-1) $CD8^+$ T cell transfer and OVA challenge whereas adoptive transfer of CD27-/- OT-1 $CD8^+$ T cells did not elicit protective anti-tumor immunity (90) indicating the contribution of CD27/CD70 pathway to anti-tumor response in this model. In theory, targeting CD27 can induce anti-tumor responses by either agonizing the effector cells or depleting the Tregs dependent on the level of expression on individual cell populations (91) similar to targeting other members of the TNFRSF. Additionally, the method of CD27 targeting (modality, engagement of FcγR etc.) is also a key issue determining the mode of action as described in more detail below. Despite providing a strong anti-tumor response, the most agonistic anti-CD27 mAb also induced activation induced cell death in the effector $CD8^+$ T cells

(91) indicating that the strength of the stimulation needs to be appropriately tuned to induce a strong primary immune response and not impair other effects such as memory generation.

Another therapeutically exciting TNFR, TNFR2, is expressed on multiple immune cells, including Tregs at high levels and has been shown to be crucial for their survival. Therefore, targeting TNFR2 to deplete Tregs was considered as a potential mechanism to boost effector T-cell responses in anti-tumor immunity. Although several studies demonstrated the possibility of such an approach (92, 93), it has recently been shown that targeting TNFR2 can also work through agonistic mechanisms in pre-clinical models. Tam and colleagues demonstrated that an agonistic anti-TNFR2 mAb could stimulate the expansion of tumor specific $CD8^+$ T cells with improved effector function. The agonistic mAb was efficacious in multiple pre-clinical solid tumor models and agonizing the effector $CD8^+$ T cells was shown to be the main mechanism of action as demonstrated by increased frequency and functionality of antigen specific $CD8^+$ T cells without the depletion of Tregs (94).

Antibody targeting of the TNFRSF

The main method for targeting the TNFRSF to date has been by using mAb. As the TNFRSF members require trimerization and higher-order clustering for optimal activation, one way that canonical bivalent mAbs can achieve this is by concurrently engaging with FcγR (Figure 2A). Depending on their isotype and subclass, mAbs interact with different FcγRs (95, 96). In mouse models, the mIgG1 isotype interacts with the inhibitory FcγRIIB with higher affinity and mediates further TNFR clustering to induce strong agonistic responses. However, depending on the tumor model, anatomical location of the tumor and microenvironmental factors, the availability of FcγRIIB can be limiting, impacting the response. In support of this observation, it has been shown in pre-clinical studies that the agonistic activity of anti-CD40 and anti-4-1BB mIgG1 antibodies relies on the availability of FcγRIIB (79, 97). It was further demonstrated that a two-fold reduction in FcγRIIB expression completely eliminated the agonistic activity of certain agonist anti-TNFR mAbs *in vivo* (98). *In vitro* studies support that for CD40 at least, if expressed at sufficient level, all FcγR can mediate increased agonism in line with their relative affinities for the given mAb isotype (97, 99). Cross-linking of the receptors is the most likely explanation for mAb induced agonism with chemical cross-linking of a mIgG2a mAb able to elicit potent agonism *in vivo*, whereas the native mIgG2a does not (100). Importantly, several studies have shown that downstream signaling from FcγRIIB is not required for its cross-linking activity (97, 98), most recently demonstrated for OX40 mAb in a mouse expressing FcγRIIB with

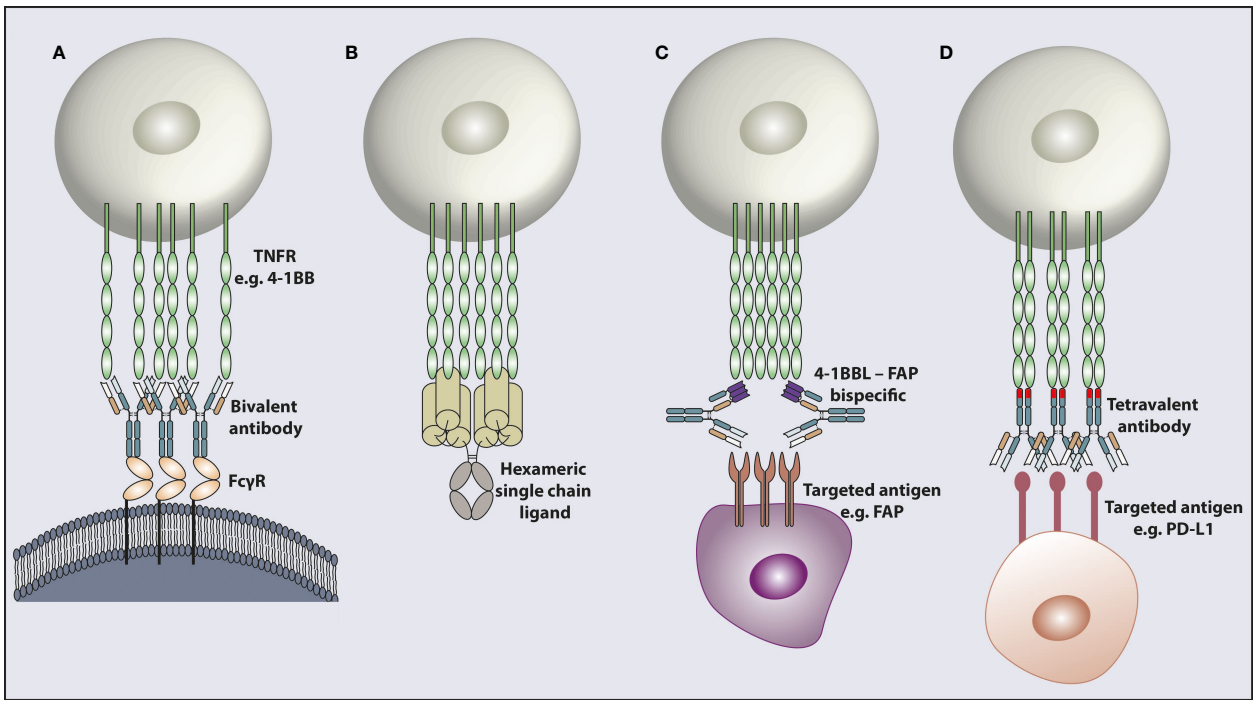

FIGURE 2
Modalities for targeting and activating TNFR. TNFR cross-linking achieved by different mechanisms. **(A)** Engagement with FcγR enables bivalent mAb cross-linking leading to target receptor clustering. **(B)** A recombinant hexameric single chain ligand inducing receptor clustering. The hexameric ligand structure is composed of a full Fc domain and six TNFSF ligand ECDs. **(C)** An antibody shaped bispecific molecule with one antigen binding arm targeting a TNFR e.g. 4-1BB and the other arm targeting a receptor e.g. FAP in the tumor microenvironment. **(D)** A bispecific molecule in a tetravalent format with two antigen binding arms targeting one receptor e.g. PD-L1 and the other two antigen binding arms in the opposite end of the molecule binding the TNFR e.g. 4-1BB, to induce receptor clustering. The 4-1BB binding domains inserted into the CH3 domain are indicated as a different color in the CH3 domain. TNFR; tumor necrosis factor receptor, FcγR; Fc gamma receptor, 4-1BBL; 4-1BB ligand, FAP; fibroblast activation protein, PD-L1; programmed death ligand 1.

a mutant, non-signaling, ITIM (101). Therefore, why FcγRIIB has this key cross-linking role in mice is not fully clear but perhaps relates to expression in the right place at the right time and the fact that multiple mouse models upregulate FcγRIIB in the tumor microenvironment, potentially due to hypoxia (102). Other variables such as the genetic background of the mouse strain may also contribute. For example, various polymorphisms in FcγRIIB have been shown to lead to reduced expression on macrophages and B cells which can increase the prevalence of autoimmune conditions (103). However, the extent of TNFR mAb agonism has not been compared in these different strains. Additionally, it has been reported in individuals with the autoimmune disorder systemic lupus erythematosus that the level of FcγRIIB expression on B cells is reduced (104) further highlighting that the level of FcγRIIB expression between individuals can vary, which could impact the agonistic activity of mAb in humans.

FcγRIIB engagement however is not the only way to elicit higher-order TNFR cross-linking. In addition to FcγR cross-linking mediated agonistic activity of TNFR mAbs, it has been shown that the human IgG2 isotype can evoke greater clustering of TNFR leading to powerful receptor activation (105). Critically, this agonism is independent of the presence of FcγR and can be achieved in mice lacking all FcγR (106), although other studies indicate that hIgG2 induced agonism may be further augmented by FcγR binding (107, 108). The hIgG2 antibody is known to undergo disulfide switching in its hinge region, producing several different isoforms, including hIgG2A, hIgG2B and hIgG2A/B (109) with the hIgG2B isoform being highly agonistic and the hIgG2A isoform agonistically inert (105). Recent analysis has confirmed that the disulfide bonding pattern in the hinge region of the more agonistic isoforms gives the antibodies a less flexible conformation leading to increased agonism whereas the isoforms with higher flexibility were found to be less agonistic (110). Although initially shown first for anti-CD40 mAb, this capability of the hIgG2(B) isotype has subsequently been confirmed for OX40 and 4-1BB and also CD28 (a member of the immunoglobulin receptor superfamily) (99, 105).

Detailed characterization of several anti-TNFR mAb has also revealed that the level of agonistic activity can depend on which domain of the receptor the antibody binds to. Antibodies binding to CRD1, the membrane distal domain, of the CD40 extracellular region induced higher agonistic activity than antibodies binding to the membrane proximal domains (111). Similar to CD40, mAb binding to membrane distal domain of CD27, CRD1, were more agonistic (112). However, mAb binding to the membrane proximal CRD4 of OX40 were found to be more potent agonists than mAb binding to other CRDs (113). It should also be noted that even within a single domain, activity of antibodies may be markedly different with some far more highly agonistic dependent upon their fine epitope and also in rare cases can be independent of their isotype. For example the anti-CD40 mAb, CP870,893 binds CRD1 (111) and is highly agonistic in any isotype, whereas 341G2, which also binds CRD1, is entirely inert as a hIgG1 and hIgG4 but maximally active and super-agonistic as a hIgG2 (106). Similar observations can also be made with other TNFRs (94). Of interest, most agonistic anti-CD40 mAbs, bind in CRD1 and so do not block ligand binding. In contrast, mAbs binding within CRD2/3 block ligand binding and are less agonistic (111). This observation may support a model whereby optimal agonists bind outside the ligand binding region. However, the above mentioned 341G2 mAb blocks ligand binding but is highly agonistic, indicating this model is incorrect. This observation is supported with other TNFR family members as an agonistic TNFR2 mAb was found to completely block ligand binding but still induce strong agonism (94). These observations suggest that binding to the same epitope as the natural ligand is not a key determinant of mAb-mediated receptor agonism but rather that certain domains and epitopes might be more generally preferable for driving agonism (such as CRD1). However, as detailed above this is likely to differ for individual receptors, according to their structure and biology.

Tolerability and response of agonistic TNFRSF targeting in clinical trials

Several agents targeting the co-stimulatory members of the TNFRSF have been tested in clinical trials. Results have demonstrated that targeting certain receptors is well tolerated whereas targeting others is limited due to toxicity. A list of the agents targeting these receptors can be found in Table 2, with specific examples outlined in further detail below.

Targeting CD27

As discussed above, CD27 is required for generating functional immune responses and targeting this receptor in pre-clinical studies has generated promising results supporting clinical evaluation. Varlilumab is a human IgG1 anti-CD27 antibody. It was well tolerated up to the maximum tested dose of 10 mg/kg with no major adverse events as a monotherapy (114, 115). Most of the toxicity related events were grade 1 or 2 with fatigue, rash, nausea, and diarrhoea the most common. Only 1 out of 56 patients had a transient grade 3 adverse event which was asymptomatic hyponatremia at 1 mg/kg. As a monotherapy, Varlilumab showed biological and clinical efficacy against tumors including hematologic malignancies, melanoma and renal cell carcinoma (114, 115). It stimulated chemokine secretion, increased the number of activated T cells and induced Treg depletion. Overall, 8 out of 56 patients had stable disease (SD) and 1 patient had a partial response (PR) (115). More recently, Varlilumab has been combined with anti-programmed cell death protein-1 (PD-1) checkpoint blockade and no additional toxicities were observed compared to anti-CD27 monotherapy. Although the initial results suggested that the combination treatment was safe and induced SD in 17% of colorectal cancers (CRC), SD in 39% of ovarian cancer (OVAC) patients, PR in 5% of CRC and PR in 10% of OVAC patients (116), more recent results revealed that the objective response rate (ORR) observed in the study was less impressive: 0% for renal cell carcinoma, 5% for CRC, 12.5% for head and neck squamous cell carcinoma and 12.5% for OVAC (117). Following promising results of a pre-clinical study demonstrating that anti-CD27 and anti-CD20 mAb in combination induced robust anti-

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TABLE 2 Co-stimulatory TNFRSF targeting agents in clinical trials.

Receptor	Drug	Modality	Clinical trial
CD27	Varlilumab	Human IgG1	NCT04081688; NCT03307746; NCT04941287; NCT02924038; NCT03688178; NCT03038672; NCT03617328
	MK-5890	Humanized IgG1	NCT03396445; NCT04924101; NCT04165096; NCT04165070
4-1BB	HLX35	EGFR – 4-1BB bispecific antibody	NCT05360381; NCT05442996
	Urelumab	Human IgG4	NCT02845323; NCT02652455
	Utomilumab	Human IgG2	NCT02554812
	YH004	Humanized IgG1	NCT05040932; NCT05564806
	ADG106	Human IgG4	NCT05236608
	ATOR-1017	Human IgG4	NCT04144842
	AGEN2373	Human IgG1	NCT04121676
	EU101	IgG1 with L234, L235 and K322 mutations	NCT04903873
	ABL503	PD-L1 – 4-1BB bispecific (Fc mutated, N299A mutation with FcγRI binding retained) human IgG1	NCT04762641
	PRS-344/S095012	PD-L1 – 4-1BB bispecific (4-1BB specific Anticalin protein), Fc silenced IgG4	NCT05159388
	GEN1046	PD-L1 – 4-1BB bispecific DuoBody (Fc silenced IgG1 antibody from human PD-L1 and humanized 4-1BB antibodies)	NCT05117242; NCT04937153
	INBRX-105	PD-L1 – 4-1BB bispecific, humanized IgG	NCT03809624
	GEN1042	CD40 – 4-1BB DuoBody, Fc silenced human IgG1 bispecific antibody	NCT05491317
	YH32367	HER-2 – 4-1BB bispecific antibody	NCT05523947
	FS222	PD-L1 – 4-1BB bispecific antibody, Fc silent human IgG1	NCT04740424
	RO7122290	FAP targeted 4-1BBL bispecific	NCT04826003
	PRS-343	HER-2 – 4-1BB bispecific (4-1BB specific Anticalin protein)	NCT05190445
	RO7227166	CD19 - 4-1BBL bispecific fusion protein	NCT04077723
	NM21-1480	PD-L1 – 4-1BB – HAS tri-specific antibody	NCT04442126
	CB307	Tri-specific Humabody targeting CD137, PSMA and HSA, not interacting with FcγR	NCT04839991
CD40	CDX-1140	Human IgG2	NCT05029999; NCT04491084; NCT04520711; NCT05349890; NCT05231122; NCT04616248; NCT05484011; NCT04364230
	LVGN7409	Antibody with enhanced FcγRIIB binding	NCT04635995; NCT05152212
	Mitazalimab	Human IgG1	NCT04888312
	2141-V11	Human IgG2 with enhanced FcγRIIB binding	NCT05126472; NCT04059588; NCT04547777
	SEA-CD40	Non-fucosylated humanized IgG1	NCT02376699; NCT04993677
	APX005M	Humanized IgG1	NCT03165994; NCT03389802; NCT04130854; NCT05419479; NCT03719430; NCT04337931; NCT02706353; NCT02600949; NCT03502330
	TQB2916	Humanized IgG2	NCT05213767
	RO7300490	FAP targeted CD40 bispecific agonist	NCT04857138
	SL-172154	SIRPα-Fc-CD40L fusion protein	NCT04406623; NCT05483933; NCT05275439

(Continued)

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TABLE 2 Continued

Receptor	Drug	Modality	Clinical trial
	MP0317	FAP - CD40 - HSA tri-specific DARPin molecule	NCT05098405
	NG-350A	Tumor selective anti-CD40 expressing adenoviral vector	NCT05165433
	LOAd703	Oncolytic adenovirus encoding trimerized CD40L and 4-1BBL	NCT03225989; NCT02705196; NCT04123470
	MEM-288	Oncolytic adenovirus encoding IFNβ and CD40L	NCT05076760
	Vaccine with tumor cells and GM.CD40L	Vaccine with cells expressing granulocyte macrophage colony stimulating factor and CD40L	NCT00101101
	HPV vaccine + anti-CD40	HPV vaccine +/- anti-CD40	NCT03418480
	CMN-001	Dendritic cell therapy, cells electroporated with RNA from tumor specimen and CD40L RNA	NCT04203901
	Selicrelumab; RO7009789*	Human IgG2	NCT03193190
OX40	MEDI6469	Mouse IgG1	NCT02274155
	BMS 986178	Human IgG1	NCT03831295; NCT03410901
	INCAGN01949	Human IgG1	NCT04387071
	BGB-A445	IgG1	NCT04215978
	HFB301001	Human IgG1	NCT05229601
	MEDI0562	Humanized IgG1	NCT03336606
	IBI101	Humanized IgG1	NCT03758001
	BAT6026	Afucosylated human IgG1	NCT05109650; NCT05105971
	PF-04518600	Humanized IgG2	NCT03092856; NCT03217747; NCT03971409; NCT02554812; NCT03390296; NCT03636503
	FS120	OX40 – 4-1BB bispecific, Fc silenced human IgG1	NCT04648202
	ES102	Hexavalent humanized IgG	NCT04991506; NCT04730843
	INBRX-106	Hexavalent IgG1	NCT04198766
	EMB-09	Tetravalent PD-L1 – OX40 bispecific antibody	NCT05263180
	SL-279252	PD-1-Fc-OX40L fusion protein (IgG4 Fc)	NCT03894618
	mRNA-2752	Lipid nanoparticle encapsulating OX40L, IL-23 and IL-36γ mRNAs	NCT03739931
	DNX-2440	Oncolytic adenovirus expressing OX40L	NCT04714983
GITR	INCAGN01876	Humanized IgG1	NCT04470024; NCT04225039
	BMS-986156	Human IgG1	NCT04021043
	REGN6569	Antibody	NCT04465487
	ASP1951	Human tetravalent antibody	NCT03799003
TNFR2	BI-1808	Human IgG1	NCT04752826
	SIM1811-03	Humanized IgG1	NCT05569057
	HFB200301	Antibody	NCT05238883

*was formerly CP870,893.

Modalities targeting the co-stimulatory receptors CD27, 4-1BB, CD40, OX40, GITR and TNFR2 are summarized in the table. The clinical trials which are active, recruiting or not yet recruiting are listed.

tumor efficacy in pre-clinical B-cell lymphoma models (118), another clinical study was designed where Varlilumab was combined with the anti-CD20 antibody Rituximab to test efficacy in relapsed or refractory B-cell lymphoma. Combination treatment was in general safe but induced a grade 3 or higher adverse event in 33% of patients. The treatment was efficacious in tumors with T-cell activated status inducing SD in 3 out of 26 patients and PR in 4 out of 26 patients (119). Another CD27 targeting agonistic mAb in development is MK-5890, which is a humanized IgG1 antibody that is being tested in the clinic as a single agent or in combination with PD-1 blocking agents in advanced solid tumors. The pre-clinical characterization of the mAb demonstrated that it could induce anti-tumor responses as a monotherapy or in combination with PD-1 blockade (120). Early results suggest an acceptable safety profile, although 24% of patients in the monotherapy group developed grade 3 or 4 adverse events related to treatment. Combination treatment did not increase the level of adverse events observed with single agent. Early signs of efficacy with MK-5890 monotherapy or combination, stimulating anti-tumor responses in patients, were observed (121). Although the mAb could induce transient upregulation of chemokine levels in patients, it also induced decreases in the level of circulating T cells (120) suggesting that identifying the right dosing regimen will be important for the successful application of this mAb. A recent study in a pre-clinical setting addressed the determinants of agonism for anti-CD27 mAb (112). It demonstrated that agonism is dictated in part by the mAb-binding domain, with the membrane distal, externally facing epitopes delivering the highest level of agonism. Additionally, the agonistic activity of hIgG1 mAb was shown to be improved by Fc engineering through either enhanced binding to FcγRIIB or hIgG2 isotype selection. The anti-CD27 mAb currently in clinic (Table 2) are unmodified hIgG1 antibodies, likely sub-optimal for agonism, and so armed with this encouraging pre-clinical data, the next generation of anti-CD27 mAb may provide greater clinical efficacy.

Targeting 4-1BB

4-1BB activation contributes to an optimal immune response and pre-clinical targeting of 4-1BB in mouse tumor models generated robust anti-tumor responses, supporting clinical evaluation. There are two mAbs that have been explored comprehensively in the clinic that target 4-1BB. Utomilumab is a human IgG2 antibody that has been shown to have a favorable safety profile, being well tolerated up to 10 mg/kg. The majority of the adverse events caused by the antibody were grade 1 or 2 including rash, dizziness, decreased appetite and fatigue in less than 10% of the patients in the study. Only 1 patient developed a grade 3/4 fatigue without increased transaminase levels. The overall ORR in solid tumors was 3.8% whereas the ORR in fifteen Merkel cell carcinoma patients was 13.3% with one PR and one complete response (CR) (122). Utomilumab has also been tested in combination with anti-CD20 treatment in patients with relapsed or refractory follicular lymphoma and $CD20^+$ non-Hodgkin lymphoma (NHL). Initial results suggested that the combination did not affect tolerability with the majority of the treatment related adverse events being grade 1 or 2. The combination treatment showed some clinical activity especially in the NHL patients (123). Additionally, safety of Utomilumab in combination with anti-PD-1 blockade was tested in patients with advanced solid tumors and the combination was found to be tolerable with mainly grade 1 or 2 toxicities and PR or CR in 6 out of 23 patients in the study (124). However, despite tolerability, clinical responses have overall been underwhelming.

Urelumab is another 4-1BB targeting agonist antibody which is of human IgG4 isotype. A study testing the safety and tolerability of Urelumab indicated that the maximum tolerated dose (MTD) of antibody given every 3 weeks was 0.1 mg/kg and higher doses induced liver toxicity in a higher percentage of patients and at higher severity above 1 mg/kg dose (125). In another study in which Urelumab was combined with Rituximab, the MTD was again found to be 0.1 mg/kg but the combination did not enhance the effect achieved by Rituximab alone (126). Several pre-clinical studies suggested that the liver toxicity induced by agonist anti-4-1BB antibody could be due to infiltration and activation of macrophages in the liver which leads to infiltration and abnormal activation of T cells, mainly $CD8^+$ T cells, leading to tissue damage (127, 128). Minimizing FcγR interactions through deglycosylation has been shown to reduce these toxicities (129).

Targeting CD40

CD40 signaling is important for APC (DC and B cell) activation and the development of strong T-cell responses. It is one of the most targeted members of the TNFRSF in clinical trials. One of the initial antibodies to be tested in multiple studies was CP870,893 which is a human IgG2 mAb. However, the antibody had to be given at low doses due to the MTD being 0.2 mg/kg. The antibody achieved modest clinical effects as a monotherapy in advanced solid tumor patients potentially due to the low doses not saturating the receptor (16). CP870,893 has also been tested in combination with multiple agents ranging from checkpoint blockade antibodies to chemotherapy. Although a significant improvement in response was not achieved with checkpoint blockade combination, combining anti-CD40 mAb with chemotherapy achieved significant responses in pancreatic ductal adenocarcinoma patients (130). Another human IgG2 anti-CD40 mAb recently developed is CDX1140. Initial studies suggested that the antibody is tolerated up to 1.5 mg/kg as a single agent or in combination with a recombinant dendritic cell growth factor, with the majority of the adverse events being low grade and early suggestion of clinical benefit in advanced solid and hematologic tumor patients (131). The 1.5 mg/kg dose is expected to give better systemic targeting of the receptor and tissue penetration compared to the MTD of CP870,893.

As described above, human IgG2 antibodies can elicit TNFR activation without requiring FcγR mediated cross-linking. However, there is also interest in developing agents with enhanced ability to bind to FcγRIIB to mediate optimal cross-linking of the antibody, leading to greater receptor clustering and activation. APX005M is a humanized IgG1 anti-CD40 antibody possessing the S267E

mutation in its Fc domain which enhances the affinity for FcγRIIB binding by 30-fold (132). Combining APX005M with anti-PD-1 blockade to treat anti-PD-1/PD-L1 refractory melanoma patients showed that the combination did not increase toxicity and the majority of adverse events were grade 1 or 2. Early results from the study are promising and indicate that the combination evokes clinical benefit (133).

Targeting OX40

Pre-clinical studies demonstrated the anti-tumor potential of reagents targeting OX40 and agonistic anti-OX40 antibodies have been shown to be well tolerated in patients. However, the response rates as a monotherapy have been low. GSK3174998 was an agonist humanized IgG1 mAb tested against advanced solid tumors but only induced 1 PR and 1 SD in 45 patients as a monotherapy and the combination with the anti-PD-1 mAb Pembroluzimab did not significantly improve the efficacy expected with Pembrolizumab alone (134). A humanized IgG2 mAb PF-04518600 was tested as a monotherapy in advanced solid tumor patients but only 1 out of 25 patients had a PR while 15 out of 25 had SD (135). In a recent study in which PF-04518600 was combined with Utomilumab, early indications were that the combination was found to be well tolerated and 7 out of 10 melanoma patients and 7 out of 20 non-small cell lung cancer (NSCLC) patients experienced SD in addition to only 1 NSCLC patient experiencing a PR (136).

Another agonistic anti-OX40 mAb being tested in clinical trials is MEDI0562 which is a humanized IgG1 antibody. As a monotherapy in advanced solid tumors, MEDI0562 was found to be safe with the majority of adverse events being grade 1 or 2. Despite the favorable safety profile, only 2 out of 55 patients experienced a PR and 24 out of 55 patients experienced SD (137). In another study where MEDI0562 was combined with anti-PD-L1 or anti-cytotoxic T-lymphocyte associated protein 4 (CTLA-4) immune checkpoint blockade in advanced solid tumors, early results indicated that the combinations induced grade 3 or 4 adverse events in a high frequency of patients and only 11.5% of patients in the anti-PD-L1 combination group showed PRs. 34.6% of patients in the anti-PD-L1 combination group and 29% in the anti-CTLA-4 combination group experienced SD (138).

Targeting GITR

GITR activation leads to the development of strong T-cell responses and mouse tumor model studies have demonstrated the anti-tumor potential of GITR targeting. Several agonistic antibodies targeting GITR have been tested in clinical trials. MK-1248 is an agonist humanized IgG4 antibody against GITR. In a study investigating the tolerability of MK-1248 as a single agent or in combination with anti-PD-1 blockade in advanced solid tumors, it was found that despite approximately 50% of patients in both arms of the study developing grade 3 or higher adverse events, the clinical benefit was very limited. No objective response was achieved with monotherapy and only 1 CR and 2 PRs were observed in the combination arm. 15% of patients receiving single agent experienced SD whereas 41% of patients receiving combination therapy experienced SD (139). Another agonistic anti-GITR agent is BMS-986156, which is a human IgG1 antibody. BMS-986156 was well tolerated as a single agent in advanced solid tumor patients with no grade 3 or higher adverse events and only 9.3% of patients in combination with anti-PD-1 experiencing grade 3 or 4 adverse events. Despite the favorable safety profile, no response was observed with BMS-986156 as a single agent and the highest ORR in the combination group was only 11.1% (140). MK-4166 is another human IgG1 anti-GITR antibody that has been recently tested in advanced solid tumor patients in combination with anti-PD-1 blockade. Although the treatments were found to be well tolerated, single agent again did not induce any clinical benefit. Comparing the checkpoint blockade treatment naïve versus pre-treated melanoma patients showed that the treatment naïve patients were responsive to MK-4166 and anti-PD-1 combination. 5 out of 13 patients had a CR and 3 out of 13 patients had a PR suggesting that the combination treatment might be efficacious in this particular group of patients (141).

Targeting TNFR2

TNFR2 targeting agonist mAbs can generate strong anti-tumor T-cell immunity but are mainly still in pre-clinical development and only recently starting clinical assessment. MM-401 is an agonist anti-human TNFR2 mAb in development. Using a mouse surrogate version of the antibody, it was found that TNFR2 agonism could generate strong anti-tumor responses by activating $CD8^+$ T cells and NK cells with activity dependent on FcγR interactions, presumably mediated by cross-linking of the receptor. In addition, the antibody synergized with checkpoint blockade (142). BI-1910 is another agonist anti-TNFR2 mAb in development following promising results from a surrogate anti-mouse TNFR2 antibody; this mAb induced strong anti-tumor responses in several pre-clinical tumor models and was effectively combined with checkpoint blockade antibodies. The dominant mechanism of action was expansion of $CD8^+$ T cells and improved $CD8^+$ to Treg ratio in the tumor site (143). BI-1808 is an alternative TNFR2 targeting mAb, classified as a deleting, ligand blocking molecule. However, pre-clinical studies with a mouse surrogate indicated intra-tumoral Treg depletion and effector T-cell expansion leading to an improved CD8:Treg ratio. Similar results were obtained with BI-1808 in pre-clinical characterization. BI-1808 was found to be well tolerated in non-human primates and is in clinical assessment (143, 144). HFB200301 is also an anti-TNFR2 agonist antibody which is already in a phase I clinical trial of advanced solid tumor patients (145). Using human TNFR2 knock-in mouse models, it was suggested that HFB200301 could stimulate anti-tumor responses through expansion of effector T cells and NK cells without depleting the Tregs. The agonistic ability of the antibody was found to be independent of FcγR mediated cross-linking (146). Although much of the data is not yet mature, with

peer review lacking for most of the pre-clinical studies, the potential of TNFR2 targeting antibodies in oncology are exciting and the initial results from clinical trials are eagerly awaited by the immuno-oncology community.

Recent approaches in targeting TNFRSF members to overcome current limitations

Fc engineering

As described above, despite success in pre-clinical studies, clinical efficacy of targeting TNFRSF members has been limited. One factor which may help to explain this is the lack of a human antibody isotype equivalent of mIgG1 with preferential binding toward FcγRIIB to facilitate agonistic activity. Therefore, in order to enhance FcγRIIB engagement, Fc engineering approaches have been developed to improve the affinity of antibodies toward hFcγRIIB. Although several mutations such as SE (S267E) and SELF (S267E-L382F) have been identified to improve affinity to hFcγRIIB, those mutations improved affinity to hFcγRIIa as well, due to sequence and structural similarity between the two receptors. Other mutations such as V9 (G237D-P238D-P271G-A330R) and V11 (G237D-P238D-H268D-P271G-A330R) however, were found to specifically improve the affinity of antibodies toward hFcγRIIB by approximately 32 and 96 fold, respectively (107). Comparing WT and Fc engineered anti-human CD40 antibodies in mice expressing hFcγRs, the variant with the V11 mutation was found to be superior to others, indicating the possibility of this approach to be taken forward for further development. Subsequent analysis demonstrated that systemic delivery of the agonistically enhanced variant could pose a risk of inducing toxicity and optimal receptor occupancy might not be reached with the MTD. Delivering the mAb *via* intra-tumoral injections was shown to ameliorate toxicity, yet retain significant tumor control even at low doses (147) indicating that where this method of delivery is practical (e.g. for localized/accessible lesions) it could provide a solution.

Another approach to overcome the requirement for mAb cross-linking could be *via* alternative, FcγR-independent, Fc domain engineering which was recently demonstrated for anti-human OX40 mAbs. Building on seminal studies showing that E345R, E345K and E430K single point mutations in the Fc region could promote "on-target" multimerization (once the mAb binds to the receptor) of the mAbs to facilitate optimal engagement of the hexa-headed C1q molecule (148, 149), Zhang et al. showed that E345R single mutation or K248E-T437R double mutations in the Fc region could induce "on-target" multimerization of agonistic OX40 antibodies, leading to activation of the receptor in an FcγR-independent way (150, 151). Although the Fc engineered antibodies were active in the absence of FcγR cross-linking, their activity could be further improved by FcγRIIB mediated cross-linking, suggesting that this approach could provide the possibility of targeting receptors in tissues without FcγRIIB availability but when FcγR are available, the activity will be further boosted.

Receptor cross-linking independent of FcγR

Although improving FcγRIIB affinity of antibodies can augment agonism, as previously mentioned the availability of FcγRIIB at the relevant anatomical site to provide the cross-linking can be a limiting factor. Thus, alternative approaches have been developed to generate agonistic agents without the requirement of FcγR mediated cross-linking. In addition to the hIgG2 isotype, soluble recombinant TNFSF ligands have been explored as a means to replicate the natural multimeric ligand-receptor interaction. The potency of soluble trimeric ligands could be improved by additional cross-linking and this approach was demonstrated for several ligands including OX40L, CD40L and 4-1BBL (13, 152). However, as the soluble trimeric ligands still require additional cross-linking, practicality of this approach *in vivo* is likely to be challenging due to possible short serum persistence of the trimers and also additional non-native sequences potentially making the products more immunogenic. To overcome this limitation, multimeric forms of soluble trimeric TNFSF ligands such as Fc fusion proteins have been developed. Multimeric ligands do not require the additional cross-linking required by the trimeric forms and the Fc fusion facilitates better *in vivo* persistence *via* its interaction with the neonatal Fc receptor (FcRn) (153). A CD27L-Fc fusion protein designed to mimic the natural CD27L activity was found to be active in *in vitro* and *in vivo* assays boosting T-cell activation (154). In that study, one CD27L extracellular domain (ECD) was fused to one Fc domain suggesting that the active product consisted of multimeric trimers of the ligand and multimers of Fc domains. More recently, a hexameric human CD27L fusion protein consisting of six CD27L ECDs and a silent human IgG1 Fc domain (not interacting with FcγR) has been reported (155). In this construct, three ECDs of the ligand are linked in a single chain format and fused to the IgG1 Fc domain with the idea of bringing two ligand trimers together upon Fc domain dimerization (Figure 2B). The fusion protein induced activation and proliferation of T cells in *in vitro* and *in vivo* experiments independently of FcγR engagement (155). Additionally, the hexameric fusion protein demonstrated anti-tumor efficacy in pre-clinical models. Hexameric Fc fusion ligand proteins in the same format have also been developed for CD40L, GITRL and 4-1BBL (156–158). Despite the promising pre-clinical results, the hexameric ligand proteins have short half-lives in circulation. Although this could be considered as a disadvantage, shorter stimulation of the immune cells can also lead to generation of a strong response and possibly could be better than chronic stimulation, which might have detrimental effects (159, 160). It has been shown in multiple studies that continuous stimulation of CD27 leads to defects in the immune cells. Continuous stimulation of CD27 by constitutive expression of CD70 on B cells resulted in increased apoptosis and depletion in NK cells (161) or T-cell immunodeficiency (159). Similarly, continuous 4-1BB stimulation leads to overactivation of $CD8^+$ T cells and macrophages which eventually results in impaired $CD8^+$ T-cell activity (160). Thus, timing and strength of stimulation are crucial

in inducing a strong immune response and avoiding immunopathology. By experimentally determining the correct dose, schedule and treatment routes the hexameric ligands might generate strong immune responses in patients. On the other hand, it is worth noting that agonistic ligand formats, specifically TNFR2 specific recombinant TNF ligand protein, have also been developed with the aim of expanding Tregs in non-cancer contexts. A nonameric version of the recombinant protein was initially found to have suboptimal serum retention *in vivo* but a newly developed version in which an Fc silent irrelevant IgG molecule is fused to two trimeric ligand units to generate a hexameric ligand showed improved pharmacokinetics and robust Treg expansion *in vivo* (162).

Recent technological advances in the field have enabled the use of computational methods to design desired structures. Using such approaches, researchers have produced antibody molecules in various oligomeric states, in a format described as "antibody nanocages". These nanocages were found to activate several receptor targets, including converting an antagonist anti-CD40 mAb into an agonist due to the ability of the designed structure to induce receptor clustering (163). This approach could potentially be applied to a plethora of different receptors to identify the best design for optimal receptor activation in each case.

Reagents targeting tumor microenvironment to induce localized TNFR activation and reduce toxicity

In addition to the variation of the availability of FcγRIIB in target tissue to provide optimal cross-linking of agonistic mAbs, off-target toxicity has also been an issue. Although some agonistic mAbs such as Varlilumab against CD27 was well tolerated, the clinical efficacy was modest. In contrast, the 4-1BB agonist Urelumab was active but found to induce liver toxicity at high doses. The mechanism behind the toxicity of Urelumab is thought to be the activation of the liver resident FcγR-expressing Kupffer cells, with the agonistic cross-linking of the anti-4-1BB mAb enabled by the high level of FcγR expressed on these myeloid cells (98) or other FcγR-expressing cells in the liver, such as FcγRIIB expressing sinusoidal liver endothelial cells (164). Activated Kupffer cells produce IL-27 which is an inflammatory cytokine involved in infiltration and expansion of other immune cells, especially T cells into the tissue (127). Hepatotoxicity following 4-1BB agonism indicated that systemic delivery of the agonistic reagents has the risk of off-target toxicity. Thus, recent efforts have focussed on eliminating the risk associated with systemic delivery in favor of targeted agonism – localizing the mAb to the desired site. One approach has been to generate recombinant proteins with a tumor targeting domain. For example, single chain fragment variable (scFv) domains of an anti-4-1BB mAb have been fused to a trimerization domain (producing a trivalent 4-1BB targeting molecule) with further fusion of a tumor targeting domain on the C-terminus to direct the trimer to the tumor site (165). Although the trimeric protein had short *in vivo* stability, the anti-tumor response generated in mouse tumor models was similar to an agonistic anti-4-1BB mAb and the trimer did not induce toxicity, which was apparent with the agonistic mAb. Additionally, repeated dosing of the trimeric protein also did not induce off-target toxicity indicating that targeted agonism approach could overcome the non-specific toxicity.

More recently, a tumor antigen targeting 4-1BB bispecific molecule was generated with one arm of the antibody designed to target a tumor antigen and the other designed to form a trimeric h4-1BBL. The bispecific molecule was generated in an Fc silent format to maintain normal antibody-like pharmacokinetics but at the same time eliminating FcγR engagement to prevent off-target toxicity. Binding of the tumor antigen specific arm at the tumor site allows accumulation of 4-1BBL in the tumor tissue to facilitate multimeric interaction between the ligand and receptor (Figure 2C) and activate the T cells in the tumor microenvironment. The bispecific molecule had a favorable pharmacokinetic profile and could accumulate in the tumor site, confirmed in non-human primates (166). Additionally, the bispecific molecule proved to be able to induce activation of T cells from human tumor tissues and also induce anti-tumor immunity in pre-clinical models. However, the main activity was observed when the bispecific molecule was used in combination with another T-cell bispecific agent stimulating the TCR and targeting a tumor antigen (166, 167), indicating that optimal co-stimulation happens in the presence of TCR stimulation. While the bispecific molecule had favorable serum stability, it did not induce toxicity indicating that it could be used in combination with other T-cell inducing treatments. Similar bispecific molecules with a scFv arm targeting a tumor associated antigen and a TNFSF ligand arm targeting a co-stimulatory receptor on the T cells have also been characterized in other studies (168). In these molecules however, a tag was inserted for purification purposes and its immunogenicity will have to be assessed further during *in vivo* validation of these reagents.

Another approach to induce TNFR clustering involves duokines, where both arms of the bispecific molecule are targeting members of the TNFRSF. Initially, the proteins were developed by either fusing one ECD protomer of a TNFSF ligand to one ECD protomer of another TNFSF ligand to allow trimerization of the ligand molecules by non-covalent interactions or by developing them as a single chain polypeptide in which three ECDs of each ligand were linked onto the same polypeptide chain separated by flexible linkers (169). Depending on the choice of ligands, this approach allows targeting of receptors in cis (on the same cell surface) or trans (on different cells) orientations. The single chain duokines were found to be more stable than non-covalently formed duokines and could induce *in vitro* and *in vivo* stimulation of T cells as co-stimulatory molecules. Using 4-1BBL-CD40L as a trans acting duokine or 4-1BBL-CD27L as a cis acting duokine, Fellermeier-Kopf and colleagues showed that both molecules could induce anti-tumor immunity in a pre-clinical melanoma model in combination with a TCR targeting bispecific antibody (169). In a subsequent study, Fc fusion proteins of the duokines were generated to facilitate enhanced stability in circulation with the active protein adopting an antibody structure with each single chain trimeric ligand domain being fused to Fc

regions and dimerization of the Fc regions bringing two trimeric ligands together (170). Although the protein was still active in combination with a TCR targeting bispecific antibody, interestingly, the Fc fusion did not improve the pharmacokinetic profile. These data demonstrated the possibility of using these duokines to target two co-stimulatory TNFR molecules to boost the anti-tumor response. By identifying the optimal combination strategies, they could potentially enhance the anti-tumor responses in the clinic.

It has been clearly observed that blocking the immune checkpoint molecules PD-1 or CTLA-4 can generate strong anti-tumor responses but the majority of the patients are either refractory or develop resistance to these therapies. In recent studies, bispecific molecules targeting the checkpoint inhibitory receptors and co-stimulatory members of the TNFRSF have been developed as a means to enhance their activity. There are multiple advantages to this approach: First, the interaction of the inhibitory checkpoint receptor and its ligand is blocked to release the suppression on the immune response. Second, the inhibitory molecules are mainly expressed in the tumor microenvironment and this ensures targeted activation of the co-stimulatory receptor at the tumor microenvironment, avoiding systemic toxicity. Third, the bispecific antibody can be generated in an Fc silent format to avoid potential systemic toxicity with co-stimulatory receptor clustering achieved by the checkpoint receptor targeting arm acting as an anchoring domain. An Fc silent IgG1 bispecific antibody in a tetravalent format with two Fab arms targeting PD-L1 and two 4-1BB targeting domains introduced into the CH3 domains, termed Fc-region with antigen binding, was recently developed (Figure 2D). The mouse surrogate version of the bispecific induced activation of T cells *in vitro* and induced anti-tumor immunity *in vivo* without hepatotoxicity. The human version of the protein induced human T-cell activation *in vitro* and toxicology studies in non-human primates, enabled by cross-reactivity between species, showed that the bispecific was well tolerated (171) and had higher activity than the combination of the single agents. Similar bispecific molecules in tetravalent formats targeting PD-L1 and CD40 or 4-1BBL have also been reported in other studies. *In vitro* characterization of these products showed PD-1/PD-L1 blockade and target receptor activation in an FcγR independent manner, supporting further validation in *in vivo* studies (172). In support of these findings with bispecific molecules, it was recently shown that an anti-PD-1/GITRL bispecific molecule induced a different mechanism of action than the combination of single agents and was more efficacious in pre-clinical studies (9). The co-stimulatory antibodies being tested in combination with checkpoint blockade antibodies to date have shown favorable tolerability in clinical trials (see above), and the recent findings support the idea that the bispecific molecules could achieve better results than the combination treatments.

Conclusion

TNFRSF members represent powerful targets for immunomodulation. Promising pre-clinical data of agonistic mAbs targeting TNFRSF has clearly demonstrated their potential to provide anti-tumor efficacy. However, the translation from the pre-clinical studies to the clinic has been difficult and lack of significant response rates or toxicity in the clinic with conventional mAbs has directed researchers to develop new strategies.

Other immunomodulatory agents such as the checkpoint blockade antibodies have shown better success than the agonistic antibodies against TNFRs. However, while the responses thus far are limited, there is an opportunity for combining the two strategies, as has been shown in pre-clinical studies. With new approaches, such as targeted agonism and bispecifics delivering two or more different mechanisms of action with a single agent, success rates may improve. The challenge however remains the same – evoking powerful, curative immune responses while avoiding toxicity. Hopefully, such innovation will finally unlock TNFR targeting for the clinic.

Author contributions

OD researched data and wrote the manuscript with MC, OD, and AE produced the figures. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Publisher's note

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Targeting CD137 (4-1BB) towards improved safety and efficacy for cancer immunotherapy

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T cells play a critical role in antitumor immunity, where T cell activation is regulated by both inhibitory and costimulatory receptor signaling that fine-tune T cell activity during different stages of T cell immune responses. Currently, cancer immunotherapy by targeting inhibitory receptors such as CTLA-4 and PD-1/L1, and their combination by antagonist antibodies, has been well established. However, developing agonist antibodies that target costimulatory receptors such as CD28 and CD137/4-1BB has faced considerable challenges, including highly publicized adverse events. Intracellular costimulatory domains of CD28 and/or CD137/4-1BB are essential for the clinical benefits of FDA-approved chimeric antigen receptor T cell (CAR-T) therapies. The major challenge is how to decouple efficacy from toxicity by systemic immune activation. This review focuses on anti-CD137 agonist monoclonal antibodies with different IgG isotypes in clinical development. It discusses CD137 biology in the context of anti-CD137 agonist drug discovery, including the binding epitope selected for anti-CD137 agonist antibody in competition or not with CD137 ligand (CD137L), the IgG isotype of antibodies selected with an impact on crosslinking by Fc gamma receptors, and the conditional activation of anti-CD137 antibodies for safe and potent engagement with CD137 in the tumor microenvironment (TME). We discuss and compare the potential mechanisms/effects of different CD137 targeting strategies and agents under development and how rational combinations could enhance antitumor activities without amplifying the toxicity of these agonist antibodies.

KEYWORDS

TNFR agonist, CD137/4-1BB, FcγR mediated cross-linking, conditional activation, cancer immunotherapy, costimulatory receptor

Introduction

T cell-mediated immunity is crucial for the host antitumor response (1). Under physiological conditions, T cell activation requires two signals: signal 1 involves TCR activation triggered by the major histocompatibility complex (MHC) presented antigenic peptide, and signal 2, a costimulatory signal, amplifies the antigen-specific signal 1 (2).

CD137 or 4-1BB, a member of the tumor necrosis factor receptor superfamily (TNFRSF), also known as TNFRSF9, is one of the key costimulatory receptors identified that has shown promise as a therapeutic target for boosting antitumor immune responses in both preclinical and clinical studies over the past two decades (3). CD137 is induced upon activation in T cells, B cells, and natural killer (NK) cells (4). Ligation of CD137 by its natural ligand, CD137L or 4-1BBL, recruits TNFR-associated factor (TRAF) 1 and TRAF2 and induces signaling through the master transcription factor NF-kB and MAPKs (5) (6), which coactivates CD8+ T cells and natural killer cells, resulting in enhanced cellular proliferation and survival, increased proinflammatory cytokine secretion, cytolytic function, and antibody-dependent cell-mediated cytotoxicity (7). As most tumors are killed by CTLs in an antigen-specific manner, agents that propel CD8+ T-cell activation and impart strong cytolytic, inflammatory, and immune-regulating properties in an antigen-specific manner are ideal candidates for enhancing antitumor immunity. Agonistic anti-CD137 mAb immunotherapy targeting CD8+ T cells meets these requisites. However, clinical development of the first fully human anti-CD137 IgG4 agonistic antibody, urelumab, was put on hold due to dose-dependent liver toxicity, including grade 3 and higher liver-related toxicities and two fatalities, despite demonstrating monotherapy efficacy in melanoma patients (3) (8). By contrast, the second clinical development of a fully human IgG2 anti-CD137 agonist antibody, utomilumab, has shown excellent safety and tolerability following a lengthy dose escalation schedule, presumably, due to the clinical safety issues for urelumab, the first agonist anti-CD137 antibody in clinics. Only modest or marginal efficacy in utomilumab monotherapy is reported for a few patients in immune-responsive Merkel cell carcinoma and for checkpoint-experienced melanoma and non-small cell lung cancer patients (9). These two extreme cases highlight the challenges in developing costimulatory receptor antibody therapies in general and anti-CD137 agonist antibodies in particular, where the preclinical observation is yet to translate into clinical reality. New generations of CD137 agonists with different targeting strategies are under development that could get around these challenges and realize the full potential of CD137 targeted immunotherapy for cancer treatment.

CD137 binding epitopes, complex conformations and IgG isotypes

The binding epitope on CD137 of an anti-CD137 antibody could have direct impact on its agonistic activity. Figure 1A illustrates that the trimeric CD137 ligand in gray binds to CRD2 and 3 on CD137 to cluster the receptor, whereas the binding epitopes of different anti-CD137 agonist antibodies can vary (10). For example, Urelumab binds to the N-terminal portion of CRD-1, utomilumab binds at the junction of CRD-3 and CRD-4 (10), and ADG106, a fully human anti-CD137 IgG4 agonist antibody developed by us, binds at the junction of CRD-2 and CRD-3, which overlaps with the CD137L binding site at CRD-2 and CRD-3 (Figure 1B) (11). Such differences in binding epitopes among different agonistic anti-CD137 antibodies explain their ligand-blocking versus non-blocking properties. Urelumab does not block CD137L interaction with CD137, whereas ADG106 strongly blocks CD137 binding to its ligand.

It should be noted that the conformation of the CD137 complex also depends on the binding epitope of the agonist antibodies in comparison with the CD137 ligand-induced complex conformation, which may play a role in CD137-mediated T cell signaling. CD137 conformations in complex with different agonist antibodies, including urelumab, utomilumab, and ADG106, in comparison with CD137 ligand seem to follow a trend that mimics their functional activities.

The CD137 binding epitope may also affect the antibody's Fc interactions with FcγRs. Such variations in epitope-oriented FcγRs engagement could further contribute to differences in the levels of agonist activity for different anti-CD137 agonistic mAbs. In addition, the difference in IgG isotypes, because of their importance in FcγRs, especially FcγRIIB-mediated crosslinking for agonistic activities, is also shown in Figure 1D, for urelumab in IgG4, utomilumab in IgG2, ADG106 in IgG4, and ADG206, a conditionally activatable ADG106 in Fc-enhanced IgG1 (more details in the following sections).

Moreover, the interactions between CD137 and CD137L not only trigger CD137-mediated costimulatory signaling, but a reverse signaling through CD137L may also be activated to regulate immune responses (12). Previous studies have indicated that blockade of CD137L reverse signaling promotes intra-tumoral differentiation of IFNγ-producing cytotoxic T cells, IL12-producing CD103+ DC, and type 1 tumor-associated macrophages to suppress tumor growth (13). Thus, the ligand-blocking anti-CD137 antibody could have additional pharmacological activities through inhibiting the CD137L-mediated reverse signaling. The ligand-blocking versus non-blocking properties of the agonist antibodies can also impact their activity in the presence and absence of CD137 ligand. For example, as a ligand non-blocking agonist, urelumab was demonstrated to be capable of inducing strong ligand-dependent CD137 clustering through cross-linking receptors trimerized by the ligand, whereas the ligand-blocking utomilumab failed to induce ligand-dependent clustering (10). Thus, the influence on ligand/receptor interaction could be a noteworthy property of an anti-CD137 agonist antibody when comprehensively evaluating its pharmacological activity.

Finally, we noted that CRD4 is tilted by Utomilumab-induced conformational change, while CRD4 is much more tilted by urelumab and ADG106, respectively, in comparison with the reference state by CD137L. This becomes quite obvious once we overlap their CD137 structures in complex with different agonist antibodies with that of CD137 (in green) in complex with CD137L based on the CRD1 and CRD2 as shown in Figure 1C.

Crosslinking by FcγR

In physiological conditions, CD137 costimulatory signaling activation requires clustering of the receptor through its natural ligand to form trimeric or larger lattice-shaped structures (14).

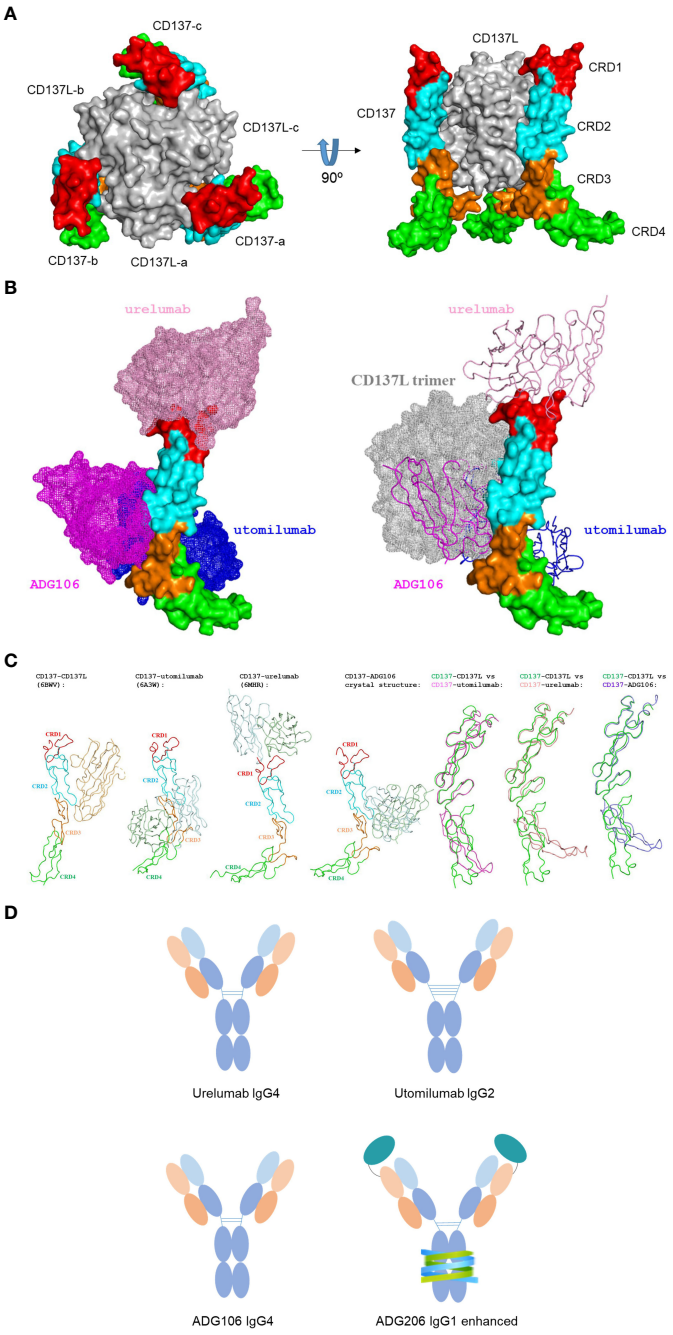

FIGURE 1
(A) The constructed structure of trimeric CD137 in complex with trimeric CD137L; **(B)** Structure of CD137 in complex with anti-CD137 agonistic mAbs with only one CD137 structure shown to illustrate the difference in CD137 binding epitopes by 3 anti-CD137 agonistic mAbs, together to show the complete overlap between ADG106 with CD137L trimers in gray; **(C)** The induced conformational changes of CD137 upon binding to CD137 ligand, three agonist antibodies and their pairwise comparison of the induced conformational change of CD137 with reference to its conformation upon binding to CD137 ligand; **(D)** the diagram illustrating the difference of IgG isotypes for urelumab, utomilumab, ADG106 and ADG206.

Similarly, agonistic antibodies activate CD137 receptor signaling by inducing cluster formation at high receptor density (15). However, the crystal structure of utomilumab in complex with CD137 indicates that utomilumab binds to monomeric or dimeric CD137 to induce limited crosslinking of the receptor (16). This explains why the Fab binding to CD137 alone by many anti-CD137 monoclonal antibodies (mAbs) is not sufficient to induce CD137 receptor activation. Most anti-CD137 agonistic antibodies require engagement of FcγRs, particularly FcγRIIb, to mediate clustering and activation of CD137. Upon Fc binding to FcγRIIb, higher order CD137 receptor clusters can be achieved, which activates CD137-mediated downstream signaling (Figure 2). Certain antibodies, such as urelumab, can directly activate CD137 receptor independent of FcγR engagement. A study comparing the agonist activity of urelumab, utomilumab, and AD106 in the CD137 Jurkat NFκB reporter cell signaling assay in the presence and absence of FcγRIIb-

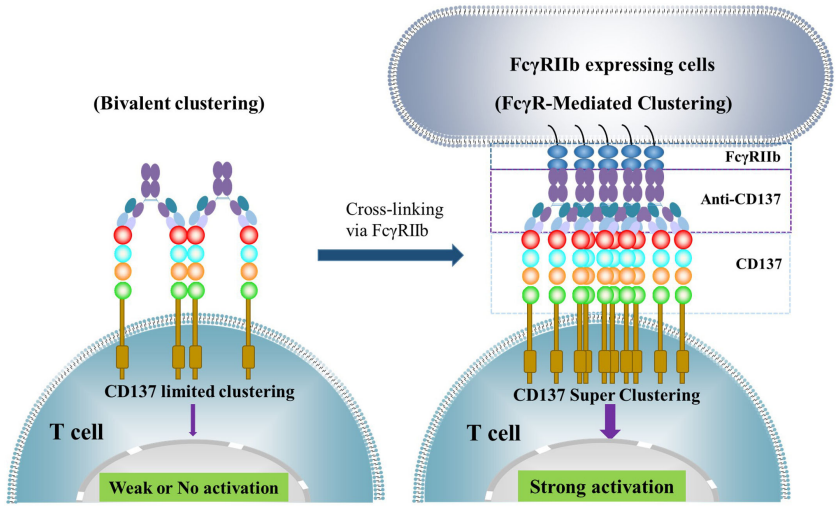

FIGURE 2
illustrates how the activation of CD137 receptor signaling is achieved through anti-CD137 agonistic monoclonal antibodies (mAbs). These antibodies bind to CD137 through their Fab region, inducing limited clustering of CD137 receptors and weak or no activation of downstream signaling. However, when the Fc region binds to FcγRs, particularly FcγRIIb, expressed on cells such as dendritic cells, macrophages, and B cells, they can induce CD137 receptor super-clustering and strong downstream signaling activation.

expressing cells demonstrated that, in the presence of FcγRIIb-expressing cells, all three antibodies stimulate CD137 and downstream signaling, with urelumab showing more potent activity than utomilumab and ADG106. However, in the absence of FcγRIIb-expressing cells, both utomilumab and ADG106 are inactive, whereas urelumab is still capable of stimulating CD137 receptor activation (11). These results suggest that the binding epitope of urelumab may allow this antibody to cluster CD137 monomers or dimers more efficiently through bivalent binding than other anti-CD137 agonists. Nonetheless, engagement of FcγRs, particularly FcγRIIb, by Fc could further enhance the clustering effect (17) (18). FcγRIIb is expressed on many types of immune cells, including B cells, dendritic cells (DCs), monocytes/macrophages, mast cells, and basophils (19). Notably, FcγRIIb is expressed on Kupffer cells, the resident liver macrophages, and liver sinusoidal endothelial cells in the liver, where it plays an important role in immune complex clearance (20). This may significantly contribute to the super-clustering and hyperactivation of CD137 by anti-CD137 agonists on immune cells in the liver, leading to hepatic inflammation and liver toxicity, as exemplified by urelumab (8).

It has been shown that the non-blocking ligand urelumab increases the clustering of CD137 receptors on cells in a ligand-dependent manner (10). This may explain how urelumab stimulates CD137 receptor activation in the absence of FcγR engagement and cause severe toxicity, particularly liver damage, in patients. Conversely, the mild agonistic activity of utomilumab may account for its weak clinical efficacy in patients.

To strengthen the activity of CD137 agonist antibodies, enhancing the clustering of CD137 receptors has been a strategy, especially for those anti-CD137 mAbs with weaker agonist activity. Fc engineering can achieve this by enhancing crosslinking by FcγRIIb. Examples include LVGN6051 (21) in an IgG4 backbone and ADG206 (22) in an IgG1 backbone. ADG206 has shown four-fold higher cross-linking potency than urelumab in the functional assay, where urelumab is the most potent anti-CD137 agonist shown clinically.

Notably, CD137 is not only expressed in activated effector T cells but has also been identified as an activation marker for antigen-specific T regulatory (Treg) cells with immunosuppressive functions (23). CD137+ cells form a major part of functional tumor Tregs (24). In one preclinical study using mouse tumor models, two mechanisms were shown to promote robust tumor rejection: tumor Treg depletion and effector T cell agonism by anti-CD137 mAbs, which, however, are competitive and dependent on antibody isotype and FcγR availability. Administration of anti-CD137 mouse IgG2a, which preferentially depletes Treg cells, followed by either agonistic anti-CD137 mouse IgG1 or anti-PD-1 mAb augmented anti-tumor responses. An antibody engineered to optimize both FcγR-dependent Treg cell depleting capacity and FcγR-independent agonism delivered enhanced anti-tumor therapy (25). Although none of the current clinical stage anti-CD137 agonist antibodies developed so far demonstrated Treg depleting capacity, such mechanism can be provided by a Treg depleting antibody, such as anti-CTLA-4 mAb (26) (27), in a combination setting.

Binding affinity and agonism

High affinity is often the desired characteristic for therapeutic antibodies, particularly for antagonistic antibodies that can neutralize or inhibit target functions. However, for agonistic antibodies, this is not always the case. Epitope and Fc-mediated crosslinking, rather than high affinity, are critical for the antitumor activity of CD137 agonist antibodies with reduced liver toxicity (28). A recent study examined the relationship between affinity and function of immunomodulatory antibodies and found that reducing

affinity can be a strategy to enhance immunomodulatory antibody agonism (29). Low rather than high affinity delivers greater activity through increased receptor clustering, independent of FcγRs. The study suggests that a faster dissociation rate, or higher off-rate rather than on-rate of the monoclonal antibody, is responsible for the increased agonistic activity of low-affinity variants. Additionally, an inert anti-CD137 mAb, utomilumab, can be transformed into an agonist. Low-affinity variants of the clinical antagonistic anti-PD-1 mAb, nivolumab, also mediated more potent signaling and affected T cell activation. Notably, low-affinity targeting by antibody binding is conducive to receptor activation but detrimental to Fc-mediated effector function, which could limit further enhanced receptor clustering through antibody engagement of FcγRs. Nonetheless, these findings provide a new avenue for agonistic antibody engineering (29).

Conditional activation

The specificity of T cell immune responses to an antigen comes from the recognition of the antigen peptide MHC complex by TCR, and not from costimulatory signaling. However, the use of CD137 agonists for cancer immunotherapy can potentially boost T cell responses triggered by any existing antigen MHC complex, including those involved in mediating autoimmune responses. This creates a dilemma between antitumor efficacy and autoimmune toxicity for costimulatory agonists with systemic agonistic activity, such as anti-CD137 agonist antibody. To address this issue, various strategies have been developed to create next-generation anti-CD137 agonists that target the agonistic activity more specifically to the tumor site while limiting the agonistic activity in normal tissues to reduce immune-toxicity (30). The primary solution is to exploit the differences between tumors and normal tissues to target tumors more specifically. Tumor-associated antigens (TAAs) are overexpressed in tumors compared to most normal tissues, and this can be leveraged to direct the antibody primarily to tumor tissues (Figure 3). Several TAAs, such as HER2 (31), FAP (32), EGFR (33) (34), Claudin18.2 (35), PSMA (36), Nectin-4 (37), Mesothelin (38), and B7-H3 (39), have been selected to construct bi- or tri-specific CD137 agonistic antibodies. These antibodies directly target the tumor-associated antigens through TAA-targeting arms and provide a costimulatory signal to enhance antitumor immune responses. The agonist activity of these constructs is dependent on TAA for crosslinking, thereby limiting immune activation primarily in tumor tissues with high levels of TAA expression. A comprehensive summary of the status of development and available clinical results of these TAA×CD137 constructs has been provided in recent reviews (40) (41).

Another major difference between tumors and normal tissues is that protease activity is generally up-regulated in tumor microenvironment through upregulation of protease expression, activation of zymogen, down-modulation of inhibitor expression, or a combination of these effects (42). Multiple extracellular proteases such as matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA), matriptase, etc. are widely overexpressed in different tumors but with limited expression in normal tissues. These proteases play important roles in many aspects of tumor biologic processes involved in tumor signaling, angiogenesis, tumor growth and metastasis (43). To take advantage of the rich protease activity in tumors, one strategy is to design a conditionally activatable agonist by blocking the antigen binding site of the antibody with a mask that is covalently attached to the antibody through a protease cleavable linker. In such antibody configuration, the masked agonist remains largely inactive in circulation and normal tissues. However, when in tumor microenvironment (TME), the masked antibody can be enriched by binding with upregulated antigen and then permanently activated through removal of masking peptides by rich proteases in the TME to expose the binding site of the antibody. This allows activation of the agonist preferentially in tumor (Figure 4). Such strategy has been tested successfully in both preclinical and clinical settings for anti-CTLA-4 antagonist antibody ADG126 (42). It has been shown, for example, a masked anti-murine CD137 agonist (Pb-Tx 1D8) has been generated with antitumor efficacy and reduced toxicity in mouse models (44). Recently, ADG206, a protease-activatable masked species cross-reactive anti-CD137 with enhanced Fc crosslinking (22), has entered clinical investigation (NCT05614258), which would provide direct assessment of the masking strategy in the costimulatory agonist therapeutics. Other approaches taking advantage of the differences between tumors and normal tissues to achieve preferential tumor activation have also been explored. For example, a conditional anti-CD137 agonistic antibody STA551 that binds and crosslink CD137 in ATP-rich condition in tumor microenvironment has been generated with improved safety in human CD137 knock-in mouse models (45).

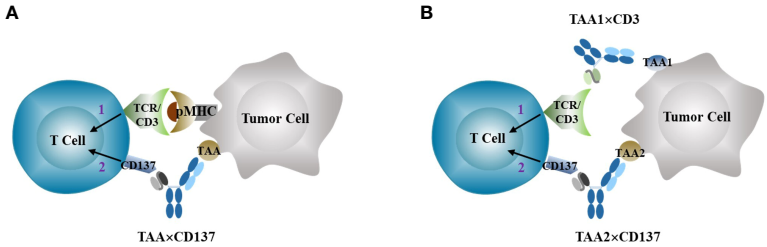

FIGURE 3
Anti-CD137 agonists (TAA×CD137 as example here) stimulates costimulatory signal 2 to enhance tumor-specific T cell activation primed by signal 1 through either neoantigen stimulated TCR activation **(A)**, or bispecific TAA×CD3 T cell engagers **(B)**.

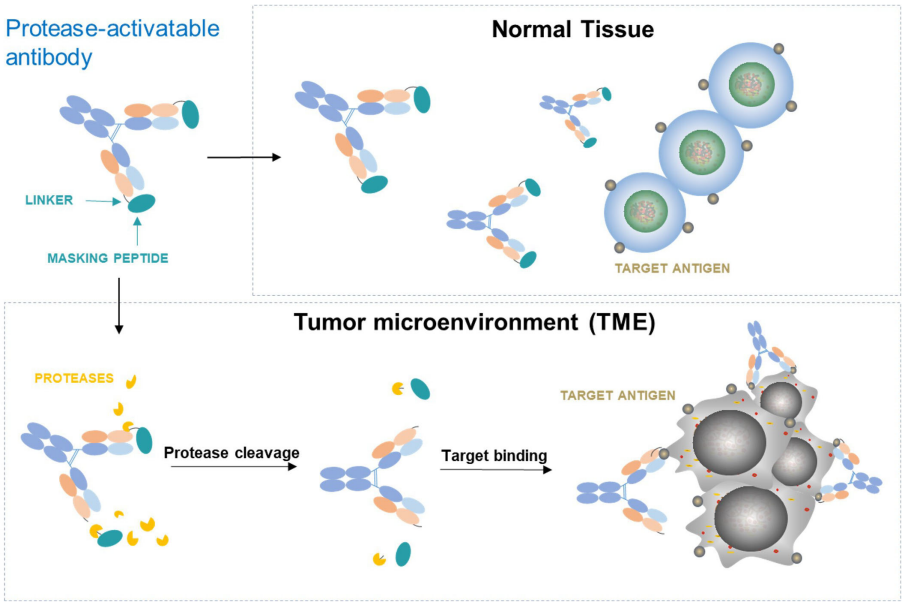

FIGURE 4
Conditionally activatable agonists controlled by antibody masking and protease-cleavable linker.

Combinations

To date, more than 20 different CD137-targeted agonistic antibodies have been developed and entered in clinical trials (40) (41). Although these trials have demonstrated various clinical responses, the overall activity in general is modest by monotherapy with these agonists. Combinations with other therapeutics are under testing, and anti-PD-1/L1 is the most common partner with the anti-CD137 agonists in the clinical investigations and improved therapeutic outcomes have been observed (3). In addition to the traditional drug-drug combinations, one other simple strategy for combination is to construct bispecific or multi-specific therapeutics, which sometimes may achieve greater activity than that of the individual drug-drug combinations. These bi- or multi-specifics targeting both CD137 and other immune-related pathways are under development and early clinical testing, such as CD137 with OX-40 (46), CD40 (47), PD-1 (48), PD-L1 (49–51), CD47 (52). Of note, various PD-L1 × CD137 bispecific antibodies have been developed. PD-L1 was used both to target PD-1 checkpoint pathway and agonistically crosslink CD137 in TME. Clinical results from the bispecific PD-L1 × CD137 antibody GEN1046 have shown signs of T cell activation and monotherapy antitumor activity in patients refractory to anti-PD-1 therapy, with 20% patients developing controllable abnormalities in liver function test (53).

The potential of the anti-CD137 and anti-CTLA-4 therapies to synergistically treat tumors has been well-established in preclinical models and confirmed by clinical trials using cross-reactive therapeutic antibodies against both targets. Combining these therapies is not only biologically compelling for their anti-tumor efficacy but also to reduce the toxicity of each monotherapy, making the anti-CD137/anti-CTLA-4 combination the top choice for clinical translation. Anti-CD137 agonists such as ADG106/206 and anti-CTLA-4 antagonists such as ADG116/ADG126, which are related to the CD137 and CTLA-4 pathways, have shown promise in preclinical studies and are being evaluated in clinical trials (11, 22, 54, 55).

Other therapeutics that can take advantage of the T cell costimulatory activity of CD137 agonists include vaccines, certain chemotherapies, and/or radiotherapy. Costimulatory signals are required for the generation of a robust and long-term T cell response, but the target specificity of this response is determined by the TCR/pMHC recognition, also known as the priming signal or Signal 1. Combining a cancer vaccine that can initiate the tumor-specific priming signal with the costimulatory agonists is a conceptually appealing approach to enhance the targeted antitumor immune response. This approach has been evaluated in mouse tumor models with promising results, showing that CD137 agonist antibodies have unique potential to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine (56). Multiple personalized or neoantigen cancer vaccines have entered clinical investigations in combination with checkpoint inhibitors, mostly anti-PD-(L)1, to overcome resistance to immune checkpoint inhibitors (57, 58). RNA-based tumor vaccines in combination with checkpoint inhibitors have also demonstrated durable objective responses in anti-PD1 experienced patients with unresectable melanoma, accompanied by the induction of strong CD4+ and CD8+ T cell immunity against the vaccine antigens (59). It is yet to be seen but worth exploring the combination of cancer vaccines and anti-CD137 agonists in a clinical setting.

CD3 bispecific T cell engagers (TCE) can directly trigger Signal 1 and combining them with the anti-CD137 agonist as Signal 2 can overcome resistance to bispecific TCE treatment in T cell-cold solid tumors in preclinical animal tumor models (60), as conceptually illustrated in Figure 3B.

Translational research

Since Melero et al. first reported that agonist anti-CD137 monoclonal antibodies can eradicate transplanted mouse tumors through enhanced CD8+ T-cell antitumor immunity over two decades ago (61), extensive studies have been conducted to evaluate the antitumor activity, toxicity, and mechanisms of anti-CD137 agonists and their combinations with other agents using syngeneic mouse tumor models, which greatly facilitated our understanding of CD137-targeted immunotherapies (55). These studies either involved mouse CD137 surrogate antibodies in wild-type mouse background or human CD137 agonists but in human CD137 knock-in mouse background due to the lack of mouse cross-reactivity of many human CD137 agonist antibodies. One caveat is that the human CD137 knock-in mice may not fully recapitulate CD137 biology, as the mouse CD137 ligand does not bind to human CD137 (62). Interestingly, ADG106, as well as its conditionally activatable, Fc-enhanced form ADG206, bind to a conserved epitope of CD137 from human, monkey, and rodents, allowing direct assessment of their pharmacological/toxicological properties in wild-type mice, which may better mimic clinical conditions. In preclinical studies, ADG106 induces robust single-agent antitumor responses in multiple syngeneic tumor models and synergizes with anti-PD-(L)1 or anti-CTLA-4 checkpoint inhibitors, without showing significant toxicity (11) (22), which translated to patient studies. In clinical trials, ADG106 stimulates CD4+ and CD8+ T-cell and NK cell proliferation and proinflammatory interferon-gamma (IFN-γ) release while displaying a low risk for adverse immune responses. Notably, ADG106 treatment alone induces significant dose-dependent increases of soluble CD137, which is further enhanced when combined with anti-PD-1 antibody (63). Similar results were observed in other anti-CD137 studies and demonstrated that soluble CD137 can be a dynamic biomarker to monitor agonist CD137 immunotherapies (9) (64).

Concluding remarks

Current checkpoint blockade therapies have shown impressive benefits in cancer treatment, but only for a minority of patients. However, recent years have seen a shift towards exploring immuno-oncology agents beyond PD1/PDL1 inhibitors in clinical trials (65). There is a pressing need for agents that can further enhance T cell immunity for cancer treatment while also having improved safety profiles. Targeting CD137, a potent T cell costimulatory receptor, with agonist antibodies shows promise for cancer immunotherapy beyond its successful application in CAR T cells (66). It is encouraging to see that multiple strategies have been employed to develop the next generation of anti-CD137 agonists that have strong agonistic activity, increased tumor specificity, and reduced agonism in normal tissues to minimize immunotoxicity. Furthermore, the success of costimulatory CD137 agonists depends on their synergistic and rational combinations with other therapeutics or CD137-based bi- or multi-specific antibodies. With many of these new agents entering clinical investigations, the next few years hold great promise for CD137-targeted immunotherapies.

Author contributions

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Conflict of interest

Both authors are employed by Adagene Inc. and have an ownership interest in Adagene.

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Heterogeneity in functional genetic screens: friend or foe?

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Functional genetic screens to uncover tumor-intrinsic nodes of immune resistance have uncovered numerous mechanisms by which tumors evade our immune system. However, due to technical limitations, tumor heterogeneity is imperfectly captured with many of these analyses. Here, we provide an overview of the nature and sources of heterogeneity that are relevant for tumor-immune interactions. We argue that this heterogeneity may actually contribute to the discovery of novel mechanisms of immune evasion, given a sufficiently large and heterogeneous set of input data. Taking advantage of tumor cell heterogeneity, we provide proof-of-concept analyses of mechanisms of TNF resistance. Thus, consideration of tumor heterogeneity is imperative to increase our understanding of immune resistance mechanisms.

KEYWORDS

heterogeneity, CRISPR-Cas9, genetic screens, therapy resistance, TNF

Introduction

The utility of functional, CRISPR-Cas9 genetic screens in understanding immune resistance mechanisms and, by extension, their value in identifying novel therapeutic targets has become increasingly clear in recent years. Multiple research groups have used such screens to elucidate immunologically active pathways in tumor cells and presented strategies to (therapeutically) exploit them to combat cancer, both *in vitro* (1–11) and *in vivo* (5, 12–15). *In vitro*, such screens have almost invariably been performed with genome-scale libraries in one (or few) tumor cell line(s), whereas *in vivo* screens have been performed using smaller, focused libraries in single tumor cell lines. The reason that screens have largely been limited to single cell lines in publications is a technical one: to ensure maintenance of library complexity (i.e., sufficient replication of each genetic perturbation), and thus fidelity and confidence of the hits identified, a(n extremely) large number of cells need to be used in such screens, making the inclusion of multiple cell lines labor-intensive. Despite this limitation, their success and fidelity were demonstrated by virtue of their identification of common pathways by several groups.

By and large they comprise the TNF, IFNγ, antigen presentation and autophagy pathways [reviewed by us (16) and others (17, 18)].

However, these genetic screens do occasionally differ in terms of the exact nodes that they discover within the identified pathways, offering glimpses at potential context-dependent vulnerabilities. This is seen most prominently in one of the few publications in which multiple cell lines were employed (5). Because the screens were performed in the same lab, technical and methodological variation is limited. In those parallel screens, the loss of *TRAF2* was able to sensitize all but one tumor cell line to T cell attack. For this gene in particular, we validated that different tumor cell lines may indeed not all be equally dependent on *TRAF2* for their immune resistance, with some cell lines relying (more) on *BIRC2*, whereas others require inactivation of both genes in order to be sensitized to T cell challenge (1). These observations thus underscore the need to scale up these screens to add to their fidelity and offer insight into the context of identified hits. Because of their limited scale, heterogeneity between tumor cell lines in terms of intrinsic immune resistance mechanisms is currently largely ignored in the design of CRISPR-Cas9 screens, limiting our understanding of immune-resistance mechanisms and preventing us from predicting which cell lines and, by extension, which tumors will respond to specific forms of immunotherapy. In this perspective we will outline sources of tumor heterogeneity, how this may negatively influence CRISPR-Cas9 screens and how to take advantage of those mechanisms in the design of these screens.

Heterogeneity: nature and causes

Tumor heterogeneity exists in different forms and is caused by multiple processes. Intertumor heterogeneity (i.e., the differences between different tumors), intratumor heterogeneity (i.e., the difference between different tumor cells/clones/populations/regions of the same tumor) and heterogeneity in the tumor micro-environment (i.e., the difference in the anatomical location and non-tumor cell infiltration between [different] tumors and/or metastases) all contribute to the *smorgasbord* we term cancer. These mechanisms of heterogeneity not only co-exist, but frequently also actively influence one another. For example, different metastases of the same tumor in distinct anatomical locations may experience different growth signals and thus display preferential outgrowth of different subpopulations (19–21). In addition, the genetic heterogeneity within tumors can surpass even that between tumors of different individuals (22, 23). Even different single cells within the same tumor can have remarkably different characteristics [reviewed in (24)].

This heterogeneity is manifested through a variety of different mechanisms. They can be summarized in four, central concepts: germline differences, genomic instability, selection by exogenous means and obligate co-dependency of tumor subpopulations. For each of these, clinical evidence illustrates how they can result in tumor heterogeneity. Germline differences are perhaps best characterized within hereditary cancers. For example, hereditary breast cancer cases generally have poorer prognoses than sporadic cases (25). Genomic instability also, a core hallmark of cancer-causing mutations and other genomic aberrations such as genetic duplications or deletions, can lead to inter- and intratumor heterogeneity. This can be driven by, for example, enhanced *APOBEC3* activity in late-stage cancers which promotes the stochastic mutation of the tumor genome (26). Furthermore, non-tumor driven selection, for example through therapy, can result in heterogeneity as tumor subclones with therapy-resistant traits are selected for (27, 28). Lastly, tumors can also evolve to be heterogeneous through the common, co-dependent evolution of different tumor cell subpopulations. In such a symbiotic relationship, one population within the tumor provides growth stimuli to another and, in some cases, this may even be reciprocated (29–31).

Heterogeneity affects immune sensitivity of tumors

Heterogeneity can also affect the sensitivity of tumors challenged by multiple different inflammatory cytokines and/or cells of the immune system. This may occur in a general sense, but could also impact specific immune effector pathways. The same general concepts of tumor heterogeneity are involved in these processes (Figure 1).

Intertumor heterogeneity is perhaps most evident for the tissue from which a tumor arises. The identity of this tissue in and of itself can already determine immune sensitivity. For example, cancers arising from intrinsically (more) hypoxic tissues, such as melanoma, have heightened expression of cIAP1. These tumors therefore display enhanced resistance against TNF (32). Extending these observations, a recent meta-analysis of tumor-intrinsic determinants of ICB sensitivity identified multiple strong predictors of response for individual tumor types, but those

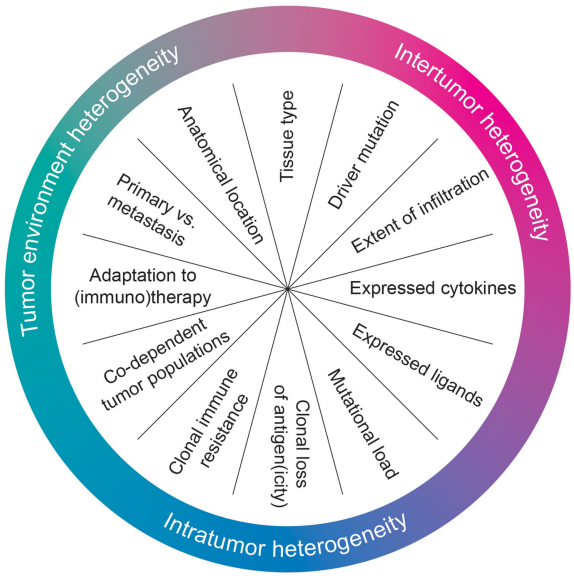

FIGURE 1
Heterogeneity in immune sensitivity mechanisms.

factors fail to predict well in a tumor type-agnostic fashion (33), implying tumor-type specific mechanisms to be at play. Intertumor heterogeneity also manifests through heterogeneity in driver mutations, which can differentially affect the antitumor immune response. An example of this is the generation of an immunosuppressive TME driven by the loss of PTEN (34). $KRAS^{G12C}$ and several p53 mutations, too, alter immune sensitivity (35, 36).

Intertumor heterogeneity can also more broadly influence immune status, being associated with both mutational load (37) and immune infiltrate (38). Each of these may influence which type of immune pressure, and of what strength, a tumor encounters. Another determinant concerns the expression of activating and inhibitory immune ligands, which also differ between tumors and/or tumor types. This heterogeneity in receptor expression may occur upon induction by signals from the TME, such as the differential strength of induction of PD-L1 in different tumors (and tumor cell lines) (39, 40). This phenomenon is particularly of interest as PD-L1, being the main ligand for the inhibitory T cell checkpoint PD-1, is a key target for immune-checkpoint blockade (41–43). Diversity in receptor expression may also be more deeply ingrained, such as the genetically-encoded, patient-specific repertoire of inhibitory receptors for NK cells (immune effector cells that rely on a combined input of activating and inhibitory ligands for their activation) (44–47). This is not only true for cell-surface bound ligands, but equally for tumor cell-derived cytokines or other soluble factors secreted (only) by specific tumors. For example, tumor cell-derived CCL2 indirectly dampens $CD8^+$ T cell responses (48) while, additionally, induction of the Wnt/β-catenin signaling pathway leads to T cell exclusion (49).

Intratumor heterogeneity can equally influence immune sensitivity. While some of the above mechanisms may also be evident within a tumor, such as local expression of cytokines and/or immune ligands, other phenomena are also at play. For example, regions within the tumor can lose components of the antigen-presentation machinery, specific T cell antigens or HLA alleles, limiting T cell recognition (50–52). At the same time, such tumor adaptations may (locally) attract otherwise absent immune cells, as was recently shown for Vδ1 and Vδ3 T cells in $B2M^{MUT}$ colorectal cancer (53). Additionally, tumor subclones can contain mutations in key immune signaling nodes, even before onset of therapy. They include mutations in *JAK1*, responsible for transmitting IFNγ signals, and in *CASP8*, responsible for the final, decisive step in the apoptotic cascade initiated by TNF (54, 55). Furthermore, different, interdependent subpopulations may contribute to intratumor heterogeneity. In a particularly elegant study, it was demonstrated that IFNγ pathway-mutant tumors are more sensitive to $CD8^+$ T cell-mediated eradication due to the loss of protection by IFNγ-induced PD-L1, but become more resistant when intermixed with PD-L1-producing wildtype tumor cells (56). This intratumor heterogeneity is enhanced once (immuno)therapy is administered to the patient tumor, with ample opportunity for selection of escape mutants (52, 57–64).

Lastly, the anatomical location of the tumor may affect immune sensitivity. First, there is a purely technical consideration: the way in which immune sensitivity mechanisms are studied influences how the biology of the pathway manifests. For example, IFNγ has seemingly opposing effects on tumor cell viability *in vitro* and *in vivo*: the cytostatic effects of IFNγ largely inhibit tumor cell growth *in vitro*, whereas *in vivo*, the induction of PD-L1 by IFNγ provides a strong, cytoprotective effect that overcomes those inhibitory effects (16, 65, 66). Additionally, and perhaps obviously, some immune pathways cannot be studied at all *in vitro* because of the use of simplified model systems: either cell types, ligands or cytokines can be missing. The influence of tumor location on heterogeneity has also been demonstrated clinically: different distant metastases may have entirely different TMEs, (neo)antigen burden and immune resistance mechanisms (19, 63, 67, 68). Along these lines, a recent meta-analysis of >2,000 patients showed that genetic alterations in IFNγ signaling components that are present prior to treatment do not necessarily diminish ICB response (69).

Approach to counteract heterogeneity in CRISPR-Cas9 immune screens

Heterogeneity thus has near limitless influence on the sensitivity of tumors to eradication by the immune system. How can we meaningfully combat, and perhaps even exploit, this heterogeneity in CRISPR-Cas9 screens for tumor-intrinsic, immune sensitivity modifiers? By integrating large amounts of functional screening and *omics* data from many different settings and contexts, one can more precisely annotate tumor cell nodes of immune sensitivity. Specifically, this integration will yield either biomarkers, which mark cell lines in which a particular immune sensitivity node is active, or will generate mechanistic hypotheses that explain why a given node is seemingly inactive in a given cell line. Based on the mechanistic sources of heterogeneity described above, ideally one would derive *omics* and screening data from as many sources as possible. These would include (epi)genomic, transcriptomic and proteomic *omics* data. At the same time, the screening data should be derived from both *in vitro* and *in vivo* screens from as many genetic backgrounds as possible [reviewed in (16)]. Such an undertaking however, would require immense investments of both time and funding.

Proof-of-concept analyses exploiting cell line-to-cell line heterogeneity

While a comprehensive catalogue of screening data is currently lacking, other domains of research have already embraced the concept of heterogeneity more comprehensively. In fact, in order to find an Achilles' heel for specific cancers, many cell lines have already been deeply characterized. A multi-decade, multi-national effort, collected within the DepMap database, has screened >1800 cell lines using genome-scale perturbation libraries to identify cancer (type)-specific dependencies. Aside from these functional genetic screens, the cell lines used in these studies have also been extensively characterized, including the collection of RNA, DNA, epigenetic, metabolic and drug-sensitivity metrics (70–72). The use

of these databases has allowed investigators to identify novel therapeutic targets in a variety of cancer indications (73–75). An important element lacking from this database then, is an annotation of which genes can be considered immune sensitivity modifiers.

Interestingly, because of the extent of this database, both in terms of cell line number and cell line characterization, we can perform a proof-of-concept analysis for immune sensitivity modifiers that exploit heterogeneity. Specifically, we can look at modifiers of TNF sensitivity. As more than 300 cell lines in the DepMap produce TNF, we can compare the effects of gene knockouts in these cell lines compared to those that do not produce TNF, to identify factors sensitizing tumor cells to TNF (which, using the excellent portal is trivial to accomplish). By performing this analysis, we could find factors whose ablation reduces viability of TNF^{Hi} cell lines specifically (Figure 2A). Indeed, many of those we had already identified and validated ourselves, including *TRAF2*, *BIRC2* (encoding cIAP1) and *RNF31* (Figures 2A, B) (1, 2). However, with such an approach we could identify also novel, potential TNF sensitivity modifiers, such as the EMC family of genes which, though currently not yet validated, we also identified in our meta-analysis of immune sensitivity screens (Figure 2A) (16).

Having established the fidelity of this approach, we could continue by also taking advantage of the size and heterogeneity of the particular database used. In our previous work, we have identified a differential reliance on *TRAF2* and *BIRC2* to establish resistance to TNF in different tumor cell lines. While it had been difficult to fully comprehend this differential sensitivity before, given that TRAF2 and cIAP1 are thought to signal in a linear fashion, we could now make transcriptomic comparisons between TNF^{Hi} cell lines in which both *TRAF2* and *BIRC2* sensitize, those in which solely *BIRC2* knockout sensitizes, those in which solely *TRAF2* knockout sensitizes, or those cell lines in which neither the loss of *TRAF2* nor the loss of *BIRC2* reduces the viability of the affected cell line (Figure 2C). These analyses can yield biomarkers of specific populations (Figures 2D–J). For example, high *HLA-F* expression marks populations that will respond solely to *TRAF2* inhibition (Figure 2D). These analyses can also provide mechanistic insight. For example, the observation that *BIRC3* expression is higher in cell lines that respond solely to the loss of *TRAF2* compared to those that respond to either *TRAF2* or *BIRC2* loss, implies that this protein compensates for the loss of its paralog *BIRC2* (Figure 2F).

As a second proof-of-concept for discovery of immune sensitivity modifiers that exploit heterogeneity using the DepMap, we performed a similar analysis for cells producing TNF-related apoptosis-inducing ligand (TRAIL, encoded by the gene *TNFSF10*; Figure 2J). Here, we identified the loss of both *CFLAR* and *RELA* to specifically sensitize those cells capable of producing TRAIL, in line with published literature (Figures 2J, K) (76, 77). Using the transcriptomic data of those same cell lines, we may even begin to speculate as to how these cells are capable of surviving in the presence of TRAIL. These cells seemingly induce transcription of genes that protect against TRAIL-induced cell death, including the aforementioned *CFLAR*, but also *TRADD* and *TNFAIP3* (Figures 2L–N) (76–78). In doing so, they may gain a previously described proliferative advantage of TRAIL signaling (78), which may explain their higher level of expression of the TRAIL receptor, *TNFRSF10A* (Figure 2O), but this prediction awaits functional validation.

Beyond these transcriptomic comparisons, we can exploit the DepMap to find ways of targeting these specific tumor cell subpopulations. Again, to probe the fidelity of such an approach, we compared the drug sensitivity between the TNF^{Lo} and TNF^{Hi} cell lines in the DepMap. With this analysis, we could, at least in part, recapitulate the genetic analysis, identifying birinapant, an inhibitor of cIAP1 to sensitize TNF^{Hi} cell lines more than TNF^{Lo} cell lines (Figures 3A, B). We validated this therapeutic approach previously in conditions of high concentrations of TNF (i.e., T cell attack) (1). Using the drug sensitivity database, we could also identify specific inhibitors for the cell lines differentially dependent on *TRAF2* and *BIRC2* for their resistance against TNF. For those cell lines that particularly depend on *BIRC2* we found that ZD-7114, a β3-adrenoceptor agonist, is a potential pharmaceutical strategy (Figure 3C). In cell lines that depend on *TRAF2*, we could find a specific sensitivity to CAY10576, an IKKε inhibitor (Figure 3D). IKKε is known interact with TRAF2, and its identification may thus have a clear mechanistic basis (79).

Considerations for the future

While the above analyses show the promise of integrating heterogeneity in target discovery, they are preliminary and marred by assumptions (e.g., can we realistically assume that TNF-producing cells are a good model for cell experiencing T cell-derived TNF? Can we assume that protein levels of TNF scale linearly with TNF mRNA expression)? Therefore, and as mentioned above, the true complexity of tumor-immune interactions, and forms and mechanisms of heterogeneity at play require more data to be integrated in these models. Firstly, and perhaps most easy to accomplish, the field should invest in performing more tumor : T cell screens, to complement those that have already been reported in key publications in the recent past (1, 3–7, 12–16, 18, 75, 80). These screens, combined with deep characterization as performed for the DepMap, should result in a more granular understanding of genotype – phenotype interactions, as demonstrated here with our proof-of-concept analyses (Figure 2). An analogous approach was already taken for NK sensitivity (75). Obviously, such screens only scratch the surface of the different types of heterogeneity outlined above. One could imagine that with time, and significant investment, the screens can be performed in parallel in a large number of settings. For example, they can be performed with different (e.g., NK cells, as was done in (75), or 'exhausted' vs. polyfunctional T cells), or more complex co-culture systems (e.g., tumor : T cell : NK cell combinations), more environmental perturbations (e.g., nutrient starvation, hypoxia, highly acidic conditions), in *in vivo* mouse models (as in (5, 12, 13), in isogenic tumor cell lines with specific alterations [as was done in (5)] or even in combination with specific therapeutics (e.g. anti-CTLA-4 or anti-PD-1). Ultimately, such genetic screens will improve our understanding of important immune resistance mechanisms,

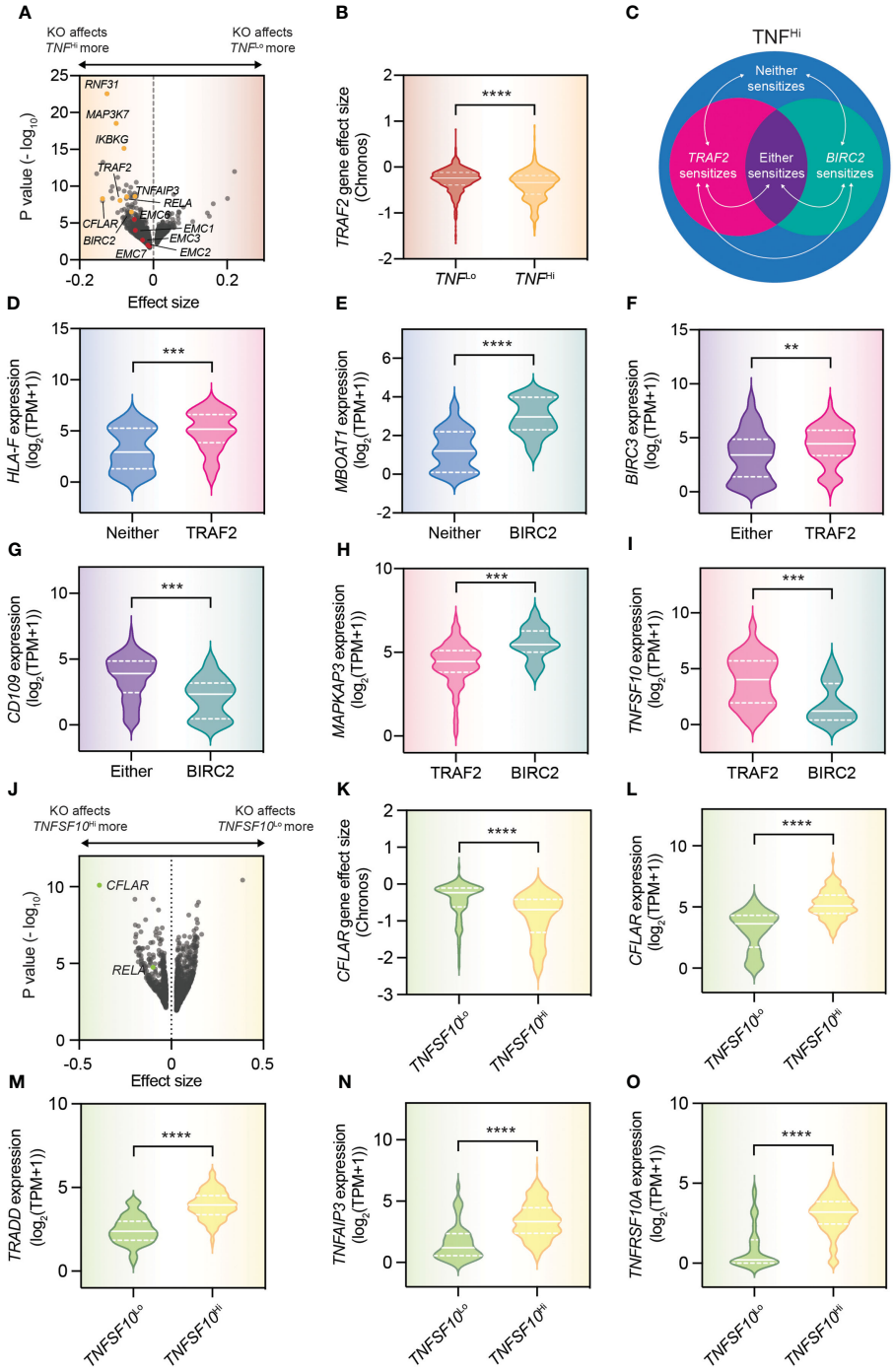

FIGURE 2
DepMap dependency analyses allow understanding of heterogeneity in immune resistance mechanisms. **(A)** Volcano plot that compares the gene perturbation effects in TNF^{Hi} [i.e., >0.5 $\log_2$(TPM+1)] and TNF^{Lo} (i.e., 0 read counts for TNF) cell lines. **(B)** Comparison of the effect of *TRAF2* knockout in TNF^{Hi} and TNF^{Lo} cell lines. **(C)** Schematic diagram indicating the populations analyzed in the panels that follow. Only TNF^{Hi} cell lines were used in the analyses. **(D–I)** Violin plots of the expression of indicated genes for the indicated populations (cell lines were deemed sensitive when their CERES score was < -0.3 and insensitive when their CERES score was > -0.1). Statistics were performed by Student t test. The solid white line indicates the population median, with the bottom and top dashed white lines indicating the first and third quartiles, respectively. **(J)** Volcano plot comparing the gene perturbation effects in *TNFSF10*^{Hi} [i.e., >5 $\log_2$(TPM+1)] and *TNFSF10*^{Lo} (i.e., 0 read counts for *TNFSF10*) cell lines. **(K)** Comparison of the effect of *CFLAR* knockout in *TNFSF10*^{Hi} and *TNFSF10*^{Lo} cell lines. **(L–O)** Violin plots of the expression of indicated genes for the indicated populations. Statistics were performed by Student t test. The solid white line indicates the population median, with the bottom and top dashed white lines indicating the first and third quartiles respectively. **p < 0.01, ***p < 0.001, ****p < 0.0001.

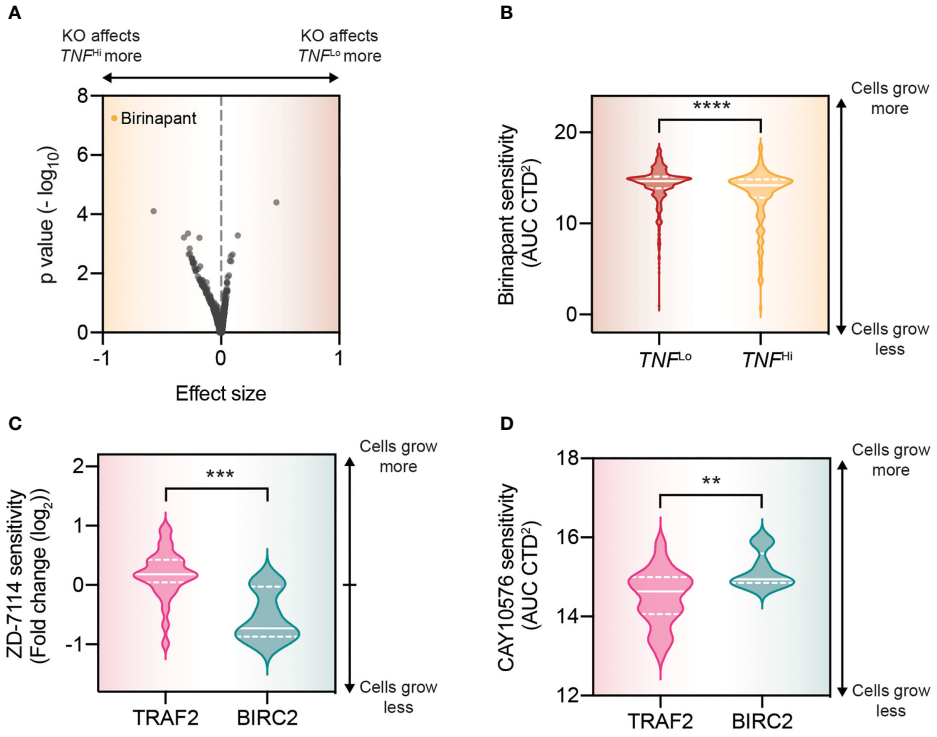

FIGURE 3
DepMap drug analyses allow for the potential exploitation of heterogeneity in immune resistance mechanisms. **(A)** Volcano plot that compares the drug treatment effects in TNF^{Hi} (i.e., >0.5 log_2(TPM+1)) and TNF^{Lo} (i.e., 0 read counts for TNF) cell lines. **(B)** Comparison of the effect of birinapant in TNF^{Hi} and TNF^{Lo} cell lines. **(C)** Violin plots of the drug effects of ZD-7114 for the indicated populations. Statistics were performed by Student t test. The solid white line indicates the population median, with the bottom and top dashed white lines indicating the first and third quartiles respectively. **(D)** As **(C)**, but for CAY10576. **p < 0.01, ***p < 0.001, **** p < 0.0001..

aiming to have as many patients as possible benefit from (personalized) immunotherapy.

Author contributions

DV performed the analyses. DV and DP wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

DP is a co-founder, shareholder and advisor of Immagene B.V. DV is currently employed at Genmab B.V., which is unrelated to this study.

Publisher's note

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Antibody-based soluble and membrane-bound TWEAK mimicking agonists with FcγR-independent activity

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Fibroblast growth factor (FGF)-inducible 14 (Fn14) activates the classical and alternative NFκB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) signaling pathway but also enhances tumor necrosis factor (TNF)-induced cell death. Fn14 expression is upregulated in non-hematopoietic cells during tissue injury and is also often highly expressed in solid cancers. In view of the latter, there were and are considerable preclinical efforts to target Fn14 for tumor therapy, either by exploiting Fn14 as a target for antibodies with cytotoxic activity (e.g. antibody-dependent cellular cytotoxicity (ADCC)-inducing IgG variants, antibody drug conjugates) or by blocking antibodies with the aim to interfere with protumoral Fn14 activities. Noteworthy, there are yet no attempts to target Fn14 with agonistic Fc effector function silenced antibodies to unleash the proinflammatory and cell death-enhancing activities of this receptor for tumor therapy. This is certainly not at least due to the fact that anti-Fn14 antibodies only act as effective agonists when they are presented bound to Fcγ receptors (FcγR). Thus, there are so far no antibodies that robustly and selectively engage Fn14 signaling without triggering unwanted FcγR-mediated activities. In this study, we investigated a panel of variants of the anti-Fn14 antibody 18D1 of different valencies and domain architectures with respect to their inherent FcγR-independent ability to trigger Fn14-associated signaling pathways. In contrast to conventional 18D1, the majority of 18D1 antibody variants with four or more Fn14 binding sites displayed a strong ability to trigger the alternative NFκB pathway and to enhance TNF-induced cell death and therefore resemble in their activity soluble (TNF)-like weak inducer of apoptosis (TWEAK), one form of the natural occurring ligand of Fn14. Noteworthy, activation of the classical NFκB pathway, which naturally is predominately triggered by membrane-bound TWEAK but not soluble TWEAK, was preferentially observed with a subset of constructs containing Fn14 binding sites at opposing sites of the IgG scaffold, e.g. IgG1-scFv fusion proteins. A superior ability of IgG1-scFv fusion proteins to

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trigger classical NFκB signaling was also observed with the anti-Fn14 antibody PDL192 suggesting that we identified generic structures for Fn14 antibody variants mimicking soluble and membrane-bound TWEAK.

KEYWORDS

agonistic antibodies, cell death, FcγR, Fn14, NFκB, TNF receptor superfamily, TWEAK

Introduction

Fibroblast growth factor (FGF)-inducible 14 (Fn14) is an unusual small member of the tumor necrosis factor (TNF) receptor superfamily (TNFRSF) with an extracellular domain only comprising a single cysteine rich domain, and an intracellular tail of 28 amino acids which contains a binding site for proteins of the TNF receptor associated factor (TRAF) family (1, 2). Fn14 is dynamically and highly expressed during development but in healthy adult organisms Fn14 expression is largely limited to heart, ovary and mesenchymal progenitor cells (1, 3). Fn14 expression is, however, strongly upregulated in non-hematopoietic cells after tissue injury irrespective of the underlying reason (4). Since tumor development is inevitably associated with tissue damage and tissue remodeling, Fn14 expression is also often high in tumor cells of non-hematopoietic origin and non-transformed non-hematopoietic cells of the tumor microenvironment (4, 5). The expression of Fn14 can be therefore considered as a bona fide marker for tissue remodeling and tissue injury. Fn14 signal transduction can be triggered by tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK), a ligand of the TNF superfamily (TNFSF) which occurs in two forms, namely as transmembrane TWEAK (memTWEAK) and as soluble TWEAK (sTWEAK) which is released from memTWEAK by proteolytic processing (6). Similar to other ligands of the TNFSF, memTWEAK and sTWEAK form homotrimeric molecules which can bind three receptor molecules (4). TWEAK expression has been shown for a variety of cell lines and cell types by immunohistochemistry and RT-PCR, but memTWEAK expression has doubtless only be demonstrated on monocytes, dendritic cells and natural killer (NK) cells and a very few tumor cell lines (4).

Importantly, sTWEAK and memTWEAK trigger different states of Fn14 activity. In response to sTWEAK Fn14 efficiently stimulates the alternative NFκB signaling pathway and sensitizes for TNF-induced cell death (4). Transmembrane TWEAK triggers the same Fn14 signaling events as sTWEAK but in addition enables Fn14 to activate also the classical NFκB pathway (4). Manifold and complex functions of the TWEAK/Fn14 system have been described in tissue repair and regeneration. For example, it has been demonstrated that the TWEAK/Fn14 system promotes regenerative responses after the injury of muscles, pancreas and the liver (7–9). However, excessive and/or chronic engagement of the TWEAK/Fn14 system can also result in tissue repair-associated adverse effects, such as fibrosis and inflammation (10–12). Thus, dependent on the context and the disease considered, both the inhibition but also the stimulation of Fn14 can elicit beneficial therapeutic effects (4).

The inhibition of the TWEAK/Fn14 system can be straightforwardly achieved by help of soluble Fn14-Fc fusion proteins, TWEAK neutralizing antibodies or blocking, effector function-dead Fn14 antibody variants (4). Specific stimulation of Fn14 signaling is, however, more challenging. Conventional sTWEAK has an extremely low serum half-life (< 20 min) (13) and oligomeric sTWEAK variants, which display memTWEAK-like activity, are modestly produced and are more challenging in translational development than antibodies. The reagents of choice to stimulate Fn14 *in vivo* are therefore agonistic antibodies but here arises two fundamental problems: First, although some anti-Fn14 IgG antibodies can promote to some extend in certain cell lines p100 processing, a hallmark of the alternative NFκB pathway, they are largely not agonistic and require anchoring to Fcγ receptors or oligomerization, e.g. by protein G or antibody crosslinking, to become fully and strongly agonistic (14–16). Antibody oligomerization by protein G or secondary antibodies, however, is no practicable translational option and the FcγR-binding dependent mode of anti-Fn14 agonism is inevitably associated with triggering FcγR effector function what can disturb the anticipated therapeutic effect. Second, if Fn14 antibodies become agonistic by the aforementioned means they mimic memTWEAK, thus mimicry of sTWEAK seems hardly possible with Fn14 antibodies.

Here, we analyzed a variety of tetra-, hexa- and octavalent antibody variants composed of Fn14-specific Fab- and scFv domains with respect to their Fn14 agonism. All the Fab-scFv chimeric multivalent anti-Fn14 antibody variants showed inherent and partly strong memTWEAK-mimicking agonism. Surprisingly, multivalent "scFv" domain-only variants preferentially mimicked sTWEAK activity on Fn14. In sum, these novel potent antibody-based Fn14 agonists with FcγR-independent activity offer an

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; FBS, fetal bovine serum; FcγR, Fcγ receptor; Fn14, fibroblast growth factor (FGF)-inducible 14; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; HC/LC, heavy chain/light chain; memTWEAK, transmembrane TWEAK; MSA, murine serum albumin; MTO, mouse tumor organoid; muTWEAK, murine TWEAK; NFκB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; NK cell, natural killer cell; TNFRSF, TNF receptor superfamily; sTWEAK, soluble TWEAK; TNF, tumor necrosis factor; TRAF1/2, TNF receptor associated factor 1/2; TWEAK; (TNF)-like weak inducer of apoptosis.

alternative to recombinant TWEAK molecules to evaluate the clinical potential of pure Fn14 agonism *in vivo*.

Results

Construction of tetra-, hexa- and octavalent anti-Fn14 variants

In view of the fact that anti-Fn14 antibodies can acquire memTWEAK-like activity upon crosslinking (15), we generated various multivalent variants of the anti-Fn14 antibody 18D1 (14) and analyzed the ability of these molecules to stimulate Fn14 *in vitro*. To obtain tetravalent 18D1 variants, we genetically fused a scFv domain derived of 18D1 to the C-terminus of the heavy chain (HC) or light chain (LC) of 18D1-IgG1(N297A) (Figure 1A, construct 18D1-(1)), a 18D1-IgG1 variant with a point mutation destroying/reducing FcγR binding, resulting in the constructs 18D1-(2) and 18D1-(3) shown in Figure 1A. Alternatively, to have a tetravalent variant with four similarly oriented Fn14 binding sites on the same side of the antibody scaffold, we replaced the variable domains of the heavy (VH) and light chain (VL) of the parental 18D1-IgG1 antibody by scFv:18D1 domains (Figure 1A, construct 18D1-(5)). Hexameric 18D1 variants were furthermore generated by fusing the scFv:18D1 domain to the C-termini of the heavy and the light chain of 18D1-(1) (Figure 1A, construct 18D1-(4)) and by fusing this domain to the C-terminus of the heavy or light chain of construct 18D1-(5) (Figure 1A, constructs 18D1-(6) and 18D1-(7)). Finally, an octameric variant was obtained by fusing the scFv:18D1 domain to the C-terminus of both the heavy and light chain of construct 18D1-(5) (Figure 1A, construct 18D1-(8)). All antibody constructs were produced by transient co-transfection of HEK293 cells with expression plasmids encoding the corresponding Flag-tagged LC and HC variants. Productivity of the parental 18D1 antibody and all variants derived thereof was largely comparable (Figures 1B, C). Western blot analysis under non-reducing conditions suggested, furthermore, that constructs (1) to (4) undergo efficient disulfide bond dimerization while the scFv:18D1-"only" variants showed significant fractions of non-paired light chains (Figure 1C).

Oligovalent 18D1 variants enhance TNF-induced toxicity and trigger the alternative NFκB pathway but substantially differ in their ability to induce IL8

With exception of the parental bivalent 18D1-(1) antibody variant, all anti-Fn14 constructs enhanced TNF-induced toxicity to a comparable extent as sTWEAK with ED50-values below 100 ng/ml (Figure 2A). All the oligovalent 18D1 constructs triggered furthermore robust p100 processing starting at concentrations of app. 20 ng/ml for 18D1-(2) to 18D1-(4) and of app. 200 ng/ml for 18D1-(5) to 18D1-(8). The conventional antibody variant (1), however, remained inactive in this respect and showed no p100 processing even at the highest concentrations of 2 µg/ml (Figure 2B). Similarly, with exception of 18D1-(1) all constructs upregulated TRAF1 expression which is controlled by the alternative NFκB pathway (Figure 2B). With respect to the induction of IL8, however, there was a clear difference between the constructs. IL8 is a prototypic target of the classical NFκB pathway and is accordingly not or only poorly induced by sTWEAK but efficiently by oligomerized sTWEAK and memTWEAK (17). Construct 18D1-(2) and especially construct 18D1-(4) displayed varying but significant and robust IL8 induction and reached in the case of the hexameric construct 18D1-(4) the maximum response that is induced by anti-Flag oligomerized Flag-sTWEAK (Figure 2C). In contrast, all 18D1-derived constructs with replacement of the VH and VL domains by the scFv:18D1 domain (18D1-(5) to 18D1-(8)) remained largely inactive (Figure 2C). Moreover, 18D1-(5) to 18D1-(8), despite their ability to trigger alternative NFκB signaling and enhancement of TNF-induced cell death (Figures 2A, B), inhibited IL8 induction by memTWEAK expressing transfectants and hexameric Fc-sTWEAK which has memTWEAK like activity (Figures 3A, B). In this respect, these constructs again resemble soluble TWEAK which also inhibits the memTWEAK-induced IL8 response (Supplemental Data Figure S1). The IKK2-specific inhibitor TPCA-1 efficiently inhibited IL8 induction by oligomerized sTWEAK and 18D1-(4) but showed no effect on p100 processing (Figures 3C, D) confirming that Fn14-mediated IL8 induction reflected activation of the classical NFκB pathway.

So far, we analyzed the activities of the various 18D1 variants by help of supernatants of cells producing these molecules. As mentioned above, the latent agonistic activity of anti-Fn14 antibodies can be unleashed by antibody crosslinking/oligomerizing reagents such as protein G (15). Therefore, to verify that the agonistic properties observed for the oligomeric 18D1 variants are not due to aggregation of the molecules and indeed mirrors intrinsic activity, we purified the parental antibody along with the prototypic variants 18D-(2) and 18D1-(4) and analyzed them by gel filtration (Figures 4A, B). All three proteins eluted in gel filtration largely as a single molecular species with MW well corresponding to Fc domain-dimerized molecules (Figure 4B). Functional analysis revealed furthermore that purification did not affect the functional properties of the molecules. The purified proteins 18D1-(2) and 18D1-(4) still enhanced TNF-induced cell death in HeLa-RIPK3-FADD$_{KO}$ cells (Figure 4C) and classical (Figure 4D, Supplemental Data Figure S2) and alternative NFκB signaling (Figure 4E) in HT1080 cells.

Format (2) and (4) variants of the anti-Fn14 mAb PDL192 also displays memTWEAK-like activity

To proof that genetic fusion of Fn14-specific scFv domains to anti-Fn14 antibodies generally favors the realization of memTWEAK mimicking agonism, we generated and evaluated construct types (2) and (4) of a second anti-Fn14 antibodies, namely PDL192. Previous studies showed that 18D1 and PDL192 recognize different epitopes on Fn14 (14). Moreover, the two

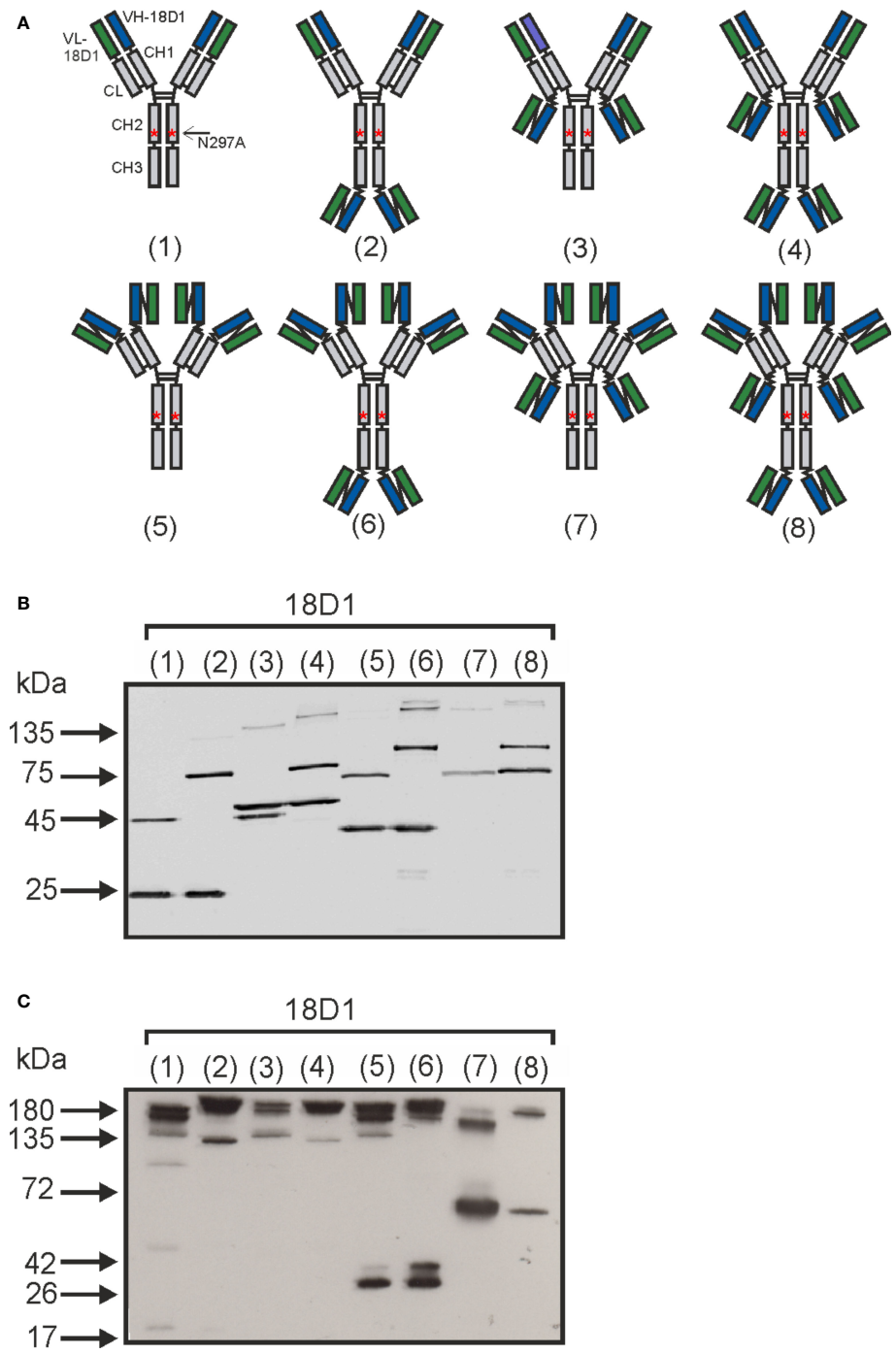

FIGURE 1
Antibody variants of the anti-Fn14-antibody 18D1. **(A)** Scheme of the domain architecture of the various 18D1 variants used in this study. **(B, C)** Western blot analysis of 10 µl supernatant of HEK293 cells transiently expressing the proteins shown in **(A)**. Samples in **(C)** were dissolved in Laemmli buffer without β-mercaptoethanol, thus without reduction.

antibodies also differ in the ability to block TWEAK binding. While 18D1 inhibits TWEAK binding, PDL192 does not compete with ligand binding (14). As reported previously, the IgG1(N297A) version of PDL192 showed no agonism while its tetravalent and hexavalent derivatives, however, stimulated p100 processing and enhanced TNF-induced cell death (Figures 5A, B). Both constructs also elicited strong stimulation of IL8 induction (Figure 5C). These data suggest that type (2) and (4) antibody variants generally confer FcγR-independent memTWEAK-like agonism. To rule out again that the observed agonistic activity of the type (2) and (4) constructs of PDL192 did not result from unspecific aggregation, we purified both constructs by anti-Flag agarose affinity chromatography (Figure 5D). All PDL192 variants proteins were efficiently purified and showed almost no high molecular weight species (Figure 5D). Thus, the observed agonism of type (2) and type (4) anti-Fn14 variants is molecule intrinsic, too.

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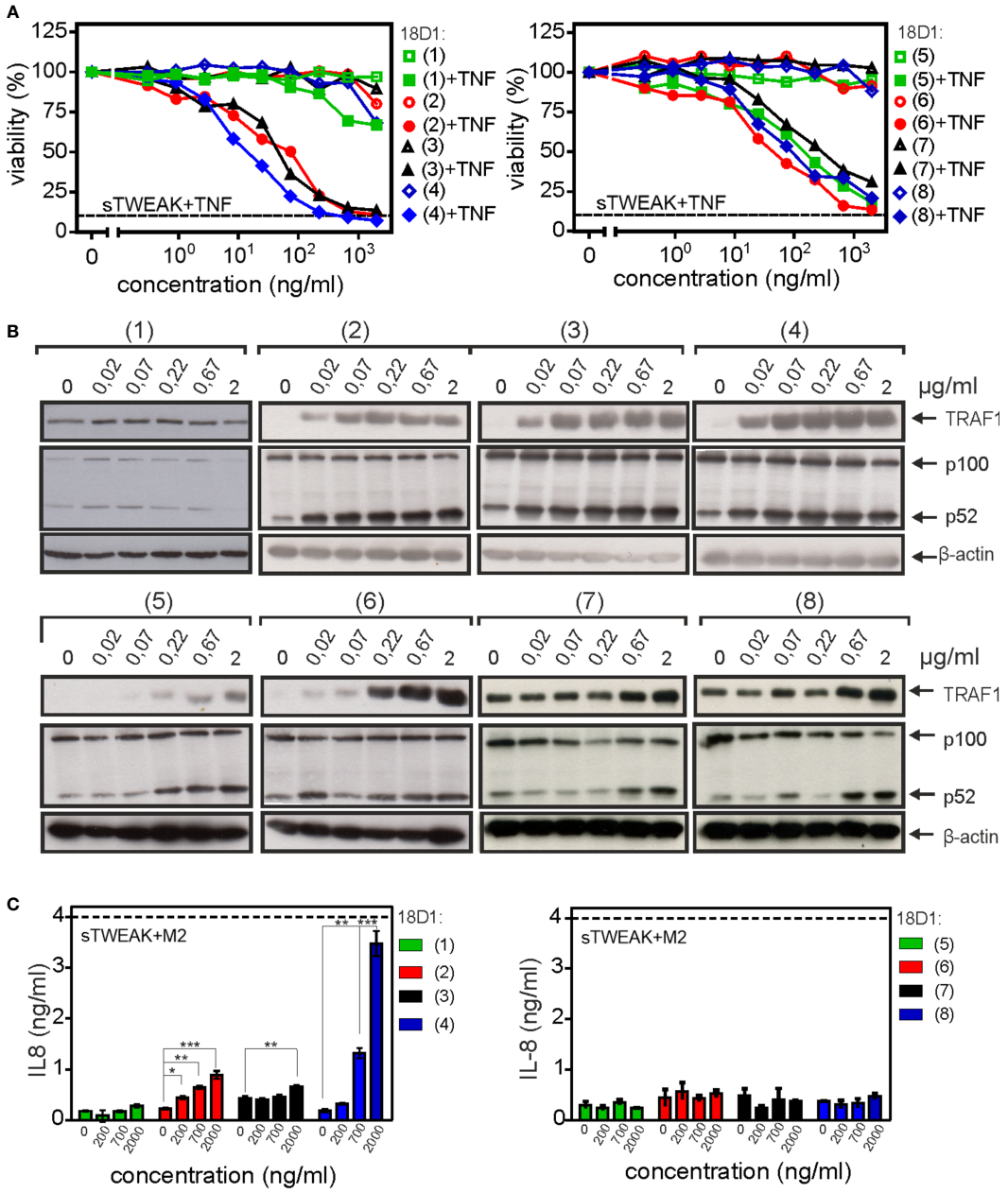

FIGURE 2
Triggering of Fn14 signaling by oligovalent 18D1 constructs. **(A)** HeLa-RIPK3-FADD$_{KO}$ cells were treated with 1 ng/ml TNF which alone induces no or only modest cell death. Cells were treated in addition with cell culture supernatants containing the indicated 18D1 constructs. Cotreatment with (200 ng/ml) Flag-sTWEAK was performed to define maximum TNF killing in TWEAK-sensitized cells indicated by the dotted line. One representative experiment of three is shown. **(B)** HT1080 cells were treated overnight with cell culture supernatants containing the various 18D1 constructs or with Flag-sTWEAK. Total cell lysates were analyzed by western blotting with respect to p100 processing and expression of the alternative NFκB pathway target TRAF1. **(C)** HT1080 cells were treated with the various 18D1 constructs or with anti-Flag M2 oligomerized Flag-sTWEAK over night. IL8 concentrations were evaluate by ELISA. Data shown were averaged from three independent experiments and were analyzed by one way ANOVA and Bonferroni post test. *p < 0.05, **p < 0.01, ***p < 0.001.

Antitumoral activity of 18D1-(2)

Fn14 expressing mouse tumor organoids (MTOs) (Supplemental Data Figure S3), derived from tumors originating from *Apc*$^{ko/ko}$, *Kras*$^{LSL-G12D}$, *Tgfbr2*$^{ko/ko}$ and *Trp53*$^{ko/ko}$ intestinal stem cells that imitate the microenvironment and severity of human colorectal cancer (18) were expanded *in vitro* and injected subcutaneously into the flank of syngeneic C57BL/6J recipients. Two weeks after organoid injections, when they developed into macroscopically established tumors, mice were treated three times per week for two weeks with 200 µg of 18D1-(2) or 18D1-(4) (Figure 6A). For comparison, mice were treated with MSA-muTWEAK, a fusion protein of murine sTWEAK with murine serum albumin (MSA) and thus prolonged serum retention, and Fc-muTWEAK, a

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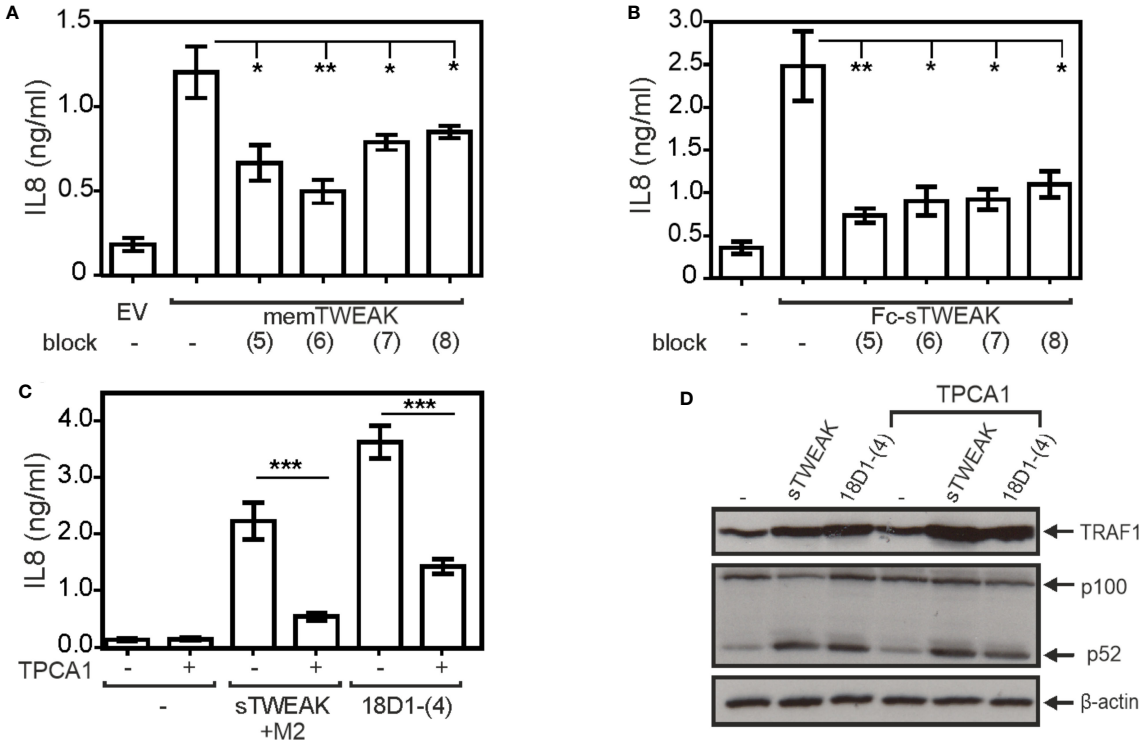

FIGURE 3
18D1-(5) to 18D1-(8) inhibit memTWEAK-induced classical NFκB signaling pathway-mediated IL8 expression. **(A, B)** HT1080 cells were pretreated with 30 µg/ml of the indicated 18D1 variants and were then challenged overnight with memTWEAK and, as a negative control, empty vector (EV) transfected HEK293 cells **(A)** or with 500 ng/ml Fc-sTWEAK **(B)**. Shown are the average of 7 **(A)** and 4 **(B)** independent experiments. Each construct type ((5), (6), (7) and (8)) was compared with the control using two-tailed t-test. *p < 0.05, **p < 0.01 **(C)** HT1080 cells were stimulated with anti-Flag antibody M2 (0.5 µg/ml) oligomerized Flag-sTWEAK (200 ng/ml) or 1 µg/ml 18D1-(4) in the presence and absence of the IKK2 inhibitor TPCA-1 (20 µM). Next day, IL8 production was again determined by ELISA. The effect of TPCA-1 on basal, sTWEAK/M2- and 18D1-(4)-induced IL8 production was evaluated in each case by the two-tailed t-test. ***p < 0.001. **(D)** HT1080 cells were stimulated with 200 ng/ml Flag-sTWEAK (200 ng/ml) or 1 µg/ml 18D1-(4) in the presence and absence of TPCA-1 (20 µM) and the next day, p100 processing and TRAF1 induction were assayed by western blotting.

hexameric murine sTWEAK variant with membrane TWEAK-like activity. Tumor weight and tumor mass were significantly reduced in mice treated with 18D1-(2) and there was also significantly reduced tumor mass after Fc-muTWEAK treatment (Figures 6B, C). MSA-muTWEAK and 18D1-(4) treated mice showed on average reduced tumor growth but this did not attain statistical significance (Figures 6B, C).

Discussion

Against the background that Fn14 is regularly expressed at high levels on tumor cells of non-hematopoietic origin but also on various cells of the tumor microenvironment (4, 5), it is considered as a promising target for cancer therapy. In order to use the tumor-associated Fn14 expression therapeutically, three different approaches can be considered.

First, killing of $Fn14^+$ tumor cells with anti-Fn14 antibodies with natural cytotoxic effector activity, such as ADCC (antibody-dependent cellular cytotoxicity), ADCP (antibody-dependent cellular phagocytosis) and CDC (complement-dependent cytotoxicity) or an artificial cytotoxic payload, such as a conjugated antitumor drug. Indeed, antitumoral efficacy has been demonstrated in preclinical *in vivo* models with the ADCC-inducing anti-Fn14 antibodies PDL192/Enavatuzumab and P4A8/BIIB036 (19–22) but also with a 18D1 version with enhanced ADCC activity (14). Noteworthy, there is evidence that the antitumoral effects of PDL192/Enavatuzumab and P4A8/BIIB036 were not only based on ADCC but also involve Fn14-mediated NFκB activation although *in vitro* these antibodies have no (or only very low) agonistic activity or even act as antagonists (14, 15, 19, 20, 22). Thus, triggering of Fn14 signaling by FcγR-bound antibody molecules may have contributed to the antitumoral effects reported for PDL192/Enavatuzumab and P4A8/BIIB036 *in vivo*. Antitumoral efficacy has also been demonstrated with antibody drug conjugates and a granzyme-B Fc fusion protein with an Fn14-specific scFv targeting domain (23–27). The second strategy to target the TWEAK-Fn14 system for tumor therapy is to block TWEAK-induced Fn14 activation to interfere with Fn14-mediated protumoral activities. This approach was successful in some murine tumor models (14, 28).

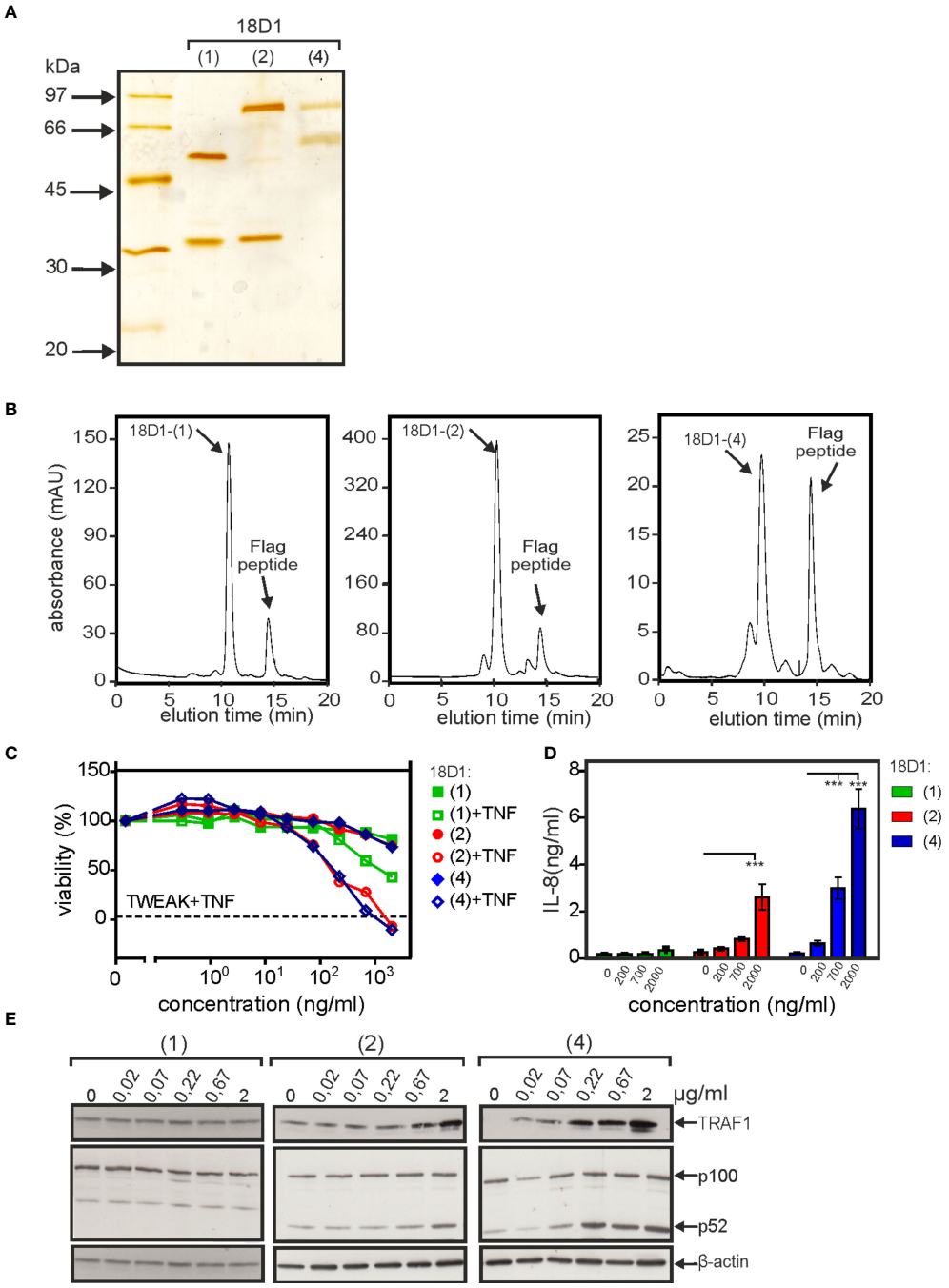

FIGURE 4
Purification and analysis of tetra- and hexavalent anti-Fn14 18D1 variants. **(A)** Purified proteins were separated by SDS-PAGE and visualized by silverstaining. **(B)** Gelfiltration analysis of purifed proteins. **(C–E)** Analysis of the ability of purified proteins from part B to trigger enhancement of TNF-induced toxicity in HeLa-RIPK3-FADD$_{KO}$ cells **(C)** and IL8 production **(D)** and p100 processing with TRAF1 induction in HT1080 cells **(E)**. IL8 data **(D)** were averaged from seven independent experiments and were analyzed by one way ANOVA and Bonferroni post test. ***p < 0.001.

The third approach is the use of membrane TWEAK-mimicking agonists to unleash the full range of proinflammatory Fn14 activities. The rational basis for this approach is that membrane TWEAK, which potently activates the classical NFκB pathway, is very efficiently processed by furine proteases to release the by far less proinflammatory soluble form of TWEAK, which only poorly activates the classical NFκB pathway. Therefore, it has to be assumed that sTWEAK is *in vivo* the dominant form of TWEAK resulting in low inflammatory activity of Fn14. Treatment with a memTWEAK-mimicking Fn14 agonist should therefore induce a comprehensive and supraphysiological strong inflammatory Fn14 response with an antitumoral net effect. Indeed, as already discussed above, there is evidence that the agonism of FcγR-bound anti-Fn14 antibodies contributes to the antitumoral effects of ADCC-

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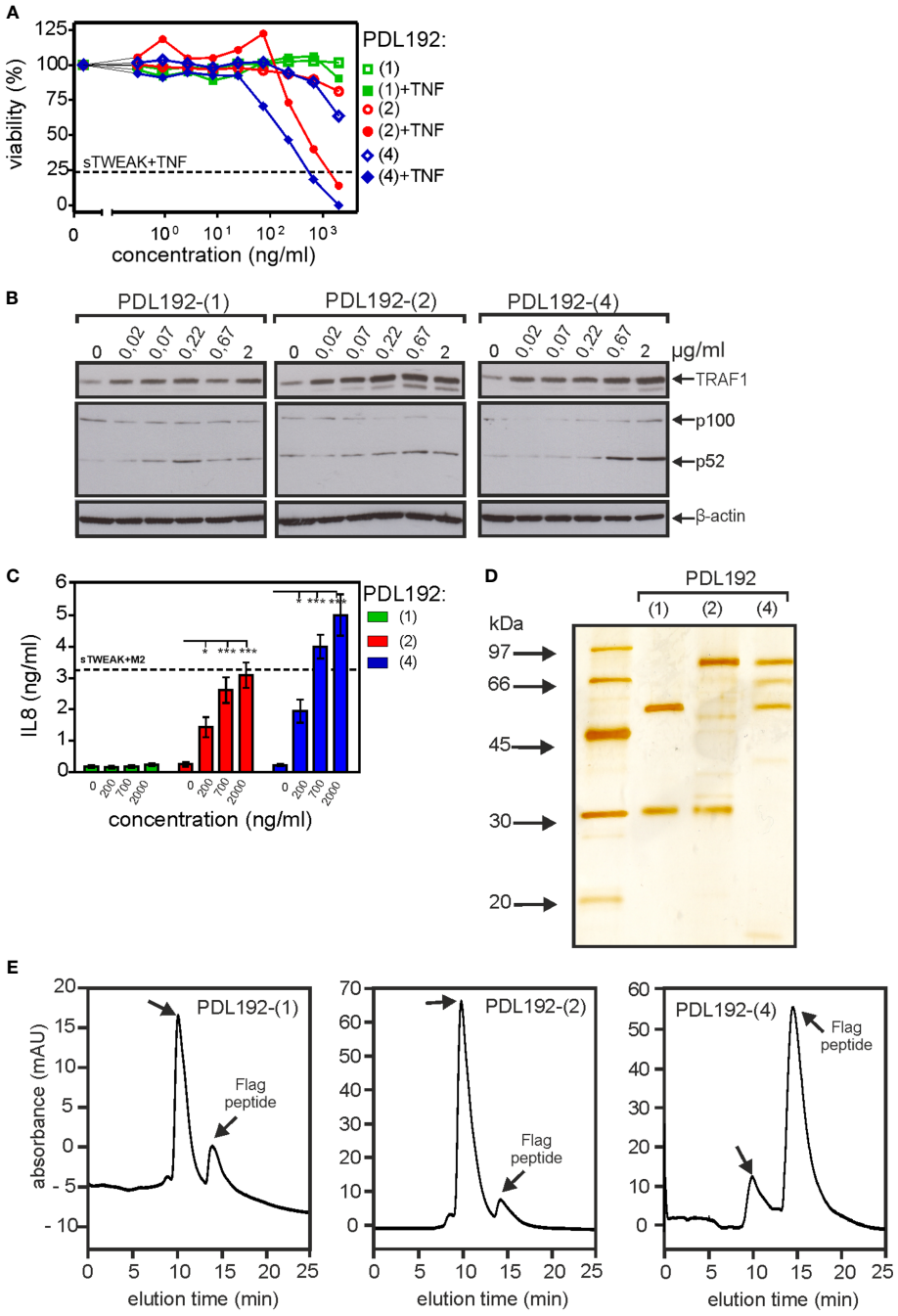

FIGURE 5
Triggering of Fn14 signaling by PDL192 type (2) and type (4) constructs. **(A-C)** The activity of PDL192-(2) and PDL192-(4) were analyzed as in Figure 2 with respect to enhancement of TNF-induced cell death in HeLa-RIPK3-FADD$_{KO}$ cells **(A)**, stimulation of p100 processing and TRAF1 indcution **(B)** and upregulation of IL8 production **(C)**. IL8 data were averaged from seven independent experiments and were analyzed by one way ANOVA and Bonferroni post test. *p < 0.05, ***p < 0.001. **(D, E)** Purified PDL192-(2) and PDL192-(4) along with the parental antibody PDL192 were analyzed by SDS-PAGE and silverstaining **(A)** or gelfiltration analysis **(E)**.

stimulating anti-Fn14 antibodies. Another important antitumoral effect of membrane but also soluble TWEAK-mimicking agonists is potentially the depletion of cytoplasmic TRAF2 pools and sensitization for TNF-induced cell death (4, 5). In this respect, two independent groundbreaking studies give strong evidence that tumor cell-expressed TRAF2 antagonizes the efficacy of checkpoint inhibitor therapies by protection against cell death induction by CD8$^+$-derived TNF (29, 30). In sum, comprehensive and selective Fn14 engagement appears as a very interesting and promising avenue to treat cancer and could also be of possible value to exploit the Fn14-mediated regenerative responses after muscle, pancreas or liver injury (7–9). However, there are yet no Fn14 antibodies available with strong and FcγR-independent, thus purely Fn14-specific agonistic activity.

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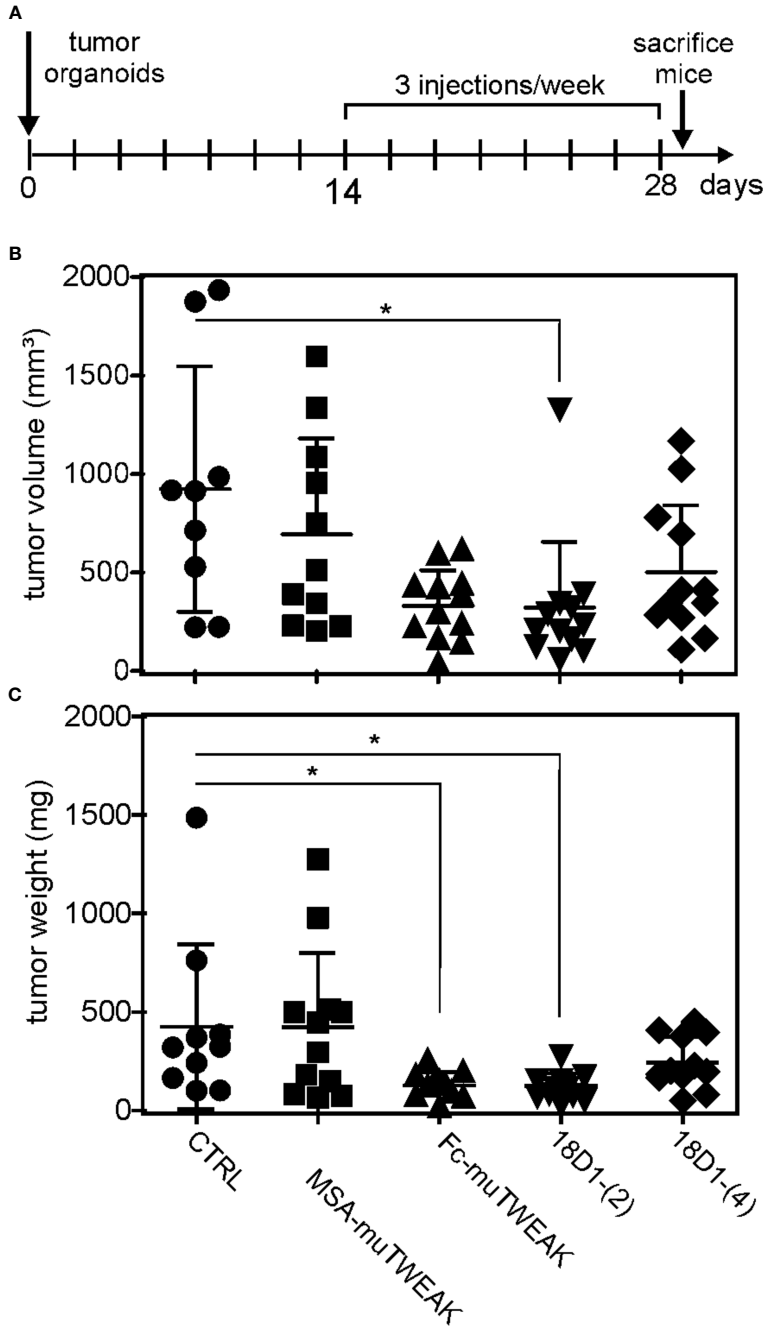

FIGURE 6
Fc-TWEAK and 18D1-(2) inhibit the growth of established murine tumor organoid-induced tumors. **(A)** Scheme of animal treatment. **(B, C)** Tumor weight and tumor volume of mice treated with fusion proteins of murine soluble TWEAK (muTWEAK) with murine serum albumin (MSA-muTWEAK) or human IgG1-derived Fc (Fc-muTWEAK), 18D1-(2) and 18D1-(4) were compared with the tumor weight and volume of control mice treated with NaCl physiological solution (CTRL) using the non parametric Kruska-Wallis test with Dunn's multiple comparison post test. N = 9-12; *p < 0.05.

In this study, we identified oligovalent variants of the Fn14-specific antibody 18D1 which mimic the activity of sTWEAK or memTWEAK independent from FcγR binding (Figures 2, 5) and which have good antitumoral activity (Figure 6). In contrast to conventional anti-Fn14 antibodies, the agonism of these novel Fn14 agonists is not limited by the availability of FcγR-expressing immune cells or competition with endogenous irrelevant antibodies for FcγR binding. Unexpectedly, although all oligovalent anti-Fn14 variants efficiently induced p100 processing and enhancement of TNF-induced cell death, there were considerable differences in the induction of IL8, a target of the classical NFκB pathway. Constructs only harboring multiple copies of the scFv:18D1 domain largely failed to activate IL8 production while constructs with similar valency but composed of Fab and scFv

domains did this very efficiently (Figure 2C). Thus, the number and type of the Fn14 binding domains within an oligovalent 18D1 construct seem to decide whether sTWEAK- or memTWEAK-like activity is mimicked. The pathway selective agonism mirrors the activities of sTWEAK and memTWEAK, the natural ligands of Fn14, but is yet without precedence for anti-Fn14 antibodies. Formation of trimeric Fn14 complexes induced by sTWEAK enables the recruitment of TRAF2 and thus reduces its availability for the inhibition of the alternative NFκB pathway and TNF-induced cell killing but this is not sufficient to ensure activation of the classical NFκB pathway. However, the latter can be achieved by membrane TWEAK and FcγR-bound anti-Fn14 antibodies (15, 17, 31). Studies on Fn14-related receptors of the TNFRSF suggest furthermore that membrane-bound ligand molecules and FcγR-bound antibodies have a superior ability compared to soluble ligands and free antibodies to promote secondary clustering of initially formed trimeric TNF receptor complexes (32). It is therefore tempting to speculate that the differential classical NFκB pathway agonism of Fab/scFv domain versus scFv-only domain anti-Fn14 variants reflects a different ability to trigger supramolecular clustering. However, this remained to be clarified in future more detailed studies on the molecular mode of action of the novel Fn14 agonists, we have identified in this study.

Materials and methods

Cell lines, reagents and statistics

HEK293T and HT1080 cells were from the American Type Culture Collection (ATCC) (Rockville, MD, USA) or the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany). HeLa-RIPK3-FADD$_{KO}$ cells were described elsewhere (33). All cell lines were cultivated in RPMI 1640 medium (Thermo Fischer Scientific, GB, #21875-034) supplemented with 10% fetal bovine serum (FBS) (Thermo Fischer Scientific, GB, #10270-106). Expression plasmids encoding the heavy and light chains of the various recombinant proteins (Supplemental Data Table SI) were produced by standard cloning techniques into pCR3 (Invitrogen, Germany). Antibodies used in the study were purchased from following suppliers: Abcam (anti-Fn14 EPR3179, # ab109365), Sigma-Aldrich, Germany (anti-GAPDH 71.1, #G9295; anti-FLAG® M2, #F3165; anti-ß-actin AC-15, #A1978; anti-NFκB p52, #05-361), Cell Signaling, GB (anti-IκBα L35A5, #4818S; anti-phospho-IκBα (Ser32) 14D4, #28592; anti-TRAF-1 45D3, 70745), LI-COR Biosciences, Lincoln, USA (IRDye® 800CW anti-mouse IgG, #926-32210), Dako, Glostrup, Denmark (rabbit anti-mouse IgG with horseradish peroxidase (HRP) #P0260, goat anti-rabbit IgG with HRP, #P0448). Production and properties of the soluble TWEAK variants Flag-sTWEAK (TWEAK) and Fc-Flag-sTWEAK (Fc-TWEAK) has been described before (17) and TNF was a kind gift from Prof. Daniela Männel (University of Regensburg, Germany). Statistical analyses were performed with the corresponding functions of the GraphPad Prism software.

Production and purification of recombinant proteins

HEK293T cells were transiently transfected with expression plasmids (ratio 1:1) encoding the heavy and light chain variants of the antibody variant of interest (Supplemental Data Table SII) using polyethylenimine (PEI, Polyscience Inc., Warrington, USA, #23966) essentially as described elsewhere (34). One day after adding the medium containing PEI/DNA mixture, the latter was replaced by RPMI 1640 medium supplemented with 2% FBS containing 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Germany, #P4333). After additional 5-7 days supernatants were collected, cleared by centrifugation (10 min, 4600 x g) and initially assayed for the presence of recombinant proteins by western blot detection (primary antibody: anti-FLAG M2; secondary antibody anti-murine IgG IRDye 800CW). The concentration of the Flag-tagged proteins in the supernatants (SN) were estimated by anti-Flag western blot and comparison of the band intensities in the SN samples and purified Flag-tagged antibodies of known concentration used as standard. The various antibody variants were purified by anti-Flag affinity chromatography as described elsewhere (34). Concentrations and purity of purified proteins were analyzed by SDS-PAGE and silver staining of the protein gel with the Pierce Silver Stain Kit (Thermo Fischer Scientific, USA) and comparison with the protein standards of the LMW Calibration Kit for SDS Electrophoresis from Amersham (GE Healthcare). The purity and integrity of purified recombinant antibodies were furthermore analyzed using High Performance Liquid Chromatography (HPLC) (UltiMate 3000, Thermo Fischer Scientific, USA) using a MabPac SEC-1 column (Thermo Fisher, #088460).

Western blot analysis

HT1080 cells were seeded in 12-well cell culture plates (Greiner Bio-One, Germany, #665180) (2 x 10^5 cells/well). Next day, medium was replaced by fresh medium supplemented with the antibodies of interest. As a positive control cells were challenged with 200 ng/ml Flag-TWEAK. After 20-24 h, total cell lysates were prepared by suspending the cell pellet in Laemmli sample buffer containing, if not otherwise stated, 5% β-mercaptoethanol, sonification for 25 seconds and heating for 5 minutes at 95°C. Lysates were separated by SDS-PAGE and proteins were transferred to nitrocellulose for western blot analysis of p100 to p52 processing (primary antibody: anti-NFκB p52; secondary antibody: HRP-labeled goat anti-mouse IgG), TRAF1 induction (primary antibody: rabbit anti-TRAF1; secondary antibody: HRP-labeled goat anti-rabbit IgG) and IκBα phosphorylation (primary antibody: rabbit anti-IκBα; secondary antibody: HRP-labeled goat anti-rabbit IgG). Protein loads were controlled by detection of β-actin (primary antibody: anti-ß-actin; secondary antibody: HRP-labeled anti-mouse IgG). To analyze Fn14 expression in murine tumor organoids (MTOs), MTO lysates were separated by SDS-PAGE and transferred to

nitrocellulose as described above and analyzed for Fn14 (primary antibody: rabbit anti-Fn14; secondary antibody: HRP-labeled goat anti-rabbit IgG) and GAPDH (peroxidase-labeled mouse monoclonal antibody) expression.

Analysis of IL8 induction

HT1080 cells were seeded in 96-well cell culture plates (Sarstedt, Germany, #83.3924) (2 x 10^4 cells/well) and the next day cells were challenged with reagents of interest for an additional 24 h. As a positive control, cells were stimulated with anti-Flag antibody M2 oligomerized Flag-TWEAK which mimics the activity of membrane TWEAK (17). Cell culture supernatants were collected and analyzed with respect to their IL8 content using the human IL8 ELISA Kit (BD Biosciences, Heidelberg, Germany, #555244).

Enhancement of TNF-induced cell death

To analyze the ability of the various Fn14-specific antibody variants to enhance TNF-induced toxicity, HeLa-RIPK3-$FADD_{KO}$ cells were seeded in the 96-well cell culture plates (Sarstedt) (2,5 x 10^4 cells/well). The next day, the medium was replaced by fresh medium containing the Fn14-specific reagents of interest with or without 1 ng/ml of TNF (Männel, University of Regensburg, Germany). The next day, cell viability was finally analyzed by crystal violet staining. Viability values were normalized according to untreated cells (100% viability) and cells incubated with a toxic mixture of reagents (0% viability).

Animal experiments

Male C57Bl/6J mice (6 weeks) were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and housed at a controlled temperature of 21 ± 1°C with a dark/light cycle of 12h/12h. Mice had access to a standard chow diet and water *ad libitum*. After an adjustment period of two weeks, 5 x 10^5 MTOs cultured as described by (18) were injected subcutaneously in the flank region, and grown over two weeks to palpable tumors. Subsequently, tumor-bearing mice received 3 intraperitoneal injections per week of MSA-muTWEAK, Fc-muTWEAK, 18D1-(2) or 18D1-(4) (all 200 µg) or NaCl (0.9%) physiological solution as a control treatment for two weeks. All animal experiments were approved by the Regierung von Unterfranken (license number AZ 2-1442) and complied with the German animal protection law.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Regierung von Unterfranken.

Author contributions

OZ, ML, MA, TZ and KK produced and purified the various proteins and performed the *in vitro* assays. OZ and AH performed the *in vivo* experiments. OZ, AH, AW, CO and HW analyzed the data and wrote the manuscript. AW advised on the animal experiments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The University of Würzburg filed a patent describing novel Fn14 agonists with authors OZ, CO and HW as co-inventors.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1194610/full#supplementary-material

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EGFR-selective activation of CD27 co-stimulatory signaling by a bispecific antibody enhances anti-tumor activity of T cells

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A higher density of tumor infiltrating lymphocytes (TILs) in the tumor microenvironment, particularly cytotoxic $CD8^+$ T cells, is associated with improved clinical outcome in various cancers. However, local inhibitory factors can suppress T cell activity and hinder anti-tumor immunity. Notably, TILs from various cancer types express the co-stimulatory Tumor Necrosis Factor receptor CD27, making it a potential target for co-stimulation and re-activation of tumor-infiltrated and tumor-reactive T cells. Anti-cancer therapeutics based on exploiting CD27-mediated T cell co-stimulation have proven safe, but clinical responses remain limited. This is likely because current monoclonal antibodies fail to effectively activate CD27 signaling, as this receptor requires higher-order receptor cross-linking. Here, we report on a bispecific antibody, CD27xEGFR, that targets both CD27 and the tumor antigen, epidermal growth factor receptor (EGFR). By targeting EGFR, which is commonly expressed on carcinomas, CD27xEGFR induced cancer cell-localized crosslinking and activation of CD27. The design of CD27xEGFR includes an Fc-silent domain, which is designed to minimize potential toxicity by reducing Fc gamma receptor-mediated binding and activation of immune cells. CD27xEGFR bound to both of its targets simultaneously and triggered EGFR-restricted co-stimulation of T cells as measured by T cell proliferation, T cell activation markers, cytotoxicity and IFN-γ release. Further, CD27xEGFR augmented T cell cytotoxicity in a panel of artificial antigen-presenting carcinoma cell line models, leading to Effector-to-Target ratio-dependent elimination of cancer cells. Taken together, we present the *in vitro* characterization of a novel bispecific antibody that re-activates T cell immunity in EGFR-expressing cancers through targeted co-stimulation of CD27.

KEYWORDS

immunotherapy, bispecific antibody, CD27, EGFR, T cell, co-stimulation

1 Introduction

The re-activation of tumor-reactive T cells with so-called immune checkpoint inhibitors (ICIs) has translated into remarkable clinical breakthroughs. Specifically, antibodies directed against CTLA-4 and PD-L1/PD-1 have improved therapeutic outcomes, including complete remissions in many solid as well as hematological cancers (as reviewed in (1–3)). ICIs prevent negative feedback on tumor-reactive T cells and re-enable the eradication of cancer cells upon binding of the T cell receptor (TCR) complex to tumor-specific peptides presented in the major histocompatibility complex (MHC). However, not all patients or cancer types respond to current ICI therapies (as reviewed in (4–6)).

One possible explanation for the limited activity of ICI therapy in certain patients and cancer types may be the absence of additional co-stimulatory signals that stimulate tumor-reactive T cells in the tumor microenvironment (TME) (7–9). For example, the inhibition of the co-stimulatory CD40-CD40L axis diminished the effects of PD-L1 checkpoint treatment on exhausted $CD8^+$ T cells (10). Moreover, the lack of CD28 co-stimulation has been postulated to be a strong determinant of PD-1 blockade resistance (as reviewed in (11)). In order to provide sufficient co-stimulation, so-called immune co-stimulators (ICS) that target and activate prominent co-stimulatory receptors (e.g., CD28, CD40, 4-1BB, CD27, and OX40) have been developed and are currently undergoing clinical evaluation (12–18).

A prominent co-stimulatory receptor family involved in T cell activation is the Tumor Necrosis Factor Receptor Super Family (TNFRSF). Within this superfamily, CD27 (TNFRSF7) has emerged as a potential target for co-stimulatory therapy, yielding clinical benefits in a select group of hematological and solid tumors (19–22). CD27 is not only constitutively expressed on the majority of both $CD4^+$ and $CD8^+$ T cells, but is also highly expressed on the majority of tumor infiltrating lymphocytes (TILs). Therefore, the activation of CD27 signaling is regarded as a potentially effective therapeutic anti-cancer strategy (23–27).

When activated by its ligand CD70, CD27 promotes the proliferation of T cells and their differentiation into effector and memory T cells (28–31). Importantly, CD27 co-stimulatory signaling is only efficiently activated upon the simultaneous occurrence of two events: (1) TCR-mediated recognition of and binding to tumor-specific peptides presented in the MHC of antigen presenting cells; and (2) crosslinking of CD27 (13, 32, 33). For instance, treatment with the CD27 agonistic antibody Varlilumab upregulated cytokine secretion upon continuous TCR-triggering, whereas pre-activated T cells without continuous TCR-triggering did not respond to Varlilumab (22, 33). Consequently, immunotherapies targeting CD27 have resulted in safer therapeutic outcomes than co-stimulatory approaches that can also operate TCR-independently, such as CD28 co-stimulation (34, 35). However, treatment with Varlilumab yielded only one (1/10) complete response and one stable disease (SD) in Hodgkin lymphoma and three (3/18) SD in B cell non-Hodgkin lymphoma (21). Further, in a trial with 31 patients with advanced solid tumors, Varlilumab yielded one partial response, with eight patients experiencing SD (22). Thus, the therapeutic effect of single CD27 targeting with Varlilumab in patients is limited.

The disappointing clinical results with Varlilumab may be attributable to suboptimal receptor crosslinking, as effective CD27 downstream signaling requires a hexameric ligand format and a hexameric CD27 complex (32, 36). Furthermore, CD27 receptor hexamerization and agonism is dependent on targeting specific extracellular CD27 epitopes and the application of Fc-engineering strategies that amplify affinity to Fc gamma receptors (FcγRs) (37). Current CD27-agonistic monoclonal antibodies (mAbs) do not efficiently promote CD27 hexamerization as single agents and require a scaffold, such as Fc receptor (FcR)-expressing immune cells. For instance, MK-5890, a novel CD27 agonistic antibody, showed increased agonistic activity with the occurrence of Fc-FcγR interactions (38). A potential approach to overcome this limitation is selective receptor crosslinking using a bispecific antibody (bsAb). Binding of a bsAb to a tumor-associated antigen can serve as a cross-linking platform for CD27 on T cells (39). Indeed, in preclinical studies, CDX-527, a tetravalent PD-L1 and CD27-targeting bsAb, induced CD27-mediated T cell co-stimulation through cross-linking by PD-L1 more effectively than the parental antibodies combined (40).

In the current study, we aimed to evaluate whether CD27 co-stimulation could be restricted to epidermal growth factor receptor (EGFR)-positive cancer ($EGFR^+$). While EGFR is ubiquitously expressed, EGFR expression is upregulated in several carcinomas and associates with tumor progression and angiogenesis (as reviewed in (41–43)). Hereto, we created an ICS-bsAb in a Dual-Variable Domain Immunoglobulin (DVD-Ig) format (44) consisting of two antigen-binding fragments (scFv1F5 and scFv425) targeting CD27 and EGFR, respectively. This antibody, termed CD27xEGFR, features an Fc-domain with LALAPG point mutations to yield an Fc-silent human IgG1, reducing FcR mediated antibody effector functions. In this format, the bsAb CD27xEGFR is designed to be minimally active 'en route'. Once CD27xEGFR binds to $EGFR^+$ cancer cells, the CD27-targeting domain can provide multivalent and tumor-localized crosslinking of CD27, potentially reducing off-tumor side effects. Besides serving as a cross-linking platform to facilitate CD27 co-stimulatory signaling, the EGFR targeting moiety of CD27xEGFR may also contribute through direct EGFR blocking, which is already a well-established therapeutic strategy for several epithelial tumors (45, 46). Hence, CD27xEGFR is designed to re-activate anti-tumor immunity safely and effectively in $EGFR^+$ cancer cells.

Abbreviations: ADCC, Antibody-Dependent Cell-Mediated Cytotoxicity; bsAb, Bispecific Antibody; DVD-Ig, Dual-Variable Domain Immunoglobulin; E:T ratio, Effector: Target ratio; EGFR, Epidermal Growth Factor Receptor; FcR, Fc Receptor; FcγR, Fc Gamma Receptor; ICI, Immune Checkpoint Inhibitor; ICS, Immune Co-Stimulators; mAb, Monoclonal Antibody; MHC, Major Histocompatibility Complex; NSCLC, Non-Small Cell Lung Cancer; PBMCs, Peripheral Blood Mononuclear Cells; scFvCD3, UchtV1 anti-CD3 antibody fragment; SD, Stable Disease; TCR, T Cell Receptor; TILs, Tumor Infiltrating Lymphocytes; TME, Tumor Microenvironment; TNFRSF, Tumor Necrosis Factor Receptor Superfamily; T_{regs}, Regulatory T cells.

2 Materials and methods

2.1 The Cancer Genome Atlas dataset analysis

A PanImmune Feature Matrix of Immune Characteristics as described in (47) was used to obtain lymphocytic infiltrate signature scores (based on the following 18 markers defined in (48): CCL5, CD19, CD37, CD3D, CD3E, CD3G, CD3Z, CD79A, CD79B, CD8A, CD8B1, IGHG3, IGJ, IGLC1, CD14, LCK, LTB, MS4A1) across multiple tumor-samples, containing The Cancer Genome Atlas (TCGA) Participant Barcodes. Batch effect normalized TCGA PAN CANCER CD27 and EGFR expression levels were obtained *via* Xena Hub (49), which consisted of TCGA data from broad GDAC firehose, that was normalized by RSEM (RNA-seq by Expectation-Maximization) and batch corrected *via* EB++ (Empirical Bayes++) (synapse ID: syn4976363). Expression levels were matched with lymphocytic infiltrate signature scores based on TCGA Participant Barcodes, and linear regressions were performed in GraphPad Prism 8.0.2. To visualize EGFR expression across multiple tumor-types, violin plots were generated.

2.2 Single cell mRNA sequencing data analysis

The dataset from the Tumor Immune Cell Atlas study (50) consisting of 13 different cancer types, 217 patients, and 526,261 cells was downloaded in the form of a RDS file containing the Seurat object. The data was ingested into Seurat V4 in R language version 4.0.3. The integrated single-cell RNA sequencing (scRNA-seq) data sets were collected as described before by Nieto et al. (50). In brief, after integration, the cells were divided into 25 clusters representing major immune cell types including 12 T cell types. To verify the robustness of the clusters and the associated signatures, a random forest classifier was used to assign cell annotations. A fivefold cross-validation was performed to assess biases and variance (50). The following T cell types were included in our study: Regulatory T cells, T helper cells, Th17 cells, recently activated $CD4^+$ T cells, Naïve-memory $CD4^+$ T cells, Transitional memory $CD4^+$ T cells, Naïve T cells, Proliferative T cells, Pre-exhausted $CD8^+$ T cells, Cytotoxic $CD8^+$ T cells, Effector memory $CD8^+$ T cells, and Terminally exhausted $CD8^+$ T cells (For the key markers per subtype, see Supplementary Table 1). Differential expression was calculated by using the FindMarkers function from Seurat with MAST as the method of choice (51).

2.3 Antibodies

Polyclonal antibody (pAb) Goat anti-human Ig-PE (cat# 2040-09, Southern Biotech, Birmingham, AL, USA), monoclonal antibodies (mAb): anti-CD27-APC (cat# 302810, clone O323, BioLegend, San Diego, CA, USA) (also used as CD27 mAb), anti-EGFR-FITC (cat# sc-120 FITC, clone 528, Santa Cruz Biotechnology, Dallas, TX, USA), anti-CD25-APC (cat# 302610, clone BC96, BioLegend), anti-CD4-FITC (cat# 300506, clone RPA-T4, BioLegend), anti-CD8-Brilliant Violet 421 (cat# 344748, clone SK1, BioLegend), mouse (IgG2A) (mAb 425) (Cat# EWI020, Kerafast, Boston, MA, USA), anti-Myc mAb Alexa Fluor 647 (cat# 2233, clone 9B11, Cell Signaling, Danvers, MA, USA). Atezolizumab was obtained from the pharmacy of the UMCG (Groningen, the Netherlands).

2.4 Cell lines and transfectants

The following wild type (WT) cell lines were obtained from the American Type Culture Collection (ATCC): A431, MDA-MB-231, ES-2, DLD-1, FaDu, and OVCAR-3. OVCAR-3.EGFR knock-out (KO) cells were a kind gift from prof. dr. Helfrich (UMCG/Dept of Surgery, Groningen, the Netherlands). HT1080.CD27 is previously described in (39) and is a kind gift from prof. dr. Harald Wajant (University of Wuerzburg, Wuerzburg, Germany). An overview of the cell lines, including tissue, cell type, cancer type, species, source and transduced genes, can be found in Table 1. Cells were cultured in RPMI-1640 (cat# 21875034, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) or DMEM (cat# 11965092, Gibco, Thermo Fisher Scientific), supplemented with 10% fetal calf serum (FCS) (cat# F7524, Thermo Fisher Scientific) at 37°C/5% CO_2. The artificial scFvCD3 (UchtV1 anti-CD3 antibody fragment)-presenting cell lines MDA-MB-$231^{scFvCD3}$, ES-$2^{scFvCD3}$, DLD-$1^{scFvCD3}$, $FaDu^{scFvCD3}$, OVCAR-$3^{scFvCD3}$, and OVCAR-$3^{scFvCD3}EGFR^{KO}$ are based on the lentiviral synNotch receptor construct pHR_PGK_antiCD19_ synNotch_Gal4VP64, which was a gift from Wendell Lim (Addgene plasmid #79125; http://n2t.net/addgene:79125; RRID : Addgene_79125) (52). The anti-CD19 scFv was replaced with the scFvCD3 UCHT-1v9 using Gibson cloning (cat# E5510S, New England BioLabs, Ipswich, MA, USA), yielding pHR_PGK_scFvCD3_synNotch_Gal4VP64. Lentivirus was produced by transient transfection of HEK293T cells with transfer vector, psPAX2, and pCMV-VSV-G packaging system using FuGENE (cat# E2312, Promega, Madison, WI, USA) according to manufacturer's recommendations. Viral supernatant was collected and filtered through a 0.45 µm filter (cat# SLHVR13SL, Millipore, Burlington, MA, USA). Transduction was performed by adding 1.5 mL viral supernatant to 1.5 mL of RPMI containing 2.5×10^5 pre-seeded cells in a 6 well tissue culture plate (cat# 3516, Corning Inc., Corning, NY, USA) in the presence of 4 µg/mL polybrene (cat# TR-1003, Sigma-Aldrich, Saint Louis, MO, USA). Transduced cells were sorted for expression of a Myc-tag (present at the N-terminus of the CD3 scFv) with a cell sorter model SH-800s (Sony Biotechnology, San Jose, CA, USA). Before each experiment, ES-$2^{scFvCD3}$, DLD-$1^{scFvCD3}$, $FaDu^{scFvCD3}$, OVCAR-$3^{scFvCD3}$ and OVCAR-3 ^{scFvCD3}.$EGFR^{KO}$ cells stably expressing scFvCD3 were characterized for their expression of EGFR, scFvCD3 and CD27, maintaining a consistent fold change from the isotype (Supplementary Figure 3). Cells were also transduced with lentivirus containing vector pLKO.1 mCherry, which was a gift from Oskar Laur (Addgene plasmid #128073; http://n2t.net/

TABLE 1 Characteristics of cell lines employed in this study.

Cell line	Tissue	Cell type	Cancer type	Species	Source	Transduced genes
A431	Skin	Epithelial	Epidermoid Carcinoma	Human	ATCC	
HT1080	Connective tissue	Epithelial	Fibrosarcoma	Human	prof. dr. Harald Wajant*	CD27
MDA-MB-231	Breast	Epithelial	Adenocarcinoma	Human	ATCC	scFvCD3 synNotch
ES-2	Ovary	Fibroblast	Clear cell Carcinoma	Human	ATCC	scFvCD3 synNotch, mCherry
DLD-1	Large intestine; Colon	Epithelial	Adenocarcinoma	Human	ATCC	scFvCD3 synNotch, mCherry
FaDu	Pharynx	Epithelial	Squamous Cell Carcinoma	Human	ATCC	scFvCD3 synNotch, mCherry
OVCAR-3	Ovary	Epithelial	Adenocarcinoma	Human	ATCC	scFvCD3 synNotch, mCherry
OVCAR-3.EGFR^{KO}	Ovary	Epithelial	Adenocarcinoma	Human	prof. dr. Helfrich**	scFvCD3 synNotch, mCherry

*Provided by Prof. Dr. Harald Wajant, University of Wuerzburg, Wuerzburg, Germany.
**Provided by Prof. Dr. Helfrich, UMCG/Dept of Surgery, Groningen, the Netherlands.
Further details can be found in Materials and Methods (Section 2.4). ATCC, American Type Culture Collection.

addgene:128073; RRID : Addgene_128073), producing the corresponding mCherry-expressing cells lines for visualization in the cytotoxicity assays.

2.5 Construction of CD27xEGFR

The bsAb CD27xEGFR was constructed in an scFv-scFv-IgG1 format, containing the antigen-binding fragments scFv1F5, targeting CD27, and scFv425, targeting EGFR. These two scFvs were connected by a flexible glycine-serine (GS) linker, consisting of a $(GGGS)_3$ sequence. The Fc domain of the antibody was designed with LALAPG mutations (L234A, L235A, and P329G) (53) in order to create an effector silent IgG molecule. Another GS linker, with the same $(GGGGS)_3$ sequence, connects the scFv EGFR to the IgG1 Fc domain. The antibody was produced by Evitria (Schlieren, Switzerland). Supernatant was harvested by centrifugation, filtered (0.2 µm filter), whereupon antibody was purified using MabSelect SuRe (cat# GE17-5438-01, Merck KGaA, Darmstadt, Germany). Purity was evaluated by analytical size exclusion chromatography with an AdvanceBio SEC column (300A 2.7 um 7.8 x 300 mm) (cat# PL1180-5301, Agilent, Santa Clara, CA, USA) and Dulbecco's phosphate-buffered saline (DPBS) (cat# 14190144, Gibco, Thermo Fisher Scientific) as running buffer at 0.8 mL/min. CD27xEGFR was successfully purified up to 98.6% with only minor amounts of degradation product (Supplementary Figure 1A). Endotoxin content was measured with the Charles River Endosafe PTS system (cat# PTS150K, Wilmington, MA, USA) and was < 1 EU/mg.

2.6 Biolayer interferometry assay

Binding of His-CD27 or His-EGFR to CD27xEGFR was analyzed using the BLItz system from ForteBio (cat# 45-5000, ForteBio, Menlo Park, CA, USA). His-CD27 (cat# 10039-H08B1, SinoBiological, Beijing, China), produced using the Baculovirus-Insect Cell expression system, encodes for the extracellular domain of human CD27 (Met1-Ile192) and included a C-terminal polyhistidine (His) tag. His-EGFR (cat# Z03194, GenScript, Rijswijk, the Netherlands), generated with the Sf9 insect cell expression system, encodes for the extracellular domain of human EGFR (Leu25-Ser645), and also featured a C-terminal His tag.

Octet protein A biosensors (cat# 18-5010, Satorius, Göttingen, Germany) were wetted for at least 10 min before use in 100 mM Tris-HCl pH 8 and all samples were diluted in the same buffer. In short, a baseline was run for 30 sec, followed by loading of 8 µg/mL of CD27xEGFR for 120 sec, baseline for 30 sec, association of either 125 nM His-EGFR and/or 500 nM His-CD27 for 120 sec, and dissociation for 120 sec. Atezolizumab (50 µg/mL) was used as a control.

The same protocol was used for binding of CD27xEGFR to immobilized His-CD27 and His-EGFR, with the following exceptions: The use of Octet HIS1K biosensors (cat# 18-5120, Sartorius), loading of 500 nM His-EGFR or His-CD27 and association of CD27xEGFR (50 µg/mL). Step corrections were applied to both the start of association and dissociation. Finally, the individual experiments were aligned to the start of association (x=y=0 for t=180 sec).

2.7 Isolation of peripheral blood mononuclear cells and T cells

Buffy coats were purchased from Sanquin (nr. NVT0465), and all donors gave informed consent (Sanquin Blood Supply, Groningen, the Netherlands). Human peripheral blood mononuclear cells (PBMCs) were isolated *via* density gradient centrifugation using lymphoprep (cat# 07851/07861, STEMCELL Technologies, Vancouver, Canada) and frozen until the day of the assay. T cells were isolated from fresh PBMCs using an autoMACS Pro Separator (Miltenyi Biotec, Bergisch Galdbach, Germany) and a Pan T Cell Isolation Kit (cat# 130-096-535, Miltenyi Biotec) following the manufacturer's recommendations. After isolation, T cells were frozen until the day of the assay.

2.8 CD27xEGFR binding studies

Binding of CD27xEGFR to CD27 and EGFR was evaluated using cell lines A431, HT1080.CD27, and primary human T cells. In brief, $5x10^4$ cells were incubated with CD27xEGFR (0.01–10 µg/mL, 45 min at 4°C) in a 96 well plate (cat# 3799, Corning Inc.) washed 3 times with DPBS (cat# 14190144, Gibco, Thermo Fisher Scientific), and then incubated with anti-human-IgG-PE pAb (45 min at 4°C). Following 3 washes with DPBS, cells were evaluated by flow cytometry (Accuri C6 Plus Flow Cytometer, BD, Franklin Lakes, NJ, USA). Binding to primary human T cells was performed analogously, but in the presence of FcR blocking reagent (cat# 130-059-901, Miltenyi Biotec) in all incubation steps. To demonstrate EGFR-specific binding of CD27xEGFR, HT1080.CD27 cells were pre-incubated with a 10-fold molar excess of mAb 425 for 15 min at 4°C. To demonstrate CD27-specific binding of CD27xEGFR, HT1080.CD27 cells were pre-incubated with a 10-fold molar excess of CD27 mAb for 15 min at 4°C. Binding of CD27xEGFR to HT1080.CD27 was blocked by pre-incubation with a 10-fold molar excess of CD27 mAb and mAb 425. The mean fluorescent intensity (MFI) was normalized to the highest obtained MFI (which was set at 100%) with the 0 µg/mL CD27xEGFR condition being set at 0%. Doublet formation between A431 tumor cells and primary human T cells upon addition of CD27xEGFR was analyzed by pre-labeling A431 cancer cells with Vybrant DiD Cell-Labeling Solution (cat# V22887, Thermo Fisher Scientific) and primary human T cells with CellTrace Violet reagent (cat# C34557, Thermo Fisher Scientific) both according to manufacturer's protocol). Cells were subsequently mixed at a 1:1 ratio ($2x10^5$ cells) with or without the addition of 10 µg/mL CD27xEGFR for 45 min at 4°C. Doublet formation was analyzed by flow cytometry (CytoFLEX V5-B5-R3, Beckman Coulter Life Sciences, Indianapolis, IN, USA).

2.9 Validation of scFvCD3 T cell activation system

In a 96 well plate (cat# 167008, Thermo Fisher Scientific), 100 µL of media containing $1x10^4$ primary PBMCs were added to 100 µL of media with or without CD27xEGFR (10 µg/mL) containing MDA-MB-231^{WT} or MDA-MB-231^{scFvCD3} cells previously incubated overnight at Effector : Target (E:T) ratios of 1:1, 1:2 and 1:5. After a 24-hour incubation, images were taken at 5x magnification using an EVOS FLoid Imaging System (cat# 4471136, Thermo Fisher Scientific) to visualize T cell clustering. Furthermore, PBMC cells were collected, stained for CD3 and CD25 and the CD25 expression of CD3$^+$ cells was measured using flow cytometry (CytoFLEX V5-B5-R3).

2.10 T cell proliferation assay

In a 96 well plate (cat# 167008, Thermo Fisher Scientific), 100 µL of media containing $4x10^4$ primary human T cells labeled with CellTrace Violet reagent with or without CD27xEGFR (10 µg/mL) were added to 100 µL of media containing $2x10^3$ ES-2^{scFvCD3}, DLD-1^{scFvCD3} or FaDu^{scFvCD3} cells previously incubated overnight. Proliferation was measured using flow cytometry (CytoFLEX V5-B5-R3) on day 5 and quantified using FlowJo Software version 10.8.1.

2.11 T cell activation assay

In a 96 well plate (cat# 167008, Thermo Fisher Scientific), 100 µL of media containing primary human T cells with or without CD27xEGFR (10 µg/mL) were added to 100 µL of media containing $2x10^3$ ES-2^{scFvCD3}, DLD-1^{scFvCD3} or FaDu^{scFvCD3} cells previously incubated overnight in the E:T ratios indicated (5:1, 10:1, 20:1). After 4 days, T cells were collected from the co-culture and stained for CD4, CD8, CD25, and Zombie NIR (cat# 423106, BioLegend) (to distinguish between alive and dead cells) and analyzed by flow cytometry (CytoFLEX V5-B5-R3). Supernatants were harvested and IFN-γ secretion was quantified using an IFN-γ ELISA kit (cat# 31673539, ImmunoTools, Friesoythe, Germany).

2.12 Cytotoxicity assay

In a 96 well plate (cat# 167008, Thermo Fisher Scientific), 100 µL of media containing primary human T cells with or without CD27xEGFR (10 µg/mL) were added to 100 µL of media containing $2x10^3$ ES-2^{scFvCD3}, DLD-1^{scFvCD3}, FaDu^{scFvCD3}, OVCAR-3^{scFvCD3}, or OVCAR-3^{scFvCD3}EGFR^{KO} cells incubated overnight in the E:T ratios indicated (0:1, 1:1, 2:1, 5:1, 10:1, 20:1). Experiments were imaged for mCherry fluorescence for up to 7 days using the Incucyte S3 live-imaging system (Essen BioScience, Royston, UK) and analyzed using Incucyte S3 software v2021A. Four pictures of each well for each of three technical replicates were acquired and analyzed based on the Top-Hat segmentation method (Radius 50 µm, Threshold 0.0950, Edge Split On, Edge Sensitivity 5, Hole Fill 0 µm^2, Adjusted Size 7 pixels, Filters: min Area 210 µm^2, min Integrated Intensity 50). As a measure of cytotoxicity, cell survival was calculated as the mCherry area (µm²/image) from the sample at the indicated time point/mCherry area (µm²/image) from the cancer cells only control at the indicated time point. To evaluate direct EGFR-blocking anti-carcinoma activity of CD27xEGFR, $2x10^3$ mCherry expressing FaDu^{scFvCD3} cells were seeded in a 96 well plate (cat# 167008, Thermo Fisher Scientific) and treated with CD27xEGFR (10 µg/mL) or mAb425 (10 µg/mL) for 3 days.

2.13 Statistical analysis

Data are presented as mean + SD as stated in the figure legends. For the before-after plots, each pair of observations represents independent experiments with different T cell donors. Statistical significance was determined as indicated in the figure legends, with a p-value of < 0.05 considered statistically significant.

For the proportions of $CD27^+$ cells, a two-sample test for equality of proportions with Bonferroni correction was applied for comparing the proportions of CD27 among different T cell types. The corresponding p-values are documented in Supplementary Table 2.

For CD27xEGFR binding, binding blockade, doublet formation, T cell proliferation, cytotoxicity, and IFN-γ secretion, experiments were performed with T cells from different donors on different days, and each experiment was treated as independent. The normality of flow cytometry data was assessed through visual inspection of flow cytometry histograms. For the proliferation and IFN-γ secretion data, normal distribution was assumed. For the cytotoxicity experiments, the Shapiro-Wilk test was employed to determine the normality of the data on three independent experiments consisting of three technical replicates.

For the relationship between EGFR or scFvCD3 expression and difference of cancer cell survival between CD27xEGFR and medium control, a simple linear regression was performed. Each data point in the analysis represents the mean of all independent experiments at the E:T 5:1, conducted for each cell line with different T cell donors.

3 Results

3.1 CD27 is a target for re-activation of tumor infiltrating cytotoxic and exhausted lymphocytes

To delineate the potential applicability of EGFR-targeted activation of CD27 agonism, the TCGA PAN CANCER dataset was analyzed for concurrent CD27 and EGFR expression. Compared to non-epithelial cancers, lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), and uveal melanoma (UVM), all 20 epithelial cancers expressed high levels of EGFR mRNA (Figure 1A, black for epithelial and gray for non-epithelial cancers). Furthermore, lymphocytic infiltrates across all 20 epithelial cancers expressed CD27 mRNA, demonstrating a clear correlation between lymphocyte infiltration score and CD27 (R^2 = 0.6895) within the whole epithelial cancer set, confirming that the strategy of EGFR-mediated crosslinking of CD27 could be employed within epithelial cancers (Figure 1B, see Supplementary Figure 2 for individual tumor types). In an established single-cell tumor immune atlas from a range of cancer types, CD27 expression was prominent in a subpopulation of regulatory T cells (T_{regs}), terminally exhausted $CD8^+$ T cells, as well as cytotoxic $CD8^+$ T cells (Figure 1C). Notably, tumor-reactive $CD8^+$ T cells - inclusive of cytotoxic, terminally exhausted, and pre-exhausted cells - present a higher proportion of $CD27^+$ cells in comparison to all $CD4^+$ T cells, Th17 cells, and naïve T cells (Figure 1D). Upon comparing $CD27^+$ T cells proportions (Figure 1D, Supplementary Table 2), T_{regs} and terminally exhausted $CD8^+$ T cells have proportions of 63.25% and 51.14% that are significantly higher than those observed in other T cell types. Notably, the proportions of $CD27^+$ cells within cytotoxic and pre-exhausted $CD8^+$ T cells are similar, with no significant difference observed between these two groups (Supplementary Table 2). Subsequent differential gene expression analysis in terminally exhausted $CD8^+$ and cytotoxic $CD8^+$ T cell subsets revealed that several exhaustion genes (GZMK, HAVCR2, TIGIT, and LAG3) and cytotoxicity genes, (TNFRSF9, CST7, and CD28) are significantly upregulated in the $CD27^+$ fraction (Figures 1E, F). In addition, expression of genes associated with tissue residency, trafficking, adhesion, and migration (VCAM1, CXCR3, ITGA4, CXCL13, and CCR7) are also elevated (Figures 1E, F). Therefore, the concurrent expression of CD27 and EGFR in various epithelial cancers, along with the gene expression signatures related to exhaustion, cytotoxicity, and tissue residency of CD27-expressing T cells, underscores the potential of EGFR-targeted activation of CD27 agonism for enhancing the re-activation of tumor-infiltrated and tumor-reactive T cells in EGFR-expressing cancers.

3.2 Bispecific antibody CD27xEGFR binds selectively and simultaneously to EGFR and CD27

To exploit the above-described concurrent expression of EGFR and CD27 for targeted activation of CD27 signaling, the ICS-bsAb CD27xEGFR was constructed. CD27xEGFR consists of an N-terminal CD27 targeting antibody fragment (scFv1F5) fused *via* a $(GGGGS)_3$ linker to the EGFR-targeting antibody fragment scFv425, with a silent human IgG1 containing LALAPG mutations (Figure 2A), which prevent Fc-FcR mediated antibody effector functions. The specific binding activity of CD27xEGFR to soluble CD27 and EGFR individually was confirmed using biolayer interferometry (Figure 2B). Moreover, the association rate (as defined by the gradient of the initial association curve) increased when both antigens were combined compared to a single antigen, supporting the proposed mechanism of action of dual binding (Figure 2B). Reversely, upon immobilization of CD27 or EGFR onto the biosensor, CD27xEGFR also specifically bound to both targets (Supplementary Figure 1B).

In a cell-based assay, CD27xEGFR dose-dependently bound to the $EGFR^+$ epidermoid carcinoma cell line A431 (Figure 2C). Similarly, dose-dependent binding of CD27xEGFR was detected on $CD27^+$ primary human T cells that expressed CD27 but not EGFR (Figure 2D, Supplementary Figure 1C). Further, CD27xEGFR also bound to the $EGFR^+$ fibrosarcoma cell line HT1080, engineered to ectopically express CD27, with binding only partly inhibited by pre-incubation with mAb 425 (anti-EGFR mAb) or anti-CD27 mAb alone (Figure 2E). However, CD27xEGFR binding was abrogated after a combined pre-incubation with mAb 425 and anti-CD27 mAb (Figure 2E), demonstrating that CD27xEGFR binds to both antigens when they are present on the same cell surface. Moreover, CD27xEGFR induced the formation of doublets between $EGFR^+$ and $CD27^+$ target cells in a mixed culture of A431 and primary human T cells. The percentage of doublets increased significantly from ~15% in the medium control up to ~40% in the CD27xEGFR-treated condition (Figure 2F), demonstrating CD27xEGFR simultaneously interacted with EGFR and CD27 expressed on distinct cells. In conclusion, the designed bsAb CD27xEGFR exhibited selective and simultaneous binding to both targets (CD27 and EGFR).

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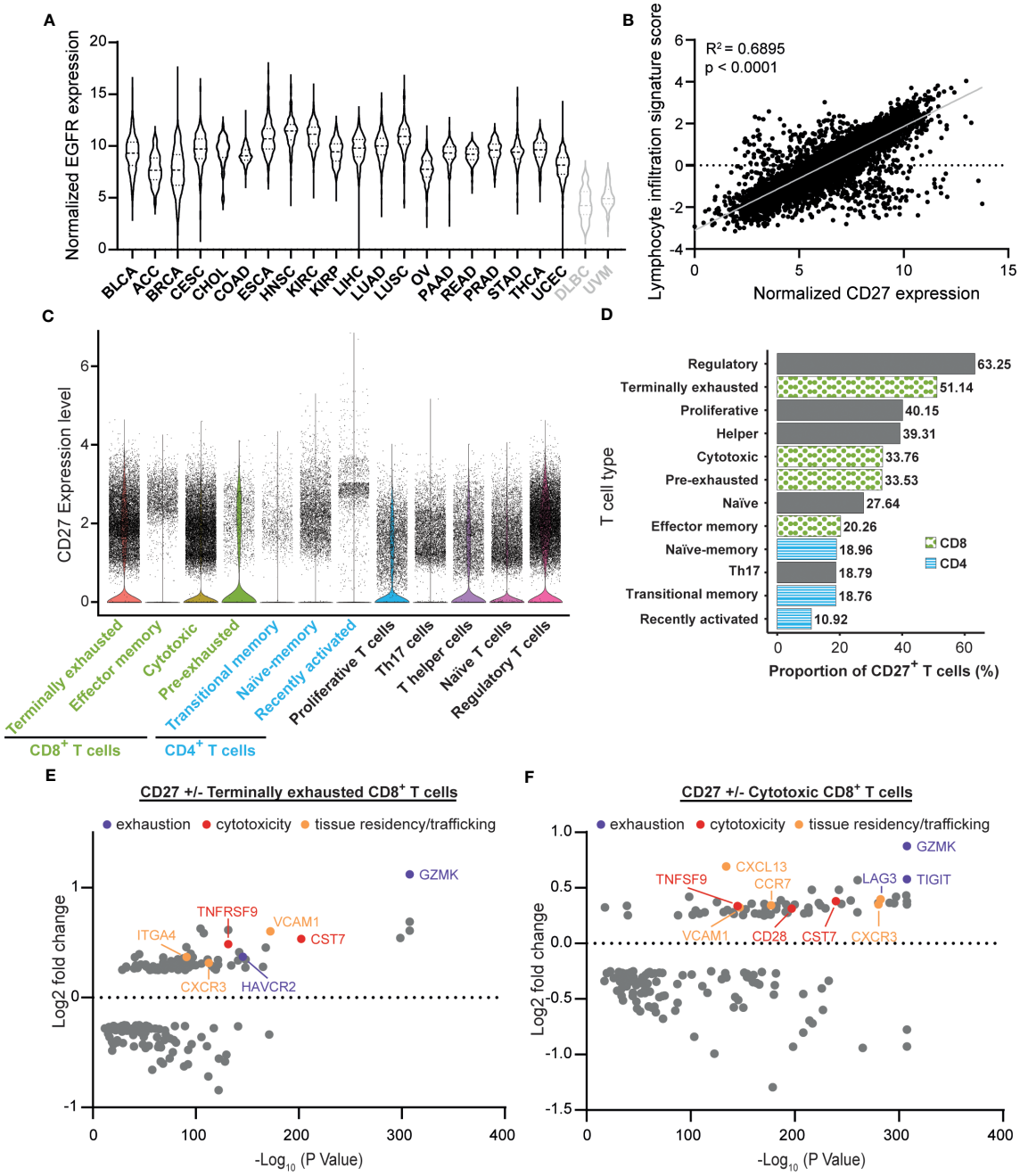

FIGURE 1
CD27 is a target for re-activation of tumor infiltrating cytotoxic and exhausted lymphocytes. **(A)** Normalized TCGA PAN CANCER epidermal growth factor receptor (EGFR) expression levels from epithelial cancers (black): BLCA (Bladder urothelial carcinoma), ACC (Adrenocortical carcinoma), BRCA (Breast invasive carcinoma), CESC (Cervical squamous cell carcinoma and endocervical adenocarcinoma), CHOL (Cholangiocarcinoma), COAD (Colon adenocarcinoma), ESCA (Esophageal carcinoma), HNSC (Head and Neck squamous cell carcinoma), KIRC (Kidney renal clear cell carcinoma), KIRP (Kidney renal papillary cell carcinoma), LIHC (Liver hepatocellular carcinoma), LUAD (Lung adenocarcinoma), LUSC (Lung squamous cell carcinoma), OV (Ovarian serous cystadenocarcinoma), PAAD (Pancreatic adenocarcinoma), READ (Rectum adenocarcinoma), PRAD (Prostate adenocarcinoma), STAD (Stomach adenocarcinoma), THCA (Thyroid carcinoma), UCEC (Uterine Corpus Endometrial Carcinoma), and non- epithelial cancers (gray): DLBC (Diffuse large B-cell lymphoma) and UVM (Uveal melanoma) were plotted in violin plots to visualize their relative EGFR expression. **(B)** Normalized CD27 expression levels from all 20 epithelial cancer types described in **(A)** were matched with lymphocytic infiltration signature scores *via* TCGA participant barcodes and plotted against each other. A linear regression was performed to visualize the correlation between CD27 expression and the lymphocytic infiltration signature score (R-squared = 0.6895, $p < 0.0001$). Statistical significance was determined using an F-test. **(C)** Single-cell tumor immune atlas RNA sequencing dataset based on 526,261 cells from 217 patients and 13 cancer types, revealing CD27 expression within different immune cell subtypes. **(D)** Proportion of $CD27^+$ cells in each T cell type described in **(C)**, statistical comparisons are shown in Supplementary Table 2. **(E)** Volcano plots of the differential gene expression analysis in $CD27^+$ vs $CD27^-$ terminally exhausted and **(F)** cytotoxic $CD8^+$ T cells calculated using the FindMarkers function from Seurat with MAST as the method of choice.

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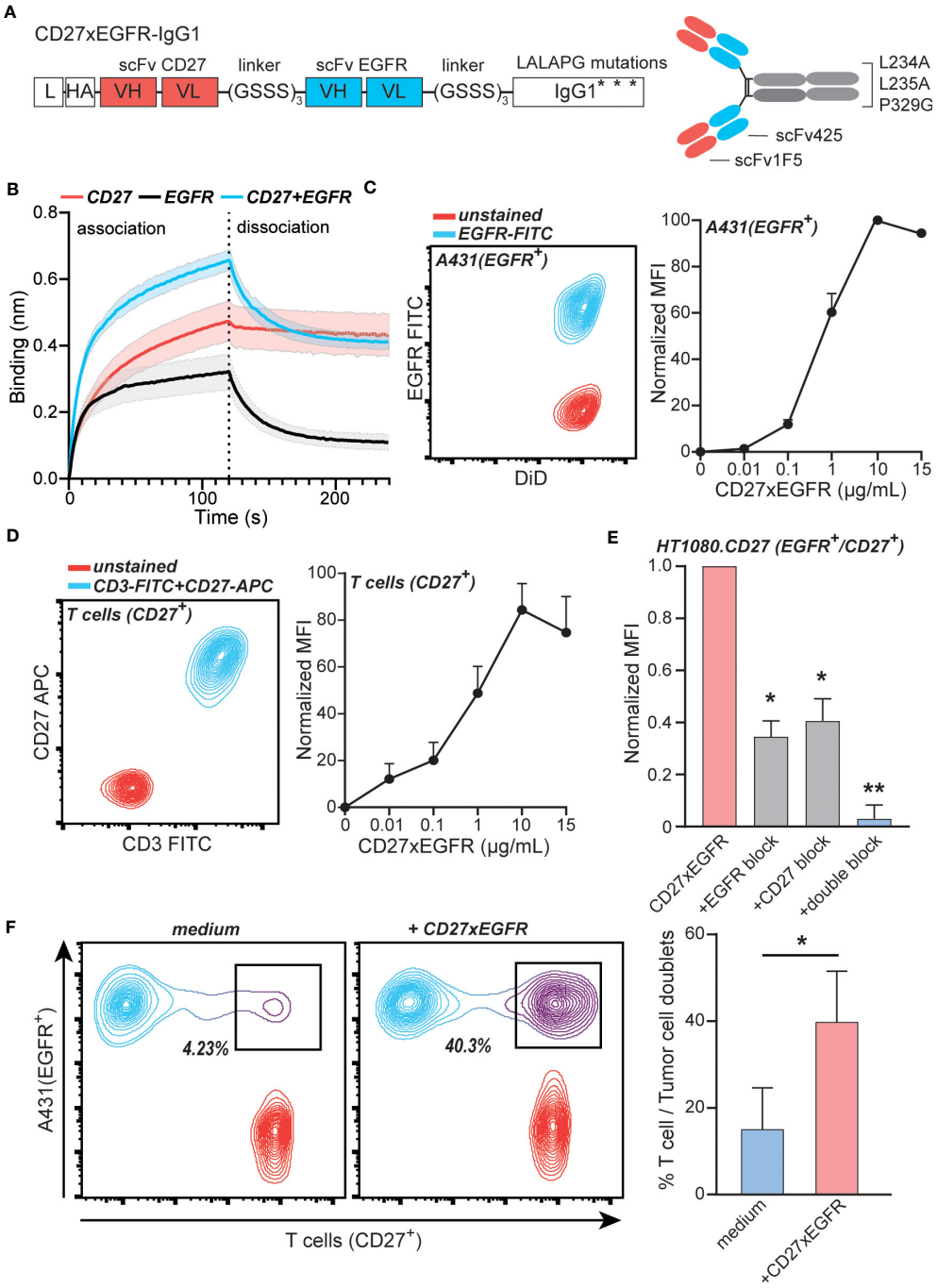

FIGURE 2
CD27xEGFR selectively binds EGFR and CD27 on tumor cells and T cells. **(A)** CD27xEGFR is designed in a scFv-scFv-IgG1 format with binding domains targeting CD27 (scFv1F5) and EGFR (scFv425) connected to an IgG1 tail containing LALAPG Fc mutations L234A, L235A, and P329G. **(B)** Association and dissociation of His-CD27 (500 nM) and/or His-epidermal-growth-factor-receptor (EGFR) (125 nM) against surface bound CD27xEGFR (8 µg/mL) as measured by biolayer interferometry (n = 3). **(C)** Flow cytometry plot displaying EGFR expression of stained (DiD) A431 cells (left). Dose-dependent binding (represented as normalized mean fluorescent intensity (MFI) to the highest MFI value) of CD27xEGFR on A431 tumor cells (n = 3) (right). **(D)** Flow cytometry plot displaying CD3 and CD27 expression on primary human T cells (left) Dose-dependent binding (represented as normalized MFI to the highest MFI value) of CD27xEGFR on primary human T cells (n = 5) (right). **(E)** Binding (represented as normalized MFI to the highest MFI value) of CD27xEGFR to HT1080 tumor cells ectopically expressing CD27 and its (partial) binding abrogation by pre-incubation of excess amounts of mAb 425 (EGFR block), an anti-CD27 mAb (CD27 block) or both (double block) (n = 3). Statistical significance was determined using one-way ANOVA test with Dunnett's correction **(F)** Representative doublet formation between EGFR$^+$ A431 tumor cells and CD27$^+$ primary human T cells upon incubation with CD27xEGFR with the corresponding bar graph on the right (n = 3). Statistical analyses were done using a paired t-test. Data are presented as mean with shaded areas and error bars denoting standard deviation. "**" indicates ($p < 0.01$), "*" indicates ($p < 0.05$).

3.3 CD27xEGFR enhances T cell proliferation and activation upon TCR stimulation

To evaluate the EGFR-restricted co-stimulation of T cells by CD27xEGFR, the carcinoma cell lines MDA-MB-231, ES-2, DLD-1, and FaDu were engineered to express a UchtV1 anti-CD3 antibody fragment (scFvCD3) on their surface, which enabled activation of TCR signaling in allogeneic T cells independent of MHC presentation (Figure 3A). The system was validated by treating a culture of MDA-MB-231^{scFvCD3} with peripheral blood mononuclear cells (PBMCs), which clearly activated T cells, as evidenced by cluster formation in the MDA-MB-231^{scFvCD3} co-culture (Figure 3A, bottom left). The addition of CD27xEGFR to this co-culture increased cluster formation further (Figure 3A, bottom right). Using flow cytometry, an increase in CD25 expression was observed in T cells within the PBMC population upon treatment with CD27xEGFR compared to the medium control (Supplementary Figure 1D). In contrast, no cluster formation was detected in the co-culture of MDA-MB-231^{WT} with PBMCs with or without CD27xEGFR treatment (Figure 3A, top left and right), validating that scFvCD3 can activate T cells MHC-independently. To specifically study the effects of CD27xEGFR on T cells, T cells were isolated from PBMCs in further studies. In line with the cluster formation, a prominent proliferation of T cells was detected in CD27xEGFR-treated mixed cultures with ES-2^{scFvCD3} cells, with up to 5 proliferation peaks detected (Figure 3B, bottom). In contrast, minimal proliferation was detected in the mixed cultures of ES-2^{scFvCD3} and T cells in the absence of CD27xEGFR (Figure 3B, top). Upon quantification, a significant increase in T cell proliferation was detected in CD27xEGFR treated ES-2^{scFvCD3} co-cultures, as evidenced by a significantly reduced percentage of cells in the parental peak and an increased percentage of cells in the proliferation peaks compared to medium control co-cultures (Figure 3C). A similar co-stimulatory activity of T cells by CD27xEGFR was detected in mixed cultures with DLD-1^{scFvCD3} and FaDu^{scFvCD3}, with a significant increase in T cell proliferation in either culture upon CD27xEGFR treatment (Figures 3D, E, respectively). Consistent with this increase in proliferation, CD27xEGFR treatment of ES-2^{scFvCD3}, DLD-1^{scFvCD3}, and FaDu^{scFvCD3} co-cultures increased the expression of CD25 on T cells compared to medium control co-cultures (Figures 3F–H, respectively), both on CD4$^+$ and CD8$^+$ T cells, and at different E:T ratios. The largest increase was detected at an E:T ratio of 20:1, with a 20–25% increase. Finally, CD27xEGFR treatment increased pro-inflammatory cytokine IFN-γ secretion in FaDu^{scFvCD3} co-cultures at 10:1 and 20:1 E:T ratios compared to medium control co-cultures (Figure 3I). Taken together, this data provides evidence that CD27xEGFR can effectively co-stimulate T cells in co-cultures with a wide range of EGFR$^+$ cell lines.

3.4 CD27xEGFR boosts T cell anti-tumor cytotoxic potential and has EGFR blocking anti-proliferative effects

In view of the clear co-stimulatory activity of CD27xEGFR, potential anti-tumor T cell activity induced by CD27xEGFR was evaluated next. As a measure of T cells cytotoxicity, cancer cell survival was determined by using the fluorescence of mCherry transduced cancer cells. After three days, treatment with CD27xEGFR strongly reduced the survival of mCherry-expressing ES-2^{scFvCD3} cells compared to medium control in co-culture experiments with primary human T cells (Figure 4A). To quantify this data, cell survival was measured over time. After approximately 24 hours of co-culture of T cells with mCherry-expressing ES-2^{scFvCD3} cells, T cell-mediated killing was observed, which continued to near complete eradication of cancer cells after 160 hours of treatment with CD27xEGFR (Figure 4B). In contrast, mCherry-expressing ES-2^{scFvCD3} started to grow back after approximately 96 hours when co-cultured with medium control and T cells (Figure 4B). Cell survival at this time point was normalized to that of cancer cells only and measured at different E:T ratios (0:1, 5:1, 10:1, and 20:1) (Figure 4C). An E:T ratio dependent reduction of cancer cell survival was detected, in which treatment with CD27xEGFR significantly reduced cancer cell numbers, with a maximum decrease of ~40% at an E:T ratio of 5:1 (Figure 4C). A similar reduction in cancer cell numbers in mCherry-expressing DLD-1^{scFvCD3} and mCherry-expressing FaDu^{scFvCD3} cells upon CD27xEGFR treatment was detected, with a maximum effect of ~20% and ~30% at a 2:1 E:T ratio for mCherry-expressing DLD-1^{scFvCD3} and mCherry-expressing FaDu^{scFvCD3} cells, respectively (Figures 4D, E). Of note, treatment of a monoculture of mCherry-expressing cancer cells (ES-2^{scFvCD3}, DLD-1^{scFvCD3}, and FaDu^{scFvCD3}) with CD27xEGFR slightly reduced the survival of the cancer cells compared to the medium controls (Figures 4C–E, 0:1 E:T ratios, ~2-7%). This effect is most likely caused by the EGFR-growth inhibitory activity of CD27xEGFR, which proved to be reminiscent of EGFR blocking with mAb 425 (Figure 4F). Notably, in a co-culture of T cells with mCherry-expressing EGFR$^+$ OVCAR-3^{scFvCD3}, treatment with CD27xEGFR increased cancer cell killing by T cells (Figure 4G, ~25% reduction at an E:T ratio of 5:1) whereas with the corresponding EGFR^{KO} cells, treatment with CD27xEGFR had no effect on cancer cell survival when compared to medium control (Figure 4H). The EGFR and scFvCD3 expression levels varied between the cell lines (Supplementary Figure 3), with EGFR expression having a significant, positive correlation with the cytotoxicity of CD27xEGFR at an E:T ratio of 5:1 (Figure 4I). No correlation was identified for scFvCD3 levels (Supplementary Figure 4). Taken together, CD27xEGFR has anti-cancer activity both by co-stimulation of T cells at the sites of EGFR expression as well as by directly blocking EGFR on cancer cells.

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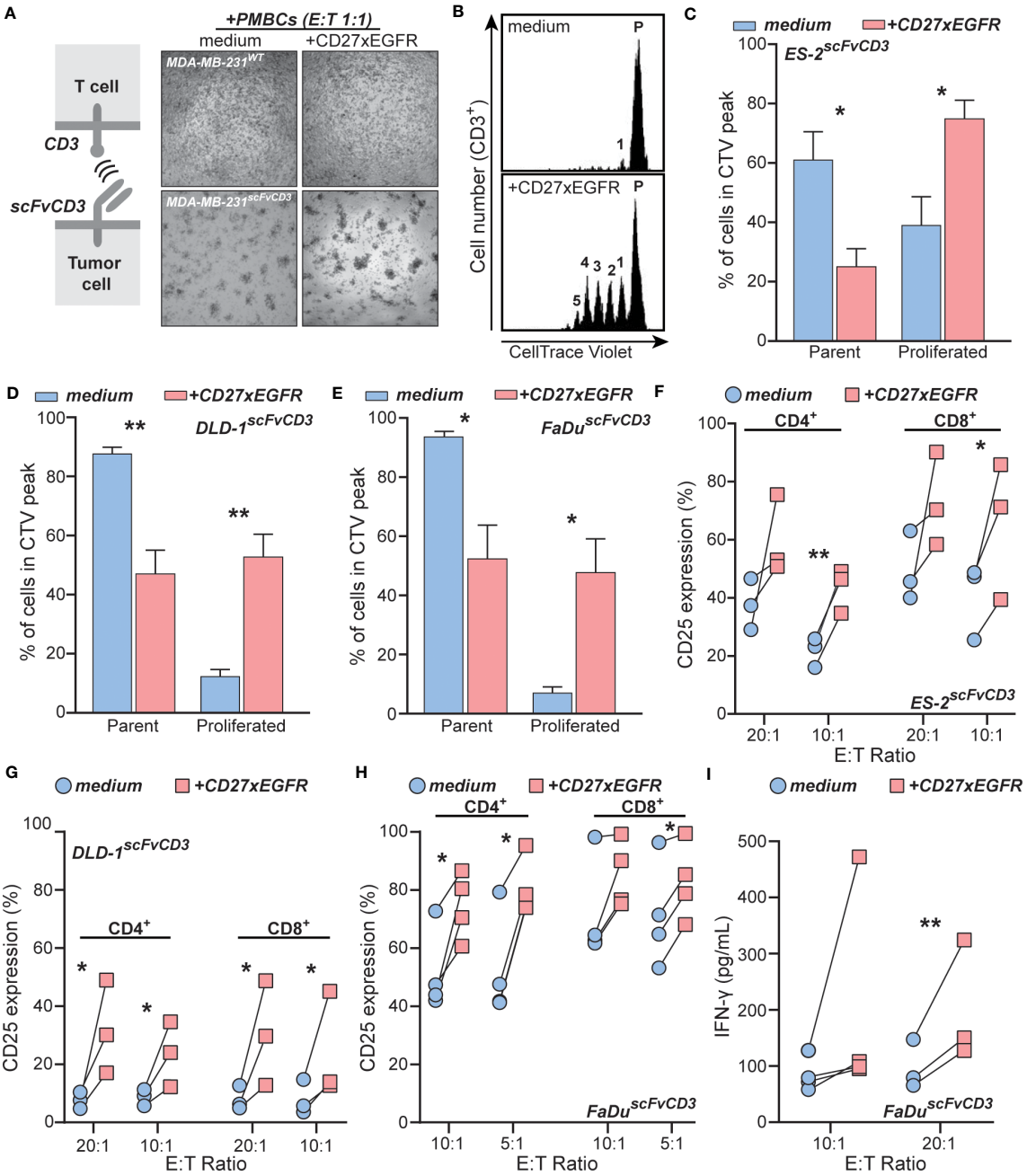

FIGURE 3
CD27xEGFR enhances T cell proliferation and activation upon TCR stimulation. **(A)** Schematic representation of the anti-CD3 (scFvCD3) T cell activation system (left). Microscopy images of MDA-MB-231^{scFvCD3} or MDA-MB-231^{WT} cells co-cultured with peripheral blood mononuclear cells (PBMCs) with or without the addition of CD27xEGFR (10 µg/mL) at an Effector : Target (E:T) ratio of 1:1 for 24 hours (right). **(B)** An exemplary co-culture and proliferation analysis of CellTrace Violet-labeled primary human T cells and ES-2^{scFvCD3} for 5 days with or without the addition of CD27xEGFR (10 µg/mL). Quantification analysis of proliferation peaks in co-culture experiments of primary human T cells with **(C)** ES-2^{scFvCD3} (n = 6), **(D)** DLD-1^{scFvCD3} (n = 3), and **(E)** FaDu^{scFvCD3} (n = 3) with (red bars) or without (blue bars) CD27xEGFR (10 µg/mL). Analysis of CD25 expression on CD4$^+$ and CD8$^+$ T cells after a 4-day co-culture experiment of **(F)** ES-2^{scFvCD3} (n = 3), **(G)** DLD-1^{scFvCD3} (n = 3), and **(H)** FaDu^{scFvCD3} (n = 4) with (red squares) or without (blue circles) the addition of CD27xEGFR (10 µg/mL) at the indicated E:T ratios. **(I)** ELISA analysis of co-culture supernatants for IFN-γ secretion by primary human T cells after a 4-day co-culture of FaDuscFvCD3 cells with (red squares) or without (blue circles) CD27xEGFR (10 µg/mL) at the indicated E:T ratios (n = 3 or 4). Significance was determined using paired t-tests. Data are presented as mean with error bars indicating standard deviation. "**" indicates ($p < 0.01$),"*" indicates ($p < 0.05$).

4 Discussion

In this study, we identified that cytotoxic and exhausted CD8$^+$ TILs with a tumor-reactive phenotype express the co-stimulatory receptor CD27 across various EGFR$^+$ cancer subtypes. Our findings revealed that these CD27$^+$CD8$^+$ T cells display a cytotoxic, exhausted, and tumor-reactive profile, closely matching the reported dysfunctional profile of the tumor-reactive immune

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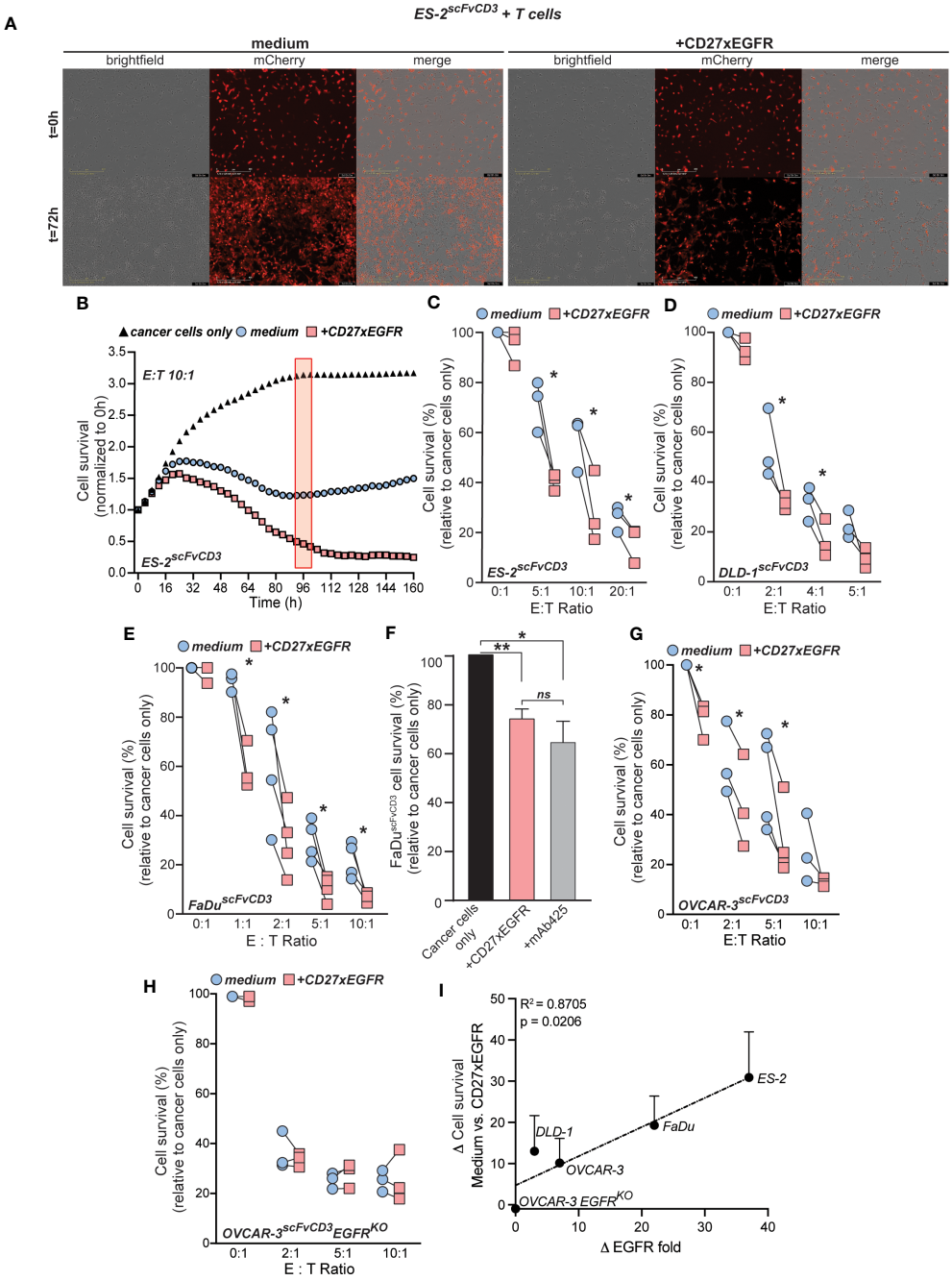

FIGURE 4
CD27xEGFR enhances T cell anti-tumor cytotoxic potential. **(A)** Exemplary brightfield microscopy images of a co-culture of mCherry-expressing ES-$2^{scFvCD3}$ cells (overlayed in red) with primary human T cells for three days with or without the addition of CD27xEGFR (10 µg/mL) **(B)** ES-$2^{scFvCD3}$ mCherry intensity (normalized to 0 h) over time using the IncuCyte S3 system (Essen BioScience) and analyzed at 96 h (red rectangle) using the IncuCyte 2021A software. The cancer cell survival (relative to cancer cells only, black triangles) of mCherry-expressing **(C)** ES-$2^{scFvCD3}$ (n=3), **(D)** DLD-$1^{scFvCD3}$ (n = 3), and **(E)** FaDu^{scFvCD3} (n = 3 or 4) after a 4 (ES-$2^{scFvCD3}$), 4 (DLD-$1^{scFvCD3}$), or 7 (FaDu^{scFvCD3}) day co-culture with (red squares) or without (blue circles) the addition of CD27xEGFR (10 µg/mL) at the indicated Effector : Target (E:T) ratios. For each plot, pairs of data points represent independent experiments, each utilizing T cells from a unique donor. Statistical significance was determined using paired t-tests. **(F)** mCherry-expressing FaDu^{scFvCD3} cancer cell survival (relative to cancer cells only) in a 3-day co-culture treated with CD27xEGFR (10 µg/mL) or mAb 425 (10 µg/mL) (n = 4). Statistical significance was determined using a one-way ANOVA test with Tukey's correction. **(G, H)** mCherry-expressing OVCAR-$3^{scFvCD3}$ (n = 3 or 4) or OVCAR-$3^{scFvCD3}$EGFR^{KO} (n = 3) cancer cell survival (% of cancer cells only) after a 3-day co-culture with primary human T cells with (red squares) or without (blue circles) the addition of CD27xEGFR (10 µg/mL) at the indicated E:T ratios. Statistical significance was determined using paired t-tests. **(I)** Linear regression depicting the positive correlation between higher EGFR expression levels and increased delta values between CD27xEGFR and medium control (R-squared = 0.8705, p = 0.0206). Each point on the graph represents an individual pair difference at the 5:1 E:T ratio. Statistical significance was determined using an F-test. Data are presented as mean with error bars indicating standard deviation. "**" indicates ($p < 0.01$), "*" indicates ($p < 0.05$), "n.s." indicates non-significant differences.

repertoire (54, 55).This observation underscores their potential as a target for immunotherapy and suggests that EGFR-targeted re-activation of CD27 co-stimulatory signaling T cells may have a broad applicability across a diverse range of carcinomas (54, 55). To therapeutically exploit this observation, we developed an Fc-silent ICS-bsAb that targets CD27 and EGFR. This bsAb was designed to be minimally active 'en route', while providing multivalent and tumor-localized crosslinking of CD27 when bound to $EGFR^+$ cancer cells. Given the ubiquitous and abundant expression of EGFR in epithelial cells, it is important to account for potential toxicities that may arise from off-tumor targeting. Nevertheless, as the associated toxicities with targeting EGFR have remained within acceptable limits in the context of four clinically approved anti-EGFR mAbs (56), it is expected that side effects of co-stimulating CD27 through EGFR-targeting should not exceed these established thresholds. Supporting this concept, studies using a similar bispecific approach (CD28xEGFR), showed no independent stimulation of the immune system in the absence of TCR engagement with the MHC of cancer cells, as demonstrated in both cynomolgus monkeys and genetically engineered triple-humanized mice (57). In alignment with this data, our *in vitro* studies demonstrated CD27xEGFR simultaneously bound to both targets, enhanced T cell activation, increased T cell proliferation, and selectively potentiated anti-cancer T cell cytotoxicity. Additionally, based on the antagonistic properties of EGFR scFv, it is plausible to hypothesize that CD27xEGFR may have the potential to directly inhibit cell growth.

The tetravalent DVD-Ig antibody design of CD27xEGFR carries two EGFR and two CD27 antibody fragment domains (scFv 425 and scFv 1F5 targeting EGFR and CD27, respectively), facilitating bivalent binding to both targets. These specific scFvs were chosen based on their unique abilities to bind with and inhibit cell growth and trigger CD27 co-stimulation. Prior research by Murthy et al. established the efficacy of murine monoclonal antibody 425 in inhibiting the binding of EGF to its receptor, EGFR (58). The scFv variant of this antibody demonstrated moderate affinity to EGFR (200 nM < Kd < 400 nM) and exhibited growth inhibitory activity, as reported in previous studies (59–61). Similarly, the scFv 1F5, adapted from the agonistic mAb varlilumab (1F5), has the ability to block binding of soluble human CD70. It has exhibited significant preclinical activity, and is currently under evaluation in clinical trials (NCT03038672, and NCT04081688) (38). Therefore, although not formally investigated in this study, CD27xEGFR is expected to competitively inhibit ligand binding to the cognate receptors.

In the current study, CD27xEGFR was demonstrated to have high-affinity binding to EGFR on $EGFR^+$ tumor cells and to CD27 on $CD27^+$ T cells. Since the carcinoma and T cell binding domains are in-frame on either side, a potential concern could be that binding of one domain to its target cell would preclude binding to the second cell or domain. Although the two variable domains are indeed linked in tandem, the high domain flexibility of the DVD-Ig format was previously shown to allow for antigen binding of the inner domain with minimal steric hindrance (62). In line with this, CD27xEGFR demonstrated selective and simultaneous binding to EGFR and CD27, with clear doublet formation of carcinoma and T cells and inhibition of binding upon pre-incubation with excess amounts of mAb 425 and CD27 mAb. However, the possibility of T cell-to-T cell doublet formation as a result of CD27 binding on two different T cells has yet to be tested. Furthermore, CD27xEGFR had an increased association rate when exposed to both antigens, compared to a single antigen, as observed with biolayer interferometry, confirming the ability of CD27xEGFR to bind both targets simultaneously. This bsAb format is similar to recently described bsAbs that restrict immune checkpoint blockade of PD-1/PD-L1 or CD47 in an EGFR-restricted manner, leading to enhanced selectivity and efficacy of PD-L1 or CD47 blockade (63, 64). Based on the data presented here, CD27xEGFR may provide tumor-localized binding and crosslinking of CD27 on T cells for $EGFR^+$ carcinomas.

CD27xEGFR mediated T cell proliferation and activation upon TCR stimulation occurred only in co-cultures with $EGFR^+$ target cells, as evidenced by the increase in proliferating T cell peaks, the upregulation of CD25 expression in both $CD4^+$ and $CD8^+$ T cells, and increase in IFN-γ secretion. These results are consistent with previous studies showing that the CD27-targeting antibody Varlilumab upregulated T cell cytokine secretion (e.g., IFN-γ) and induced T cell proliferation at comparable levels in co-culture experiments (15, 33). Similarly, a tetravalent PD-L1 and CD27-targeting bsAb (CDX-527) induced IL-2 production upon TCR stimulation on a plate coated with OKT3 mAb and soluble PD-L1 (40). The functional activity of CDX-527 was further demonstrated using a CD27-NFκB reporter cell line, revealing enhanced activity compared to parental antibodies and further augmentation with the addition of recombinant soluble FcγR. However, CDX-527 relied on both PD-L1 expression and FcR interactions for CD27 crosslinking and activation, potentially unleashing strong on-target but off-tumor activity (65). CD27xEGFR's FcR independence, relying on $EGFR^+$ cancer cells to provide a CD27 cross-linking platform, could avoid these unwanted effects. Indeed, the combination of a T cell engager with the bsAb CD28xEGFR has successfully demonstrated the safe triggering of CD28 co-stimulatory signaling *via* EGFR crosslinking of co-stimulatory molecules, such as CD28 (57). Given that Varlilumab recently yielded synergistic anti-tumor activity in multiple tumor models when used in combination with PD-1/PD-L1 blockade (66), it would be worthwhile to further examine the activity of CD27xEGFR in combination with PD-1/PD-L1 blockade.

The antigen-dependent and tumor-selective cross-linking with CD27 was previously reported to functionally replace the FcγR dependent agonistic activity reported for several TNFRSF targeting antibodies (13, 67, 68). However, in a study that combined an EGFR-targeted bispecific T cell engager with several EpCAM-targeted TNFRSF bsAbs (41BB, OX40, TL1A, and CD27), the bsAb 41BBxEpCAM showed the highest activity (69). Therefore, it would be valuable to develop additional bsAbs with the same EGFR-selective tetravalent DVD-Ig antibody design but targeting different TNFRSF receptors, as these may provide even higher co-stimulatory activity than CD27.

CD27xEGFR-mediated cancer cell reduction was observed in different carcinoma cell line settings across a range of E:T ratios. The EGFR-dependent crosslinking of CD27 facilitated these effects, as the Fc domain of CD27xEGFR was designed with LALAPG mutations in order to create an effector silent IgG molecule to

reduce off-target activity *via* FcγR-expressing cells. In line with the CD27 crosslinking requirements, previous studies with syngeneic mouse tumor models have shown that Varlilumab induces FcR-engagement-dependent tumor regression and facilitates long-term anti-tumor immunity (13). As silencing of the Fc domain in CD27xEGFR also excludes effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity effects, a side-by-side comparison of Fc-silent CD27xEGFR to Fc-functional CD27xEGFR and their parental antibodies in co-culture experiments with PBMC populations should be conducted, next to experiments with isolated T cells. These molecules should also be evaluated as mouse surrogate molecules or in transgenic mice expressing human CD27 and EGFR, to further characterize the functional characteristics and safety profile of CD27xEGFR.

CD27xEGFR also had anti-proliferative effects, likely induced by blocking EGFR-mediated signaling, which was the strongest in 3-day treatments and comparable to the effects induced by mAb 425. This is consistent with an earlier report where mAb 425 and bsAb PD-L1xEGFR were compared for their ability to inhibit EGFR-mediated cancer cell proliferation (63). The extent of EGFR inhibitory effects varied among each of the carcinoma cell lines, which is in line with the varying levels of sensitivity to EGFR-inhibition reported for different tumor types (as reviewed in (56)). Furthermore, the activity of CD27xEGFR directly correlated with the EGFR expression in each cell line. This correlation could be attributed to enhanced growth inhibitory effects induced by EGFR blockade, or to heightened CD27 co-stimulation facilitated by greater CD27xEGFR binding. Importantly, blocking EGFR signaling can induce remodeling of the tumor microenvironment (TME) towards an immunoresponsive phenotype in non-small cell lung cancer (NSCLC) and inflammatory breast cancer (70–72). Thus, the potential antiangiogenic activity of EGFR-restricted CD27 co-stimulation warrants further investigation.

In addition to CD27 being expressed in cytotoxic and exhausted TILs, CD27 mRNA expression was also detected in tumor infiltrating regulatory T cells (T_{regs}), suggesting possible unwanted co-stimulatory effects on T_{regs} by CD27xEGFR. In this respect, the development of T_{regs} and increased T_{regs} activity in the TME are linked to CD27 agonism by $CD70^+$ tumor cells (73, 74). Upon prolonged T_{reg} stimulation, however, CD27 expression is downregulated and CD70 upregulated, leading to subsequent CD70-mediated T cell co-stimulation (75). Notably, in NSCLC tumors that develop EGFR-TKI refractory disease, CD70 is upregulated by refractory cancer cells (76). In tumor-bearing mice, this CD70 interacts constitutively with $CD27^+$ T_{regs} during tumor development, thereby promoting T_{reg} expansion and preventing cytotoxic T cell responses (77, 78). Therefore, it will be important to study the specific effects of CD27 co-stimulation on T_{regs} in the context of restricted co-stimulation to a tumor antigen such as EGFR in relevant murine models and in combination with T_{reg} depleting strategies, including sorafenib treatment (79).

In clinical studies, the active Fc domain of Varlilumab induced ADCC-mediated $CD27^+$ T_{reg} depletion, while providing co-stimulation to effector T cells in both hematological and solid tumors (21, 22). Indeed, in some patients, Varlilumab even triggered the development of *de novo* $CD8^+$ anti-tumor responses (22). Hence, as CD27xEGFR has an inactive Fc domain, the effector function of T_{reg} depletion and its subsequent effects, such as possible *de novo* $CD8^+$ responses, would be expected to be absent. However, a study inducing transient and deliberate CD27 agonism in $CD27^+$ T_{regs} through dendritic cells demonstrated that T_{regs} partially lost their suppressive function and converted into $CD4^+$ Th1 cells (80). Furthermore, CD27 co-stimulation is critical for the protection of $CD8^+$ T cells against subsequent T_{reg} suppression and is necessary for the priming of new T cells (25, 80). Therefore, CD27 agonism is anticipated to be a beneficial intervention, even in malignancies with T_{regs}. Moreover, CD27 agonism also enhances NK cell activation and proliferation, suggesting that these two additional anti-tumor mechanisms could also be explored in the context of CD27xEGFR treatment in follow-up studies (81, 82).

In conclusion, CD27xEGFR is a novel DVD-Ig bsAb targeting CD27 and EGFR, that has the potential to re-activate T cell immunity in $EGFR^+$ carcinomas through its interaction with tumor-reactive and exhausted $CD27^+CD8^+$ TILs. Moreover, the Fc-silent format of CD27xEGFR enables tumor-localized binding and crosslinking of CD27 only at $EGFR^+$ tumor sites, potentially enhancing its specificity and safety profile. These unique features of CD27xEGFR offer a compelling rationale for its further exploration in preclinical and clinical settings as a promising immunotherapeutic agent for $EGFR^+$ tumors.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE158803, GSE158803.

Ethics statement

All blood donors gave informed consent (nr. NVT0465, Sanquin Blood Supply, Groningen, the Netherlands).

Author contributions

VM, LN, MV, VW, GH, MdB, and EB contributed to the conception of the study. VM, LN, MV, VW, and EB contributed to investigation. VM, HL, and VB provided bioinformatic analysis. VM, LN, MV, HL, and EB contributed to data curation and formal analysis of the study.VM, LN, MV, and EB wrote the manuscript and were involved in manuscript revision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1191866/full#supplementary-material

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FcγR requirements and costimulatory capacity of Urelumab, Utomilumab, and Varlilumab

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Introduction: Targeting costimulatory receptors of the tumor necrosis factor receptor (TNFR) superfamily with agonistic antibodies is a promising approach in cancer immuno therapy. It is known that their efficacy strongly depends on FcγR cross-linking.

Methods: In this study, we made use of a Jurkat-based reporter platform to analyze the influence of individual FcγRs on the costimulatory activity of the 41BB agonists, Urelumab and Utomilumab, and the CD27 agonist, Varlilumab.

Results: We found that Urelumab (IgG4) can activate 41BB-NFκB signaling without FcγR cross-linking, but the presence of the FcγRs (CD32A, CD32B, CD64) augments the agonistic activity of Urelumab. The human IgG2 antibody Utomilumab exerts agonistic function only when crosslinked via CD32A and CD32B. The human IgG1 antibody Varlilumab showed strong agonistic activity with all FcγRs tested. In addition, we analyzed the costimulatory effects of Urelumab, Utomilumab, and Varlilumab in primary human peripheral blood mononuclear cells (PBMCs). Interestingly, we observed a very weak capacity of Varlilumab to enhance cytokine production and proliferation of CD4 and CD8 T cells. In the presence of Varlilumab the percentage of annexin V positive T cells was increased, indicating that this antibody mediated FcγR-dependent cytotoxic effects.

Conclusion: Collectively, our data underscore the importance to perform studies in reductionist systems as well as in primary PBMC samples to get a comprehensive understanding of the activity of costimulation agonists.

KEYWORDS

human T cell costimulation, 41BB, CD137, CD27, agonistic antibodies, Urelumab, Utomilumab, Varlilumab

Introduction

In the last years, antibody-based T cell directed immunotherapy has improved cancer treatment. In addition to so-called immune checkpoint inhibitors (ICIs), which block coinhibitory receptors such as PD1 and CTLA-4, the engagement of costimulatory pathways with agonistic antibodies is a promising approach to enhance T cell mediated antitumor immunity (1–5). Receptors of the tumor necrosis factor receptor (TNFR) superfamily (TNFRSF) are considered the most promising targets for costimulation agonists, and antibodies to 41BB, CD27, OX40, and GITR, have already entered clinical trials (1, 6–13).

41BB (CD137, TNFRSF9) is an inducible costimulatory receptor and is expressed on activated CD4 and CD8 T cells (1, 14). Engagement via its natural ligand 41BBL or agonistic antibodies leads to the activation of multiple signaling pathways, resulting in the activation of NFκB and MAPK (15–17). 41BB induces intracellular signals that mediate T cell proliferation, cytokine production, and effector functions, such as cytotoxicity (18, 19). Currently, ten "classical" 41BB agonistic antibodies and around thirty additional 41BB agonists, such as bi-specifics have entered Phase I clinical trials (20). Urelumab (BMS-663513), a fully humanized IgG4 antibody that does not block 41BB – 41BBL interaction, and the ligand-interaction blocking human IgG2 antibody, Utomilumab (PF-05082566), can be considered as the first generation of 41BB agonists for cancer immunotherapy (20–23). Several *in vivo* and *in vitro* studies demonstrate, that both antibodies enhance T cell function and elicit anti-tumor immunity (24, 25). However, severe side effects such as liver inflammation and limited efficacy have hampered the clinical development of Urelumab and Utomilumab, respectively, and their clinical development has been discontinued (11, 20, 26, 27). We have observed that 41BB agonists have the potential to promote the activation of bystander CD8 T cells, which could also contribute to the unwanted effects of 41BB antibodies (28).

CD27 (TNFRSF7) is another attractive candidate target to improve tumor immune response. Unlike several other TNFRs, CD27 is constitutively expressed by the majority of T cells. CD27 costimulation promotes T cell activation, proliferation, generation of effector cells, and maintenance of memory cell function (29, 30). Currently, Varlilumab (CDX-1127), a fully humanized IgG1 CD27 antibody, is applied in clinical trials (31–33). Other CD27 agonists, such as MK-5890, are also in clinical development (34, 35). Varlilumab acts agonistically by interacting with the CD70 binding site of CD27 (31). The potent anti-tumor activity of this antibody was shown in preclinical and clinical studies, where targeting CD27 in hematologic and solid tumors led to increased survival and stable disease (32, 33, 36, 37). It is well known that the activity of agonistic antibodies is critically modulated by Fc - FcγR interactions since oligomerization via cell surface expressed FcγRs influences their immunomodulatory efficacy (38–40). Furthermore, interaction with FcγRs is also implicated in immune abnormalities and toxic side effects, and the clinical development of 41BB antibodies was restricted by severe hepatoxicity linked to FcγRs-induced cross-linking (41–43). In addition, FcγRs can transduce activating signals, resulting in the production of proinflammatory cytokines, but also antibody-dependent cellular cytotoxicity and phagocytosis (ADCC and ADCP) towards cells expressing the target antigens (42, 44). Furthermore, certain IgG subclasses can also mediate complement-dependent cytotoxicity (CDC). A better understanding of how FcγRs and other components of the immune system influence the effect of agonistic antibodies may help to optimize their efficacy and to prevent adverse effects.

In this study, we have assessed the individual contribution of different human FcγR classes on the agonistic activity of Urelumab, Utomilumab, and Varlilumab using a Jurkat reporter system in conjunction with stimulator cells expressing individual human Fcγ receptors. In addition, we have analyzed the capacity of Urelumab, Utomilumab, and Varlilumab to augment proliferation and cytokine production in human peripheral blood mononuclear cells (PBMCs) stimulation cultures *in vitro*.

Materials and methods

Sample collection

The study was approved by the ethical committee of the Medical University of Vienna (1183/2016). The study abides by the Declaration of Helsinki principles. PBMCs were isolated from buffy coats or heparinized blood obtained from healthy volunteer donors by using Ficoll-Hypaque (GE Healthcare Life Sciences, Pittsburgh, PA, USA) density gradient centrifugation.

Cell culture, antibodies, flow cytometry

The mouse thymoma cell line Bw5417 (short designation within this work Bw) and Jurkat E6.1 (JE6.1), were cultured as described (45). Triple parameter reporter cell lines (TPR) and the monoreporter cell line are based on the JE6.1 Jurkat cell line, stably expressing NFκB::eCFP, NFAT::eGFP, and AP-1::mCherry reporter constructs or NFκB::eGFP, respectively as described (46).

T cell stimulator cells (TCS) used in this study are Bw5147 cells that stably express membrane-bound single chain antibody fragments derived from the CD3 antibodies (mb-α-CD3) UCHT1 or OKT3 on their surface (47, 48).

A CD14 mAb antibody was used to stain the surface expression of aCD3scFv which were expressed on the cell surface via a c-terminal CD14 sequence (49). To exclude the TCS in the reporter assays, an mCD45 antibody was used.

The following flow cytometry antibodies were used in this study: PE-Isotype control (MPOC-21), PE-41BBL (5F4), PE-CD70 (113–16), PE-OX40L (11C3.1), PE-41BB (CD137, 4B4-1), PE-CD27(M-T271), PE-GITR (621), PE-OX40 (CD134, ACT35), APC-CD16 (3G8), APC-CD32 (FUN2), APC-CD64 (10.1), APC-mCD45 (104), APC-CD14 (63D3), PE-CD14 (63D3), FITC-CD56 (HCD56), BV421-CD19 (HIB19), BV421-CD4 (OKT4), PerCP-CD8 (HIT8a, all from Biolegend, San Diego, CA, USA), and PE-GITRL (REA841, Miltenyi Biotec).

For CFSE proliferation assays a functional grade CD3 mAb (UCHT1, Biolegend) was used. For annexin V assays, an FcR silenced CD3 mAb (REA613, Miltenyi Biotec) was used. Agonistic 41BB antibodies - Urelumab (BMS-663513), Utomilumab (PF-05082566), and the CD27 agonist mAb Varlilumab (CDX-1127) were purchased from Creative Biolabs (NY, USA).

For blocking of Fc receptors, cells were incubated for 20 minutes at 4°C with 20 mg/ml Beriglobin (CSL Behring). Flow cytometry analysis was performed using FACSCalibur™ or LSRFortessa™ flow cytometers (BD Bioscience, Franklin Lakes, NJ). FlowJo software (version 10.4.1. Tree Star, Ashland, OR) was used for flow cytometry data analysis.

Generation of reporter and T cell stimulator cell lines

The sequences encoding for CD27 (UniProt P26842), 41BB (UniProt Q07011), GITR (UniProt Q9Y5U5), and OX40 (UniProt P43489) were cloned into the lentiviral expression vector pHR and stably expressed on Jurkat reporter cell lines. The sequences encoding for low affine CD16A (FcgRIIIA, UniProt P08637), the high affine natural variant of CD16A 176V (FcgRIIIA 176V, UniProt P08637 VAR_003960, short designation in this work CD16A F176V), CD32A (FcgRIIA, UniProt P12318), CD32B (FcgRIIB, UniProt P31994), CD64 (FcgRI, UniProt P12314) were introduced into the lentiviral expression vector pHR and stably expressed in the T cell stimulator cells (TCS) (50). The sequences encoding for the TNFR ligands OX40L (UniProt P23510), CD70 (UniProt P32970), 41BBL (UniProt P41273), GITRL (UniProt Q9UNG2) were cloned into the retroviral expression vector pCJK2 and stably expressed on the T cell stimulator cells as described (47).

Reporter assay

Jurkat reporter cells ($5x10^4$) were stimulated with TCS ($2x10^4$) for 18-24h. In some experiments, 41BB (Urelumab, Utomilumab) or CD27 (Varlilumab) agonistic antibodies were added in different concentrations (as indicated in Figures). Subsequently, reporter activity was analyzed by flow cytometry as described previously (49). The gating strategy used is depicted in Supplementary Figure 1. Reporter gene induction is shown as gMFI (geometric mean fluorescence intensity). For some experiments, reporter gene induction in response to stimulation was normalized to control-stimulated reporter cells as indicated and expressed as fold induction.

CFSE proliferation assay

Human PBMCs were CFSE (Molecular Probes) labeled as described previously (51). $1x10^5$ labeled cells were stimulated with soluble CD3 mAb UCHT1 (final concentration 30 ng/ml or 10 ng/ml) or plate-bound CD3 mAb UCHT1 (final concentration 1 µg/ml) in the presence or absence of soluble Urelumab, Utomilumab, or Varlilumab (concentration used at 0.03, 0.1, 0.3, 1 µg/ml as indicated). For the plate-bound assays, ELISA plates were coated with 1 µg/ml CD3 mAb in PBS overnight at 4°C, followed by two washing steps with PBS. For proliferation assays with T cell stimulator cells, TCS were pretreated with Mitomycin C (final concentration 20 µg/ml, Carl Roth) as described previously (50). Following 5 days of stimulation, the percentage of $CFSE^{low}$ in gated CD4 and CD8 T cells was determined by flow cytometry. Flow cytometry analysis was performed using constant cell volumes, flow rates, and acquisition time for all samples (20 sec at medium flow).

Annexin V staining

For apoptosis assay, PBMCs were stimulated with plate-bound Fc-silenced CD3 mAb (1 µg/ml final) together with soluble Urelumab, Utomilumab, or Varlilumab (final 1 µg/ml) for 24 and 48 hours. Subsequently, cells were harvested and resuspended in 50 µl of Annexin V binding buffer (Biolegend). Annexin V-FITC (Biolegend) was diluted 1:100 from stock, 5 µl were added to each tube and cells were incubated for 15 min in the dark at room temperature. Finally, another 50 µl of Annexin-V binding buffer was added to a total volume of 105 µl. Flow cytometry analysis was performed using constant cell volumes, flow rates, and acquisition time for all samples (30 sec at medium flow).

Cytokine measurement

Supernatants of stimulations assays for annexin V and CFSE proliferation assays were harvested after 48h or at day 5, respectively. GM-CSF, IFN-γ, TNF-α, IL-13, and IL-2 were measured with the Luminex 100 system (Luminex Inc., Texas, USA) according to the manufacturer's instructions.

Statistics

Statistical analyses were performed using GraphPad Prism (Version 9, GraphPad Software, Inc., La Jolla, CA, USA). Statistics were calculated using the Friedman test followed by Dunn's multiple comparison test (compared to a control group), One-way ANOVA followed by Tukey's multiple comparison or 2-way ANOVA with Dunnett's multiple comparison test. The EC_{50} values and the 95% confidence intervals were determined using the four-parameter nonlinear regression. Levels of significance were categorized as follows: ns, not significant; ns > 0.05, *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Creation of schemes

BioRender was used for the creation of schematics.

Results

Evaluation of 41BB, CD27, OX40, and GITR signaling in a Jurkat-based reporter cell system

In the first set of experiments, we evaluated the capacity of four important T cell costimulatory members of the TNFRSF, 41BB, CD27, OX40, and GITR to activate transcription factors that play major roles in T cell activation, namely NFκB, NFAT, and AP-1. Therefore, we made use of a Jurkat-based triple parameter T cell reporter cell line (TPR) where each of these transcription factors drives the expression of a distinct fluorophore (NFκB::eCFP, NFAT::eGFP, and AP-1::mCherry) (46). A schematic of the experimental design is given in Figure 1A.

Since Jurkat cells do not express these TNFR endogenously (Supplementary Figure 1), they are well suited for gain-of-function studies. We introduced 41BB, CD27, OX40, and GITR into the Triple-reporter cells (Figure 1B). The Jurkat reporter cells can be activated with stimulator cells expressing membrane-bound anti-CD3 single chain fragments (T cell stimulator cells, TCS) (46). TCS expressing 41BBL, CD70, OX40L, or GITRL were generated to stimulate the TNFR-expressing reporter cells in the presence of their respective ligands (Figure 1A). Cell surface expression of the membrane-bound anti-CD3 single chain fragments (mb-α-CD3) and the respective TNFR-ligands in the TCS was verified by flow cytometry (Figure 1C).

The TPR-TNFR cell lines were co-cultured with stimulator cells expressing their respective ligands. TCS expressing no costimulatory ligand (TCS-ctrl) were included as controls.

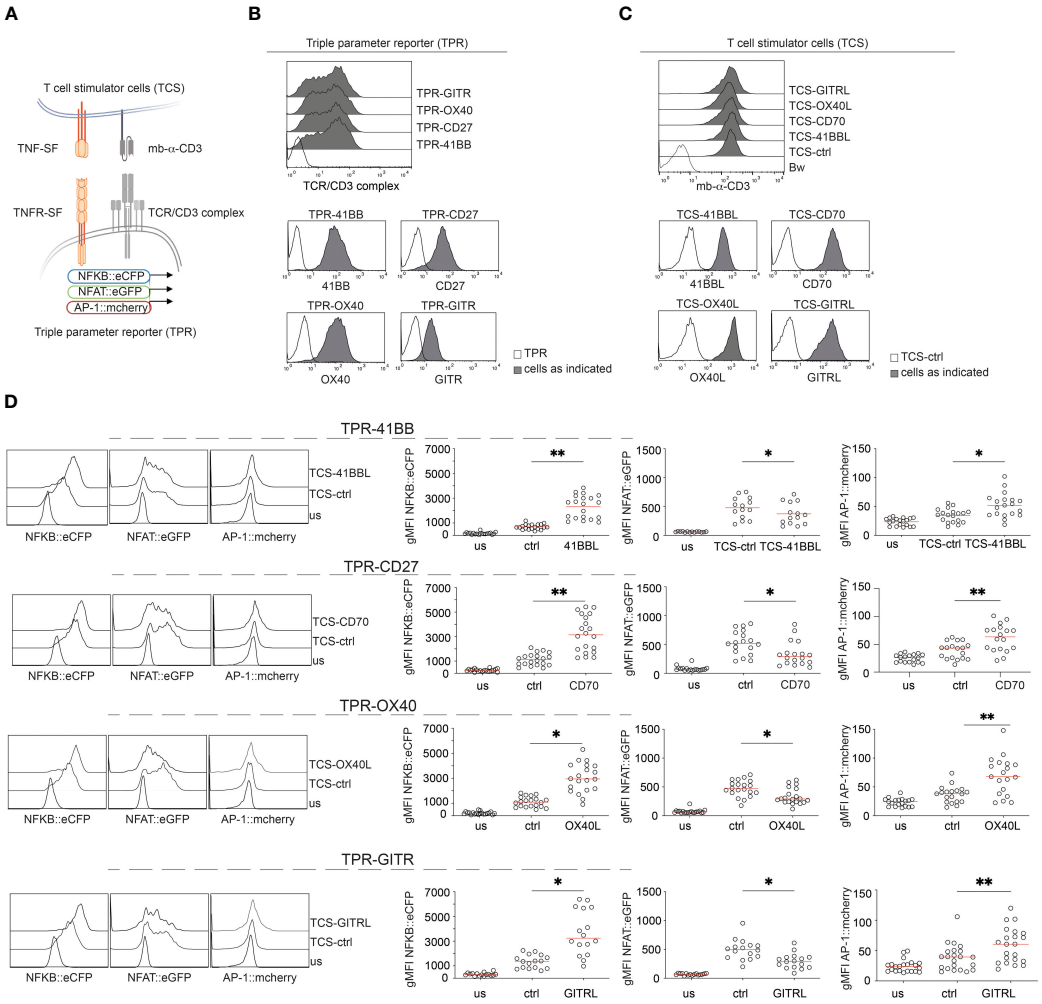

FIGURE 1
Evaluation of 41BB, CD27, OX40, and GITR signaling in a Jurkat-based triple parameter reporter system. **(A)** Schematic of the Jurkat reporter – T cell stimulator cell system. **(B)** Flow cytometry staining of Jurkat reporter cells. **(C)** Flow cytometry staining of T cell stimulator cells (TCS). Upper panel: expression of the membrane-bound anti-human CD3 single chain fragment (mb-αCD3) on the indicated TCS; the paternal Bw cell line was used as control. Lower panels: expression of TNFR-ligands on TCS. Filled histogram: expression level on the indicated TCS; open histograms: staining of control TCS. **(D)** Jurkat-TPR expressing the indicated TNF receptor were stimulated with TCS control or with TCS expressing the corresponding ligand or left unstimulated (us). Reporter gene expression (NFκB::eCFP, NFAT::eGFP, and AP-1::mCherry) was assessed via flow cytometry. Left panel: Histograms show data from one representative experiment. Right panel: summarized data are shown (n=18 for CD27, n=16 for GITR, n=20 for OX40 and 41BB), each dot represents the mean of triplicate measurement, red line shows median; geometric mean fluorescence intensity (gMFI). The statistics were calculated using the Friedman test followed by Dunn's multiple comparison test. *p ≤ 0.05; **p ≤ 0.01.

Reporter gene expression was assessed by flow cytometry (Figure 1D). The gating strategy is shown in Figure S1B. Compared to control stimulation, the activation of NFκB and AP1 was strongly enhanced when TPR-TNFR were stimulated with TCS expressing their respective ligands (Figure 1D). Interestingly, we observed that signaling via these TNFRSF members significantly reduced NFAT reporter gene expression (Figure 1D). The parental TPR cell line expressing no TNFR did not respond to any of these TNFR ligands (Supplementary Figure 1C). Collectively, in our T cell reporter system, 41BB, CD27, OX40, and GITR exerted similar costimulatory effects and we did not observe a significant difference in their capacity to induce the activation of NFκB and AP1 (Supplementary Figure 2).

Influence of human FcγRs on the agonistic activity of Urelumab and Utomilumab

The 41BB agonistic antibodies Urelumab and Utomilumab represent the first generation of 4-1BB agonists. It is known that the agonistic potential of these antibodies strongly depends on Fcγ receptor cross-linking. We assessed the Fc receptor dependency of Urelumab (human IgG4 antibody) and Utomilumab (human IgG2 antibody) with our reporter system as outlined in Figure 2A. Therefore, we used highly sensitive NFκB::eGFP reporter cells expressing 41BB in conjunction with stimulator cells expressing Fcγ receptors (FcγRs). The NFκB::eGFP reporter cells are based on the Jurkat JE6-1 line. Stimulation with TCS-41BBL confirmed that the NFκB::eGFP-41BB reporter cells strongly responded to 4-1BB costimulation (Figure 2B).

Stimulator cells equipped with one of the following human Fcγ receptors were used: CD16A (FcγRIIIA), CD16A F176V natural variant (FcγRIIIA F176V), CD32A (FcγRIIA), CD32B (FcγRIIB), and CD64 (FcγRI) (Supplementary Figure 3).

NFκB::eGFP-41BB reporter cells were co-cultured with different concentrations of Urelumab or Utomilumab (ranging from 0.001 µg/ml to 3.16 µg/ml) in the presence of TCS-ctrl (no FcγR present) or stimulator cells expressing CD16A, CD16A F176V, CD32A, CD32B, or CD64 (Figures 2C, D and Supplementary Figure 4). Reporter gene activation was assessed in flow cytometry.

Stimulation with Urelumab yielded an increase of reporter gene induction in a dose-dependent manner without FcγR-mediated cross-linking. However, compared to stimulation with its natural ligand 41BBL (indicated by a dotted line), the activation induced by Urelumab alone was considerably lower. The agonistic potential of Urelumab was augmented by cross-linking via CD32A, CD32B, and CD64, but only when cross-linked via CD32B Urelumab induced a stronger activation signal than its natural cell-surface expressed ligand 41BBL (Figure 2C middle panel). The strong costimulatory activity of 41BBL is due to hyperclustering mediated by the cell surface expression as soluble trimeric 41BBL is less active than Urelumab. The introduction of trimerization domains or crosslinking 41BBL via targeting to tumor or tumorstroma antigens can greatly enhance the costimulatory activity of soluble 41BBL (43, 52, 53). In contrast, the human IgG2 antibody Utomilumab did not act agonistically without FcγR-mediated cross-linking. Furthermore, this antibody only exerted a good agonistic function when cross-linked via CD32B and only a minor agonistic activity when cross-linked via CD32A. Cross-linking via CD64 had no effect (Figure 2D). Of note, compared to engagement by 41BBL or Urelumab, the activation signals induced by Utomilumab were substantially lower per se. The presence of CD16A or CD16A F176V on the TCS did not induce NFκB signaling through Urelumab and Utomilumab (Supplementary Figure 4A). The EC50 values and the 95% confidence intervals (CI) calculated from the reporter gene activation signal for Urelumab and Utomilumab are shown in Figure 2 (C and D lower panel) and summarized in Table 1. In the absence of CD3 stimulation, Urelumab also exerted a weak agonistic activity on 41BB expressing reporter cells, whereas Utomilumab had no effect as expected (Supplementary Figure 4).

Taken together, Urelumab was found to functionally engage 41BB much stronger than Utomilumab. Furthermore, we observed that Urelumab can also exert costimulatory activity in the absence of FcγRs.

Influence of human FcγRs on the agonistic activity of the CD27 antibody Varlilumab

Next, we wanted to examine the FcγR requirements of the CD27 agonist Varlilumab (human IgG1) with our Jurkat-based reporter system as depicted in Figure 3A.

CD27 was expressed on NFκB::eGFP reporter cells (Figure 3B and Supplementary Figure 3). Stimulation with TCS-CD70 confirmed that the NFκB::eGFP-CD27 reporter cells strongly responded to CD70 costimulation (Figure 3B). Next, cells were stimulated with different concentrations (ranging from 0.0003 µg/ml to 3.16 µg/ml) of Varlilumab in the absence of FcγRs (TCS-ctrl) or by TCS expressing CD16A, CD16A F176V, CD32A, CD32B, or CD64 (expression shown in Supplementary Figure 3). Reporter gene induction was analyzed by flow cytometry (Figure 3C).

Varlilumab did not show any effect in the presence of TCS control, whereas it dose-dependently enhanced reporter activation in the presence of all FcγRs tested (Figure 3C and Supplementary Figure 4). CD16A F176V and CD32B had the strongest effect and Varlilumab-mediated reporter activation in the presence of TCS expressing these Fc-receptors was much stronger than reporter activation mediated by TCS expressing CD70, the natural CD27 ligand (Figure 3C middle panel).

The EC50 values and 95% CI obtained from the reporter gene activation signal for Varlilumab are summarized in Figure 3C and Table 1.

Effects of Urelumab, Utomilumab, and Varlilumab on proliferation and cytokine production of primary human T cells

It is known that 41BB and CD27 are potent costimulatory receptors in CD4 and CD8 T cells. Whereas CD27 is constitutively

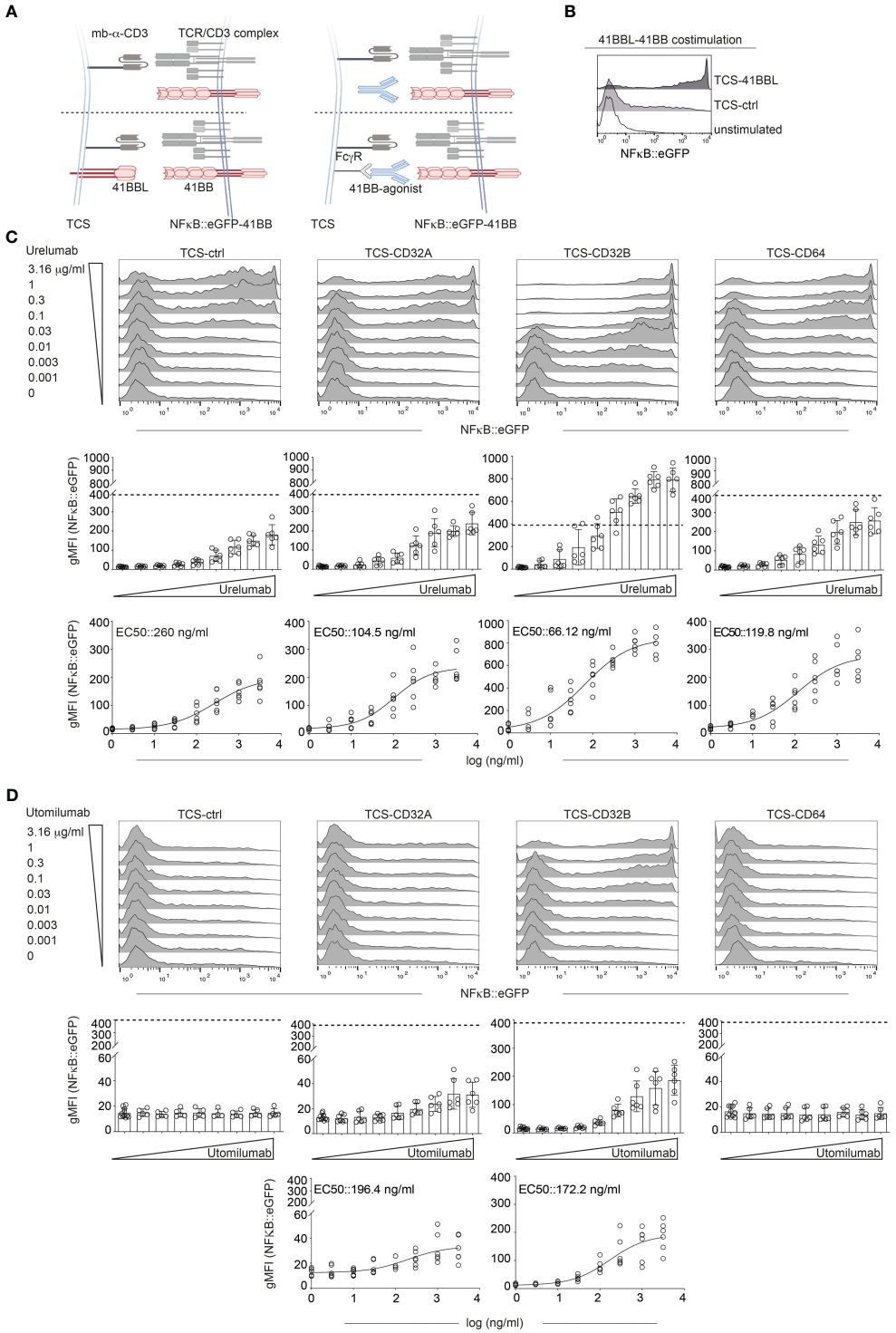

FIGURE 2
Influence of human Fcγ receptors on the agonistic activity of Urelumab and Utomilumab. **(A)** Schematic of the experimental set up. Jurkat-NFκB::eGFP expressing 41BB were stimulated either with TCS control, TCS expressing 41BBL (TCS-41BBL) or TCS expressing one of the indicated Fcγ receptors in the presence of different concentrations of 41BB agonistic antibodies. **(B)** Jurkat-41BB reporters were stimulated with TCS control (TCS-ctrl) or TCS-41BBL or left unstimulated. NFκB::eGFP reporter gene activation was analyzed by flow cytometry. **(C, D)** Jurkat-41BB reporters were stimulated with TCS control (TCS-ctrl) or TCS expressing the indicated Fcγ receptors (TCS-CD32A, TCS-CD32B, TCS-CD64) in the presence of different concentrations (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, or 3.16 µg/ml) of Urelumab **(C)** or Utomilumab **(D)**. Reporter gene induction was analyzed by flow cytometry. Upper panels: histograms show the results of one representative experiment. Middle: Summarized data +/- SD are shown (n=3, each performed in triplicates), dotted line indicates reporter gene expression upon stimulation via TCS-41BBL. Lower panels: stimulation curves and half-maximum effective concentration (EC50) were calculated as described in material and methods (n=3, performed in duplicates).

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TABLE 1 EC50 values and CI intervals for Urelumab, Utomilumab and Varlilumab.

Mab	Fc Receptor	EC50 ng/ml	95% CI
Urelumab	None	155.3	84.84 - 284.3
	CD32A	104.5	53.14 - 205.5
	CD32B	66.12	37.74 - 115.8
	CD64	119.8	52.41 - 273.8
Utomilumab	CD32A	196.4	47.52 - 812.2
	CD32B	172.2	78.51 - 377.5
Varlilumab	CD16A	249.2	149.4 - 415.5
	CD16A F176V	90.89	79.39 - 104.1
	CD32A	157.5	116.5 - 212.8
	CD32B	27.66	23.47 - 32.59
	CD64	14.56	8.642 - 24.52

EC50 values and the 95% confidence intervals (CI) were determined for Urelumab, Utomilumab, and Varlilumab for their ability to induce 41BB-NFκB or CD27-NFκB signaling respectively in a functional assay. Data from three independent experiments performed in duplicates were used to calculate the EC50 values.

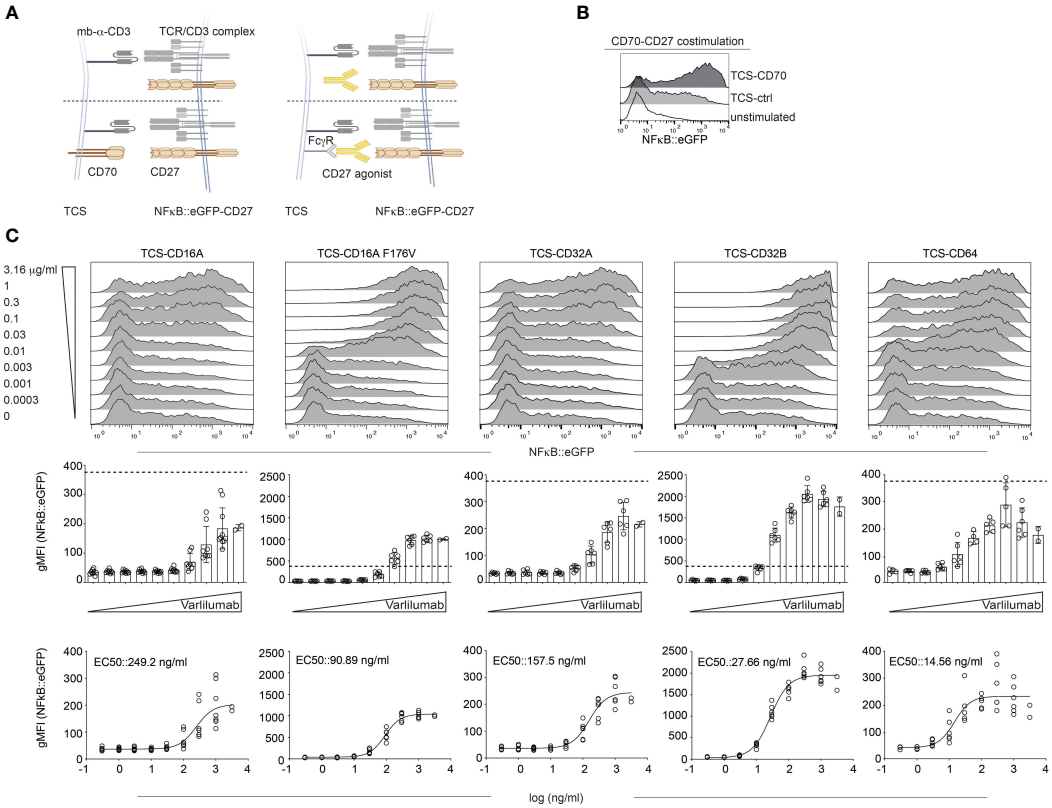

FIGURE 3
Influence of human Fcγ receptors on the agonistic activity of Varlilumab. **(A)** Schematic of the experimental set up. Jurkat-NFκB::eGFP expressing CD27 were stimulated either with TCS control (mb-α-CD3), TCS expressing CD70, or with TCS expressing one of the indicated Fcγ receptors in the presence of different concentrations of CD27 agonistic antibody. **(B)** Jurkat-CD27 reporters were stimulated with TCS control (TCS-ctrl), TCS-CD70, or left unstimulated. NFκB::eGFP reporter gene activation was analyzed by flow cytometry. **(C)** Jurkat-CD27 reporters were stimulated with TCS control or TCS expressing one of the indicated Fcγ receptors (TCS-CD16A, TCS-CD16A F176V, TCS-CD32A, TCS-CD32B, or TCS-CD64) in the presence of different concentrations of Varlilumab. Reporter gene induction was analyzed by flow cytometry. Upper panel: histogram overlay show results of one representative experiment. Middle: Summarized data +/- SD are shown (n=3, each performed in triplicates), dotted line depicts reporter gene expression upon stimulation with TCS-CD70. Lower panel: stimulation curves and half-maximum effective concentration (EC50) were calculated as described in material and methods (n=3, performed in duplicates).

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expressed in the majority of CD4 and CD8 T cells, 41BB is not expressed in resting cells, but upregulated upon activation on both CD4 and CD8 T cells (54). Since FcγR critically modulate the activity of agonistic antibodies we analyzed their expression in freshly isolated and *in vitro* stimulated PBMCs (Supplementary Figure 5). In line with previous reports, CD16 was expressed in approximately 60% of NK cells ($CD56^+$) as well as in a smaller subset of monocytes/macrophages ($CD14^+$). CD32 was highly expressed in B cells ($CD19^+$) and to a lower degree in monocytes/macrophages. CD64 was highly expressed in monocytes/macrophages (Supplementary Figure 5). To compare the capacity of Urelumab, Utomilumab, and Varlilumab to costimulate proliferation and cytokine production of CD4 and CD8 T cells *in vitro* human PBMCs were CFSE-labeled and stimulated with CD3 antibodies (30 ng/ml) alone or in combination with these antibodies. Proliferation (CFSE dilution) was analyzed in gated CD4 and CD8 T cell populations on day 5 by flow cytometry (Figure 4A). In both populations, the 41BB agonists, Urelumab and Utomilumab, induced significantly higher proliferation compared to the CD3 antibody alone. In contrast, the CD27 agonist Varlilumab failed to significantly increase the percentage of proliferated CD4 and CD8 T cells (Figure 4B).

In parallel, we also analyzed the content of GM-CSF, IFN-γ, TNF-α, and IL-13 in the supernatants of stimulation cultures (Figure 4C). Stimulation with Urelumab significantly enhanced GM-CSF, IFN-γ and IL-13 production whereas TNF-α was

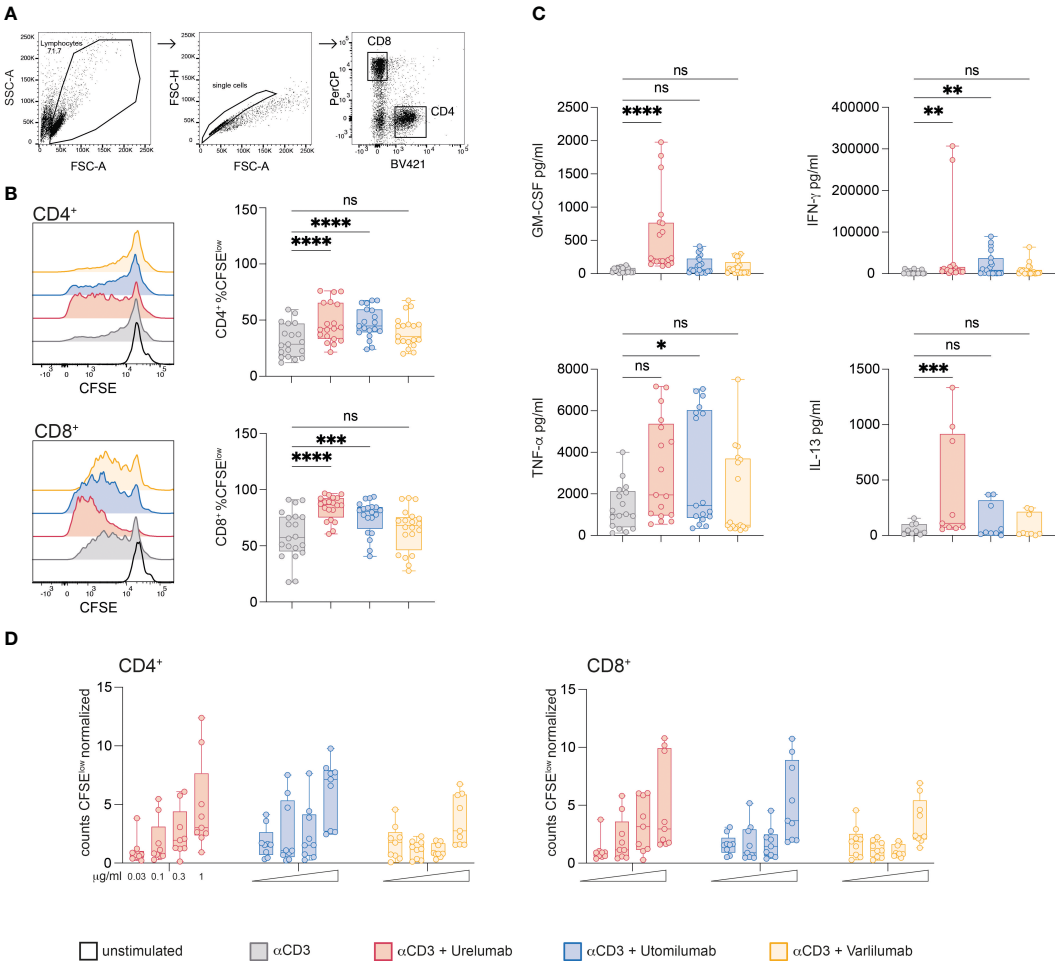

FIGURE 4
Effects of Urelumab, Utomilumab, and Varlilumab on the proliferation and cytokine production of primary human T cells. **(A)** Gating strategy used. **(B, C)** CFSE labeled human PBMCs were stimulated with CD3 antibodies (final 30 ng/ml) in the presence or absence of Urelumab, Utomilumab, or Varlilumab (soluble, all used at a final concentration of 1 µg/ml) for 5 days. **(B)** CFSE dilution was analyzed in gated CD4 and CD8 T cell populations. Left panel: Histogram overlay shows CFSE dilution in CD4 and CD8 T cells of one representative donor; right panels: box plots show summarized data from all donors. **(C)** Cytokine content (IFN-γ, GM-CSF, TNF-α and IL-13) of stimulation cultures was assessed using a Luminex-based assay. **(B, C)** Summarized data are shown. n=7, each performed in triplicates. For statistical analysis, the Friedman test followed by Dunn's multiple comparison correction were used. ns, not significant, *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$. **(D)** CFSE-labeled human PBMCs were stimulated with plate-bound CD3 antibodies in the presence or absence of Urelumab, Utomilumab, or Varlilumab (soluble, used at 0.03, 0.1, 0.3, or 1 µg/ml) for 5 days. Counts of $CFSE^{low}$ cells were analyzed in gated CD4 and CD8 T cell populations. Flow cytometry analysis was performed using constant cell volumes, flow rates, and acquisition time for all samples. Counts of $CFSE^{low}$ CD4 or CD8 cells are depicted normalized to control stimulated cells (CD3 antibody alone). Summarized data of 3 donors are shown (n=3, each performed in triplicates).

increased, but the difference did not reach statistical significance. The presence of Utomilumab induced significant IFN-γ and TNF-α levels, whereas GM-CSF, and IL-13 were slightly increased compared to stimulation with CD3 antibodies. Varlilumab did not significantly augment the production of any of the tested cytokines compared to CD3 stimulation alone. Similar results were obtained in the presence of weaker CD3 stimulation (10 ng/ml; Supplementary Figure 6). We also analyzed the effects of Urelumab, Utomilumab, and Varilumab in different concentrations (0.03; 0.1; 0.3, and 1 µg/ml) in conjunction with plate-bound CD3 antibodies. We observed a dose-dependent increase of proliferated ($CFSE^{low}$) CD4 and CD8 T cells for all antibodies tested. Varlilumab had the weakest effect also in these experiments. In contrast to Urelumab and Utomilumab, which were effective also at lower concentrations (0.1 and 0.3 µg/ml), Varlilumab only increased the number of proliferated T cells at the highest concentration (1 µg/ml) (Figure 4D). Use at higher concentrations did not further increase the costimulatory effect of Urelumab and Utomilumab and Varilumab (data not shown). In general, the effect of Varlilumab on proliferation and cytokine production in primary human PBMCs was weak. This is in strong contrast to the results obtained with the Jurkat-reporter system, where Varlilumab strongly induced NFκB signaling. This discrepancy might be due to cytotoxic effects such as ADCC towards T cells triggered via its unmodified human IgG1 Fc part.

Varlilumab induced apoptosis in CD4 and CD8 T cells

To test this, we performed annexin V staining of PBMCs stimulated with immobilized CD3 antibodies. An Fc-silenced CD3 antibody was used in these experiments to preclude a potential interference with TNFR-agonist-FcγR interaction. Following 48h of stimulation the cells were harvested stained with annexin V and analyzed by flow cytometry. Indeed, we observed a significant increase in the percentages of annexin V-positive CD4 and CD8 T cells (Figures 5A, B). Furthermore, in stimulation cultures containing Varlilumab, the number of CD4 and CD8 cells was significantly reduced (Figure 5B). Cytokine measurements indicated a weak stimulatory capacity of Varlilumab in these experimental conditions, but the differences did not reach statistical significance (Figure 5C).

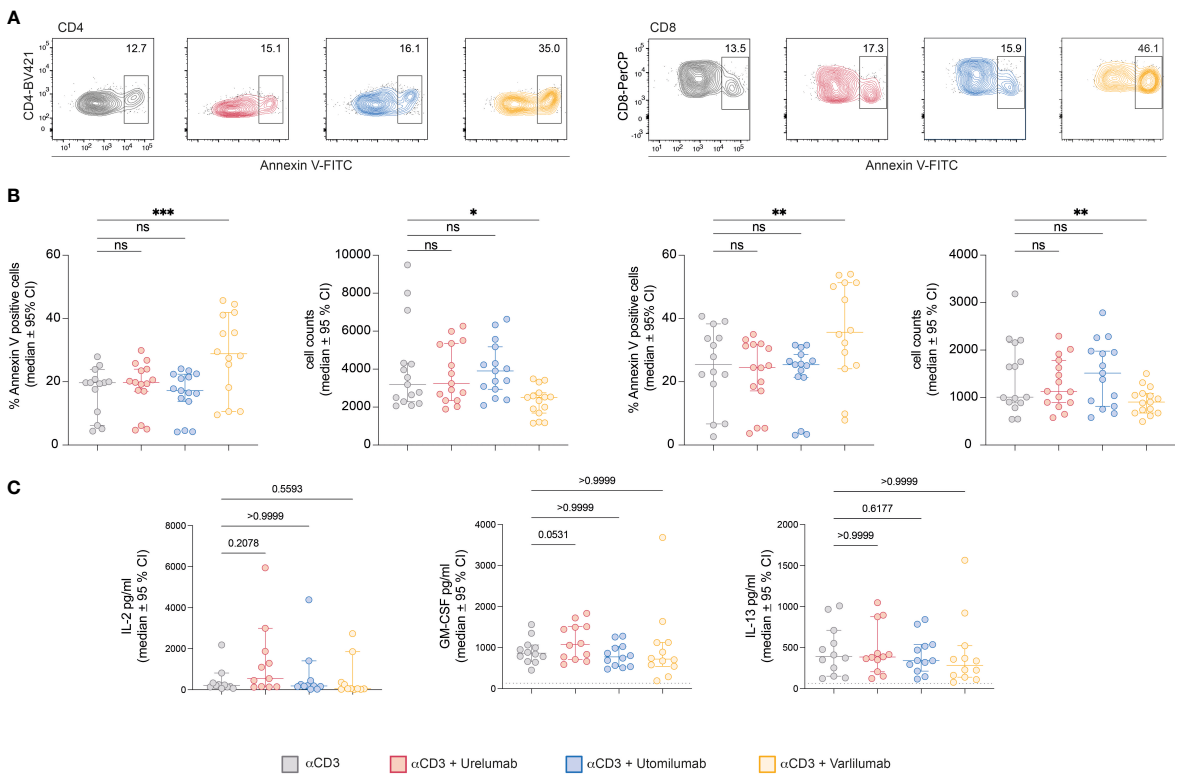

FIGURE 5
Varlilumab-induced apoptosis in CD4 and CD8 T cells. Human PBMCs were stimulated with plate-bound CD3 antibodies in the presence or absence of Urelumab, Utomilumab, or Varlilumab (1 µg/ml) for 48h. **(A)** Annexin V expression was analyzed in gated CD4 and CD8 T cells. Flow cytometry analysis was performed using constant cell volumes, flow rates, and acquisition time for all samples. **(B)** Summarized data of annexin V staining and cell counts of all donors are shown (n=5, each performed in triplicates). **(C)** Cytokine content (IL-2, GM-CSF, IL-13) of stimulation cultures was assessed using a Luminex-based assay. B-C) For statistical analysis, the Friedman test followed by Dunn's multiple comparison correction were used. Median and +/- 95% CI is shown. ns, not significant; ns > 0.05, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

CD16A F176V, CD32B, and CD64 mediate strong costimulatory effects of Varlilumab on purified T cells

In an attempt to dissect immunostimulatory effects mediated by FcγR interaction and effects such as ADCC mediated by the interaction of TNFR-agonist with cytotoxic effector cell populations, we performed 5-day co-culture experiments with purified T cells in the presence of FcγR-expressing TCS. In parallel, we also performed stimulation experiments with PBMCs from the same donor (Figure 6A). In the T cell samples, significant costimulatory effects of the 41BB agonists Urelumab and Utomilumab were only observed in stimulation cultures with TCS expressing CD32B. TCS expressing CD16A F176V, CD32B, and CD64 mediated Varlilumab costimulation and significantly increased the percentage of proliferated CD4 and CD8 T cells (Figure 6B). In PBMC samples, significant costimulatory effects of Urelumab and Utomilumab were again only observed in the presence of CD32B. By contrast, in the PBMC stimulation cultures, the presence of Varlilumab significantly enhanced the percentage of $CFSE^{low}$ CD4 and CD8 T cells irrespective of the TCS used (Figure 6C). However, Varlilumab also mediated a significant reduction in CD4 and CD8 T cell numbers under most conditions. In cultures with TCS expressing FcγR that mediated strong costimulation of Varlilumab, this effect was less pronounced or absent (Figure 6C). This could indicate that strong T cell proliferation mediated by the interaction of Varlilumab with TCS-expressed CD16A F176V, CD32B, or CD64 partially or fully compensates for T cell loss caused by this antibody.

Discussion

Targeting T cell costimulatory TNFR with agonistic antibodies is of potential therapeutic benefit in cancer immunotherapy. Currently, antibodies against several TNFRs have been evaluated in clinical trials (2, 12, 13, 55, 56). Many factors, including affinity, avidity, and epitope, determine the agonistic activity of an antibody (57, 58). Furthermore, its potency is influenced by its isotype and the FcγRs present in the tumor environment (39, 59). FcγR engagement can potentiate the agonistic activity but therapeutic complications and limitations such as off-target toxicity and severe liver inflammation have also been associated with FcγR binding (41, 42, 60). FcγRs can also mediate cytotoxic effects of agonistic antibodies towards T cells such as activation-induced cell death (AICD) as well as ADCC and ADCP. Although, it is well-established that Fc - FcγR interactions modulate costimulation agonists there is still limited knowledge of how individual FcγRs enhance the activity of therapeutic antibodies (61–63).

Here, we have used T cell stimulator cells expressing different FcγRs in conjunction with T cell reporter cells expressing different TNFR to evaluate and compare their agonistic activity. First, we analyzed the costimulatory capability of 41BB, CD27, GITR, and OX40 to induce NFκB, NFAT, and AP-1 transcription factors, upon ligation by their natural ligands. Engagement of these receptors activated NFκB and AP-1 to a similar extent, whereas NFAT signaling was downregulated. Previously, we have used TCS expressing the ligands for 41BB, CD27, OX40, and GITR to stimulate primary human T cells. While our results pointed to the strong costimulatory activity of each of these receptors, we observed considerable differences between their costimulatory capacity: signals via 41BB, CD27, and OX40 mediated sustained activation and proliferation in primary human T cells whereas the costimulatory activity of GITR was considerable weaker (54).

Next, we compared the costimulatory capacity of Urelumab and Utomilumab in a highly sensitive Jurkat-NFκB::eGFP-monoreporter system. Consistent with previous results, Urelumab activated 41BB signaling independently of FcγRs, although the effect was quite moderate (22, 42). This was potentiated when cross-linked via CD32A, CD32B, and CD64. In the presence of CD32B, Urelumab had the lowest EC50 value (66.12 ng/ml) and induced the strongest reporter activation. Unlike Urelumab, the activity of Utomilumab fully depended on the presence of FcγRs. Only in the presence of CD32A and CD32B Utomilumab was able to induce reporter activation, but the costimulatory activity of this antibody was low. The results obtained with the reporter system indicated, that Urelumab is a much stronger agonist than Utomilumab, which is in line with earlier studies (11, 42). The superiority of Urelumab is likely due to its epitope and its interaction with Fc-receptors since these factors have been shown to be critical for the activity of 41BB agonists (64).

In agreement with previous reports, we found that the CD27 agonist Varlilumab requires co-engagement with FcγR to activate CD27 signaling (31, 34, 37). Varlilumab had the highest costimulatory potency as reflected by the EC50 values and the maximal reporter induction for each FcγR tested in our study. This agonist is a fully human IgG1 antibody and consequently also strongly interacted with the FcγR CD16A and its high-affinity variant CD16A F176V.

We also compared the activity of Urelumab, Utomilumab, and Varlilumab in human PBMC samples regarding their ability to induce T cell proliferation and cytokine production *in vitro*. Urelumab augmented proliferation and cytokine production in primary human T cells more strongly than Utomilumab. However, compared to the results obtained in our T cell reporter system, this effect was much less pronounced in the PBMC cultures. These divergent results could potentially be due to over-activation resulting in AICD, induced upon Urelumab – 41BB ligation. There have been reports regarding 41BB agonist-induced cytotoxicity and the strong activation and high induction of IL-2 production could potentially induce AICD in T cells exposed to Urelumab (65–68). However, we did not observe evidence of enhanced cell death or reduced T cell numbers in stimulation cultures when Urelumab was present.

Despite its potent agonistic activity in the reporter system, Varlilumab had a very low capacity to augment proliferation and cytokine production in PBMC stimulation cultures. This might be due to cytotoxic effects triggered via its unmodified human IgG1 Fc part. In contrast to Utomilumab and Urelumab, Varlilumab has the capability to engage CD16, which mediates ADCC by NK cells but also by monocytes that express this FcγR (69). This antibody was

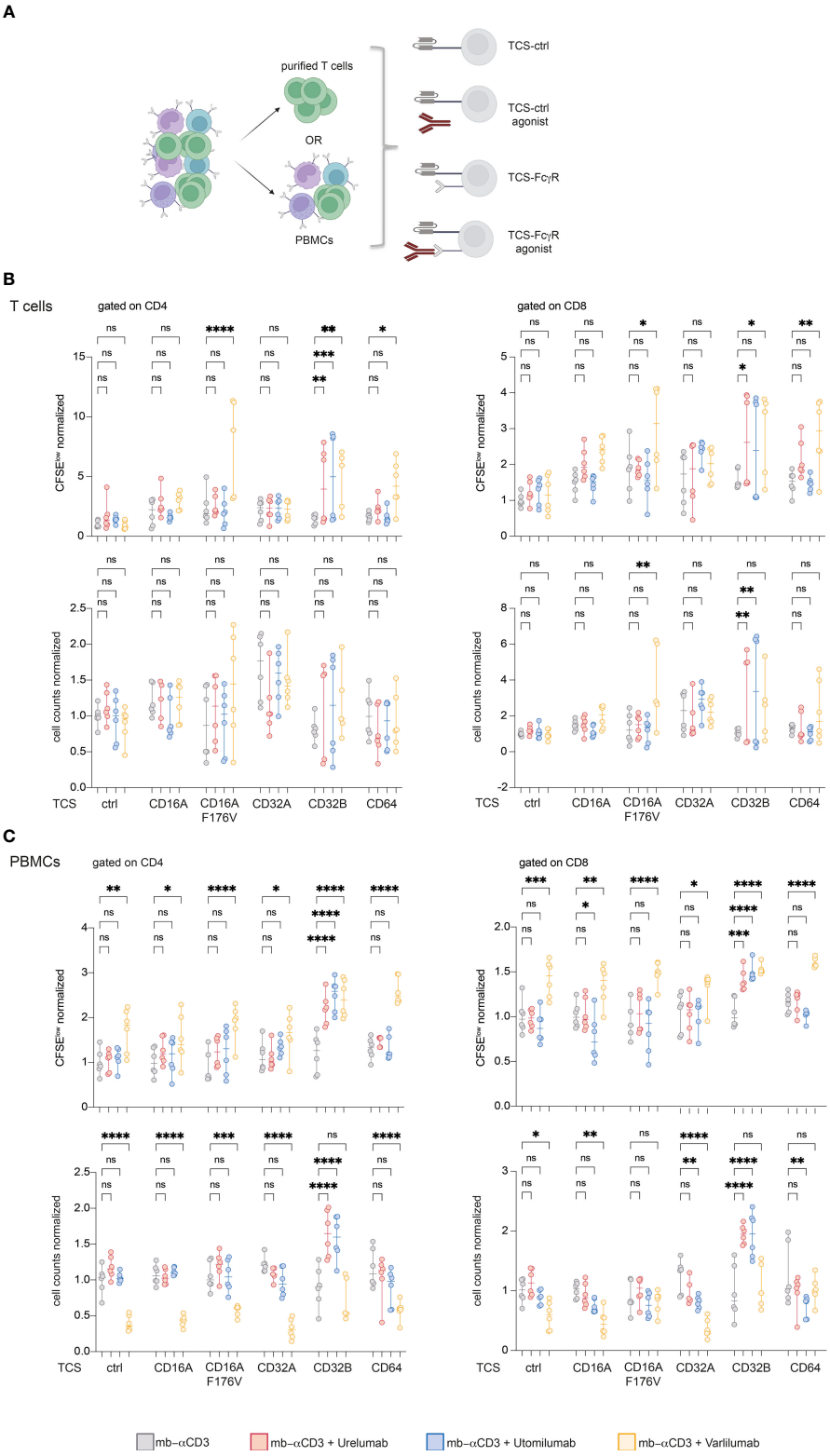

FIGURE 6
CD16A F176V, CD32B, and CD64 mediate strong costimulatory effects of Varlilumab on purified T cells. **(A)** Schematic of the experimental design. CFSE-labeled purified T cells and human PBMCs from the same donor were stimulated with TCS-control and TCS-expressing FcγR in the absence or presence of agonistic antibodies for 5 days. **(B, C)** $CFSE^{low}$ cells (upper panel) and cell counts (lower panel) were analyzed in gated CD4 and CD8 cells within purified T cells **(B)** and PBMCs **(C)**. FACS analysis was performed using constant cell volumes, flow rates, and acquisition time for all samples. Numbers of $CFSE^{low}$ cells and cell counts are depicted normalized to the values obtained with respective TCS without the addition of agonists ("mb-αCD3"). Summarized data of two donors each performed in triplicates is shown. 2-way ANOVA with Dunnett's multiple comparison test was performed. Median and +/- 95% CI is shown. ns, not significant; ns > 0.05, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

shown to exert antitumor immunity as well as direct killing of $CD27^+$ tumor cells in animal models and it is currently evaluated in patients with hematologic malignancies (32). Varlilumab has a dual role as a costimulation agonist and cytotoxic agent and could potentially enhance T cell responses as well as ADCC towards $CD27^+$ tumor cells. There are few studies that have analyzed the effects of Varlilumab *in vitro*. Ramakrishna et al. reported that Varlilumab strongly activated human T cells in the context of TCR stimulation (70). Importantly, by immobilizing this antibody, they investigated its costimulatory activity under conditions where it could not exert cytotoxic effects. We have performed annexin V staining and found evidence for enhanced percentages of apoptotic CD4 and CD8 T cells in PBMC stimulation cultures when Varlilumab was present. Furthermore, we found the number of CD4 and CD8 T cells to be reduced in these cultures. When used with purified T cells in the presence of TCS expressing CD16A F176V, CD32B, or CD64, this antibody exerted a strong costimulatory effect similar to the results with the Jurkat reporter T cells. Taken together our data indicate that the strong cytotoxic effects of Varlilumab counteract its capability to augment T cell responses.

Of all FcγRs tested, cross-linking via CD32B mediated the strongest agonistic activity of Urelumab, Utomilumab, and Varlilumab, whereas cross-linking via CD32A had a weaker effect on costimulation. Only Urelumab showed weak FcγR independent agonism, whereas Utomilumab and Varlilumab did not lead to activation in the absence of a TCR signal. Furthermore, in the presence of the high-affinity FcγR CD64, only Urelumab and Varlilumab induced NFκB signaling in the reporter system. To our knowledge, our study is the first to comprehensively analyze the contribution of individual FcγRs to the agonistic effect of 41BB (Urelumab, Utomilumab) and CD27 (Varlilumab) antibodies in a T cell reporter system. We believe that this platform has the ability to analyze and compare the FcγR-dependent and independent costimulatory activity of antibodies targeting different costimulatory receptors. Our results also highlight the need to complement studies in reductionist systems with studies in primary human PBMCs to account for effects such as ADCC and AICD which critically impact the activity of costimulation agonists.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the ethical committee of the Medical University of Vienna (1183/2016). The study abides by the Declaration of Helsinki principles. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats or heparinized blood obtained from healthy volunteer donors. The participants provided their written informed consent to participate in this study.

Author contributions

JL performed the majority of experiments, supervised experimental work, designed the study, and wrote the manuscript. RE performed experiments shown in Figure 1. PW-S and KG-P performed cytokine measurements. PS supervised experimental work, designed the study, and wrote the manuscript. All authors critically revised the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1208631/full#supplementary-material

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Agonism of 4-1BB for immune therapy: a perspective on possibilities and complications

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Costimulatory receptors on immune cells represent attractive targets for immunotherapy given that these molecules can increase the frequency of individual protective immune cell populations and their longevity, as well as enhance various effector functions. 4-1BB, a member of the TNF receptor superfamily, also known as CD137 and TNFRSF9, is one such molecule that is inducible on several cell types, including T cells and NK cells. Preclinical studies in animal models have validated the notion that stimulating 4-1BB with agonist reagents or its natural ligand could be useful to augment conventional T cell and NK cell immunity to protect against tumor growth and against viral infection. Additionally, stimulating 4-1BB can enhance regulatory T cell function and might be useful in the right context for suppressing autoimmunity. Two human agonist antibodies to 4-1BB have been produced and tested in clinical trials for cancer, with variable results, leading to the production of a wealth of second-generation antibody constructs, including bi- and multi-specifics, with the hope of optimizing activity and selectivity. Here, we review the progress to date in agonism of 4-1BB, discuss the complications in targeting the immune system appropriately to elicit the desired activity, together with challenges in engineering agonists, and highlight the untapped potential of manipulating this molecule in infectious disease and autoimmunity.

KEYWORDS

4-1BB (CD137), agonist, cancer immunotherapy, vaccination, clinical trials, TNFR, autoimmunity

Abbreviations: TAA, tumor associated antigen; TME, tumor microenvironment; TIL, tumor infiltrating lymphocyte; TCE, T cell engager; HCV, hepatitis C virus; HSV, herpes simplex virus; HIV, human immunodeficiency virus; VACV, vaccinia virus; RSV, respiratory syncytial virus; CHIKV, chikungunya virus; HCMV, human cytomegalovirus; MAYV, mayaro virus; FV, friend virus.

Introduction

Pioneering work from Byoung Kwon, who discovered 4-1BB (1, 2); Lieping Chen, Robert Mittler, and Ignacio Melero with the first stimulatory antibodies to 4-1BB (3); and the latter together with Tania Watts with over-expression of 4-1BBL (4, 5), established the concept that agonist targeting of 4-1BB can promote responses of T cells and NK cells that are favorable for protecting against tumor growth. Other data initiated by studies from Tania Watts, and from Yang-Xin Fu, Lieping Chen, and Robert Mittler, respectively, further raised the possibility of agonizing 4-1BB to protect against viral infection (6) and to suppress autoimmunity (7, 8). As described in prior reviews (9–14), 4-1BB is an attractive target for immunotherapy firstly because it can be expressed on conventional T cells (both CD8 and CD4) and NK cells, where it's signals can promote their proliferation and survival, and hence accumulation in numbers, as well as enhance the production of effector molecules such as IFN-γ, TNF, perforin, and granzyme. All of these activities contribute to protective immunity against tumors and viruses, and in the case of certain self-reactive regulatory CTL populations (15–17) might be relevant for augmenting a suppressive immune response that limits autoimmunity.

There are some advantages, but several disadvantages, when considering targeting 4-1BB. 1) 4-1BB is transiently inducible on most of the cells that are desirable to stimulate, including conventional CD8 and CD4 T cells and NK cells, driven primarily by antigen recognition but aided by cytokine action, which is a potential advantage as it might minimize prolonged and off-target activities. 2) Studies of the tumor microenvironment (TME) however promoted the concept that expression of 4-1BB on the aforementioned cells can be negatively regulated, likely from signals from suppressive cytokines or coinhibitory receptors such as PD-1. Thus, together with its naturally brief expression pattern, this presents significant complications in being able to engage 4-1BB on the appropriate cell and to elicit the desired response depending on the context of targeting. 3) Ligation of 4-1BB in isolation on T cells and NK cells may result in some functional effects, such as enhancing their capacity to survive, but its action in driving proliferation or production of effector molecules is primarily as a cosignal, either synergizing on T cells with the T cell receptor when recognizing antigen or synergizing on NK cells with receptors for cytokines such as IL-2, IL-15 or IL-21. Therefore, the full effects of 4-1BB will only be revealed if agonism is provided in these contexts. 4) Other cell types can bear 4-1BB on their membranes, including dendritic cells and macrophages, that may be pro- or anti-inflammatory (18–21), as well as both thymic and peripherally-induced CD4 regulatory T cells (22), and non-hematopoietic cells such as vascular endothelial cells (23–25). Consequently, the activity of several cell types can be elicited by agonist reagents that might or might not be desirable when attempting to augment anti-tumor responses, vaccinate against infectious disease, or treat autoimmunity. 5) The structure of 4-1BB and its mechanism of signaling requires several 4-1BB monomers to be in close proximity and multimerized in order to produce a significant biological effect. This means that to engage it successfully, and strongly stimulate target cells, agonistic biologics need to induce a degree of aggregation on the cell membrane that may not be provided by many simple soluble molecules, such as most conventional antibodies, unless they are clustered on other cells.

Thus, while the concept of stimulating 4-1BB for therapeutic intervention is well grounded, there are considerable hurdles to surmount to be able to do this in a manner that: a) has specificity in targeting the appropriate cell; b) can achieve an appropriate biological effect that is therapeutically relevant; and c) minimizes off-target activity that either results in toxicity, or elicits an immune response that is inappropriate or antagonistic toward the response that needs to be induced. Here, we summarize some of the major clinical efforts agonizing 4-1BB to date in immuno-oncology, provide a perspective on strategies that are being attempted to generate greater specificity in targeting and biological activity, and highlight opportunities in other clinical arenas such as viral vaccines and autoimmunity that have yet to be pursued.

4-1BB structure and signaling and agonist biologics

4-1BB is a monomeric type I transmembrane receptor composed of 4 extracellular cysteine-rich domains (CRD's), a single-pass transmembrane domain and an intracellular signaling domain (Figure 1). Upon binding, *via* the internal face of CRD's 1, 2, and 3, to its ligand, 4-1BBL (TNFSF9), which is a covalent homodimer in mice (26) and a non-covalent homotrimer in humans (27–29), 4-1BB monomers need to cluster together to allow the intracellular signaling domains to bind effectively to trimeric adaptor proteins, TNF receptor associated factors (TRAF) 1-3. This initiates several downstream signaling pathways, including NF-κB, ERK, and p38 MAPK, which control cellular proliferation, survival, and cytokine production (30). In normal physiology, 4-1BBL is displayed on the surface of cells, and when binding to 4-1BB on another cell, this results in aggregation and allows higher-order clustering of 4-1BB monomers to occur. Since mouse 4-1BBL is only able to dimerize 4-1BB, secondary factors are required to potently cluster monomers, such as by binding Galectin-9 (Gal-9) (31). Gal-9, a tandem-repeat protein, binds to terminal galactose residues of N-linked glycans, and since the N-glycans on 4-1BB are outside the binding site for 4-1BBL in CRD4, Gal-9 is able to secondarily cluster 4-1BB monomers, thereby increasing the valency of the 4-1BB/4-1BBL signaling unit. As the human Gal-9/4-1BB interaction is conserved (31), this might also be important for aiding clustering and signaling on human cells. In addition, human 4-1BB can form covalent dimers, which could also lead to secondary clustering of individual 4-1BBL/4-1BB signaling units (27). Therefore, the 4-1BB signal strength depends on the level of aggregation of 4-1BB monomers, with higher-order multimers of dimers and trimers leading to greater activation of pathways such as NF-κB.

4-1BB agonist targeting can be achieved using the natural ligand, IgG based modalities, or alternate scaffolds such as small cyclic peptides, anticalin's, and DARPin's. Its natural human

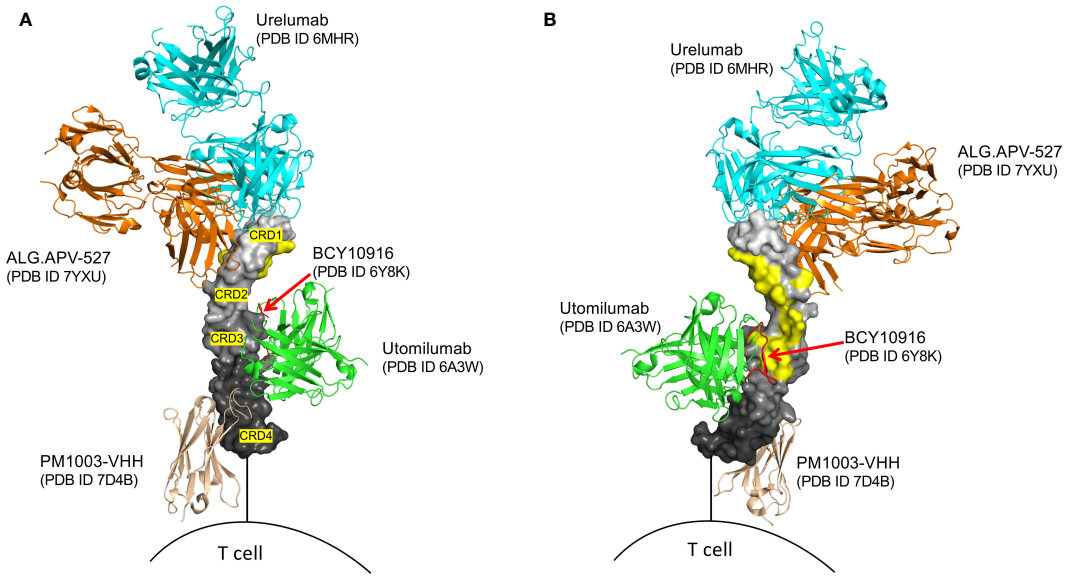

FIGURE 1
Structural superposition of several agonist modalities targeting human 4-1BB. 4-1BB in graded grey surface representation, with individual cysteine-rich domains (CRD's) labeled. **(A)** outer view of 4-1BB. **(B)** inner view of 4-1BB, highlighting the 4-1BBL binding sites in yellow. Only utomilumab and the BCY10916 peptide block binding of 4-1BBL to 4-1BB, while urelumab, ALG.APV-527, and PM1003-VHH, allow for simultaneous binding with 4-1BBL. BCY10916 is a close analog of the cyclic 4-1BB binding peptide found in the EphA2/4-1BB bispecific BCY12491.

trimeric ligand, when in soluble form, is unlikely to drive the multimerization needed for effective signaling because it cannot be aggregated, and needs to be displayed on an Fc to allow it to exhibit any significant functional agonist activity (32, 33). Similarly, conventional antibodies, because of their bivalent nature, might not engage sufficient monomers for maximizing 4-1BB's costimulatory signal, even if 4-1BB is naturally clustered on a cell through Gal-9 or covalent interactions. In fact, it is now generally recognized that engagement of the Fc domain of most conventional antibodies to FcγR (particularly FcγRIIB) on a separate cell (e.g. tumor cell or macrophage) is required to cluster enough 4-1BB monomers on a neighboring T or NK cell for effective induction of functional activity (34, 35), an observation shared with agonist antibodies to other TNFRs such as CD40 (36). It is also important to note that the epitope, rather than the binding affinity of 4-1BB antibodies to individual monomers, is another factor that can be important in determining the extent of 4-1BB activation, leading to some exceptions regarding the FcR-dependency, exemplified by urelumab described below. Thus, when considering creating an agonist of 4-1BB, it is not as simple as making a molecule that only binds one 4-1BB monomer.

To date all efforts to clinically agonize 4-1BB have been in oncology. Two antibodies were originally produced, urelumab (BMS-663513, IgG4) and utomilumab (PF-05082566, IgG2), that target different domains of 4-1BB (Figure 1). Urelumab binds at the tip of CRD1 and does not compete for natural 4-1BBL binding and is a strong agonist. In contrast, utomilumab binds CRD2 and 3 and competes for 4-1BBL binding, thereby reducing the potential of secondary clustering of individual 4-1BBL/4-1BB signaling units, leading to its weak agonist activity (28, 37). Similar observations had been made for anti-CD40 antibodies, where targeting the membrane-distal CRD1 region led to potent agonists, whereas those antibodies targeting CRD2-4, especially those that block CD40L binding, were weak agonists or potent antagonists of CD40 activity (36). Although IgG4 was chosen for urelumab allowing FcγRIIB binding, it is not clear it needs FcR engagement for its activity, however IgG4 and IgG2 (utomilumab) antibodies are also able to engage FcγRIIa and FcγRIIIa, and in addition to 4-1BB clustering, have the possibility to mediate ADCC (38–40) further complicating the development of pure agonist antibodies. While IgG4 can also engage FcγRI, its high affinity to monomeric IgG will result in saturation of the receptor by the high levels of serum IgG, meaning the lower-dosed therapeutic IgG4 antibodies are less likely to engage this FcR (41). As detailed below, in clinical trials of cancer, urelumab, although effective, had issues with off-tumor targeting activities and toxicity, while utomilumab demonstrated weak clinical activity as a monotherapy due to it being a weak agonist. More mono-specific 4-1BB antibodies in addition to urelumab and utomilumab have also been produced with enhanced or reduced FcR binding and other modifications (Figures 1, 2) to allow the 'optimal' level of 4-1BB engagement for agonism, but it is still not clear what characteristics an antibody needs to possess to provide this optimum. Furthermore, none of these approaches address the issue of specificity or selective agonism, i.e. being able to target the right cell type in the right location. Additional approaches have then been deemed desirable, leading to the development of a wealth of second-generation modalities aimed at maximizing agonism while engendering specificity. These are described in detail in several excellent recent reviews of cancer immunotherapy (42, 43) and are listed in Figure 2, and further discussed in general terms below.

One approach is tumor-targeting, covering both heme malignancies (CD19) and solid tumors (PD-L1, Her2, FAP,

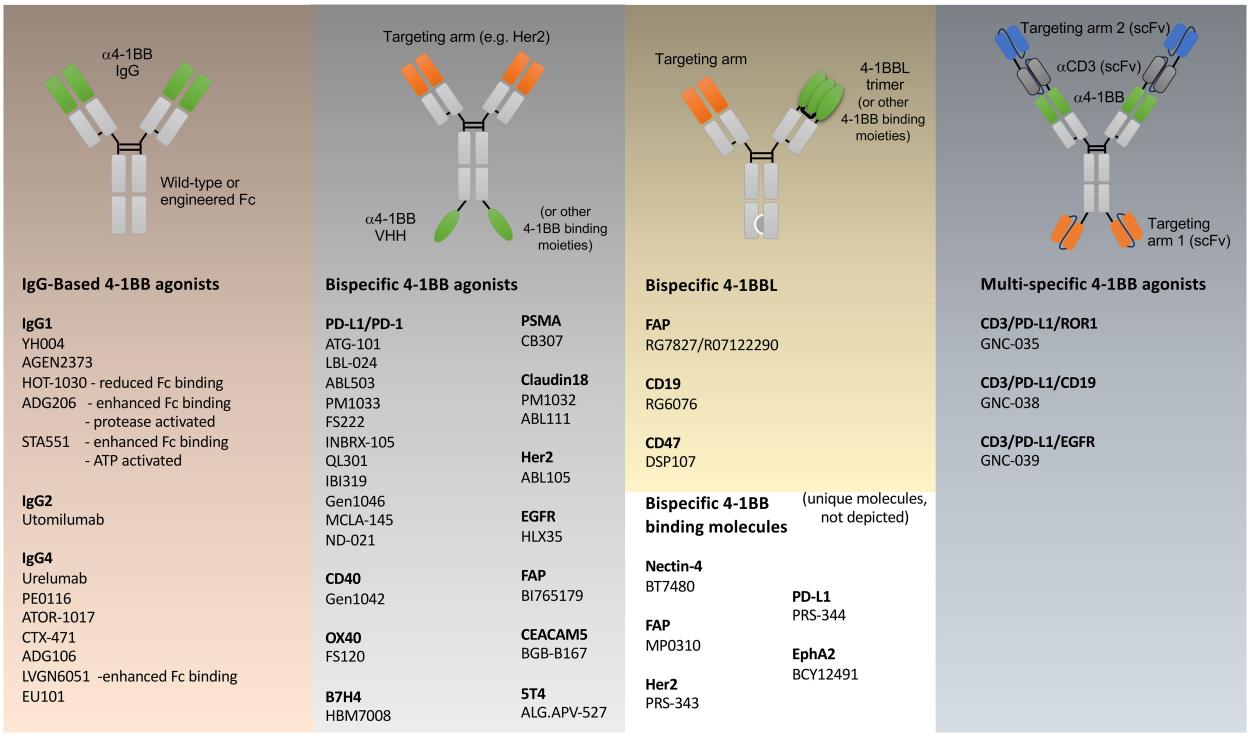

FIGURE 2
Agonist modalities that have been generated against 4-1BB. Examples of various antibody like constructs targeting 4-1BB are shown, engineered primarily for immuno-oncology, grouped into: simple IgG based biologics; bispecific biologics incorporating binding regions of 4-1BB antibodies in green, that additionally target other functional molecules (PD-1/PD-L1, CD40, OX40, B7H4) or tumor-expressed antigens (PSMA, Claudin18, Her2, EGFR, FAP, CEACAM5, 5T4) in orange; bispecific biologics incorporating 4-1BBL or alternative 4-1BB-binding molecules; and multi-specific T cell engager biologics incorporating binding regions of 4-1BB and CD3 antibodies (grey), and tumor targeting (blue). 4-1BB binding moieties beyond IgG-derived fragments include VHH's (e.g. HBM7008), 4-1BBL, peptides (BCY12491), DARPins (MP0310) anti-calins (PRS-343,-344). Each model represents an idealized example of the various classes of biologic. Individual reagents that have been produced will vary in structure. Individual structures that are not following an Ig-based scaffold are not depicted and include molecules such as DSP107 (4-1BBL-SIRPα fusion trimers), bicyclic peptides (BT7480), human serum albumin containing fusion proteins (CB307, ND021, NM21-1480). More specific details regarding each agonist modality can be found in Claus et al. (42).

EGFR, PSMA, Cldn18.2, Nectin 4, B7H4, CEACAM5, 5T4, EphA2). Most tumor-targeted modalities are bispecific antibodies where a single arm engages the tumor-associated antigen (TAA) on the tumor cell, while the other arm targets 4-1BB, with the aim of only activating tumor-infiltrating lymphocytes (TILs) present in the tumor microenvironment (TME) (44–50). Another concept is to target 4-1BB exclusively on T cells through the simultaneous binding of two T cell proteins, such as 4-1BB and OX40 (51), or 4-1BB and PD-1 (52). Both PD-1 and OX40 are upregulated on antigen-responding T cells, allowing more specific engagement of 4-1BB on only subsets of T cells that hopefully are relevant for tumor elimination. A third approach combines 4-1BB costimulation with T cell engagers (TCEs). Traditionally, T cell engagers bridge T cells and tumor cells *via* simultaneous TAA and CD3 binding, leading to the activation of all T cells regardless of their antigen specificity (53). This modality circumvents the need for TCR recognition of an MHC-presented peptide and has been successfully used in heme malignancies. However, solid tumors pose a challenge for T cell engagers, either due to the lack of T cell infiltration, the lack of a durable and potent T cell response, or T cell exhaustion due to the immune suppressive microenvironment that limits T cell cytotoxicity. In an attempt to overcome some of these challenges, 4-1BB antibodies have been combined with a TCE in a single molecule (42, 54). Several multi-specific TCE's that contain 4-1BB antigen-binding have been produced (Figure 2), with GNC-035 being in clinical trials for breast cancer (NCT05160545) and hematologic malignancies (NCT05104775) and GNC-038 in trial for central nervous system lymphoma (NCT05485753). Although pre-clinical data has not been published, the design of these molecules are complex, containing 2 binding domains each against CD3, 4-1BB, PD-L1, and ROR1 or CD19. In summary, clearly novel, and highly complex, protein engineering ideas are fueling the field of 4-1BB agonism, but which is the best tactic is still to be determined, and one that might vary depending on the cellular target and goal in terms of disease modification.

Clinical targeting of 4-1BB in cancer

The rationale for agonizing 4-1BB in cancer is strong, given the ability of 4-1BB to drive CD8 T cell and NK cytotoxic activity (3–5, 10, 12), and was spearheaded by the first-generation agonist antibodies, urelumab (BMS-663513) and utomilumab (PF-05082566) that were evaluated both as monotherapies and

combined with other therapies (28, 37, 55–62). Urelumab monotherapy demonstrated activity, but modest clinical response rates have prompted the exploration of combination therapies. In a trial of several B cell lymphomas, urelumab monotherapy achieved objective response rates (ORR) of 6-17% and disease control rates (DCR) of 19-42% depending on the tumor type, and the combination of urelumab with rituximab had improved outcomes of 10-35% ORR and 24-71% DCR (63). Utomilumab treatment alone has shown less apparent activity in clinical trials as a monotherapy, although it is difficult to compare the two antibodies as the types of cancer targeted and patient populations treated have varied. For example, a phase 1 trial in patients with advanced solid tumors reported an ORR of 4% in 53 patients, but stable disease in 25% and a disease control rate (DCR) of 28% (60), and a phase 1 trial in advanced melanoma or NSCLC reported ORRs of 2% and 0%, respectively, although again 23-50% of patients showed stable disease (62). Utomilumab in combination with pembrolizumab in 23 patients with advanced solid tumors gave an ORR of 26% and a DCR of 70% (64), and when combined with rituximab, an ORR of 21% in patients with non-Hodgkin lymphomas was reported (61). However, although the combination treatments displayed more efficaciousness than the anti-4-1BB antibodies alone, it was not clear if response rates were significantly different than historically seen with the partner drugs.

Urelumab was associated with a higher incidence of immune-related adverse events (irAEs) than utomilumab, including cytokine release syndrome (CRS), immune-mediated colitis, hepatotoxicity, and dermatologic reactions (58). Deaths associated with urelumab treatment have also been reported, primarily due to severe cytokine release syndrome and hepatotoxicity. Although the exact mechanisms underlying the observed toxicities are not fully elucidated, preclinical data with agonists with similar properties to urelumab have shown that T cells are required, and associated with anti-4-1BB upregulating IFN-γ, TNF, and IL-6, and systemic inflammation and organ damage. This is presumably as a result of presentation of autoantigens that might be available in various tissues, given that 4-1BB ligation on T cells in the absence of an antigen-induced T cell receptor signal is unlikely to result in significant cytokine production. Also, studies of the liver have suggested that 4-1BB can be expressed on infiltrating monocytes and tissue-resident Kupffer cells, which when ligated can further contribute to inflammatory cytokine production, enhance antigen presentation to T cells, and lead to hepatotoxicity. Furthermore, engagement of anti-4-1BB by FcγR expressed on these myeloid cells or other similar cells has been suggested to be critical for toxicity, which may additionally amplify the T cell effects, as well as lead to other direct or indirect activities such as promoting the expression of Fas on liver cells, rendering them susceptible to Fas-mediated apoptosis (21, 40, 65–68). Considering the challenges associated with toxicity, the development of urelumab as a monotherapy has largely been discontinued. Similarly, the development of utomilumab as monotherapy has also been discontinued, driven by strategic decisions and the pursuit of more efficaciousness.

One idea put forward is that alternative dosing regimens for antibodies to 4-1BB may achieve a better balance between therapeutic efficacy and manageable toxicities (40, 42, 43, 69). These regimens include dose escalation, dose fractionation, or intermittent dosing. However, while dose optimization is conceptually viable, practical and commercial feasibility presents significant challenges. The inherent heterogeneity among patients and cancer types and the complex interplay of various factors influencing treatment response make establishing universally appropriate dosing regimens challenging. Tailoring dosing regimens individually may require substantial resources, including comprehensive patient profiling, ongoing monitoring, and dose adjustments, and be time-consuming, costly, and impractical. Additionally, changing the dose does not alter the intrinsic agonist activity of an individual antibody on a cell, based on its 4-1BB epitope binding and affinity, and does not circumvent the potential for off-target effects due to the expression of 4-1BB on cells in other organs or on suppressive cells such as Treg. Thus, while dose optimization may have some value, it is unlikely to strongly lead to greater efficacy and improved safety while accounting for the biological, logistical, and economic considerations.

Nevertheless, the knowledge gained from these early studies has provided valuable insights for developing next-generation 4-1BB biologics that specifically aim to overcome limitations in agonism and decouple efficacy from toxicity (Figure 2). Moreover, because 4-1BB can be expressed on cells, such as endothelial cells, dendritic cells, macrophages, and regulatory T cells, that might counter anti-tumor activity, attempts are being made to provide greater specificity in cell targeting (40, 70–78). While the merits of these ideas are discussed below in general terms, most of the second-generation agonists mentioned in Figure 2 are still in early clinical development with only a small amount of data from phase I studies being reported at present in peer-reviewed publications (49, 50, 79, 80).

Modified binding affinity and specificity

One approach being explored involves the development of 4-1BB agonists with modified binding characteristics (affinity or epitope specificity) (43, 74, 81). Preclinical studies have provided some insights into the potential success of this approach. By carefully screening antibodies with broad epitope coverage and fine-tuning their binding affinity through site-directed mutagenesis, enhanced antitumor immune responses and reduced immune-related adverse events have been reported in some animal models (74, 81). However, a major challenge, both theoretically and practically, is the idea of identifying the optimal or "magic" epitope on 4-1BB that might selectively activate the desired signaling pathways in the desired target cell without either triggering excessive immune activation or associated toxicities. The intricate nature of 4-1BB receptor regulation and ligand interaction makes this task complex and potentially futile. It has been suggested that retaining 4-1BBL binding by not interfering with its binding sites, as with urelumab and ALG.APV-527 (Figure 1), will aid agonism, which is logical given the need for 4-1BB monomers to cluster and potentially for the clustering of 4-1BBL-organized trimers. However, it is unlikely that this will circumvent off-target activities. Moreover, varying the binding

epitope of 4-1BB antibodies to be outside of the 4-1BBL-binding region (e.g. in CRD1 or 4) and with the goal of inducing different functional outcomes, is akin to threading a needle, especially given that 4-1BB expression on any individual cell can vary, both in density and in intrinsic clustering from covalent interactions or *via* coreceptor proteins such as Galectin-9. Furthermore, preclinical studies of these reagents in mice are complicated given differences in 4-1BB receptor expression and clustering between mice and humans, and that mouse 4-1BBL is a dimer and not a trimer which will influence overall signaling and functional outcomes.

Fc engineering

Another approach to generate a better agonist has involved removing the Fc domain (81, 82) or altering the antibody isotype to one with reduced binding to FcR (83), to try to circumvent toxic side effects such as liver damage thought dependent at least in part on clustering of anti-4-1BB on FcR on Kupffer cells (35). However, it is important to note that these modifications may impact the antibody's effector function and antitumor activity. A different approach is to modify the Fc glycan structure of the 4-1BB antibody, e.g. through afucosylation, which reduces binding to FcγRIIIa, and has been reported to decrease liver toxicity while maintaining antitumor activity in mice (40, 84). Point mutations in the Fc region also can modulate the antibody's affinity for Fc receptors, such as decreasing binding to FcγRIIB, which was reported to reduce the risk of thrombocytopenia while preserving antitumor activity (85). Half-life extension without the use of the Fc region is an additional strategy where the antigen binding region of the 4-1BB antibody is linked with another protein, such as serum albumin, altering the antibody's pharmacokinetics, including its half-life. Again, in mice, this has been reported to result in enhanced antitumor activity while minimizing toxicity (78). However, it is important to acknowledge the complexities and differences that may affect the translatability of these findings to humans (35). Humans exhibit distinct patterns of Fc receptor expression on immune cells compared to mice, and humans possess FcRs, such as FcRn, with different affinities for various IgGs compared to mice. Fc glycosylation also differs between mice and humans, which can impact the degree of activation induced by Fc-engineered antibodies. Given these variabilities, achieving a transformative impact with Fc-engineered 4-1BB antibodies in the clinic remains challenging, and evaluating the translatability of mouse findings likely will require humanized mouse models and conducting thorough pharmacokinetic and pharmacodynamic studies in human subjects. Studies in non-human primates, while essential, also have their drawbacks. While FcγRs are similar to those in humans, their distribution differs (86). In addition, other differences also confound interpretation of antibody effects, as illustrated by urelumab exhibiting minimal toxicity in non-human primates as opposed to humans, possibly related to the affinity of urelumab for macaque 4-1BB being lower than for human 4-1BB.

Selective agonism

Other arenas are aiming to create strategies for selective agonism of anti-4-1BB drugs, to specifically activate 4-1BB signaling pathways only in the TME and on desirable target cells, such as CTL, while minimizing off-target effects and toxicity. These may hold the greatest chances for success. As mentioned above, bi-specific antibodies designed to bind 4-1BB and a tumor antigen simultaneously, such as Her2, EGFR, or CEACAM5, should result in the selective activation of 4-1BB pathways in the TME (43, 81). Based on preclinical and early clinical studies, CD19 and CD20 bi-specific antibodies have potential for selectively enhancing 4-1BB signaling in B-cell malignancies (44). Another approach involves using antibodies engineered to be activated exclusively in the tumor microenvironment. These antibodies are designed with a tumor-specific trigger, such as enzymatic activation or pH-dependent conformational changes, initiating their activation and 4-1BB engagement in the TME (77, 87, 88). Furthermore, targeted delivery systems are being developed, using nanoparticles, liposomes, or other conjugates specifically designed to reach the tumor site (89). Despite this, their efficacy may be limited by the restricted distribution and penetration of the treatment within the tumor. Tumors often exhibit heterogeneous characteristics, including variations in antigen expression, immune cell infiltration, and vasculature. Consequently, exclusively targeting 4-1BB agonists to the tumor may only partially engage all relevant immune cells or tumor subpopulations.

Another limitation of these approaches is the likelihood that 4-1BB agonists might also need to have important activity in lymphoid tissues, particularly tumor-draining lymph nodes. 4-1BB signaling in lymph nodes can reactivate non-exhausted memory T cells, or even new naive T cells, specific for tumor neoantigens. Therefore, restricting 4-1BB agonist activity solely to the TME could potentially limit the full activation and expansion of tumor protective T cells. As 4-1BB is primarily induced on T cells by antigen recognition, in this case there is an argument for a vaccine-type approach, using neoantigen administration in combination with 4-1BB agonism, to effectively engage relevant T cells outside of the TME. Additionally, the restricted expression of 4-1BB in the tumor microenvironment is an important consideration. It is clear that 4-1BB expression is often confined to a small subset of tumor-resident T cells (most often less than 20%), and many of these can be Treg, or it is only seen in certain tumor types and not others, while most conventional CD8 and CD4 T cells within tumors may lack 4-1BB (90–94). To overcome this limitation, combination strategies are likely needed to try to induce 4-1BB expression on a broader population of T cells within the tumor. For example, immune checkpoint inhibitors that block coinhibitory receptors, such as PD-1 or CTLA-4, have resulted in enhanced 4-1BB expression on T cells (95–99). Combining checkpoint inhibitors with tumor-targeted agents in bi-specific or multi-specific formats could increase the number of T cells capable of responding to 4-1BB agonism (45, 49, 52, 75, 100). Other immunomodulatory agents,

including cytokines like IL-12 and IL-15, or immune stimulatory molecules such as TLR agonists (101–106), also can modulate directly or indirectly 4-1BB expression on T cells, and are potentially good approaches for combination therapy. However, the optimal strategy and their efficacy for increasing the availability of 4-1BB on the appropriate cell type, not the inappropriate one, require further investigation and validation through preclinical and clinical studies.

CD3 T cell engagers

A variant example of selective agonism is the creation of CD3 T cell engagers (TCEs) that incorporate antibody binding regions of 4-1BB with CD3, and checkpoint blockade (PD-L1), and a tumor target such as CD19 or EGFR (Figure 2), with the aim of only engaging 4-1BB on a T cell in the TME (53, 107). While TCEs are an interesting concept, published work on any incorporating 4-1BB binding is limited, and such a multivalent modality has many potential drawbacks and still risks having off-tumor and off-target effects. A major consideration is whether the targeting arms specific for a TAA (depending on how they are displayed in the construct) are sufficient for ensuring localization to the TME. As such, this modality might have a similar liver toxicity risk as traditional 4-1BB antibodies. Similarly, would both 4-1BB targeting arms be sufficient for strongly activating 4-1BB expressing cells. If they are not, this may negate toxicity but also mean little efficacy. Moreover, will T cells be activated without binding to the TAA, since the TCE could simultaneously engage two CD3 molecules and two 4-1BB molecules on the same T cell. In addition, the molecule would have to be engineered to favor cis-binding of both 4-1BB and CD3 on the same T cell, rather than providing signal 1 to one T cell and signal 2 to the other T cell which would ultimately reduce the potency of the molecule (108). Thus, while the notion of complex TCEs providing specificity, targeting, and limiting toxicity, all in one, is good, generating the appropriate construct that exhibits all of the relevant activities may be very challenging.

Other combination therapies

Yet another approach to maximize the potential of 4-1BB agonism is to combine 4-1BB antibodies with other therapies. Preclinical or clinical studies have shown promise with various combinations, including chemotherapy or radiation treatment, T cell engagers, CAR T cells, cytokines such as IL-2, antibodies to checkpoints such as anti-PD-1, or antibodies to other costimulatory molecules such as anti-OX40 (43). It is important to note that the incidence and severity of adverse events have either been found to, or are likely to, vary depending on the specific combination, dosing, patient population, and prior treatment history, but most importantly that adverse events will be similar to those with 4-1BB antibody monotherapy, with none of these combinations at present mitigating the off-target effects of anti-4-1BB or providing selective agonism. Perhaps future evaluation of the second-generation bi-specific and multi-specific reagents with some of these combinations will provide the level of agonism desired to harness the potential of 4-1BB antibodies to enhance antitumor immune responses while minimizing toxicities.

Additional considerations to maximize 4-1BB agonism in cancer

As alluded to before, one major restriction is whether 4-1BB is expressed on the cell type(s) most desirable to target for cancer immunotherapy. Biomarker-based patient selection approaches can help to identify specific biological markers associated with improved response rates (109). Obviously, the most promising biomarker is the expression of 4-1BB itself in the tumor microenvironment and tumor draining lymph nodes. Studies in patients with certain tumors have demonstrated that high levels of 4-1BB expression are associated with a higher response rate to therapy (109–111). Anecdotally, it has also been suggested that the presence of TILs will be associated with an improved response to 4-1BB-targeted therapies, and patients with a higher density of TILs may exhibit a higher response rate to 4-1BB agonism. In addition, biomarkers such as PD-L1 or IFN-γ appear to indicate the presence of an active antitumor immune response that could be further enhanced by 4-1BB-targeted therapy, and patients with high levels of tumor IFN-γ may then demonstrate a higher response rate. Although incorporating biomarkers that predict treatment response is crucial, it is equally important to identify biomarkers that might predict the risk of toxicity. However, identifying and validating reliable biomarkers requires extensive research and clinical studies and the complexity of the immune system and TME presents a challenge in accurately assessing treatment response and predicting toxicities. Moreover, the heterogeneity of patient populations and tumor types poses a challenge for biomarker-based patient selection, as the predictive biomarkers may vary among different cancer types. Technological advances, such as high-throughput sequencing and proteomics, artificial intelligence, and machine learning algorithms, offer opportunities to identify novel biomarkers as well as provide longitudinal insights into treatment response dynamics. By leveraging technological advancements, collaborative efforts, and innovative approaches, the future development of biomarker-based patient selection holds tremendous potential to optimize 4-1BB-targeted therapies and improve patient response.

Agonizing 4-1BB in viral vaccines

As well as cancer immunotherapy, an obvious but still underappreciated application of agonizing 4-1BB is in vaccination against viral infections, given the importance of T cells and NK cells in protective immunity. However, many of the same issues discussed above also apply in this arena. Targeting 4-1BB therapeutically during active acute infections has many practical challenges, not least of which are treating patients within the critical short time period when 4-1BB will be induced on virus-responding T cells or virally activated NK cells and the feasibility of

administering an agonist such as an antibody to those patients. Also, the potential for driving unwanted virus-induced pathology by excessively triggering CTL activity during an active infection, as is naturally seen in some patients with SARS-CoV-2 or influenza virus, is a true risk that would limit the therapeutic use of 4-1BB agonists.

However, incorporating an agonist into a prophylactic vaccine has much appeal. Following the initial demonstration that an agonist antibody to 4-1BB could increase the frequency of LCMV-reactive CD8 T cells in mice vaccinated with an LCMV peptide (6), a number of other studies with vaccine protocols using viral peptides, live or attenuated viruses, or DNA plasmid vectors encoding viral proteins (Table 1), have demonstrated a similar phenomenon with agonist antibodies to 4-1BB (112–119). This has been seen with responses to influenza virus, HCV, HSV, Friend virus, VACV, RSV, and CHIKV in mice, not only promoting a greater magnitude of acute CD8 and/or CD4 effector T cell responses but also enhancing protective T cell memory against

TABLE 1 Summary of major studies demonstrating that agonist antibodies to 4-1BB, or forced expression of 4-1BBL, can enhance T cell priming and memory T cell responses, and protective immunity, in vaccine protocols with virus infection, or immunization with viral peptides or vectors encoding viral antigens.

Virus or viral antigen	Organism or cells	4-1BB agonist and delivery	Functional Effect of Stimulating 4-1BB	Reference
LCMV NP peptide	Mice	Antibody i.p	Increased primary splenic NP-specific IFNγ CD8 T cells	Tan, 2000
Influenza PR8	Mice	Antibody i.p	Increased # primary lung flu-specific CD8 T cells and cytotoxicity	Halstead, 2002
Influenza HKx31	Mice	Antibody i.p	Increased # primary and memory splenic flu-specific CD8 T cells and cytotoxicity	Bertram, 2004
Influenza M1 peptide; EBV BMLF1 peptide	Human	Adenovirus encoded 4-1BBL in monocytes	Increased flu-specific IFNγ, TNF, and cytotoxic memory CD8 T cells	Bukczynski, 2004
HIV env, nef, gag peptides	Human	Adenovirus encoded 4-1BBL in monocytes	Increased # memory HIV-specific CD8 T cells, and cytotoxicity	Bukczynski, 2005
Adenovirus encoded HCV-NS3	Mice	Antibody i.p	Increased NS3-specific CD4 IFNγ, and CD8 cytotoxicity, and protection against HCV infection	Arribillaga, 2005
HSV-1	Mice	Antibody i.p	Increased # primary and memory LN HSV gB-specific CD8 T cells and cytotoxicity, and protection against HSV-1 reinfection	Kim, 2005
HIV A/E gag/pol	Mice	Fowlpox virus encoded 4-1BBL i.m	Increased # splenic HIV-specific CD8 T cells and IFNγ	Harrison, 2006
HCMV pp65	Human	pcDNA3 encoded 4-1BBL in fibroblasts	Increased # HCMV-specific CD8 T cells	Waller, 2007
FV	Mice	Antibody i.p	Increased # primary splenic FV-specific CD8 T cells, IFNγ and cytotoxicity, and reduced virus replication	Robertson, 2008
pGA1 and MVA encoded HIV gag/pol/env	Mice	Antibody i.p and pGA1 encoded 4-1BBL i.m	Increased # primary and memory HIV-specific CD4 T cells and IFNγ CD8 T cells	Ganguly, 2010
Adenovirus encoded influenza NP	Mice	Adenovirus encoded 4-1BBL i.m and i.n	Increased # primary and memory lung, splenic, and LN influenza-specific CD8 T cells and cytotoxicity, and protection against influenza infection	Moraes, 2011
VACV-WR; VACV-Lister; VACV B8R, N2L and B16R peptides	Mice	Antibody i.p	Increased # primary and memory splenic and lung VACV-specific CD8 T cells and TNF and IFNγ, and protection against VACV infection	Zhao, 2012
pcDNA3 encoded HIV gag	Mice	pcDNA3 encoded 4-1BBL and SF protein D i.m	Increased # primary and memory HIV-specific IFNγ CD8 T cells	Kanagavelu, 2012
RSV M2 peptide, anti-CD40, polyIC	Mice	Antibody i.p	Increased % blood and lung RSV-specific CD8 T cells, IFNγ, and cytotoxicity, and increased protection against lung RSV infection	Lee, 2014
Adenovirus encoded HIV gag	Mice	Adenovirus encoded 4-1BBL in dendritic cells i.v	Increased # HIV-specific CD8 T cells	Wang, 2015
Adenovirus encoded influenza NP	Mice	Adenovirus encoded 4-1BBL i.n	Increased # stable lung memory influenza-specific CD8 T cells, IFNγ and cytotoxicity, and protection against influenza infection	Zhou, 2017
CHIKV; MAYV	Mice	Antibody i.p	Reduced T cell dependent primary splenic and LN GC B cells and viral RNA	Hong, 2019

re-infection, as well as in some cases broadening the repertoire of anti-viral specific T cells. Other studies (Table 1) have used 4-1BBL to deliver the agonist signal in mice or tested *in vitro* with human cells, with 4-1BBL either incorporated into adenoviral or other DNA vectors for direct injection, or with plasmid transfection into monocytes, fibroblasts, or dendritic cells for cell therapy, all with similar results on enhancing T cell immunity to viral antigens (120–128). In total, these results have then created a very strong argument that agonizing 4-1BB would be highly useful and effective against infection with multiple viruses if integrated into a vaccination strategy. Moreover, the ability of 4-1BB to drive persistently high numbers of memory T cells, including those resident memory cells that accumulate in peripheral tissues (101, 128, 129), and to overcome defects in T cell immunity associated with aging (118, 130), is highly relevant given the current conversations around persistence of T cell memory and effectiveness of COVID mRNA vaccines in adults and older people.

As yet, no clinical trials of viral vaccination have attempted to agonize 4-1BB. One study more than 10 years ago tested an agonist antibody to 4-1BB in NHP given an intramuscular SIV DNA vaccine (131). This resulted in an increase in the SIV-specific CD8 T cell response and a decrease in viral titers after the SIV challenge, as predicted. In contrast, another study with 4-1BBL in DNA plasmid or viral vectors again showed increased CD8 T cell responses in mice, but in a limited study in NHP, mixing a plasmodium antigen-encoding vector with another vector encoding 4-1BBL, intramuscularly, resulted in no enhancement of IFN-γ producing cells (132). The latter could have reflected a need for 4-1BBL to be co-expressed in the same vector with antigen or the lack of another adjuvant activity. Although little work has since moved away from the mouse, this field is particularly ready and appropriate for translation to humans if the right vehicle and adjuvant system can be found to agonize 4-1BB, especially given the recent focus and success of mRNA vaccination.

Off-target effects are again a potential and likely problem with agonist antibodies, as illustrated by repeated injections of anti-4-1BB into HBV-transgenic mice resulting in hepatitis, fibrosis, and liver cirrhosis, mimicking liver disease during natural chronic HBV infection (67). This is similar to the issues with cancer immunotherapy. However, in the case of viral vaccines, the development of bi- or multi-specific antibodies to surmount targeting the wrong cell type in the wrong location is far more of a challenge than when making use of the TME and tumor-associated proteins, given that viruses can replicate in multiple organs and many cell types. Thus, promoting the expression of 4-1BBL encoded in DNA or mRNA with viral antigen would be preferable to minimize off-target adverse events, given that it is likely that subcutaneous, intradermal, or intramuscular administration of these vaccines would focus 4-1BBL more specifically to the cells that present viral peptides directly to T cells. Some studies in mice comparing anti-4-1BB to 4-1BBL delivered in a DNA vaccine support the idea that vaccines encoding 4-1BBL could limit such adverse events (124). 4-1BBL-transduced dendritic cells or macrophages bearing viral antigens used in adoptive cell vaccines, or extracellular vesicles (EVs) derived from these cells, could be an alternative to DNA or mRNA delivery. This may again create more specificity in delivering 4-1BB signals to the appropriate cells, but these methods are not yet attractive for large-scale vaccination efforts.

What would be the best vaccine centered around 4-1BBL is still to be determined. Agonists to 4-1BB were initially shown to perform much better in preclinical studies when combined with the TLR3 adjuvant poly I:C in terms of their ability to increase the magnitude of the T cell response that can persist (101). However, whether this TLR is the preferred partner to maximize 4-1BB activity is not clear given that other studies have reported apparent synergies with ligands of TLR4, TLR7/8 or TLR9 (103, 104, 106). IL-15 can also induce or prolong 4-1BB expression on T cells, suggesting its incorporation into a vaccine would help with enhancing or prolonging 4-1BB signaling (102, 105), and IL-7 can promote TRAF1 levels in T cells which would further aid the ability of 4-1BB to signal (133). As detailed above, synergistic activities of agonizing 4-1BB and blocking PD-L1 have also been reported in several tumor studies, leading to the current bispecific constructs reviewed earlier, and this synergy has additionally been seen in models of chronic virus infection (133, 134). Therefore, DNA or mRNA viral antigen vaccines encoding 4-1BBL with one or several of these factors are likely to be more effective than simply combining 4-1BBL with the viral antigen alone. One novel way of delivering 4-1BBL was reported, constructing what was termed a synTac, a dimeric Fc fusion protein incorporating 4-1BBL with MHC complexes of peptides of HIV or CMV (135). This could represent a further approach to increase specific targeting of relevant T cell populations whilst minimizing off-target effects, and such constructs can be further modified by adding cytokines such as IL-7 and IL-15, or TLR ligands, to complement the adjuvant activity.

Agonizing 4-1BB in autoimmunity

Lastly, an unexpected finding that has been revealed in the field of 4-1BB agonism is the ability to shut off or limit autoimmune and other inflammatory reactions. This has been seen with agonist antibodies injected into murine models of SLE (7, 8, 136), MS (31, 137), RA (16, 138), conjunctivitis (139, 140), IBD (141), uveitis (142, 143), asthma (31, 144, 145), type I diabetes (146), chronic GVHD (147), diet-induced obesity (148), psoriasis (149), and Sjogren's syndrome (150). In general, suppression driven by anti-4-1BB has been seen during the initiation phases of the disease rather than with therapeutic intervention during active disease, although in some models, therapeutic activity has been noted (8, 138).

Two primary mechanisms of 4-1BB-driven suppression have been suggested, either promoting the accumulation and activity of conventional $CD4^+Foxp3^+$ Treg that can express 4-1BB, or more

in-line with the tumor and virus literature on 4-1BB, inducing the differentiation or reactivation of CD8 T cells, either into CTL or a type of $CD8^+$ Treg that makes IFN-γ and can suppress the normal inflammatory response of CD4 T cells or B cells (16, 22, 140, 141, 143, 144, 151–154). Other suppressive activities such as directly driving death of pathogenic effector T cells, expanding MDSC, or promoting regulatory activities in dendritic cells, have also been suggested (19, 20, 142, 147, 155).

While the literature on this aspect of 4-1BB agonism is extensive, translating the research to humans is an opportunity that has not been pursued as yet, primarily because there are many targeting hurdles to overcome. A major concern if contemplating using agonist antibodies in patients with active autoimmune or inflammatory disease is whether stimulating 4-1BB could trigger pathogenic effector CD4 or CD8 T cells or other inflammatory cell types such as pro-inflammatory macrophages and exacerbate the specific disease. This has been seen in some mouse disease models (156–158), and would be a problem with an antibody to 4-1BB as well as with simple injection of 4-1BBL in soluble or vector form. Engineered bi- or multi-specific antibody constructs are again unlikely to circumvent this issue unless a targeting partner can be found that is only expressed on the regulatory/suppressive cell type whose activity needs to be enhanced. As discussed above, this could be a $CD4^+Foxp3^+$ Treg, a regulatory DC or MDSC, or a $CD8^+$ Treg that can kill or suppress pathogenic effector cells. At present, it is not clear if such markers exist or can be found that truly distinguish these cells from non-regulatory cells. However, continued screening efforts with single-cell RNA-seq and CITE-seq might be able to reveal a protein or proteins whose targeting could be incorporated into a second-generation agonist.

Another potential path forward that, on paper, is more feasible is a vaccine-like strategy that incorporates an antigen into an mRNA or DNA vaccine utilizing 4-1BBL. This could again minimize off-target effects and focus 4-1BBL on cells that present antigen directly to regulatory CD4 or CD8 T cells. The question here is whether a relevant antigen or peptide epitope can be defined that would only be recognized by the Treg. A significant literature exists on what sometimes have been termed Tregitopes. These can be epitopes of proteins that have been argued to be specifically recognized by thymic Treg or regulatory CD8 T cells, and have been described to range from peptides in the Vβ regions of TCRs of autoreactive T cells to peptides presented on non-classical MHC molecules, to conserved repeat regions in the Fc domain of IgG (15, 17, 159–163). If these can be shown in humans to truly be specific for pre-existing Treg or for driving the differentiation of newly formed Treg, this can potentially harness the utility of agonizing 4-1BB in an mRNA or DNA formulation used in prophylactic or therapeutic vaccination.

An easy alternative to *in vivo* agonism of 4-1BB, of course, could be to exploit the ability of 4-1BB signals to expand Treg or CTL *in vitro* (164–168). These could be used in adoptive cell therapy of autoimmune disease, although this is not as attractive for widespread therapeutic treatment. In this case, knowledge of a relevant autoantigen for CD4 Treg, and a relevant antigen for regulatory CD8 T cells, such as a peptide of a dominant Vβ TCR expressed on autoreactive CD4 T cells or a peptide presented on non-classical MHC recognized by suppressor CD8 T cells, would again likely be needed to allow specific targeting and inhibition of the T cells that drive autoimmunity or other inflammatory diseases (169). Engineering Treg with specific TCRs of autoantigens, if they can be identified, would be another option. Lastly, attempts are already underway to use CAR Treg therapy for autoimmune disease, along with identifying specific antigens that can be used to mobilize these Treg (169), which could be further expanded in number with agonists to 4-1BB. Although only indirectly relevant for the current discussion, incorporation of the intracellular domain of 4-1BB has already been established to be beneficial in such cells.

Concluding remarks

The fact that interest in 4-1BB as a therapeutic target has persisted and even expanded in the past few years, given the less-than-compelling results in clinical trials with agonist antibodies, is a testament to the potential that has been raised for this molecule from basic research in preclinical studies. Although we have provided opinions for and against strategies that might or might not be fruitful in oncology, and also in infectious disease and autoimmunity, our enthusiasm for 4-1BB agonism is still extremely high. The use of 4-1BB agonist antibodies in cancer treatment has shown promise in enhancing antitumor immune responses, but several challenges and limitations must be addressed. Optimal dosing and treatment regimens are a primary challenge. Balancing immune activation and toxicity avoidance is complex. Monitoring and managing treatment-related toxicities are crucial. Biomarker-based patient selection approaches, including predictive biomarkers of treatment response and toxicity, are important to understand the heterogeneity of patient populations and how individual groups will benefit. The complexity of the tumor microenvironment and its immunosuppressive mechanisms pose challenges, as does the complexity of autoimmune and inflammatory disease. Advances in technology and our understanding of immune system dynamics can optimize treatment strategies, and integrating immune cell phenotyping and genetic profiling can aid in patient selection and personalized treatment approaches.

Ongoing research aims to identify novel 4-1BB agonists with next-generation bi-specific or multi-specific platforms targeting the 4-1BB pathway together with other pathways, and applying predictive modeling and machine learning algorithms can assist

in tailoring therapy to individual patients. Thus, despite the many challenges, future development in 4-1BB agonist biologics holds substantial potential. The varied concepts proposed in multi-specific targeting through protein engineering, in academia and especially in industry, demonstrate the wealth of talent available to bypass and solve the complexities of the immune system. With good science, trial and error with the many great ideas that have arisen in this area, and some fortune, we remain confident that targeting 4-1BB will ultimately be productive and therapeutically efficacious.

Author contributions

All authors equally contributed to writing and editing the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

SS-A is employed by Yz Consulting.

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The benefits of clustering in TNF receptor superfamily signaling

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The tumor necrosis factor (TNF) receptor superfamily is a structurally and functionally related group of cell surface receptors that play crucial roles in various cellular processes, including apoptosis, cell survival, and immune regulation. This review paper synthesizes key findings from recent studies, highlighting the importance of clustering in TNF receptor superfamily signaling. We discuss the underlying molecular mechanisms of signaling, the functional consequences of receptor clustering, and potential therapeutic implications of targeting surface structures of receptor complexes.

KEYWORDS

TNF receptor superfamily (TNFRSF), TNF signaling, receptor clustering, TNFR agonism and antagonism, signal amplification

Introduction

The TNF receptor superfamily (TNFRSF) comprises a diverse group of cell surface receptors involved in regulating immune responses, inflammation, and cell survival (1). Dysregulation of TNF signaling is implicated in various pathological conditions, including cancer, autoimmune and allergic diseases. Recent studies have highlighted the importance of receptor clustering in the activation and modulation of TNF receptor signaling. In this review, we will summarize the key findings of TNFRSF signaling, the benefits of clustering in TNFRSF function and its implications for therapeutics development.

Receptor classification and mechanism of action

Members of the TNFRSF are type I, single pass membrane proteins with their C-terminal end anchored in the membrane. Their elongated ectodomains contain 1-6 cysteine rich domains (CRDs). TNF receptors can be grouped into three distinct groups (see Table 1): the first group contains receptors with a death domain (DD), essential for the initiation of apoptosis, though receptors in this group can also activate chronic inflammatory pathways. The second group of TNFRSF members interact with TNF receptor associated factors (TRAF) to initiate cell survival and proliferation via the canonical or non-canonical NFkB pathways. The third group contains decoy receptors that lack a functional cytoplasmic domain and instead act as decoys by binding to TNFSF

TABLE 1 TNFRSF receptors, their ligands and intracellular binding partners.

TNFRSF Receptor (TNFRSF No.)	Number of CRD	Intracellular Binding Partner	TNFSF Ligand (TNFSF No.)	Receptor Stem Region (AA)
Death Receptors				
TNFR1 (1a)	4	TRADD, FADD, RIP	TNF (2), LTα (1), LTβ (3)	197-211
Fas (6)	3	FADD	FasL (6)	167-173
TRAILR1 (10A)	3¶	FADD, TRADD, RIP	TRAIL/Apo2L (10)	230-239
TRAILR2 (10B)	3¶	FADD, TRADD, RIP	TRAIL/Apo2L (10)	179-210
NGFR (16)	4	NADE	NGF (not a TNFSF member)	191-250
DR3 (25 or 12)	4¶	TRADD, FADD	TL1A (15), TWEAK (12)	193-199
DR6 (21)	4	TRADD, RIP	N-APP (not a TNFSF member)	212-349
EDAR	3¶	EDARADD	EDA-A1	149-187
Receptors with TRAF-interacting motif				
TNFR2 (1b)	4	TRAF1-3	TNF (2), LTα (1)	202-257
LTβR (3)	4	TRAF2-4, TRAF5	LTα (1), LTβ (3) as $LT\alpha\beta_2$, $LT\alpha_2\beta$	212-227
OX40 (4)	4¶	TRAF1-3, TRAF5, TRAF6	OX40L (4)	168-214
CD40 (5)	4	TRAF1-3, TRAF5, TRAF6	CD40L (5)	188-193
CD27 (7)	3	TRAF2, TRAF3, TRAF5	CD27L (7)	142-191
CD30 (8)	6	TRAF1-3, TRAF5	CD30L (8)	326-385
4-1BB (9)	4	TRAF1-3	4-1BBL (9)	160-186
RANK (11A)	4	TRAF1-3, TRAF5, TRAF6	RANKL (11)	195-212
Fn14 (12A)	1	TRAF2, TRAF6	TWEAK (12)	68-80
TACI (13B)	2	TRAF2-3, TRAF5, TRAF6	APRIL (13)	105-165
BAFFR (13C)	1	TRAF2, TRAF3, TRAF6	BAFF (13B/20)	36-78
HVEM (14)	3	TRAF1-3, TRAF5	LIGHT (14), LT-α (1)	163-202
BCMA (17)	1	TRAF1-3, TRAF5, TRAF6	APRIL (13), BAFF (13B/20)	42-54
GITR (18)	3	TRAF1-3	GITRL (18)	154-162
TROY (19)	3¶	TRAF1-3, TRAF5	LTα (1)	150-170
RELT (19L)	1	TRAF1	not known	110-162
XEDAR (27)	3¶	TRAF1, TRAF3, TRAF6	EDA-A2	119-138
Decoy receptors				
TRAILR3 (10C)	3¶	none	TRAIL/Apo2L (10)	
TRAILR4 (10D)	3¶	none	TRAIL/Apo2L (10)	
OPG (11B)	4	none	TRAIL/Apo2L (10), RANKL (11)	
DcR3 (6B)	4	none	FasL (6), TL1A (15), LIGHT (14)	

¶: Contains truncated CRD domains.

ligands and prevent them from binding to other functional receptors. Ligands of the TNF superfamily (TNFSF) are type II membrane proteins with their N-terminal end anchored in the membrane. They share 20-30% sequence homology and a structurally conserved TNF homology domain (THD) (2). TNFSF ligands form non-covalent trimers and bind to three monomers of their corresponding receptors (Figure 1A). Efficient signaling in the TNFRSF requires that the receptors preassemble on the cell surface to form hexagonal honeycomb clusters (4–6). In return, the downstream signaling components assume the same hexagonal clustering geometry (7, 8). Free receptor monomers interact through the so-called pre-ligand assembly domain (PLAD) formed by the N-terminal and the CRD1 domains of the receptor (9–13). We have earlier proposed a uniform model that can apply to

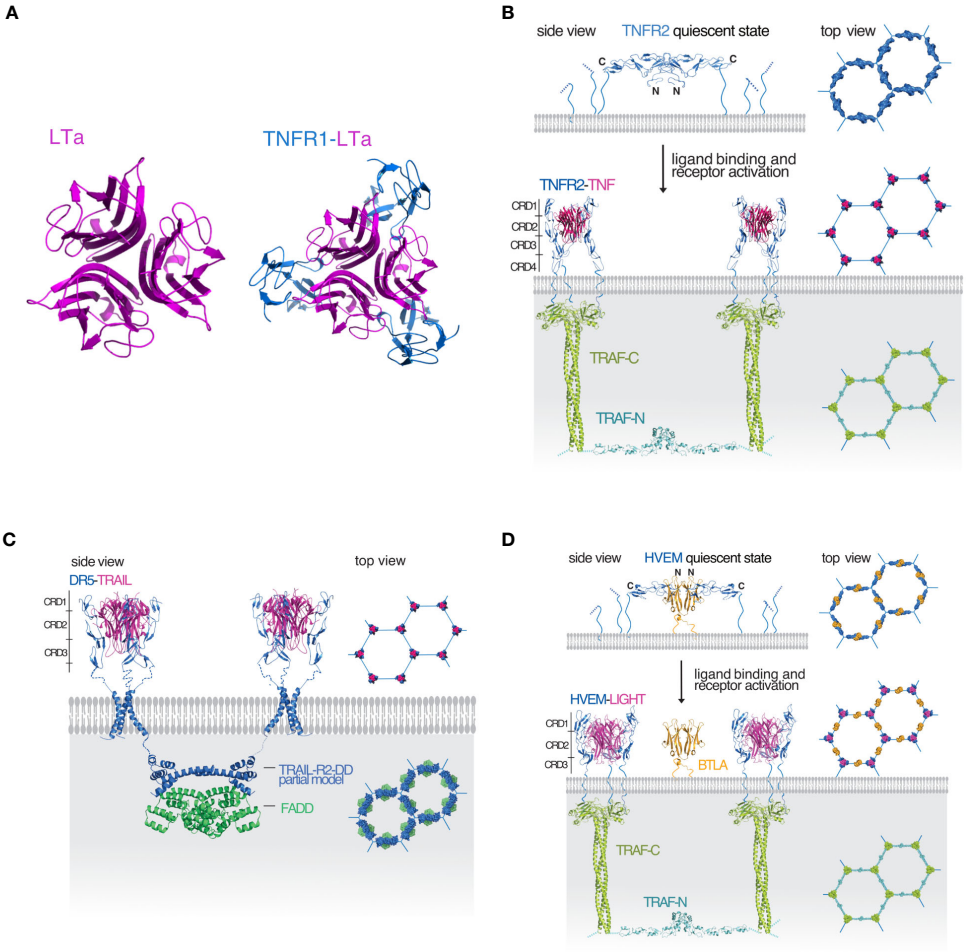

FIGURE 1
Illustration of the mechanism of signaling in the TNF receptor superfamily **(A)** Molecular representations of trimeric lymphotoxin (LTa) shown in magenta (top view) and the LTa (magenta)-TNF receptor 1 (TNFR1, blue) complex (top view). **(B)** A TRAF-interacting TNFRSF receptor represented by a model of the TNF/TNFR2/TRAF2 signaling complex. In the quiescent state (top panel), the receptor antiparallel dimers (blue) are arranged in a hexagonal lattice. TNF (magenta) binding breaks up the dimer interface and activated TNFR2 trimers recruit TRAF2 (green) resulting in the dimerization of the TRAF2 N-terminal RING domains (cyan) and activation of further downstream events. The hexagonal lattice of the downstream components mirrors the hexagonal lattice of the receptors. **(C)** Death receptor 5 (DR5, blue) in complex with its ligand TRAIL also forms a hexagonal cluster. After DR5 activation, TRAIL-R2-DD (blue) dimerizes and recruits a FADD dimer (green) also forming a hexagonal lattice. **(D)** For receptors like HVEM (blue) that are unable to dimerize on their own, hexagonal lattice formation is aided and controlled by the dimeric IgSF member, BTLA (orange). Upon binding of the ligand (magenta) the activated receptors recruit a TRAF homolog resulting in RING dimerization and activation of further downstream events. The program PyMOL was used for creating all molecular representations (3).

both the DD containing and TRAF-interacting receptors of the TNFRSF (4) (Figures 1B, C). To briefly summarize: the model assumes that receptors initially assume a quiescent state on the cell surface where trimers of antiparallel dimers form a hexagonal honeycomb cluster. The antiparallel dimer form partially buries the ligand binding surface and therefore unable to bind the ligand until activated. The activated receptors maintain the same clustering geometry and recruit the downstream signaling partners. The major benefit of this model is that the honeycomb lattice of the surface receptors is the same as the assembled honeycomb lattice of the downstream components so there is no need for major movement in the membrane upon activation. Since the downstream signaling partners form weak interactions, preassembly of the receptors on the cell surface enables the downstream partners to bind more efficiently once the receptors are activated by their ligands. This model accommodates both death receptors and TRAF-interacting receptors into one model.

One potential issue with this model is that the number and size of the extracellular CRD domains of TNFRSFs vary and not all members have been shown to be able to form interactions via their PLAD. This raises the question whether the proposed model can hold for TNFRSF receptors that are unable to form stable dimers on their own. The answer has been provided by the structure of herpesvirus entry mediator (HVEM, TNFRSF14) in complex with the regulatory protein B- and T-lymphocyte attenuator (BTLA) (14).

Immunoglobulin superfamily members aid TNFRSF clustering and regulate function

BTLA is the member of the Immunoglobulin (Ig) superfamily (IgSF) that comprises of proteins that play crucial roles in the immune system and other biological processes. These proteins are characterized by the presence of one or more Ig domains, which are structurally conserved regions that contain about 70-110 amino acids arranged in a sandwich-like structure (15). BTLA functions as an inhibitory receptor on T lymphocytes similar to well-known IgSF members such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD-1). BTLA interacts with the TNFRSF member HVEM that modulates B and T lymphocyte activation (16), dendritic cell proliferation (17) and protects mucosal epithelia from damage during inflammation (18). In the cytosol, HVEM interacts with several TRAF homologs including TRAF2 to induce NFkB activation. LIGHT and LT- α are the two canonical TNFSF ligands that activate HVEM. BTLA and CD160, another IgSF protein, regulate HVEM function.

The crystal structure of the HVEM-BTLA complex is a heterotetramer consisting of an antiparallel dimer of HVEM on the outside and a BTLA dimer on the inside (14) (Figure 1D). The complex closely resembles the antiparallel dimer structure of TNFR1 (19) and the modeled structure of TNFR2 shown in Figure 1B. Based on these data, we have earlier proposed that the HVEM antiparallel complex with BTLA may arrange in a hexagonal lattice on the cell surface representing the receptor quiescent state similar to TNFR1/2 (20). Since BTLA is a type I transmembrane protein similar to HVEM, both can be co-expressed and anchored to the cell by their C-terminal ends. In the *cis* configuration BTLA does not interfere with LIGHT or LT-α binding, instead it serves to facilitate HVEM oligomerization on the cell surface and to inhibit the ligand independent activation of HVEM. BTLA rather than being a true ligand of HVEM as previously proposed, serves as a regulatory protein modulating HVEM oligomerization and controls receptor activation. There are several TNFRSF receptors with three or fewer CRD domains that could potentially utilize a co-regulatory receptor to aid their oligomerization on the cell surface (see Table 1) and the HVEM/BTLA complex structure can serve as a template for such interactions.

We now have a unified model of TNFRSF that shows the receptors arranged in a honeycomb cluster both in their free and ligand bound states and this model can accommodate all members of the TNFRSF regardless of function or the size of their ectodomains. The size of the hexagonal lattice may vary from receptor to receptor but is expected to be the same for receptors that interact with the same downstream signaling partners. Next, we are going to discuss how the formation of the honeycomb cluster can improve signaling in the TNFRSF.

Clustering enables cooperativity and leads to signal amplification

Cooperativity in the TNFRSF requires that at least two signaling trimers are placed close enough for an interaction to occur. Signal is transmitted first vertically after the binding of the ligand to the intracellular binding partners. For members of the TRAF-interacting receptors such as TNFR2 and HVEM, their cytoplasmic tails recruit TRAF homologs and it is TRAF dimerization via the N-terminal RING domains that enables cooperativity between signaling units (Figures 1B, D). TRAF binding proteins such as cIAP1/2 can also dimerize and facilitate cooperative signaling. In case of death receptors, such as CD95 (Fas) or death receptor 5 (DR5), their DDs recruit the Fas associated death domain (FADD) and it is FADD dimerization that enables cooperativity between two signaling units as seen in Figure 1C. In all instances, cooperativity requires that the two TNFRSF receptor trimers on the cell surface are activated by their ligands to create a logical AND gate.

We have shown earlier that cooperative signaling networks can be represented as planar graphs with nodes (***n***) and edges (***e***), where ***n*** represents the input signal and ***e*** the output signal (21). Cooperativity requires that at least two ***n*** input nodes are placed near each other at the right distance to create an ***e*** output. This generates one output signal from two input signals at a 50% loss of signaling efficiency. Even if thousands of TNFRSF signaling pairs are added onto the cell surface their efficiency remains at 0.5. However, if we order six input signaling units into a closed loop we end up with equal number of nodes and edges where $\boldsymbol{e/n} = 1$. This closed signaling unit can be represented by a regular hexagon. Further clustering can then be illustrated by tiled regular hexagons. Tessellation or tiling refers to the process of covering a surface with one or more geometric shapes called tiles with no overlaps and no gaps. Mathematically, it means that the graph representing such system is a simple graph with no self-loops or multiple edges. A regular hexagon is one of only three regular polygons that can be tiled by themselves in two-dimension, the other two regular polygons are equilateral triangles and squares. As we have shown earlier, as the cluster size of tessellated polygons grow, the output/input signal ratio increases but can never exceed 3 (21). The maximum is also inversely proportional to the degree of the tiled polygon, therefore smallest in a hexagonal cluster and largest in a clustered system of tiled triangles where it can reach 300% of the original amplitude leading to the maximum value shown in Eq. 1, where **e** represents the sum of all edges and **n** represents the sum of all nodes in the cluster:

$$\frac{\textbf{output signal}}{\textbf{input signal}} = \frac{\mathbf{e}}{\mathbf{n}} \leq \mathbf{3} \qquad (1)$$

The formula derived in Eq. 1 is the consequence of Euler's polyhedron formula (22). It illustrates that the signal in a clustered network can be amplified. We have also shown that the amplification depends on cluster size and clustering geometry and it can broadly apply to all clustered cooperative signaling systems beyond the TNFRSF regardless of their molecular makeup (21).

In Figure 2 we provide examples of signal amplification in hexagonal clustering relevant to the TNFRSF. Ligand bound activated receptors represent the input signal that can be illustrated by the vertices or nodes. The dimerization of the TRAF RING domains or DD dimerization represent the output signal that is illustrated by the edges of the hexagon. The signal amplitude depends on the geometry of the honeycomb cluster. Tiling the hexagons in a more or less symmetrical fashion in each direction is

the most efficient, leading to the highest **e/n** or input/output signal ratio compared to hexagons tiled in a linear fashion. This is because the **e/n** ratio is maximized when most hexagons are surrounded by other hexagons (21).

As the cluster size grows the **e/n** ratio increases and reaches a plateau. For hexagonal clusters 90% of the maximum signal amplification can be achieved in a cluster of 100 signaling units, and 400 units are required to reach 95% signal amplification. This is important because experimental data indicates that signaling receptors tend to form small clusters on the cell surface around 300-500 nm in diameter (23–25). Optimal cluster size may also dependent on the size of the cell-to-cell interface.

Model of ligand activation of clustered receptors

Now that we understand the optimal arrangement of receptor clusters on the cell surface, we can examine how ligand binding affects receptor activation and signaling. Ligands of the TNFSF are expressed as transmembrane proteins with their N terminal end anchored in the membrane. The ligands are cleaved to create a soluble form that is generally less effective than the membrane bound ligand across most of TNFSF. For instance, membrane bound TNF (memTNF) can activate TNFR2 very effectively but soluble TNF (sTNF) is a weak activator of TNFR2. We can illustrate how clustering can potentially explain these differences. Figure 3A illustrates a hexagonal lattice with receptor trimers represented as nodes in a hexagonal honeycomb grid. In this example, each activated (ligand bound) node is shown in dark blue, inactive nodes are in light blue, dark blue edges connect two active nodes, while all other edges are shown in light blue. In Figure 3C the amplitude (the ratio of the active edges over the total number of edges in the cluster) is calculated for 50% initial occupancy (red line) and 95% initial occupancy (blue line). The program then randomly activates a certain percentage of remaining inactive nodes until all are activated. When soluble ligands bind to their receptors, they bind more or less randomly with low overall amplitude. This is because even at 50% occupancy, not all activated receptor will be connected to other active trimers to create an output signal. When the ligands are bound to the membrane with the same geometry as the receptors (Figure 3B), the ligand trimers are going to line up with the receptor trimers and a much higher portion of receptors will be activated creating a strong signal similar to what is seen at high occupancy. The membrane bound ligands will generate a narrow and high amplitude, digital-like ON signal for activation. The soluble ligands on the other hand generate a low amplitude signal spread out over time as illustrated in Figure 3C. This is in remarkable agreement with experimental data using a DNA origami platform with immobilized FasL ligands arranged in different geometries to test the effect of ligand clustering on apoptosis efficiency in cells overexpressing the Fas receptor. Hexagonally arranged ligands generated a high amplitude signal in contrast to the low amplitude, broad signal generated by ligands with the wrong geometry (26). Super-resolution imaging has confirmed the importance of clustering *in vivo* in a Fas/FasL model but there is disagreement of the state of the ligand-free receptors (27). The Fas receptors appear largely monomeric and dimeric in the ligand-free state as observed by fluorescence energy transfer studies of C-terminal labeled Fas-fluorescence protein (Fas-FP) receptors. Fas-FP could appear monomeric even in the clustered state because the C-terminal ends of receptors in the ligand-free state maybe separated by a distance larger than the Förster distance of the FP pairs.

A low amplitude signal may not reach the threshold of activation and may result in not just quantitatively but

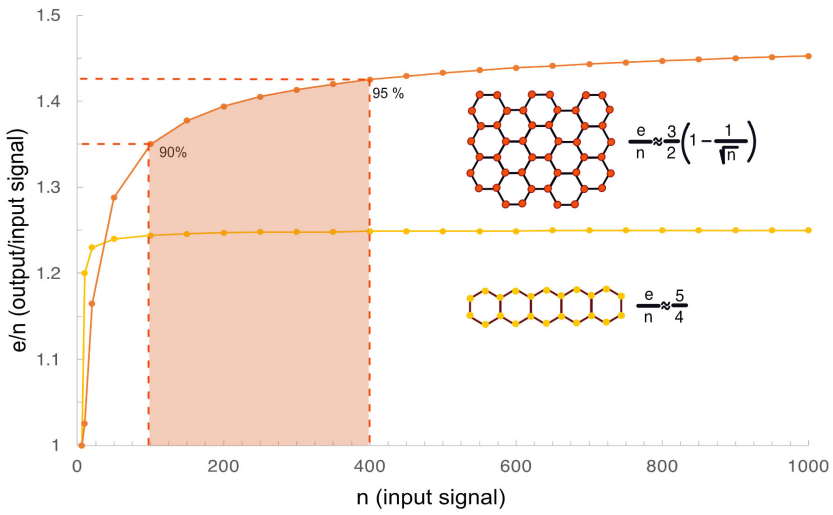

FIGURE 2
Illustration of signal amplification in hexagonal clusters. **(A)** Signal amplification represented by the **e/n** ratio is calculated for two examples of regular tiled hexagons with different geometries and plotted against **n**. It is higher in a hexagonal lattice that grows equally in both direction in the plane (shown in red) over a lattice tiled in only one direction (yellow). The red shaded area illustrates that 90-95% of the maximum signal amplification can be achieved with a cluster of 100-400 receptors in agreement with experimental data on the average size of receptor nano clusters in cells.

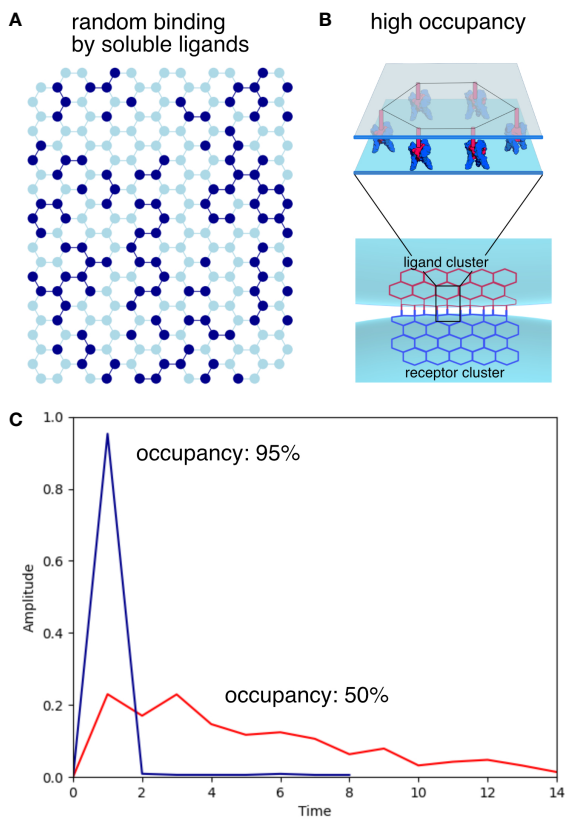

FIGURE 3
Illustration of simulated signal amplitudes with soluble or membrane-bound ligands. **(A)** Representation of a honeycomb receptor cluster with 50% occupancy. Activated receptors are represented as dark blue nodes, inactive receptors shown as light blue nodes. Edges between active nodes are shown in dark blue while all other edges are colored light blue. **(B)** In cell-to-cell interactions, clustered ligands attached to the membrane allow the simultaneous activation of clustered receptors with the same geometry, resulting in maximum signaling efficiency. **(C)** The amplitude is calculated as the ratio of active/inactive edges for 50% and 95% occupancies. High occupancy, illustrating membrane-bound ligand activation results in a sharp, high amplitude signal, while lower occupancy by soluble ligands results in a low amplitude signal spread out over time.

qualitatively different signaling outcomes. This could explain how activation of the same receptor can result in different outcomes in the TNFRSF. Interestingly a recent paper on DR5 signaling provides an explanation of how the long isoform of the FLICE-like inhibitory protein (FLIP(L)) can act as both an inhibitor and promoter of caspase-8 at the death-inducing signaling complex (DISC) (28). The outcomes depend on the ratio of FLIP(L):caspase-8. When caspase-8 concentration is higher than FLIP(L) concentration apoptosis is accelerated. This can be explained by the proposed model of receptor activation of DR5 and the different outcomes generated by low vs high occupancy receptor clusters. Procaspase-8 binding to the DR5-DD-FADD complex activates caspase-8 and simultaneous activation of clustered DR5 with memTRAIL will lead to much higher concentrations of activated caspase-8 and a higher amplitude signal. Random activation of the receptor by sTRAIL could lead to much lower active concentrations of caspase-8 tilting the ratio in favor of FLIP(L) and result in apoptosis blockade.

FLIP has three functionally different isoforms. In addition to FLIP(L), two shorter isoforms FLIP(R) and FLIP(S) also exist and together they play a pivotal role in switching between cell survival, apoptosis and necroptosis. While FLIP(L) plays an important role in modulating apoptosis, FLIP(s) is important for assembling the necrosome to induce necroptosis. Necroptosis is the caspase independent regulated inflammatory form of cell death via cell lysis and necrosis. Receptor-interacting serine/threonine protein kinase-1 and 3 (RIPK1/3) and mixed lineage kinase domain–like (MLKL) are central mediators of TNF induced necrosis via TNF receptor 1 (TNFR1). Interestingly, TNFR1 internalization is an important first step in necroptosis (29). As we discussed earlier, receptor clustering and cluster stabilization can influence receptor internalization and therefore may also affect necroptosis. The ultimate outcome of cell fate is the result of a complex interplay of different cellular components and the activation or inhibition of several pathways. We believe that receptor clustering and the differential activation of clustered receptors play an important but not yet appreciated role in these processes.

Clustering may not explain all the differences between membrane and soluble TNFSF ligands regarding their ability to activate the receptor. The stem (or stalk) region of the receptor plays an important role as well. In most cases, the stem region is defined by the sequence between the last CRD domain and the transmembrane domain of the receptor. When the stem regions of TNFR1 and TNFR2 are switched, sTNF can readily activate TNFR2 but not TNFR1 (30). TNFR1 has a short stem region (15 AA), while TNFR2 has a longer proline rich stem region (56 AA) (Figure 4A). sTRAIL can also more easily activate DR4 that have a short stem region but not DR5 that has a longer stem (Figure 4A) (31). LTβR with a short stem region also belongs to receptors that are known to be readily activated by their respective soluble ligand. On the other hand, OX40, CD27, 4-1BB and TACI have longer proline rich stems and are less readily activated by their respective soluble ligands (30). Table 1 lists the stem size for all TNFRSF members and there seems to be a clear correlation between stem size, rigidity and responsiveness to soluble TNFSF ligand. In the quiescent state model, trimers of antiparallel dimers setup the honeycomb cluster. The antiparallel dimer acts as a ruler to position receptor trimers at the right distance away from each other and to also sequester the ligand binding site. The dimer interactions are needed to create the right lattice of the honeycomb cluster. In the inactive, lateral state the stem region of each receptor is exposed and may directly interact with the TNFSF ligand (soluble of membrane bound) to initiate the conformational transition of the receptor from horizontal (inactive) to vertical (active) position (Figure 4B). This kind of transition is not unusual. In Munc13 that also forms hexagonal clusters, the Munc13 core (Munc13C) transitions between upright (open) conformation to lateral (closed) (32). We believe it warrants further research to address how the size and rigidity of the stem region may affect receptor activation by TNFSF ligands for other members of the TNFRSF.

We are now going to illustrate how taking into account the natural 3D structure of an antigen on the cell surface can guide the successful development of therapeutics with examples from the TNFRSF.

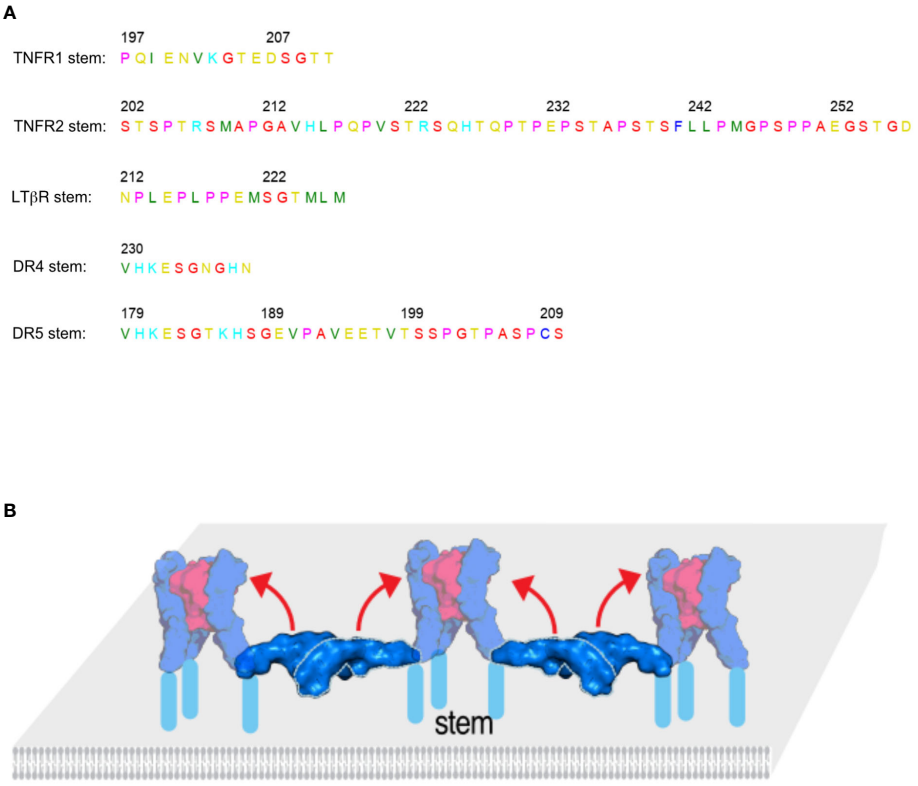

FIGURE 4
The size and rigidity of the stem region plays a role in receptor activation by the ligand. **(A)** The stem regions of several TNF receptors are listed. Soluble TNF can more readily activate TNFR1 and LTβR with short stem regions than TNFR2 that has a long stem sequence. Similarly, TRAIL can more readily activate DR4 with a short stem than DR5 that has a longer stem. **(B)** In the TNFR activation model the stem regions shown in light blue are exposed and may directly interact with the ligand or play an otherwise important role in receptor activation. The structures ligand bound complexes are shown in 50% transparency to indicate the final state after conformational change.

Understanding the surface structures of the TNFRSF can aid the development of better therapeutics

It is important to highlight that early antibody development was done in the absence of high-resolution structures of the target antigen. Often, the exact mechanism of action of the therapeutic antibodies were unknown as well resulting in surprises decades later. Over the years research has shown that the target epitope can influence the function of antibodies and they can act both as agonists or antagonists. To untangle this relationship requires a more detailed understanding of the antigen structure and the relationship between the target epitope and antibody function. While several very successful anti-TNF therapeutics have been launched to treat rheumatoid arthritis, psoriatic arthritis and other autoimmune conditions (33), their mechanism of action still holds surprises even two decades after development. As an example, the anti-TNF antibody, adalimumab has only recently been shown to paradoxically function as a TNFR2 agonist (34). Adalimumab not only has been shown to bind to TNF but surprisingly to increase its expression on the surface of monocytes. As the authors wrote: "The mechanism that may underlie this surprising result is unclear, but one possibility is that adalimumab stabilizes membrane TNF at the cell surface and prevents recycling or cleavage to soluble TNF" (34). In the context of clustering, we propose that adalimumab may aid the formation and stabilization of TNF clusters on the cell surface that in turn may facilitate better signaling via the also clustered TNFR2 on the surface of cells. This is in agreement with experimental data showing the higher order complexes of anti-TNF antibodies in complex with TNF (35). Before this information became available, it was widely believed that TNF blockade and not TNFR2 activation was responsible for T_{reg} expansion. On the receptor side, it has been challenging to create therapeutic antibodies against the TNFRSF. Antibodies against the TNFRSF can either block signaling and function as antagonists or promote signaling to function as agonists. It is only during the last few years that we have begun to understand how antibodies binding to different epitopes and surface structures can achieve these opposing functions (4–6, 36, 37).

For agonism, the stabilization of the hexagonal cluster of upright (free or ligand bound) receptors by antibodies that bind on the outside of the receptor, opposite the ligand binding site, may provide the best solution (6, 38) (Figure 5A). This strategy has been observed in an agonist antibody targeting DR5 (6) and also by an agonist targeting TNFR2 (38), highlighting that these strategies may be uniform regardless of the receptor type and their downstream partners. These antibodies link two receptor trimers together

therefore both Fab arms are necessary. Stabilizing the receptor cluster may allow prolonged ligand binding and receptor activation or may directly activate the receptors in the absence of exogenous ligand. An additional potential benefit maybe the inhibition of receptor cleavage and/or receptor internalization. This seems to be the case in a recently developed artificial protein scaffold (39) that uses an inducible two-component system to produce hexagonal arrays to which receptors can be attached, thus allowing the study of geometry on signaling behavior. An important finding of the study is that the artificial protein scaffolds can modulate the internalization of the attached receptors with array size playing an important role in inhibiting endocytosis (39). A naturally occurring receptor also appears to employ this mechanism. In the epidermal growth factor receptor (EGFR) the transmembrane GxxxG motif plays an important role in oligomerization induced internalization and signal attenuation (40). The artificial 2D scaffold blocks receptor oligomerization and inhibits receptor internalization without inducing signaling (39), which could be important for therapeutic applications. Interestingly, several, but not all, TNFR members also contain the transmembrane GxxxG motif (41) that could play a similar role in modulating receptor internalization and signal attenuation in these members of the TNFRSF. Therefore, agonist antibodies against TNFRSF members that cross-link neighboring receptors greatly improve receptor stability and signaling (6, 38), and may inhibit receptor internalization by maintaining the separation of individual receptor trimers in the hexagonal lattice.

There are conflicting data in the literature on the need for Fcγ recruitment but it is not an absolute requirement for receptor agonism (4, 6, 38, 42–44). Neither the anti-DR5 nor the anti-TNFR2 agonist antibodies require Fcγ engagement for agonism (6, 38). Further examples include anti-CD40 and anti-Fn14 agonist antibodies that similarly do not require Fcγ engagement (45, 46). At times antibodies that function via Fcγ recruitment have been

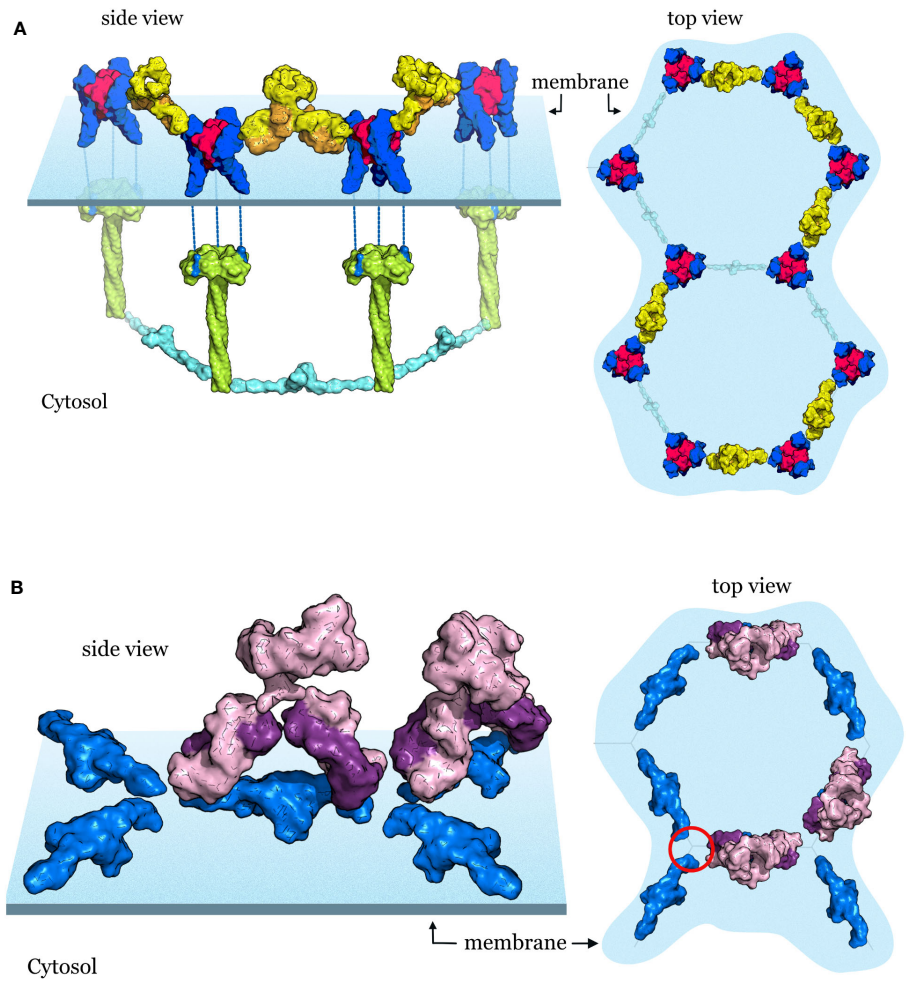

FIGURE 5
Antibody targeting strategies of TNF receptors **(A)** Agonist antibodies stabilize the hexagonal signaling complex TNF (magenta)-TNFR2 (blue) complexes are arranged on the cell surface in a hexagonal lattice. After receptor activation downstream TRAF signaling partners (shown in green and cyan) are recruited with matching hexagonal geometry. Agonist antibodies (shown in yellow and orange) stabilize the receptor clusters and improve signaling. **(B)** Antagonist antibodies stabilize the quiescent state and block the activation of receptors. Receptor dimers (blue) in the quiescent state are arranged in a hexagonal lattice on the cell surface. Antagonist antibodies shown in purple and violet lock in the ligand-free state and block ligand binding, receptor activation and the recruitment of downstream signaling partners.

designated as agonists despite clearly blocking ligand binding. These antibodies should be more properly designated as therapeutics functioning via antibody dependent cell cytotoxicity (ADCC) and their function should be separated from true receptor agonism that does not depend on Fcγ involvement.

Historically, it has been difficult to create effective antagonist antibodies against the TNFRSF. For those working in the field, it is broadly appreciated by trial and error that the natural ligands to the TNFRSF not only have high affinity but also high avidity and can, in most cases, successfully compete against antagonist antibodies in challenge assays. For instance, antagonist antibodies raised against TNFR2 can block TNF binding with different effectiveness (36). Characterization of several antagonist antibodies to TNFR2 have shown that strong (or dominant) antagonists targeting the CRD3-CRD4 domain could effectively block TNFR2 signaling even in the presence of increasing concentrations of TNF, while weak (or recessive) antagonist antibodies target the CRD1-CRD2 domain and compete poorly with TNF (36). Further characterization revealed that, only the full antibody or the $F(ab')_2$ structure is able to successfully block TNF binding. The data supports a mechanism where the best antagonists bind to the antiparallel dimer form of the receptor locking in the non-signaling form of the receptor (4, 36) (Figure 5B). To highlight the case that structural homology can translate these findings to other members of the TNFRSF, an antagonist antibody to CD40 has also been shown to bind to the antiparallel dimer form of the receptor (47). In this case, the antibody binds as a single Fab domain making interactions to CRD1 of both CD40 monomers in the dimer. Interestingly, a mutation that abolishes binding to the antiparallel dimer form and results in the mutant antibody binding to a single CD40 monomer turns this antibody into a functional agonist proving that binding to the antiparallel dimer form is required for antagonistic activity (47).

Several groups have mapped the surface of TNF receptors to see how the epitopes influence function. As the above examples show, there is no clear connection between the epitopes position on the CRD and agonism or antagonism. However, the consensus that seems to be emerging is that the best agonists are bivalent or multivalent antibodies that cross-link and stabilize receptor complexes in the hexagonal cluster (6, 38) and the best antagonists are stabilizing the antiparallel form of the receptor (36, 47).

The antibody isotype can also have a huge influence on the function of antibodies both for agonism and antagonism. Several anti-CD40 agonist antibodies have been shown to benefit from isotype switching from the IgG1 to IgG2 isotype (48, 49). The IgG2 isotype has also improved the function of an anti-TNFR2 antagonist antibody (50). Structural and biophysical studies have shown that the IgG2 isotype is the most rigid of all the IgG isotypes with a narrower range in Fab movement and separation distance (51, 52). These studies suggest that both agonism and antagonism can benefit from the IgG2 isotype presumably by better stabilizing the hexagonal cluster with a less flexible antibody.

For ligand-based therapeutics targeting the TNFRSF, approaches that mimic the membrane-bound form result in much improved signaling. For a good review on different strategies see De Miguel et al. (53). The minimum requirement is the stabilization of the ligands by creating stable covalent trimers by various methods (54–56). This improves half-life and bioavailability. Generating a more stable and rigid ligand may also aid in the activation step of the receptors highlighted in Figure 4B. Linking two trimeric ligands can further improve signaling by activating neighboring receptors in the cluster (57–62). Several other ligand-fusion complexes have been created with improved signaling (53, 63–68). However, improvements in signaling by fusion constructs have to be carefully balanced against the risk of immunogenicity by unnatural looking complexes that the immune system may recognize as foreign. Indeed, many ligand constructs that have shown promise in the lab have never made it to the clinic for this reason.

Conclusions

We have reviewed the current state of our understanding of TNFRSF signaling mechanism. We have shown that TNFRSF signaling can be described by a unified model that orders the receptors and ligands into a honeycomb cluster. The hexagonal lattice of TNF receptors is optimized for signal transduction as it provides the most economical way to build a stable scaffold. Clustering also results in signal amplification that depends on cluster size and geometry in agreement with experimental data showing TNF receptors are arranged in small nanoclusters on the cell surface. We have shown that high occupancy of a receptor cluster by ligands leads to a sharp, high amplitude signal, while random occupancy leads to broad low amplitude signal that is directly proportional to the concentration of RING dimers or caspase-8 generated and could explain differences in signaling outcomes between membrane and soluble TNFSF ligands. Building of more detailed signaling models in the future combined with experiments will further improve our understanding of the intricacies of TNFRSF signaling.

Beyond the TNFRSF, there are a growing number of hexagonal biological systems that indicate this may be a common arrangement of signaling networks in general. In addition to TNF receptors and their downstream signaling partners, chemo- or phototaxis receptors also cluster into hexagonal core complexes, consisting of trimers of dimers that further assemble to form large hexagonal arrays (69–71). Signal amplification has been observed in these systems and it has been proposed that the amplification is the result of cooperativity in the clustered arrays (71–73). Mimicking natural receptor clustering, artificial two-dimensional scaffolds have now been developed that utilize hexagonal lattices to modulate cell responses (39). The numerous available examples indicate the ordered clustering of surface proteins is more frequent in biological systems than previously appreciated, and most likely

represents the rule and not the exception. It can also provide an optimal solution to the processing of biological information (21).

As the examples have shown a better understanding of receptor conformations on the cell surface can lead to the development of more effective therapeutics. Beyond antibody- and ligand-based therapeutics, the detailed knowledge of cell surface structures could also aid the development of small molecule drugs. Due to the high structural homology among members of the TNFRSF, strategies that work for the targeting of one receptor can be applied to others.

Author contributions

EV and DF contributed to the design, writing and revision of the article. EV and DF approved the final content of the article.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Therapeutic potential of TNFR2 agonists: a mechanistic perspective

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TNFR2 agonists have been investigated as potential therapies for inflammatory diseases due to their ability to activate and expand immunosuppressive CD4+Foxp3+ Treg cells and myeloid-derived suppressor cells (MDSCs). Despite TNFR2 being predominantly expressed in Treg cells at high levels, activated effector T cells also exhibit a certain degree of TNFR2 expression. Consequently, the role of TNFR2 signaling in coordinating immune or inflammatory responses under different pathological conditions is complex. In this review article, we analyze possible factors that may determine the therapeutic outcomes of TNFR2 agonism, including the levels of TNFR2 expression on different cell types, the biological properties of TNFR2 agonists, and disease status. Based on recent progress in the understanding of TNFR2 biology and the study of TNFR2 agonistic agents, we discuss the future direction of developing TNFR2 agonists as a therapeutic agents.

KEYWORDS

Treg - regulatory T cell, TNFR2 agonism, TNFR2, tumor, autoimmune diseases

Introduction

Tumor necrosis factor (TNF) is one of the most potent pro-inflammatory cytokines that cause cell death and promote inflammatory responses, and high levels of TNF are attributable to the pathogenesis of autoimmune disease (1, 2). Anti-TNF therapeutics have been used as a first-line biological treatment of a variety of inflammatory diseases, including rheumatoid arthritis (RA), psoriasis, and inflammatory bowel disease (IBD) (3–5). However, paradoxically autoimmune inflammation frequently occurred in a subset of patients who received anti-TNF treatment. For example, anti-TNF therapy can increase the incidence of multiple sclerosis (6). These observations brought intense interest in elucidating the cause and mechanism. TNF receptor type I (TNFR1) and TNFR2 are two different receptors that mediated the biological function of TNF (7). A recent report

showed that polymorphisms in TNFR2 frequently occurred in patients with IBD or RA (8, 9), which suggests that TNFR2 signaling plays an essential role in preventing these diseases.

TNFR2 is preferentially expressed by immunosuppressive cells, including Tregs, MDSCs, and some endothelial progenitor cells (EPCs) (10). Now, there is compelling evidence showing that TNF-TNFR2 signaling plays an important role in curbing pro-inflammatory responses and promoting tissue regeneration. It was reported that TNFR2 deficiency aggravated autoimmune inflammatory responses in collagen-induced arthritis (CIA) (11), experimental autoimmune encephalomyelitis (EAE) (12), graft-versus-host diseases (GVHD) (13), and psoriasis (14). Moreover, several studies have shown that TNFR2 agonists protect mice from autoimmune inflammatory diseases and degenerative diseases (15–17). Thus, TNFR2 agonists have been proposed as a novel strategy for the treatment of autoimmune diseases, by mainly activating and expanding TNFR2-expressing Tregs, and MDSCs (18–20). However, some studies indicate that TNFR2 plays an important role in the activation of conventional T cells (Tcon cells) and CD8 T cells (21, 22). Furthermore, antibodies that can trigger the TNFR2 signal in vitro were shown to promote antitumor immune responses by activating CD8 T cells, Tcon cells, or NK cells (23). And consequently, TNFR2 agonists have been developed for the treatment of human cancers (19). Thus, the role of TNFR2 signal in orchestrating the inflammatory responses in autoimmune diseases or immune responses in cancers is complicated, and the mechanism may be involved in the activation of Tregs, MDSCs, $CD8^+$ T cells and ADCC, or inversely, depletion of Tregs in tumor environment (summarized in Table 1). The contradictory pro-inflammatory and anti-inflammatory properties of TNFR2

TABLE 1 The application of TNFR2 agonist in autoimmunity and cancer.

Category	Class	Agent	In vitro activity	In vivo activities
Autoimmunity				
	TNFR2 agonistic antibody	"TNFR2 agonist"	a) Promote the expansion, immunosuppressive function, and phenotypic stability of human Tregs (24).	N/A
		"TNFR2 agonistic antibody"	a) Promote Treg expansion and immunosuppressive function (25) b) Promote fatty acid oxidation in Tregs (25)	N/A
		MR2-1(Isotype: mouse IgG1)	a) Promote the expansion and immunosuppressive function of Tregs (26–28). b) Promote CXCL13 expression on T follicular regulatory cells (26). c) Promote EZH2 expression in Tregs (29). d) Promote glycolysis in Tregs (23, 27). e) Promote cell death of autoreactive CD8 T cell death (30)	N/A
		TY010	a) Promote M2 polarization (12). b) Promote IFN-γ expression in NK cells (31).	N/A
	Transmembrane mimetics	STAR2	a) Promote the expansion, immunosuppressive function of Treg (15, 17, 32–34). b) Protects oligodendrocyte progenitor cells and nerouns from oxidative stress-induced cell death (35, 36).	Protects mice from collagen-induced arthritis (15), GvHD (33), , BCG-induced chronic inflammation (34)
		New STAR2 (STAR2 conjugated with IgG)	a) Promote Treg expansion and immunosuppressive function (17, 32, 37) b) Enhances Microglial Phagocytosis (17, 32)	Protects mice from Alzheimer's disease (17) and GvHD (37)
		EHD2-scTNF$_{R2}$	a) Promote the expansion, immunosuppressive function of Treg (38–40). b) Activating PI3K-PKB/Akt and NF-κB signaling (40–42)	Protects mice from neuropathic pain (39), collagen-induced arthritis (21), traumatic contusive injury (43), Experimental autoimmune encephalomyelitis (41), and Alzheimer's disease (42).
		P53-sc-mTNF$_{R2}$ and GCN4-sc-mTNF$_{R2}$	a) Promote the expansion of Treg (44).	N/A
	TNF mutants	TNF07	a) Promote the expansion and immunosuppressive function of Treg (45, 46).	Protects mice from DNFB-sensitized contact hypersensitivity (46).
	Endogenous proteins	Membrane lymphotoxin-α2β	a) transmembrane LTα2β robustly activates human TNFR2 signaling (47).	N/A

(Continued)

TABLE 1 Continued

Category	Class	Agent	In vitro activity	In vivo activities
		Progranulin (PGRN) or its derivatives.	Promotes TNF-induced Treg proliferation (48). Promotes M2 polarizaiton (49). Promotes the IL-10 expression (50, 51).	Protects mice from osteoarthritis (52, 53).
Cancer				
	TNFR2 antibody trigger TNFR2 activation in vitro	TNFR2 agonist (Y9)	Promote the activation of CD8 T cells and NK cells (54).	Inhibit the tumor growth (Require FcγR activity) (54).
		MM401	Provides T cell co-stimulation (55).	Inhibit tumor growth and deplete Treg with ADCC (56) (Require FcγR activity) (55, 56)
		BI1910	Promote CD8 T cell function and infiltration (57). Regulating the myeloid contents in tumor (57).	Inhibit tumor growth with or without IgG conjugation (57)
		HFB200301	Activates T cells, NK cells and Tregs in vitro (58).	Inhibit tumor growth without affecting Treg numbers (independent of FcγR activity) (58)
		IAT0981-231	stimulated $CD8^+$ T cell activation, proliferation and cytokine secretion (59)	Inhibit tumor growth (59)

signaling should be further clarified in future investigations. This contradictory pro-inflammatory and anti-inflammatory property of TNFR2 signaling should be further clarified in future investigation

This review will focus on the discussion of the current understanding of the effects of TNFR2 agonists on inflammatory responses and anti-tumor immune responses. The development of TNFR2 agonists is introduced and the effect of these agents on the activation of different subsets of immune cells, and factors that may determine the therapeutic outcome of TNFR2 agonists in the treatment of cancer or autoimmune diseases, are reviewed and analyzed.

Overview of TNFR2 agonists

TNF mutants

Selective mutation of residues in TNF protein significantly altered its affinity to TNF receptors. The TNF mutant (D143N-A145R) is a TNFR2-selective agonist developed for decades. TNF mutant (D143N-A145R) only binds to TNFR2 but not TNFR1 (60). However, such a TNF mutant presents a 5~30 fold lower affinity to TNFR2 in comparison with wild-type (WT) TNF (61, 62) In recent years, several new TNF mutants that selectively bind and activate TNFR2 were developed by the phage display technique (63). The SPR analysis showed that these TNF mutants bind to TNFR2 with lower affinity but had a higher association/dissociation rate in contrast with WT TNF (63), indicating TNF mutants can form a stable complex with TNFR2Moreover, an *in-vivo* study showed that the TNF mutants fused with IgG could trigger the activation of TNFR2 signaling and induce Treg proliferation in a TNFR2-dependent manner (46). These results suggested that TNFR2-selective TNF mutants exhibit different binding modes for unique biological functions.

Transmembrane TNF mimetics

Compared with TNFR1, TNFR2 can only be fully activated by transmembrane TNF (64). The monomer transmembrane TNF always forms homotrimers on the cell membrane as a consequence of self-assembly before binding with TNFR2 (65, 66). Thus, one of the strategies to enhance the affinity of TNFR2-selective TNF mutants is to construct oligomerized TNFR2-selective TNF mutants (Figure 1). STAR2, a TNFR2 agonist composed of murine TNF mutants (D221N and A223R) and trimerization domain from chicken tenascin C, displays significantly higher affinity to TNFR2 than single chain TNF mutein and can induce TNFR2 activation more effectively (33). Moreover, STAR2 treatment significantly promoted Treg expansion in the mouse GVHD model (33). Based on this idea, Fisher et al. generated several oligomerized TNFR2-selective TNF mutants by using different oligomerization domains. The results showed that dodecavalent ligands by engineering oligomerization domain from GCN4 and TNFR2-selective TNF mutants (GCN4-sc-$mTNF_{R2}$) displayed superior bioactivity and affinity than other oligomerized TNFR2-selective TNF mutants in vitro (44). Furthermore, GCN4-sc-$mTNF_{R2}$ could be less immunogenic because the structure of GCN4-sc-$mTNF_{R2}$ more resembles human protein structure (44). Although oligomerized TNFR2-selective TNF mutants represent a more effective strategy to evoke TNFR2 activation, the risk of immunogenicity cannot be neglected as the sequence of

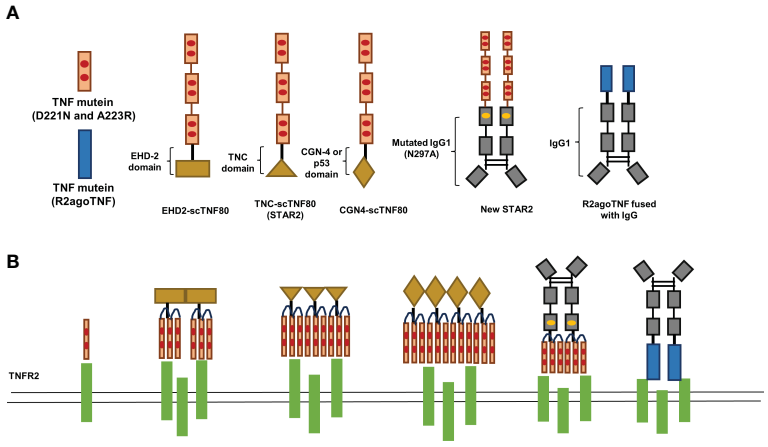

FIGURE 1
A schematic of TNF mutants and transmembrane TNF mimetics.

oligomerized TNFR2-selective TNF mutants cannot be found in nature. Thus, the immunogenicity of oligomerized TNFR2-selective TNF mutants should be carefully evaluated.

Progranulin (Endogenous proteins)

Progranulin (PGRN) is a secreted factor that regulates biological processes including inflammation, wound healing, and tissue repair (67). The initial results showed that progranulin directly interacts with and antagonizes both TNFR1 and TNFR2 (68), but subsequently, studies indicated that progranulin triggers the activation, instead of blocking TNF-TNFR2 signaling (69). Moreover, progranulin exhibits a relatively high affinity to TNFR2 (68). It has been shown that progranulin or its derivatives, Asttrin, alleviated the inflammatory responses in a TNFR2-dependent manner (50, 52, 70). However, there is contradictory evidence that progranulin may not interact with TNFRs, as progranulin failed to block the TNFR1-induced cell death (71). Furthermore, different concentrations of progranulin (2~200 ng/ml) plus IL-2 did not promote Treg proliferation, indicating that progranulin may not directly agonize TNFR2 (48). Thus, further investigation is needed to clarify if progranulin indeed promotes the activation of TNF-TNFR2 signaling.

TNFR2 agonistic antibodies

Recently, several TNFR2 antibodies with the capacity to activate TNFR2 *in vitro* have been developed for the treatment of autoimmune diseases (25) or cancers (57, 59, 72, 73). These TNFR2-stimulating antibodies were reportedly to possess either immunostimulatory or immunosuppressive *in vivo*, as its complicated nature, presumably based on different mechanism. For example, Y9, a close of agonistic anti-TNFR2 antibody, was found to be a competitive activator of TNFR2 and bind to CRD2 and CRD3 in TNFR2 (54). Other TNFR2-stimulating antibodies appeared to bind to CRD1 and CRD2 and did not compete with TNF for binding with TNFR2 (25, 57, 58). Whether the antibodies compete to bind to TNFR2 could also be important for their in vivo effect, as competitive activators sparing more TNF in local tissue, which may enhance the TNF-TNFR1 signaling. In contrast, the non-competitive TNFR2-stimulating antibodies did not affect TNF-TNFR2 interaction but may synergize with TNF in activating TNFR2 (74). Therefore, it is important to determine whether the non-competitive activator could elicit different responses with competitive activators.

Fragment crystallizable region (Fc) is another factor that profoundly affects the function of TNFR2-stimulating antibodies. For example, TNFR2 agonists may require FcγR activity for more potent agonistic function, as it confers TNFR2 agonist the transmembrane TNF-like activity (75). This was evidenced by the observation that TNFR2 agonistic antibody (BI-1910) with poor FcγR-binding activity (N297A, IgG1 mutated) exhibited a decreased antitumor effect as compared to IgG1 or IgG2a conjugated BI-1910 (57). Therefore, the engagement of FcγR could be an important factor that determine the therapeutic effect of some TNFR2 agonistic antibodies (76). Moreover, Fc conjugation can induce the antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP). For example, the antitumor effect of Y9 was reportedly dependent on the ADCC and ADCP, as its antitumor effect was diminished in Fcgr2b$^{-/-}$ and Fcer1g$^{-/-}$ mice (54). In this case, Y9 may deplete TNFR2-expressing cells rather than a TNFR2 agonist. Nevertheless, a recent study shows that a TNFR2 agonistic antibody had a more potent function in the presence of antibody that can block crosslinking activities (25), suggesting that neither ADCC nor Fc-mediated crosslink activities were required for the activity of this TNFR2 agonistic antibody.

TNFR2 agonist-induced activation of immune suppressive cells

$CD4^+Foxp3^+$ regulatory T cells

There is compelling evidence that TNFR2 plays a pivotal role in Treg activation, function, proliferation, and phenotypic stability (77, 78). Several TNFR2 agonists have been reported to alleviate inflammatory responses by promoting Treg function and expansion (15, 33, 34, 39, 40). TNFR2 agonism also represents an efficient approach to expand the Tregs from low-purity human Tregs for adoptive Treg transfer therapy. Several studies showed that TNFR2 agonistic antibody plus the standard Treg expansion protocol (in the presence of CD3/CD28, IL-2 with or without rapamycin) resulted in the expansion of homogenous stable Tregs with potent immunosuppressive function (24, 25, 33). Moreover, TNFR2 agonistic antibody treated-Treg has lower expression of CD127, IL-17A, and IFN-γ, indicating TNFR2 agonistic antibody help maintain the phenotypic stability of expanded Tregs (79). It is of great interest to examine whether TNFR2-agonist-expanded Tregs are more effective for adoptive Treg transfer therapy.

Myeloid-derived suppressor cells

MDSCs, a subset derives from pathologically activated neutrophils or monocytes, have potent immunosuppressive activity. MDSCs are considered a potential target for the treatment of cancer and autoimmune diseases (80). It was reported that TNF-TNFR2 signaling is important for the recruitment, immunosuppressive function, and survival of MDSCs (81–83). Thus, MDSCs is also a potential target of TNFR2 agonist. However, recent studies showed that the effect of TNFR2 agonists is mainly mediated by CD4 T cells, albeit with a minor effect on MDSCs. Lamontain and colleagues reported that TNFR2 agonist (TNCscTNF80) treatment promoted the expansion of MDSCs in bone marrow, but not in spleen and lymph nodes in the mouse CIA model, indicating the anti-inflammatory effect of TNCscTNF is not dependent on MDSCs (15). This result was further supported by the data from $CD4^{cre}TNFR2^{fl/fl}$ and $LysM^{cre}TNFR2^{fl/fl}$ mice. TNFR2 agonist (TNCscTNF80) suppresses T cell proliferation in $LysM^{cre}TNFR2^{fl/fl}$ mice but not in $TNFR2^{-/-}$ mice and $CD4^{cre}TNFR2^{fl/fl}$ (34), indicating the anti-inflammatory effect of TNFR2 agonist were mainly dependent on the TNFR2 expression by CD4 T cells. Further evidence is needed to support the claim that TNFR2 agonists can boost MDSC's activity to suppress inflammatory responses.

Monocytes/macrophages

Monocytes and macrophages express both TNFR1 and TNFR2. These two receptors play complicated roles in the regulation of the viability, function, and recruitment of monocytes/macrophages (84). Moreover, tissue-resident macrophages may also have different responses to TNFR2 activation, as compared with circulating monocytes/macrophages (85). Thus, the effect of TNFR2 agonism on macrophages could be tissue specific. It has been shown that administration of TNFR2 agonist (EHD2-sc-mTNFR2) increased the expression of M2 markers in macrophages and macroglia, and reduced the expression of M1 markers, but without activation of macrophage in mouse central nervous system (16, 39). However, these effects of TNFR2 agonists could be the indirect result of the activated Tregs which may suppress macrophage activity (86). A recent study showed that TNFR2 agonist (TY010) promoted M2 polarization of bone marrow-derived macrophage in TNFR2 dependent manner, indicating TNFR2 agonist may directly activate TNFR2 on macrophage and induced an immunosuppressive phenotype (49). Moreover, TNFR2 agonist (NewStar2, TNCscTNF80 fused with mutated human IgG) has been shown to enhance the phagocytosis of microglia and promote the clearance of Aβ plaques, which contributes to the alleviation of Alzheimer's diseases in mouse (17). These results indicate that macrophages/monocytes are the targets of TNFR2 agonists in the treatment of inflammatory diseases.

TNFR2 agonist-induced activation of effector immune cells

Conventional T cells

TNFR2 has been shown to promote the activation, function, differentiation, and proliferation of Tcon cells (22, 87, 88). $TNFR2^+$ Tcon cells are more resistant to Treg-mediated immunosuppression (87). However, TNFR2 was expressed much lower by Tcon cells than by Tregs in the resting state (87). Thus, TNFR2 agonists may not effectively activate TNFR2 signaling in unstimulated Tcon cells (89). Previous studies showed that the treatment with TNFR2 agonists (TNF mutants or transmembrane mimetics) inhibited Tcon cell proliferation by promoting Treg expansion (32). However, TNFR2 expression can also be upregulated by TCR stimulation (90) or pro-inflammatory cytokines (91), suggesting the activated Tcon cells could respond to TNFR2 agonists. A recent study showed that stimulation of anti-CD3 and a TNFR2 agonistic antibody (MR2-1) induces a similar alteration of transcriptome profile, albeit the alteration of the Treg cell transcriptomic profile is more obvious (23). This effect of TNFR2 agonism has shown to be pathological-relevant, as TNFR2 is expressed by tumor-infiltrating Tcon cells (89, 91–93) and proinflammatory subsets of CD4 Tcon cells (94, 95), suggesting that TNFR2 agonists could induce the activation of TNFR2 signal in Tcon cells in tumor and inflammatory diseases. In the mouse tumor model, it was reported that TNFR2 agonistic antibodies induced the expansion of $CD4^+$ Tcon cells without affecting the Treg number in vivo (58). Therefore, Tcon cells are also a potential target of TNFR2 agonists albeit with relatively lower TNFR2 expression.

CD8 T cell

As one of the co-costimulatory receptors, TNFR2 promotes the activation, function, proliferation, differentiation, and recruitment of CD8 T cells (96–98). However, TNFR2 signaling can also play a dual role in the modulation of the activation of CD8 T cells. For example, genetic ablation of TNFR2 impairs the production of effector cytokine while can also result in a more persistent activation of CD8 cells in mouse tumor and infection models (99, 100). CD8 T cells at different stages of activation may likely respond to TNFR2 activation differently. This notion is supported by the observation that the activation of TNFR2 promotes the differentiation of naïve CD8 T cells (96), while TNFR2 stimulation also selectively induced the activation-induced cell death (AICD) of the autoreactive CD8 T cells without significantly affecting the other T cell subsets (30). The different responses could be attributable to the alteration of downstream signaling. TNFR2 expression is crucial for the activation of NF-κB signaling in CD8 T cells when stimulated with anti-CD3/CD28. While a persistent activation of TNFR2 can inhibit NF-κB signaling through depleting TRAF2, an important signal component in mediating NF-κB activation (39), thereby sensitizing CD8 T cells to TNF-induced cell death (101).

Although treatment of TNFR2 agonistic antibody can stimulate the activation of tumor-infiltrating CD8 T cells (57), the time frame of TNFR2 agonist treatment in a preclinical mouse tumor model is relatively short. Such studies may not be able to reflect the effect of long-term activation of TNFR2, including activation-induced cell death (AICD) or exhaustion of CD8 T cells (100, 102). More recently, we reported that TNFR2 expression is associated with the exhaustive phenotype of CD8 T cells in human cancers (103). Thus, the role of TNFR2 in tumor-infiltrating CD8 CTLs is complex and needs further investigation. A more thorough understanding of the molecular basis underlying the effect of TNFR2 signal in CD8 CTLs is crucial to device TNFR2 agonists in tumor immunotherapy.

Natural killer cells

TNFR2 has been reported to be expressed by both human and mouse NK cells, albeit the expression pattern could be different (89). It has been shown that genetic ablation of TNFR2 has been shown to significantly decrease the expression of IFN-γ in α-galactosylceramide (α-GalCer)-treated mouse, indicating that TNFR2 signaling is also important for the activation and function of NK cells (31). TNF or TNFR2 agonist (TY010) in concert with IL-12 elevated the expression of IFN-γ in human and mouse NK cells in vitro (31, 104). The antitumor effect of TNFR2-targetingantibody (Y9) can be impaired by the depletion of NK cells, suggesting that TNFR2 agonists may also target the NK cells to elicit antitumor immune responses (54). Now several TNFR2-stimulating antibodies in clinical development have been reported to enhance NK cell activation (58, 73). However, the mechanism that how TNFR2 agonism affects tumor-infiltrating NK cells remains to be investigated.

Factors may determine the therapeutic outcome of TNFR2 agonists in the treatment of cancer or autoimmune diseases

TNFR2 expression: pro-inflammatory vs anti-inflammatory cell subsets

TNFR2 signaling can result in both anti-inflammatory or pro-inflammatory effects, depending on the cell type of TNFR2 expression and the functional status of the cells. High levels of TNFR2 are constitutively expressed by Tregs, and the activation of TNFR2 signaling in Tregs or autoreactive CD8 T cells (and other immunosuppressive cells) can cause immune suppression or elicit an anti-inflammatory effect (30, 78). However, elevated TNFR2 expression can be shown in pathogenic T cell subsets in patients with Crohn's disease (94) and rheumatoid arthritis (95), and blockade of TNF promoted cell death of pathogenic T cells (94, 95). These results indicated that TNFR2 agonism may also promote pathogenic T-cell responses. The pathogenic T cells with elevated TNFR2 expression can be more resistant to Treg-mediated immune suppression (105). Moreover, activation of TNFR2 also promotes the inflammatory responses of innate immune cells and non-immune cells (106–108). The dual role or bi-phasic effect of TNFR2 signaling is exempted by a study that shows that, in TNFR1 deficient mice, infusion of murine TNF at the initial phase of collagen-induced arthritis increased the disease severity, while the same treatment markedly alleviated the inflammation in the progression phase (109). This study demonstrated the bi-phasic effects of TNF-TNFR2 signaling in an inflammatory response.

Both humanized TNFR2 antagonists and agonists have been developed for the treatment of tumors (19, 110), based on the notion that antagonistic antibodies may eliminate the immunosuppressive Tregs, while antibodies that trigger TNFR2 signaling may activate CD8 CTLs and NK cells (54, 56–58, 72). Despite the assumptions are opposite, the results of the studies appear to support that TNFR2 antagonistic antibodies inhibit the tumor infiltration of Tregs and consequently enhance the antitumor immune responses (111–119), while antibodies that trigger the TNFR2 signal in vitro also elicited antitumor immune responses (54, 56–58, 72). It was shown that TNFR2 activation promotes the differentiation and the production of effector cytokine by CD8 T cells (96, 120). Thus, as a costimulatory molecule, TNFR2 is likely to promote the initiation of antitumor T cell immune responses. In line with this notion, preclinical studies showed that TNFR2 agonistic antibodies with diminished activity to induce ADCC can enhance the antitumor immune responses by activating CD8

T cells and NK cells. However, it should be noted that the preclinical studies about TNFR2 agonistic antibodies were based on the transplanted tumor models and the time of experiment settings is relatively short. Such studies may not be able to reflect the effect of long-term activation of TNFR2, including activation-induced cell death (AICD) or exhaustion of CD8 T cells (100, 102).

Ligand-based agonists vs agonistic antibodies

Different types of TNFR2 agonists may own distinguished features and consequently elicit different effects on immune response in vivo. We summarized the antibodies that can trigger TNFR2 signaling from the published studies (Table 1). Interestingly, some of these TNFR2 antibodies can enhance the anti-tumor immune responses even though they have been shown to significantly promote Treg expansion in vitro. While the TNFR2-selective TNF mutant or transmembrane TNF mimetics elicited anti-inflammatory responses in vitro and vivo. The paradoxical effects of antibodies were also reported in other TNFRSF members. For example, one of the GITR antibody has been shown to promote Treg expansion in vitro (121). However, this GITR antibody has also been shown to enhance the infiltration of non-Foxp3 expressing T cells into tumor tissue and enhance the antitumor immune responses (122). These results suggest the complicated nature of TNFR2-antibodies with stimulating activities. By comparing with transmembrane TNF mimetics, TNFR2-antibodies with agonistic activities also bind with TNFR2 with high affinity. However, the function of TNFR2-stimulating antibodies is significantly affected by the Fc region as aforementioned. Moreover, although there is compelling evidence that TNFR2 agonists promote the expansion of Tregs, excessive or prolonged TNFR2 activation may elicit different responses of Tregs in some circumstances. For example, tumor-infiltrating Tregs are highly activated and express high levels of TNFR2. The activation of TNFR2 may induce the TRAF2 depletion in Tregs present in the tumor environment, thus sensitizing Treg to TNF-induced cell death (123, 124). Moreover, although optimal PI3K/Akt/mTOR signaling could be important for TNF-induced Treg activation and expansion (26, 28), a high dose of TNFR2 agonistic antibody or superclustering of TNFR2 may also induce hyperactivation of PI3K/Akt/mTOR signaling pathway, which can destabilize Foxp3 expression (125). The agonistic antibody-induced Foxp3 instability and Treg cell death have been reported in other TNFRSF members with a similar co-stimulatory capacity (126, 127). Therefore, this evidence may provide an alternative interpretation of the reported anti-tumor effect of TNFR2 agonistic antibodies.

Tissue-specific responses to TNFR2 agonists

The TNFR2 agonist-regulated immune responses could be tissue-specific, as the tissue-resident immune cells and non-immune cells can express TNFR2. TNFR2 activation may induce anti-inflammatory responses or pro-inflammatory responses as well, depending on the cell types targeted. There is compelling evidence that TNFR2 signaling not only suppresses the inflammatory responses in the central nervous system (CNS) but also promotes tissue repair of the CNS system by activating TNFR2 signaling through several different cell types (35, 128, 129). Thus, TNFR2 agonism evokes immunosuppression and tissue repair in the CNS. However, TNFR2 also induced potent inflammatory responses in some organs. For example, activation of TNFR2 signaling in parenchymal cells was important for the development of hepatitis in mice (130). Blockade of TNFR2 has been shown to alleviate anti-PD-1-induced hepatic inflammation in mouse hepatic carcinoma, even infiltration of Tregs was also decreased (131). Moreover, TNFR2 signaling in some tumor cell types also contribute to tumor progression (132, 133), so the potentially undesirable effect of TNFR2 agonistic antibody should be considered. Thus, tissue-specific responses are also an important factor that needs to be considered in TNFR2 agonist therapy.

Conclusion and future perspective

Numerous studies indicated the potential of TNFR2 agonism in the treatment of autoimmune inflammatory diseases and cancer, as TNFR2 has a dual role in modulating immune responses. TNFR2 plays a decisive role in maintaining Treg function and activity, which is important for the suppression of autoimmune inflammatory responses. On the other hand, TNFR2 activation in Tcon cells or CD8 T cells also elicits pro-inflammatory responses. However, the therapeutic outcome of TNFR2 agonism could be affected by the property of agonists, the disease condition, and tissue-specific responses. To minimize the unwanted effect elicited by TNFR2 agonism, one of the strategies is to develop therapeutics that specifically target certain cell types. For example, IL2-EHD2-sc-mTNFR2, a recombinant protein fused with TNFR2 agonist (EHD2-sc-mTNFR2) and IL-2, induced a more potent Treg expansion than IL2 plus EHD2-sc-mTNFR2 (38). Moreover, combining the immunosuppressants with TNFR2 agonists could be another strategy in the treatment of autoimmune inflammatory diseases, as Tregs are more resistant to immunosuppressant-mediated cell death (134). Although several TNFR2 agonistic antibodies were demonstrated in the clinical trial, how the TNFR2 agonistic antibody elicits a different immune response in vivo remains largely unknown. Identifying the factors that affect biological consequences induced by TNFR2 agonists may pave the way to the more effective treatment of cancer or autoimmune diseases.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

YC and MJ drafted the manuscript. XC, the corresponding author, was involved in designing the frame of the manuscript and

approving the final version to be published. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Reconciling intrinsic properties of activating TNF receptors by native ligands versus synthetic agonists

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The extracellular domain of tumor necrosis factor receptors (TNFR) generally require assembly into a homotrimeric quaternary structure as a prerequisite for initiation of signaling via the cytoplasmic domains. TNF receptor homotrimers are natively activated by similarly homo-trimerized TNF ligands, but can also be activated by synthetic agonists including engineered antibodies and Fc-ligand fusion proteins. A large body of literature from pre-clinical models supports the hypothesis that synthetic agonists targeting a diverse range of TNF receptors (including 4-1BB, CD40, OX40, GITR, DR5, TNFRSF25, HVEM, LTβR, CD27, and CD30) could amplify immune responses to provide clinical benefit in patients with infectious diseases or cancer. Unfortunately, however, the pre-clinical attributes of synthetic TNF receptor agonists have not translated well in human clinical studies, and have instead raised fundamental questions regarding the intrinsic biology of TNF receptors. Clinical observations of bell-shaped dose response curves have led some to hypothesize that TNF receptor overstimulation is possible and can lead to anergy and/or activation induced cell death of target cells. Safety issues including liver toxicity and cytokine release syndrome have also been observed in humans, raising questions as to whether those toxicities are driven by overstimulation of the targeted TNF receptor, a non-TNF receptor related attribute of the synthetic agonist, or both. Together, these clinical findings have limited the development of many TNF receptor agonists, and may have prevented generation of clinical data which reflects the full potential of TNF receptor agonism. A number of recent studies have provided structural insights into how different TNF receptor agonists bind and cluster TNF receptors, and these insights aid in deconvoluting the intrinsic biology of TNF receptors with the mechanistic underpinnings of synthetic TNF receptor agonist therapeutics.

KEYWORDS

TNF superfamily, agonist, TNF receptor, TNF ligand, CD40, 41BB, OX40

Introduction

The tumor necrosis factor (TNF) superfamily (TNFSF) of 19 ligands and 29 receptors serve as critical regulators of human immunity, and modulating the activity of individual receptors and ligands for therapeutic benefit in autoimmunity and cancer has been studied for over 40 years (1–3). Activating, or agonizing, TNF receptors to enhance immunity has proven to be a far more elusive goal than inhibiting TNF receptors. Enbrel (TNFR2-Fc) and Remicade (TNFα targeted monocolonal antibody [mAb]) were approved in 1998 to inhibit TNFα, and together with Humira (TNFα targeted mAb), quickly grew to become one of the most successful drug franchises in history (4, 5). In contrast, not a single TNF receptor agonist therapy (with the exception of recombinant TNFα) has progressed to a phase 3 clinical trial to date.

The focus of this review is to highlight the structural hypotheses underlying TNF receptor trimerization and subsequent activation of various cytoplasmic signaling cascades, both following activation via native TNF ligands and also with synthetic receptor agonists. A particular emphasis is placed upon areas where the pharmacodynamic activity of a TNF receptor agonist differed between pre-clinical mouse studies and human clinical trials. Several different TNF receptor agonists and TNF receptor targets, are included in this discussion, however the analysis is focused on how the available data inform on the magnitude and specificity of receptor engagement, rather than on the cellular and mechanistic differences between individual TNF receptors themselves. For example, this review focuses on whether the cytokine release syndrome (CRS) observed in human cancer patients treated with a 41BB or CD40 agonist antibody was likely a consequence of the underlying structural features of those antibody therapeutics rather than a deep dive into the specific differences in 41BB mediated costimulation of CD8 positive T cells versus CD40 mediated costimulation of antigen presenting cells (6–8).

Many patients, patient investors and drug developers have dedicated decades of effort to translating the powerful biology of TNF receptor agonists for the benefit of human disease. Most of these efforts have not lived up to the potential shown by the pre-clinical biology, yet many important lessons have been learned along the way. An improved understanding of the structural basis of TNF receptor activation has the potential to guide future development of improved therapeutic agonists.

TNF receptor and ligand trimerization

All twenty nine TNF receptors, with the exception of DcR3, are single-pass type 1 membrane proteins, oriented with a cytoplasmic carboxy terminus and an extracellular amino terminus (9). DcR3 is a decoy receptor that evolved in higher-order primates as a secreted TNF receptor that functions as a soluble competitive inhibitor to LIGHT, TL1A and FasL (10). The extracellular domains of TNF receptors generally contain between one and four cysteine rich domains (CRDs), arranged in an elongated fashion within each monomer and which in turn are stabilized by a network of intrachain disulfide bridges. A majority of the interactions between TNF receptors and their ligands tend to involve the membrane-distal CRDs, and ligand binding occurs both through hydrophobic and polar interactions (9, 11).

Nineteen distinct TNF ligands exist in humans, and all are single-pass type 2 membrane proteins, oriented with a cytoplasmic amino terminus and an extracellular carboxy terminus (9). A conserved c-terminal TNF homology domain (THD) characterizes each TNF ligand, and mediates interaction with conserved cysteine rich domains (CRDs) in corresponding TNF receptors. The THD domain is arranged as a series of two stacked β-pleated sheets. The inner β-sheet contains the contact sites which mediate predominantly hydrophobic interactions between TNF ligand monomers, and contribute to assembly of stable TNF ligand homotrimers. The outer surface of the β-sheet structure mediates binding to the CRDs of the cognate TNF receptors (9). An underexplored aspect of TNF ligand trimerization relates to the conditions under which TNF ligand trimers assemble and degrade, and whether other cellular or matrix components are involved in the process. For example, TRAIL homotrimers were reported to assemble around a central Zn^{2+} ion, however it is unknown whether other TNF ligand trimers are similarly dependent upon cation coordination (9, 12).

In the absence of ligand, a full-length TNFR exists at the cell membrane as a mixture of monomers and dimers, whereas soluble TNFR exist primarily as monomers. Quantitative high resolution microscopy studies of cells with physiological expression of TNFR1 demonstrated that 66% of TNFR1 molecules are present as monomers and 34% are present as dimers (13–15). Following stimulation with ligand, the balance shifts to 13% TNFR1 monomers, 64% trimers, and 23% higher-order oligomers (15). Dimerization of TNFR can occur primarily as a result of non-covalent, low-affinity, interactions between pre-ligand assembly domains (PLAD), which are typically in a low micromolar affinity range (16–18). Ligand-induced trimerization of TNFR is likely influenced by a variety of non-covalent interactions, including the PLAD domains, but the quantum of signaling transmitted by the cytoplasmic domains increases when ligand-induced avidity interactions lead to trimerization, hexamerization, and higher-order network formation such as the 9-mers observed for TNFR1 (19–21).

The hypothesis that the efficiency of TNF receptor signaling is related to the degree of hexamer or higher-order network formation in the cell membrane is supported by several mechanistic studies. A minority of TNFR (including BaffR, DR3, GITR, LTβR and TNFR1) achieve activation with soluble ligand trimers, and are referred to as Category 1 TNFR. Clinical data are available for GITR agonist antibodies, but not for any of the others. For the so-called Category 2 TNF receptors (including 41BB, CD40, OX40, and others), soluble ligand trimers fail to activate downstream receptor signaling, unless those ligand trimers are cross-linked either via an Fc domain fused to the amino terminus of the TNF ligand extracellular domain, or if the soluble ligand trimers are cross-linked by an anti-TNF ligand antibody (9, 22). That the minimal signaling unit of TNF receptors is a trimer is a logical extension of the observation that the TNF receptor associated factor (TRAF) cytoplasmic adaptor complexes also require assembly into trimers to initiate signaling. Thus, a trimerized clover-like TNF ligand complex engages and facilitates

trimerization of a TNF receptor complex in order to recruit and facilitate trimerization of cytoplasmic TRAF signaling adaptor complexes. Whether or not trimerization of TNF receptors is driven primarily by ligand-induced proximity interactions or via an associated conformational change in the structure of individual TNF receptor monomers is unclear. The structural basis of a 'resting' versus 'active' state of individual TNF receptors could be influenced by a transition between low-affinity interactions between neighboring TNF receptor monomers via the PLAD domains to higher-affinity interactions in the presence of trimerized ligand. Another possibility includes the association between TNF receptors and other accessory molecules, such as galectin-9, which could influence both affinity interactions between adjacent TNF receptor monomers, or potentially higher-order avidity interactions in a higher-order network (11, 23). A corresponding higher-order structural model of TRAF family oligomerization has been reported, wherein the ultimate signal-transduction potential of TNF receptor activation would be proportional both to the number of functional membrane trimers which are engaged by ligand, and also the degree to which those trimers assemble into an approximated higher-order network (9, 24).

Experimental evidence therefore consistently demonstrates that TNF receptor signaling is facilitated by TNF ligand mediated oligomerization of TNF receptors into homotrimeric complexes, which may then form higher-order 6-mer, 9-mer and potentially higher-order networks in cell membranes (15, 21). Transitioning this understanding to synthetic agonists with *in vivo* activity has proven to be an elusive goal, however. Over the years the number of synthetic agonist compounds has expanded, and now includes: monoclonal IgG antibodies, bispecific antibodies, tetravalent antibodies, hexavalent antibodies, Fc-fusion proteins, anticalin fusion proteins, bispecific Fc-fusion proteins, and IgM antibodies (Table 1) (3, 22, 25–27, 29, 32–34, 36, 37). All of these synthetic agonists have reported activity in pre-clinical models, particularly when the model facilitates an 'array' of individual agonist molecules, but the translatability of that pre-clinical data to *in vivo* activity in human patients has been dismal. One contributing factor to this lack of translatability may be related to the ability of different types of synthetic agonists to facilitate higher-order clustering of TNF receptors, which are further discussed in the following sections.

Clinical data from trials testing bivalent TNFR agonist bivalent antibodies

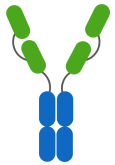
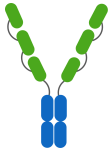
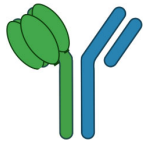
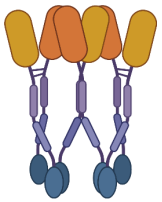
A majority of synthetic TNF receptor agonists which entered clinical trials were IgG based monoclonal antibodies being tested in oncology indications, and the most common targets in those trials included: CD40, OX40, 41BB, GITR and DR5. Dadas et al. published a recent review of these approaches, which provides a helpful background on some of the TNFR targets and a thorough description of the role played by the Fc domain of different agonist antibodies (35). The focus of the following section is thus to synthesize how the available human clinical data reflect the underlying mechanisms of mAb based agonists.

All TNF receptor agonist monospecific antibodies tested in clinical trials incorporate Fc domains with retained Fc gamma receptor (FcγR) binding activity, as this was shown to be a pre-requisite for agonist activity in most cases (28). The most common Fc domain for clinical stage agonist mAbs is IgG1, followed by IgG2, with only a few developers selecting the IgG4 isotype (38, 39). Whereas the FcγR binding activity of most targeted antibodies is required for effector function (antibody dependent cellular phagocytosis:ADCP and/or antibody dependent cellular cytotoxicity:ADCC), agonist antibodies depend on FcγR binding purely as a mechanism to immobilize antibodies such that the TNF receptor binding ends of multiple mAbs are displayed as an array on the FcγR expressing cell. FcγRIIB provides particularly efficient cross-linking of TNF receptor agonist mAbs, but other FcγR can also participate (21, 40, 41). Mouse antibodies, particularly IgG1, bind to FcγRIIB with high affinity, whereas human antibodies have universally low affinity for FcγRIIB (40, 42). One of the general observations which can be made from reviewing the last thirty years of literature on TNF receptor agonist antibodies is that the *in vivo* activity of TNFR agonist antibodies has been much more potent in mouse syngeneic models than in human clinical trials, and this relative difference in binding affinity to FcγRIIB may be a significant contributor to that learning, particularly in light of pre-clinical data showing that only a two-fold reduction in the binding affinity to FcγRIIB was sufficient to eliminate the agonist activity of several TNFR agonist mAbs (43).

The FcγR-dependent mechanism of TNFR agonist antibodies requires sub-saturating receptor occupancy on both the TNFR target and Fcγ receptors. As illustrated in Figure 1, FcγR dependent TNFR agonist antibodies must engage both target TNFR and FcγR simultaneously to promote antibody-mediated TNFR clustering in a trans orientation. This mechanism thus inherently depends upon having both free Fcγ receptor and free TNFR. The probability that FcγR bound mAbs encounter free TNFR target follows a Gaussian distribution, where a 'maximal' effect is predicted to occur when approximately 50% of the TNFR targets remain unoccupied by mAbs, assuming that both the abundance of and binding affinity to both TNFR target and Fcγ receptor are similar for a particular antibody (44). Gaussian distribution curves can also be described to have a 'bell-shaped' appearance, which is in fact the way in which the pharmacodynamic effects of many TNFR agonist antibodies tested in clinical trials have been reported. Here, high doses of TNFR agonist mAbs can independently saturate the intended TNFR and also the FcγR required to facilitate TNFR clustering, thereby eliminating agonist activity.

Amongst the clearest examples of bell-shaped dose response effects in humans treated with TNF receptor agonist antibodies include data from cancer patients treated with BMS-986178 (anti-OX40 mAb), PF-04518600 (anti-OX40 mAb) or mitazalimab (anti-CD40 mAb). Each of these antibodies are dependent on FcγR mediated cross-linking for agonist activity. The main pharmacodynamic marker reported from patients treated with the two OX40 agonist mAbs was proliferation (as indicated by Ki67 expression) of specific T cell subsets (44, 45). In both studies, a greater fold change in the proportion of Ki67+ T cells was observed in the low and mid-dose groups (~2 mg/kg or lower) than in the

TABLE 1 Molecular configurations of synthetic TNFR agonists.

Type of TNFR Agonist		Molecular Configuration	Valency to TNFR	Compounds that have entered clinical trials	References
Antibody formats	Monoclonal IgG		2	BMS-986178 PF-04518600 ADC-1013 BMS-986156	(3, 23, 25–28) Described in Figures 1–3
	Bispecific		1	GEN1042 GEN1046 FS222 PRS-343	(29) Described in Figure 4
	Tetravalent		4	TAS266 INBRX-109	(29)
	Hexavalent		6	ABBV-621 and INBRX-106	(3) Described in Figure 5
	IgM		5	IGM-8444	(3)
Fc-fusion protein formats	Contains TNF ligand trimer		3	RO7227166 RO7122290	[30, 31) Described in Figure 6
	Bispecific (hexameric TNF ligand)		6	SL-279252 SL-172154 MEDI6383 MEDI1873	(32–35) Described in Figure 7

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higher dose groups. The fold change observed in humans was also generally 3-fold or less, whereas the fold change in pre-clinical studies was approximately 5-fold or greater (44). Neither study reported corresponding changes in the actual numbers of the T cell subsets that stained positive for Ki67 expression. A phase 1 study tested mitazalimab across a dose range of 0.075-1.2 mg/kg in patients with advanced solid tumors (46), and reported a broader range of pharmacodynamic findings (47). Specifically, deep and rapid declines in the number of B cells in the peripheral blood were reported following the first dose, which was attributed to migration of B cells which are known to express CD40, and presumed to be bound by mitazalimab. Another finding included increased serum concentrations of multiple chemokines, including MCP1, IP10, MIP1α and MIP1β (46). The dose response for each of these chemokines showed a peak increase at the 0.075 or 0.2 mg/kg dose level, and lower magnitude increases in each chemokine at doses of 0.9 and 1.2 mg/kg. Wang et al. then proposed a model for the dosing of BMS-986178, wherein the optimal pharmacodynamic activity was achieved when the dose of the OX40 agonist mAb achieved approximately 50% receptor occupancy on OX40 expressing T cells (44).

A combination of inter-patient and intra-patient variability in TNF receptor abundance creates a significant challenge to selecting a single dose to advance into larger clinical trials if the mechanism of TNF receptor agonist mAbs requires sub-maximal receptor occupancy for the desired biological effect, as described above. Consider 'Patient A', who has a PBMC count of $1.4x10^6$ cells per mL, of which 80% are lymphocytes, 85% of the lymphocytes are T cells, 60% of the T cells are CD4+ T cells and 20% of those CD4+ T cells express OX40. Also consider 'Patient B', who has a PBMC count of $0.8x10^6$ cells per mL, of which 70% are lymphocytes, 70% of the lymphocytes are T cells, 50% of the T cells are CD4+ T cells and 5% of the CD4+ T cells express OX40. In both patients, these example lymphocyte/T cell percentages fall within the 'normal' range for healthy adults (the magnitude of variability can have a wider range in oncology patients who have received prior chemotherapy), yet Patient A will have approximately 10^5 CD4+OX40+ T cells per mL of peripheral blood, and Patient B will have approximately 10^4 CD4+OX40+ T cells per mL of peripheral blood. If the dose of an OX40 agonist antibody is modeled on the basis of achieving 50% receptor occupancy, the appropriate doses for Patient A and Patient B would differ by 10-fold. In a phase 1 clinical trial of SL-279252, we observed even wider variation, with the number of CD4+ T cells ranging from $2.2x10^3$ to $1.1x10^6$, and the percentage of CD4+OX40+ cells ranging from 5.1-48.6% (48, 49). Further, the example above assumes that each CD4+OX40+ cell expresses the same number of OX40 receptor molecules, which is unlikely to be the case either at baseline or through a course of therapy. In fact, both preclinical and clinical studies have reported that individual antigen-specific CD4+OX40+ T cell clones can expand more than 5-fold following treatment with an OX40 agonist, and the per-cell expression of OX40 can also increase following stimulation (13, 50, 51). Thus, a dose of an OX40 agonist antibody that achieves 50% receptor occupancy at the first dose is unlikely to be an appropriate dose several weeks later if activation of OX40+ cells has actually occurred, because both the density of OX40 on the cell surface, and the absolute number of CD4+OX40+ T cells would be expected to increase. The above example is not intended to indicate that inter- and intra- patient variability in degree to which a selected dose of a TNFR agonist agent is likely to cross an 'all or none' threshold of activation, but rather to highlight the risk that a selected dose for an agent with an expected bell-shaped dose response curve may lead to variable degrees of TNFR activation which may or may not remain in a therapeutic range.OX40 agonism is one such example, but the same mechanistic dependencies can likely be generalized to other TNF receptors including 4-1BB, CD27, CD40, GITR and others.

Additional clinical evidence is available to support this assertion from GITR agonist antibody studies, the only Category I TNFR target from which clinical data are available. GITR, as a Category 1 TNFR, is activated by soluble ligand trimers, and therefore it may be expected that bivalent antibodies would more readily activate this TNFR given the lack of a mechanistic requirement for higher-order TNFR oligomer assembly. A phase 1 clinical trial testing BMS-986156 (IgG1 Fc domain) in late stage cancer patients did not report any clear dose-dependent pharmacodynamic activity following infusion, either alone or in combination with nivolumab. In a phase 1 clinical trial with an FcγR non-binding GITR agonist, TRX518, some evidence reductions in regulatory T cells were reported both in the peripheral blood and within the tumor. The dose-dependence of this effect was not clear from the study given the limited sample size, nor was the potential mechanism of action given that GITR lacks a death domain.

Another variable to consider in selecting an optimal dose for a TNF receptor agonist antibody - based on sub-maximal receptor occupancy - involves the potential competition for Fcγ receptor binding. Two pre-clinical studies concluded that the sequencing of an OX40 agonist antibody and a PD-1 inhibitory antibody determined whether or not the combination was efficacious, despite not controlling for the fact that both antibodies were the same isotype, and thus competed with one another for Fcγ receptor binding (16, 17). The clinical impact of this specific combination is limited, given that pembrolizumab and nivolumab both have inactive IgG4 Fc domains, however there are many potential antibody combinations where it could be relevant. This antibody/antibody FcγR interference issue becomes an even greater challenge to contend with in clinical trials, due to the fact that most human or humanized antibodies have long half-lives and can be detected at therapeutically relevant levels in serum for greater than 6 months following discontinuation of therapy.

Some TNF receptor agonist antibodies have been described as 'super agonists', which is a descriptive term indicating that the functional activity of the antibody is independent on FcγR binding. Recent work has provided important insights into the potential mechanisms of action for super agonist antibodies, and suggest they relate to a combination of the specific epitope bound by the antibody, and the binding affinity of the antibody. Vanamee and Faustman proposed a model in which a TNFR agonist antibody binds to epitopes shared by adjacent dimers of a TNFR, thus cross-linking those dimers into a higher-order network in the presence of endogenous TNF ligand (Figure 2) (9). If true, this mechanism is

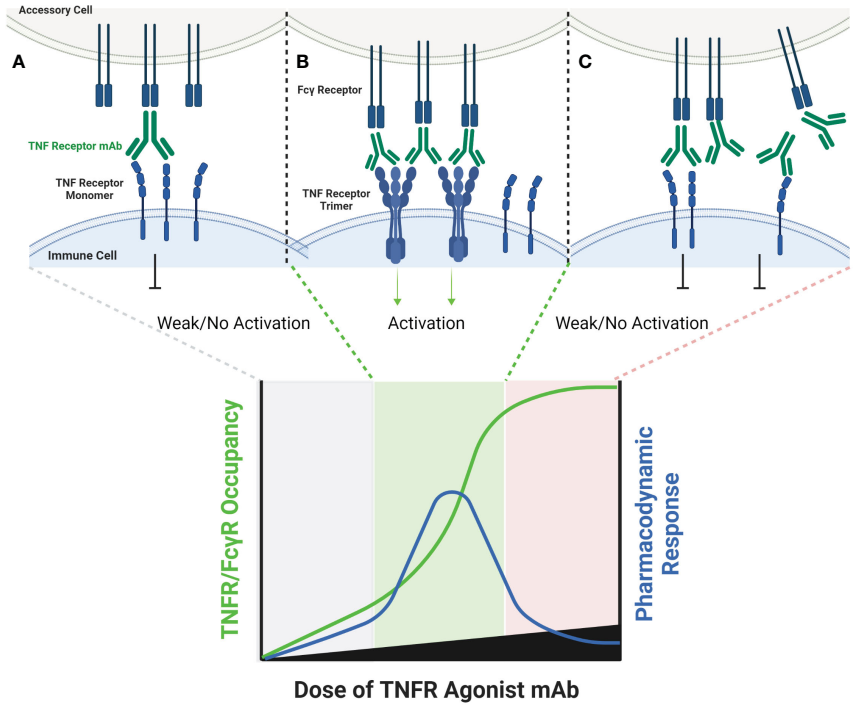

FIGURE 1
Schematic of FcγR Dependent TNFR Agonist mAb Dose Response. TNFR most commonly exist as monomers and dimers in cell membranes, and can be bound by one or both scFv domains of bivalent mAbs **(A)**. TNFR agonist mAbs commonly require FcγR binding for TNFR activation to occur, which is dependent upon the Fc domain of the TNFR agonist mAb binding to FcγR on an 'accessory cell' so that multiple TNFR agonist mAbs can be displayed as an array when binding TNFR **(B)**. An array of multiple TNFR agonist mAbs may include 4, 6, 8, etc. scFv domains in close proximity, capable of approximating the corresponding number of TNFR on a target cell if those TNFR are unoccupied. Administration of saturating concentrations of a TNFR mAb can result in a reduction in the number of free TNFR or FcγR **(C)**. If a TNFR is bound by a TNFR agonist mAb, then that TNFR is not available to bind TNFR agonist mAbs which have been 'arrayed' via FcγR binding, thus reducing activation of TNFR and the corresponding pharmacodynamic response.

also susceptible to a bell-shaped dose response curve, as depicted in Figure 2. A recent study by Yu et al. carefully investigated the relationship between affinity, FcγR binding and receptor off-rate kinetics, and demonstrated that reducing the affinity of TNFR antibody interactions was sufficient to promote increased receptor clustering and agonist function, as had been suggested previously by Ho et al. (18, 52). For both CD40 and 41BB specific antibodies, these authors demonstrated that faster off-rates improved agonist activity if the overall affinity remained approximately within the 1-300 nM range. These findings were dependent upon antibody bivalency, but only partially dependent on FcγR binding. Whether or not FcγR binding is essential is likely influenced by the specific epitope bound, and which CRD domain that epitope resides in (19, 52). Low affinity TNFR agonist mAbs could function in a model according to the one proposed by Vanamee & Faustman, but have also been shown to function in the absence of ligand *in vitro*. In either model, the low-affinity & high off-rate properties likely endowed the candidate antibody with 'toggling' characteristics, wherein receptor occupancy was never fully saturated because the antibodies were constantly associating and dissociating between membrane-proximal TNFR (Figure 3). This mechanism is unlikely to be as susceptible to a prototypical bell-shaped dose response curve in humans, as has been observed with high affinity antibodies.

Aside from the unusual bell-shaped dose response properties of TNFR agonist antibodies in humans, development of many agents has been hampered due to the emergence of dose-dependent toxicities, principally in the form of liver toxicity or cytokine release syndrome - particularly for CD40 and 41BB agonist antibodies. Liver toxicities and/or cytokine release syndrome have been reported from phase 1 clinical trials of selicrelumab, sotigalimab, mitazalimab, ChiLob7/4, and urelumab, which occurred at doses below 0.5 mg/kg, and were partially mitigated by pre-medication with corticosteroids (8, 25, 26, 47, 53–55). Another 41BB agonist antibody, utomilumab, was not found to cause liver enzyme elevations nor cytokine release syndrome, however the highest dose tested was 0.3 mg/kg and no evidence of agonist activity was reported in humans (25). The similarity in the toxicity profile of CD40 and 41BB agonist antibodies raises the question of whether these toxicities are related to CD40 or 41BB activation, a property of the agonist antibody, or a mixture of the two. Knorr et al. demonstrated that liver toxicity for a CD40 agonist antibody correlated with the strength of binding to FcγRIIB (56). Because the 'agonist' activity and anti-tumor activity of the CD40 antibody was also dependent upon FcγRIIB binding, toxicity and efficacy went hand-in-hand. A strategy to circumvent this issue involved direct injection of the antibody into tumors, thus avoiding

adsorption in the liver through first-pass metabolism. The specific cause of toxicity, whether agonism and toxicity go hand-in-hand with one another, whether toxicity results when a threshold of TNF receptor activation is exceeded, or result from the kinetics of receptor activation cannot be determined from these clinical studies, but inferences can be made through comparison to clinical results obtained with non-antibody agonists, as discussed in the next sections.

Clinical data from monovalent multi-specific TNFR agonist therapeutics

An alternative approach to relying upon FcγR to crosslink TNFR antibody domains is to pair a combination of TNFR specific antibody domain with a tumor antigen or immune checkpoint specific antibody binding domain, thereby creating a bispecific antibody (bsAb). Examples of antibodies and antibody domain containing molecules with this structure for which clinical data are available include GEN1042 (CD40x41BB bsAb), GEN1046 (PDL1x41BB), FS222 (PDL1x41BB bsAb), PRS-343 (HER2x41BB Ab/Anticalin fusion), NM21-1480 (PDL1x41BBxHAS trispecific Ab) and MP0317 (FAPxCD40xHSA trispecific Ab). This class of agents tends to lack FcγR binding, since the underlying mechanism is proposed to rely upon antigen-specific clustering of the TNFR binding domain from multiple individual bsAbs in close proximity to one another (Figure 4).

In general, this class of agents lacks clinical evidence of cytokine release syndrome, and liver enzyme elevations are mild and sporadic in comparison to clinical data for bivalent antibodies directed to the same TNFR. This observation strengthens the hypothesis that the CRS and liver tox observed with TNFR agonist bivalent antibodies is largely due to FcγR driven mechanisms. GEN1042 and GEN1046 were tested across a wide dose range (0.1-400 mg and 25-1200 mg, respectively), and PK/PD models predicted a bell-shaped dose response curve, as is expected for these agents (57). Despite not requiring FcγR binding, the monovalent TNFR targeting arms of these agents still require secondary clustering via the non-TNFR targeted arm of the agent. Thus, dose levels that lead to >50% TNFR occupancy are expected to have reduced pharmacodynamic activity than those that target approximately 50% receptor occupancy, and the dose-finding complications of this mechanism discussed above are applicable. To date, limited safety, pharmacodynamic, pharmacokinetic, and clinical outcome data has been shared from clinical trials testing FS222, PRS-343, NM21-1480, and MP0317 (58–60). Each is expected to show a similar bell-shaped dose response to that of GEN1042 and GEN1046, however additional clinical data is needed

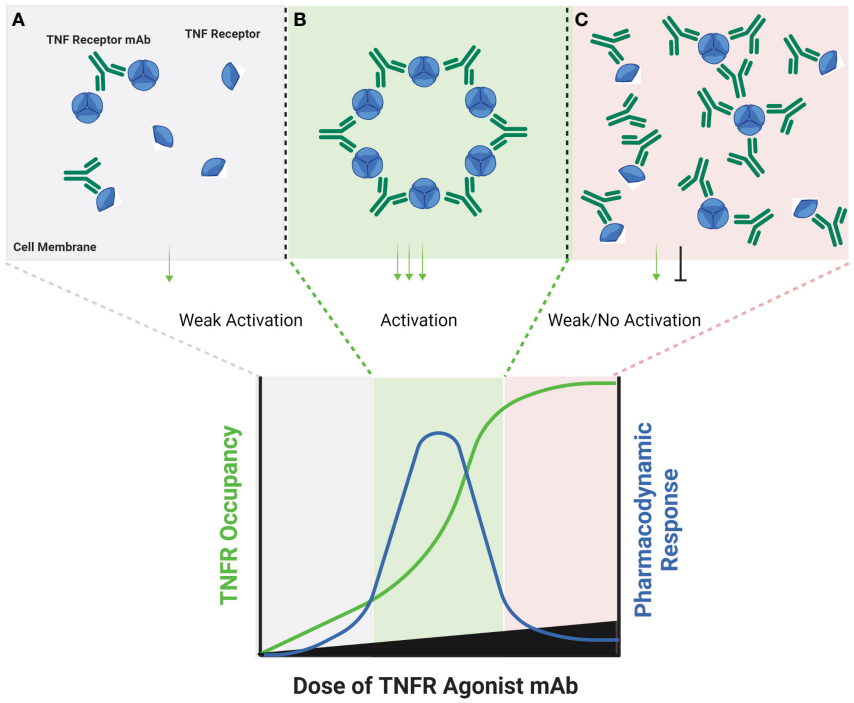

FIGURE 2
Schematic of FcγR Independent Clustering of TNFR Networks in Cell Membranes. Certain TNFR exist in cell membranes as inactive dimers, which can then assemble into trimers upon interaction with a corresponding TNF ligand. Certain TNFR agonist mAbs, may be capable of stimulating a similar response via binding particular epitopes on adjacent TNFR in cell membranes. At low antibody concentrations, TNFR agonist mAbs may bind epitopes on adjacent TNFR, and sometimes cause activation by approximating TNFR dimers or trimers into higher-order networks **(A)**. A hypothetical maximum response is predicted to occur in this model when the molar ratio or TNFR agonist mAb is equal to the number of available binding sites on each trimer of a target TNFR **(B)**. When this ratio is reached, every TNFR trimer is theoretically cross-linked to another TNFR trimer by the TNFR agonist mAb, thus creating a high-order network of TNFR. When the concentration of TNFR agonist mAb exceeds the number of available epitopes on TNFR dimers and trimers, then a TNFR bound by one arm of an antibody may not lead to cross-linking with a nearby TNFR, if that nearby TNFR is also bound by one or both arms of another TNFR agonist mAb **(C)**, thus reducing receptor activation and the corresponding pharmacodynamic responses.

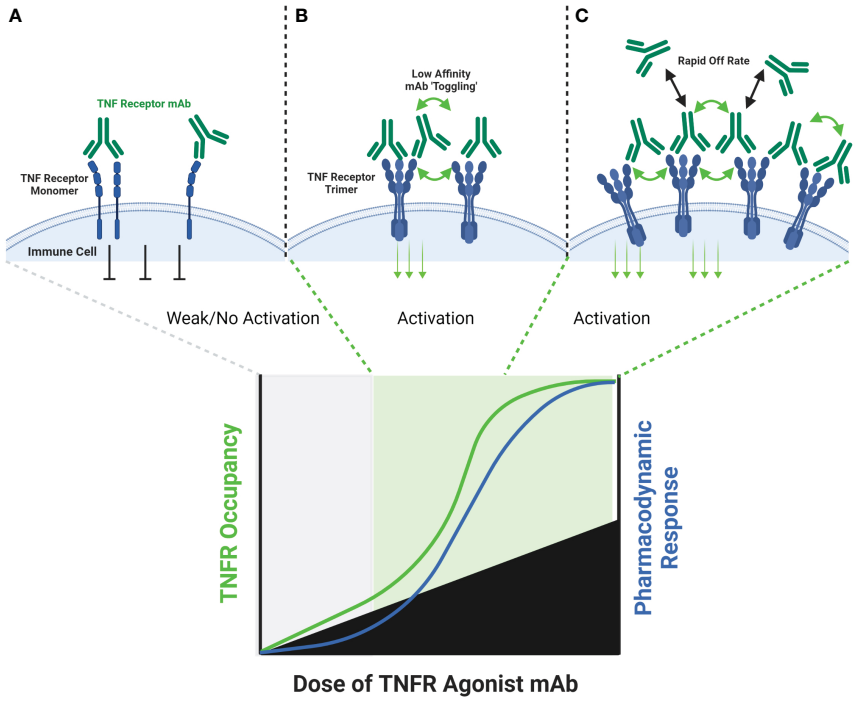

FIGURE 3
Schematic of FcγR Independent Clustering of TNFR by Low-Affinity TNFR Agonist mAbs. Both high affinity and low affinity TNFR agonist mAbs are capable of binding a TNFR monomer or dimer in a similar manner, however the low affinity antibody will have a faster 'off-rate' than the high affinity antibody **(A)**. The faster off-rate of low affinity antibodies leads to an equilibrium where antibodies are rapidly binding and releasing a TNFR target in a cell membrane. In some cases, one arm of the antibody could remain bound to a TNFR while the other arm releases and then re-binds another adjacent TNFR, leading to clustering and TNFR activation **(B)**. If the off-rate of a low affinity antibody is fast enough, then a persistent state of 'receptor occupancy' may not occur. This property could theoretically enable low-affinity antibodies to toggle on-and-off a TNFR target fast enough to cause cross-linking and activation even when the molar ratio of the TNFR agonist mAb is in excess to the number of TNFR binding sites **(C)**.

to confirm this hypothesis. A longer list of agents, including YH32367 (anti-HER2/41BB), HLX35 (anti-EGFR/41BB), CB307 (anti-PSMA/41BB), RO7300490 (anti-FAP/41BB) and FS120 (anti-OX40/41BB) have been in phase 1 clinical trials for several years, however no clinical data has been publicly shared to date.

Clinical data from trivalent, tetravalent, hexavalent and decavalent mAb-derived TNFR agonist therapeutics

Development of agonist agents which contain three or more TNFR binding domains has progressed more recently, in part due to the clinical experience obtained with the mono- and bi- valent agents described above. For category 1 TNFR, including BaffR, DR3, GITR, LTβR and TNFR1, a hexavalent agonist is likely the minimal valency to exert potent agonism (36, 61). For category 2 TNFR (41BB, BCMA, CD27, CD40, CD95, EDAR, Fn14, OX40, TACI, TNFR2, DR4 and DR5), a trimer is expected to cause signaling, however the quantum of signaling is expected to increase following assembly of hexameric or higher-order complexes (9, 32, 33, 62).

Theoretically, agonists that minimally contain a trimeric TNFR binding domain should lead to receptor activation in a soluble phase, without a requirement for cross-linking. It is tempting to speculate that these agents may not exhibit the bell-shaped dose response curves observed with monovalent and bivalent mAb formats, however emerging clinical data are suggestive of greater nuance. Of the multivalent agonists included in this discussion, some contain pre-formed ligand trimers (RO7227166 & RO7122290), some contain pre-formed ligand hexamers (SL-279252, SL-172154 & MEDI6383), and some contain a tetravalent, hexavalent or decavalent array of antibody-based TNFR binding domains. Because the pharmacodynamic activity is likely distinct between the antibody based multivalent agents, and those that contain one or more pre-formed TNF ligand trimers, the discussion between both is divided in the following sections.

A common characteristic to agents that utilize antibody derived binding domains is that those domains are capable of binding to a TNFR regardless of whether it has pre-assembled into a trimer or hexamer in a cell membrane (Figure 5). The probability that a tetravalent, hexavalent or decavalent antibody leads to cross-linking of multiple TNFRs that are nearby one another in a cell membrane is undoubtedly more probable than that with a monovalent or bivalent antibody. However, if the kinetics of binding of individual antibody domains to a target are faster than the kinetics of saturation of all binding sites within individual tetravalent or hexavalent antibodies, then bell-shaped dose response curves could still be observed (Figure 5). The agents in clinical development that could inform on this question include; eftozanermin (ABBV-621, TRAIL-R agonist), IGM-8444 (anti-DR5), GEN1053 (anti-CD27) and INBRX-106 (anti-OX40).

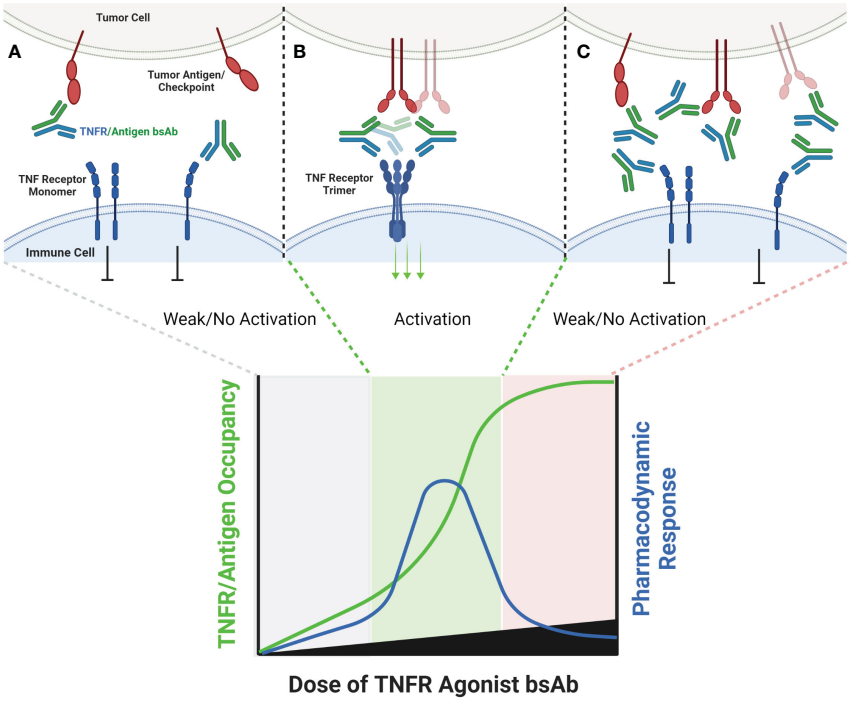

FIGURE 4
Schematic of Bispecific Antibody Mediated Clustering of TNFR. Bispecific antibodies often target an antigen expressed by a tumor cell (commonly an immune checkpoint such as PD-L1, or a tumor specific antigen such as FAP), and contain a second arm which binds a TNFR **(A)**. The TNFR binding arm of these antibodies is therefore monovalent. As the dose of the bispecific antibody is increased, the probability that multiple antibodies will cluster on the surface of an antigen positive tumor cell increases, and the probability that the monovalent TNFR binding arms from multiple antibodies will bind and approximate multiple nearby TNFR also increases **(B)**. Similar to FcγR specific mAbs, when the dose of the bispecific antibody begins to exceed approximately 50% receptor occupancy on either the tumor antigen or TNFR target, the probability that any individual antibody encounters both a free tumor antigen and TNFR target decreases, leading to a decline in TNFR clustering **(C)**.

ABBV-621 and IGM-8444 are hexa- and decavalent DR5 agonists, respectively, mechanistically designed to trigger the death domain in DR5 to cause apoptosis in target cells (27, 63). Because the goal of therapy is to kill DR5 expressing cells, including FcγR effector function is an intended attribute of the compound. Although the pharmacodynamic data are sparse, two peripheral biomarkers of apoptosis (M30 and M65) trended lower in some of the high dose groups (≥7.5 mg/kg) relative to the lower dose groups (≤2.5 mg/kg) in a phase 1 clinical trial (63). Clinical data for IGM-8444 and GEN1053 have not yet been shared, and only qualitative comments have been made regarding the performance of INBRX-106 in a phase 1 clinical trial. INBRX-106 has an IgG1 Fc domain, and thus is capable of binding to FcγR. In a phase 1 clinical trial, toxicities were observed at a relatively low dose of 0.3 mg/kg, which led to selection of the 0.1 mg/kg dose level for further study. No pharmacokinetic, pharmacodynamic or receptor occupancy data have been shared to date.

Clinical data from agents comprising one or more trimerized TNF ligand domains

Unlike multivalent TNFR agonists derived from a series of antibody-derived binding domains, agonists which contain pre-formed TNF ligand trimers are predicted to interact with TNF receptors in a unique manner, which potentially better reflects the native physiology of TNF ligand and receptor interactions. As described above, each individual TNFR binding domain in a multivalent TNFR targeted antibody can interact independently with a TNFR target, regardless of whether it is pre-assembled into a trimer or not. As such, there is no guarantee that each TNFR binding domain within an individual antibody molecule will become saturated before the TNFR itself becomes saturated, because there will be competition for free TNFR both between and within individual multivalent antibodies. Agents which contain pre-formed TNF ligand trimers, on the other hand, are expected to stimulate ligand-induced TNFR trimerization, and the stoichiometry of interaction is more likely to be 1:1 between individual ligand and receptor trimers. Emerging pharmacodynamic data from clinical trials supports this assertion, and is described below.

Englumafusp alfa (RO7227166) is a fusion construct comprised of a CD19-specific antibody domain fused to a trimerized extracellular domains of human 41BBL. This agent is being developed in conjunction with a CD3xCD20 T cell engager, for patients with relapsed or refractory B cell non-Hodgkin lymphoma. In a phase 1 study, RO7227166 had an acceptable safety profile across a dose range of 0.36 to 33 mg, without reaching a maximum tolerated dose (MTD). CRS was attributed to RO7227166 in just 4.8% of patients, and all of those events were Grade 1 in severity.

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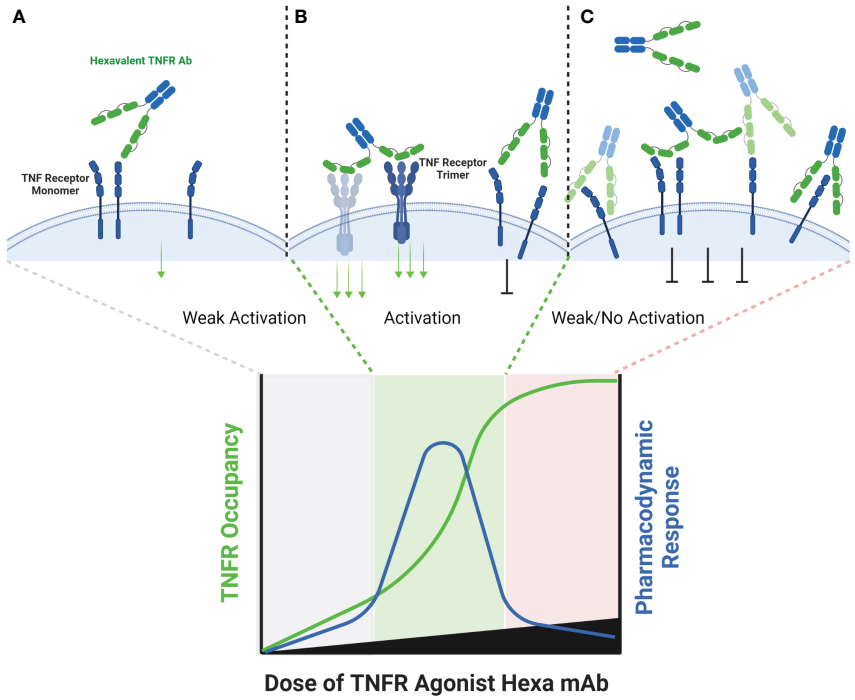

FIGURE 5
Schematic of Hexavalent mAb Binding to TNFR. Tetravalent, hexavalent and decavalent antibodies each contain sufficient TNFR binding domains to facilitate TNFR clustering in the absence of cross-linking by FcγR or a tumor antigen. Each of the binding domains of these multivalent antibodies are capable of binding to a TNFR target independently from one another, and it is possible that certain domains are more 'exposed' to find antigen than others, as illustrated for the distal domains **(A)**. At sub-saturating dose levels, each of the TNFR binding domains may occupy a TNFR target, and in the process cluster multiple TNFR targets into close proximity to one another, enabling activation **(B)**. Because each binding domain on an individual antibody can interact independently with a TNFR target, the theoretical maximum pharmacodynamic effect is most likely when the number of antibodies are at a 1:4, 1:6 or 1:10 ratio to the number of TNFR targets (for tetravalent, hexavalent or decavalent antibodies, respectively). When these ratios begin to be exceeded, then independent TNFR binding domains from separate antibodies are expected to compete with one another, thus reducing the probability that an individual antibody is capable of clustering the TNFR target **(C)**.

Importantly, expansion of primed and activated T cell subsets occurred in a dose-dependent manner, without strong evidence of a bell-shaped dose response (64).

RG7827 (RO7122290) has a similar structure to RO7227166, but anchors trimerized 41BBL to FAP instead of CD19 and is being developed for patients with advanced solid tumors in combination with atezolizumab (PD-L1 mAb). A phase 1 dose escalation study evaluated RO7227166 across a dose range of 5-2000 mg, without reaching an MTD. A single case of CRS was encountered as a dose limiting toxicity, and no evidence of liver enzyme elevations were observed in the RO7122290 monotherapy arm. The pharmacodynamic changes observed in humans for RO7227166 are substantially more pronounced than those reported for urelumab or utomilumab, and include not only increases in the proportion of T cells that express Ki67 (a marker of proliferation), but also increases in the absolute numbers of T cells following treatment in a dose-dependent manner (29, 31). Further, up to ~100-fold changes were observed in the serum concentration of IFNγ post treatment, along with multi-fold increases in IL-6 and TNFα. While there were increases in each of these pharmacodynamic markers across the entire dose range in some patients, there was a trend toward higher fold-changes in the 45-260 mg group as compared to the 500-2000 mg group. In contrast to human data with the antibody agonists, pharmacodynamic responses did not return to baseline in the higher dose groups, but instead showed more variability in the magnitude of induction which came shy of the peak elevations observed in the lower dose groups. It is possible that this phenomenon is related to primarily 'trimeric' signaling in the high dose groups, as a result of doses high enough to independently saturate FAP and 41BBL. In the lower dose groups, the increased pharmacodynamic activity could be the result of a higher proportion of molecules of RO7227166 encountering free 41BB after binding to FAP, resulting in an 'array' of 41BBL trimers and a higher probability of increased signaling due to hexamer or higher-order oligomer formation (Figure 6).

Two other classes of agonist therapeutics that have been tested in humans containing pre-formed TNF ligand trimers are single- and dual-side Fc fusion proteins. TNF ligands are type II membrane proteins, and fusion of the extracellular domain of a TNF ligand to an Fc domain requires a hinge-CH2-CH3-TNF ligand configuration to ensure unhindered folding and activity of the TNF ligand domain.

When expressed, the quaternary structure of an Fc-TNF ligand fusion protein is influenced both by the interchain disulfide bonds in the Fc region leading to covalent dimer formation, and by non-covalent interactions in the TNF ligand domains facilitating trimer formation. The resulting structure is a hexamer consisting of a 'dimer of trimers' as illustrated in Figure 7 for a dual-sided Fc fusion protein (32, 33, 36).

At least two OX40L-containing Fc fusion proteins entered clinical trials, including a single-sided Fc-OX40L (MEDI-6383) fusion and a PD1-Fc-OX40L (SL-279252) dual-sided fusion protein. Unfortunately, clinical data from a phase 1 clinical trial with MEDI-6383 has not been published. A phase 1 clinical trial testing SL-279252 in patients with a mixture of advanced solid tumors, primarily PD-1 resistant, was completed in 2023. This study examined SL-279252 across a wide dose range of 0.001 through 24 mg/kg, and was well tolerated without any treatment related grade 3 or higher adverse events and no MTD was reached. The primary pharmacodynamic finding was immediate post-dose reductions in the number of peripheral blood CD4+OX40+ T cells following each infusion, which was dose-dependent and believed to be due to migration of OX40+ T cells from the blood into tissues after activation (49). This finding was distinct from the pharmacodynamic findings in humans with OX40 agonist mAbs, where sporadic increases in the proliferation marker Ki67 were sometimes reported in subsets of CD4+ or CD8+ T cells, were not accompanied by reported changes in the actual numbers of those subsets of T cells, and generally did not provide evidence of the agonist mechanism that was predicted by pre-clinical studies (44, 45, 65, 66).

SL-172154 is a dual-sided Fc fusion protein adjoining the extracellular domains of human SIRPα and human CD40L via a mutated IgG4-derived Fc domain lacking FcγR binding. A phase 1 monotherapy dose-escalation trial was completed in patients with platinum resistant ovarian cancer, and tested SL-172154 across a dose range of 0.1 to 10 mg/kg. In contrast to prior CD40 agonist mAbs, SL-172154 had an acceptable safety profile across the dose range, with a single incidence of grade 3 LFT elevation at the 10 mg/kg dose level, and no MTD was reached. Dose-dependent infusion related reactions were common, primarily grade 1/2, but were not consistent with typical cytokine release syndrome and no elevations in IL-6 and TNFα were observed. CD40 receptor occupancy was approximately 60-80% at the 0.1 mg/kg starting dose, and full receptor occupancy and saturation was observed by the 3 mg/kg dose. The agonist activity of SL-172154 was evident post infusion with near immediate migration of CD40+ B cells and monocytes from the peripheral blood into tissues. This pharmacodynamic effect was concurrent with rapid release of cytokines and chemokines into the serum, including: IL-12, CXCL10, CCL2, CCL3, CCL4, CCL22, IL-8, IL-10 and others (30). These pharmacodynamic observations translated between species and were consistently observed in previous mouse and non-human primate studies (30, 32). This translatability has often been lacking for TNFR agonist antibodies, and in contrast to prior CD40 agonists, there was no evidence of a bell-shaped dose response for any of the pharmacodynamic findings with SL-172154. In addition, the translation of these peripheral blood findings to the tumor microenvironment was noted via a shift in myeloid cell polarization from an M2-dominated to an M1-dominated phenotype. The potency of the pharmacodynamic effects for SL-172154 exceed those reported for any prior CD40 agonist agent, and may reflect the benefit of agents containing hexamerized ligands, which potentiate ligand-induced trimerization and network formation of target TNFR (Figure 7).

Conclusions and future directions

Over the past thirty years, a tremendous level of effort, investment, innovation and hope has supported the testing of many types of TNF receptor agonists in human clinical trials. Unfortunately, the resulting clinical data did not closely resemble the biology of the TNF receptor agonism predicted by pre-clinical studies, which prompted the question of whether the failure in translation was more likely a result of the intrinsic biology of TNF receptors, or of the therapeutics used to target those receptors in patients.

Some generalizations on the necessity for TNFRs to trimerize in order to signal were made throughout this review. As with most rules, these generalizations are acknowledged to have limits and special cases where they may not apply. As an example, NGF is a TNF receptor which can be activated by neurotrophin ligands, which are dimeric. In addition, the models proposed in the figures to summarize clinical data from various TNF receptor agonist agents assume that the distribution of a particular agonist agent to its potential binding partners *in vivo* are balanced. For example, Figure 1 assumes that the TNFR agonist mAb occupies the TNFR target and Fcγ receptor targets in a roughly proportional manner. Whether or not this happens *in vivo* is influenced by multiple factors, including the binding affinity and abundance of each target. There are not any publicly available clinical data which demonstrate the relative receptor occupancy kinetics of TNFR agonist antibodies in this manner, so the models should be interpreted in a qualitative manner with these assumptions in mind.

A review of the clinical data across different TNF receptor agonist modalities reveals common themes that should be considered in advancing future agents to the clinic. These themes include the following:

1) High-affinity TNF receptor agonist antibodies which bind to Fcγ receptors have a higher likelihood of causing toxicities including cytokine release syndrome and/or liver enzyme elevation than bispecific antibodies which lack Fcγ receptor binding function.
2) Both bivalent antibodies and bispecific antibodies show bell-shaped dose response curves in humans, which likely limits the agonist potential of the modality, and creates risk that a 'recommended phase 2 dose' may not cause reproducible

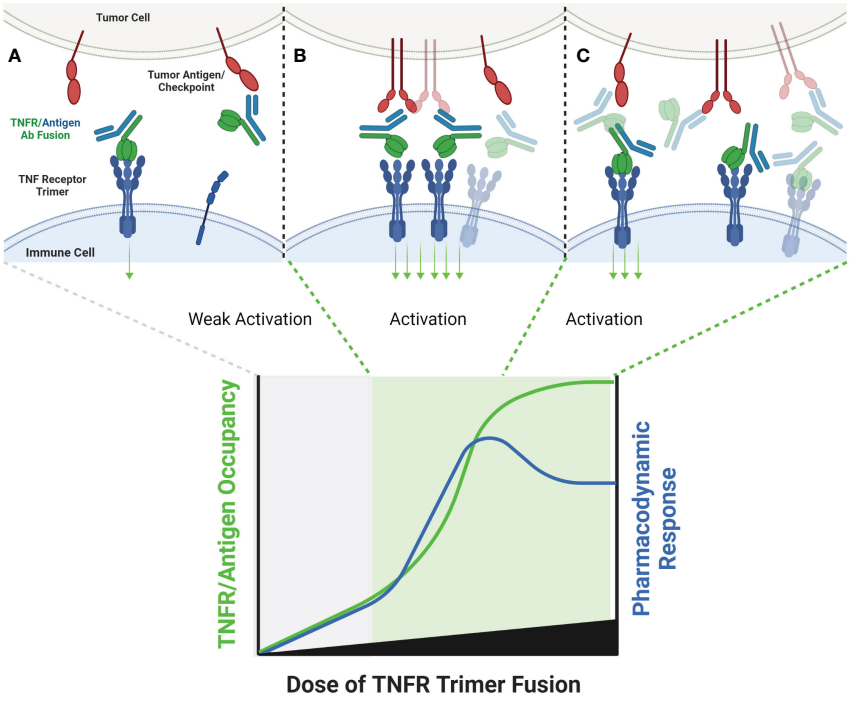

FIGURE 6
Schematic of TNF Ligand Trimer-Containing Antibody Binding to TNFR. Bispecific antibodies wherein one arm of the antibody has been replaced with a trimerized set of TNF ligand extracellular domains is capable of stimulating ligand-induced TNFR trimerization in the absence of other cross-linking or antibody clustering mechanisms, which is expected to stimulate TNFR activation even at low doses of antibody, because TNFR activation is not conditional upon clustering by the tumor antigen binding arm **(A)**. As the dose level of the antibody increases, the probability that multiple antibodies will be clustered in close proximity to one another, thus clustering multiple trimeric TNF ligand domains also increases **(B)**. If the quantum of TNFR activation is increased when the trimeric TNF ligand domains are clustered, via organizing TNFR in a higher-order network, a 'peak' pharmacodynamic effect may be observed at sub-saturating concentrations of antibody **(B)**. When the concentration of antibody is high enough to saturate both tumor antigen and TNFR target, the probability of TNFR network formation may decrease, leading to a tailing of the pharmacodyamic response curve, but only to a level which reflects the activity of a primarily TNFR trimer pharmacodynamic response **(C)**.

agonist effects due to variable starting frequencies of the TNF receptor expressing cells between patients, and to dynamic expression of the TNF receptor target within individual patients over time.

3) Antibody therapeutics containing three or more domains each capable of binding a TNF receptor target have a higher probability of agonist activity than mono- or bivalent antibody therapeutics, and do not require FcγR binding for function.

4) Antibody therapeutics containing three or more domains each capable of binding a TNF receptor target may still encounter bell-shaped dose response curves, similar to bispecific and bivalent antibodies, because TNF receptor saturation can occur in the absence of saturating the cross-linking potential of each antibody.

5) Agents containing pre-formed TNF ligand trimers demonstrate more potent evidence of agonist activity than antibody derived agents, potentially because those agents can facilitate ligand-dependent trimerization of TNF receptors.

6) Agents which contain multiple TNF ligand trimers demonstrate more sustained dose-dependent pharmacodynamic effects than those which require clustering by another mechanism to facilitate hexameric or higher-order TNF receptor network formation.

The consequences of the themes above in terms of safety and efficacy likely vary based on the specific TNF receptor being targeted. For 41BB and CD40 directed agents, cytokine release syndrome may prove to be more problematic than it ever will be for OX40 or GITR directed agents. Regardless, development of agents which require cross-linking for activity (either by FcγR or a target antigen) and thus exhibit bell-shaped dose response curves will be impractical because of the variable and dynamic nature of expression of TNF receptors between and within patients. Whether pre-clinical studies suggesting that this issue can be overcome by reducing the affinity of a TNF receptor agonist antibody will translate to clinical trials is unclear. The observation that tetravalent and hexavalent antibody agonists also exhibit bell-shaped dose response kinetics in humans should raise similar concerns about the ultimate agonist potential of these agents.

The pharmacodynamic effects of agents which contain at least one TNF ligand trimer demonstrate improved translation of pre-clinical to clinical findings in comparison to any of the antibody-based agonists. The ability of TNF ligand containing agonists to trigger ligand-induced trimerization of the target TNF receptor is a likely reason for this observation. The observed advantages of ligand-

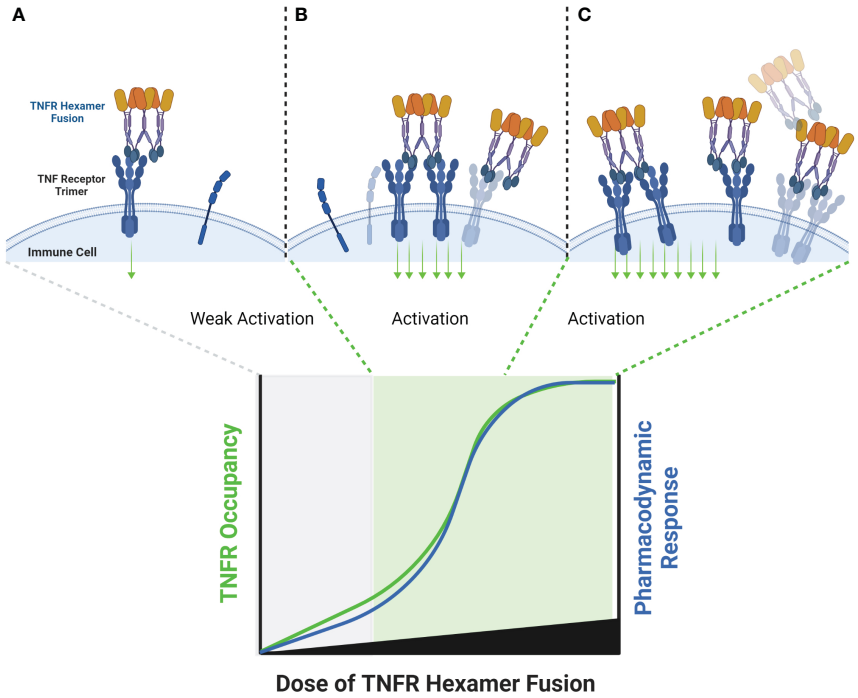

FIGURE 7
Schematic of a TNF Ligand Hexamer-Containing Fusion Protein Binding to TNFR. Fusion proteins containing two trimerized TNF ligand domains are expected to stimulate ligand-induced trimerization and TNFR hexamer network formation even at low doses of the fusion protein **(A)**. As the dose of the fusion protein increases, the probability of TNFR trimer and hexamer activation is expected to increase in proportion to the dose of the fusion protein, because the TNF ligand domains are not expected to bind TNFR monomers efficiently due to the lower avidity characteristics of the interaction **(B)**. An increasing pharmacodynamic effect is expected until the molar ratio of TNF ligand domains to trimeric TNFR is 1:1, however there is some possibility of tailing in the pharmacodynamic response if a significant proportion of the fusion proteins bind as trimers rather than hexamers, similar to the effect described in Figure 6 **(C)**.

containing agonists are balanced by the potential drawbacks of a larger molecular format, including manufacturing efficiency, stability *in vivo*, risk of immunogenicity, and potential for decreased tissue penetration. Thus far, these issues have not limited the development of englumafusp alpha, RG7827, SL-279252 or SL-172154, however more data are needed from these and other agents to gain further confidence.

Drug developers should carefully review the structural lessons that are now available after over thirty years of clinical experimentation with different TNF receptor agonist agents. While pre-clinical studies may be very important for selecting which TNF receptor to target, the structure of the TNF receptor agonist advanced into the clinic should be made on the basis of clinical data gathered across the class of TNF receptors, rather than on the activity of a particular agonist agent in pre-clinical models.

Author contributions

TS, GF and SD wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

All authors are shareholders and employed by the company Shattuck Labs, Inc.

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Fcγ receptor binding is required for maximal immunostimulation by CD70-Fc

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Introduction: T cell expressed CD27 provides costimulation upon binding to inducible membrane expressed trimeric CD70 and is required for protective CD8 T cell responses. CD27 agonists could therefore be used to bolster cellular vaccines and anti-tumour immune responses. To date, clinical development of CD27 agonists has focussed on anti-CD27 antibodies with little attention given to alternative approaches.

Methods: Here, we describe the generation and activity of soluble variants of CD70 that form either trimeric (t) or dimer-of-trimer proteins and conduct side-by-side comparisons with an agonist anti-CD27 antibody. To generate a dimer-of-trimer protein (dt), we fused three extracellular domains of CD70 to the Fc domain of mouse IgG1 in a 'string of beads' configuration (dtCD70-Fc).

Results: Whereas tCD70 failed to costimulate CD8 T cells, both dtCD70-Fc and an agonist anti-CD27 antibody were capable of enhancing T cell proliferation *in vitro*. Initial studies demonstrated that dtCD70-Fc was less efficacious than anti-CD27 in boosting a CD8 T cell vaccine response *in vivo*, concomitant with rapid clearance of dtCD70-Fc from the circulation. The accelerated plasma clearance of dtCD70-Fc was not due to the lack of neonatal Fc receptor binding but was dependent on the large population of oligomannose type glycosylation. Enzymatic treatment to reduce the oligomannose-type glycans in dtCD70-Fc improved its half-life and significantly enhanced its T cell stimulatory activity *in vivo* surpassing that of anti-CD27 antibody. We also show that whereas the ability of the anti-CD27 to boost a vaccine response was abolished in Fc gamma receptor (FcγR)-deficient mice, dtCD70-Fc remained active. By comparing the activity of dtCD70-Fc with a variant (dtCD70-Fc(D265A)) that lacks binding to FcγRs, we unexpectedly found that FcγR binding to dtCD70-Fc was required for maximal boosting of a CD8 T cell response *in vivo*. Interestingly, both dtCD70-Fc and dtCD70-Fc(D265A) were effective in prolonging the survival of mice harbouring BCL1 B cell lymphoma, demonstrating that a substantial part of the stimulatory activity of dtCD70-Fc in this setting is retained in the absence of FcγR interaction.

Discussion: These data reveal that TNFRSF ligands can be generated with a tunable activity profile and suggest that this class of immune agonists could have broad applications in immunotherapy.

KEYWORDS

CD27, TNFRSF, costimulation, T cells, cancer, vaccine, immunotherapy

Introduction

Activation of conventional T cells requires T cell receptor (TCR) recognition of peptides bound to MHC molecules as well as signals delivered by costimulatory receptors interacting with their cognate ligands on antigen presenting cells. Costimulatory molecules enhance and sustain the magnitude of the signalling pathways downstream of the TCR/CD3 complex by recruitment of adaptor proteins and kinases. Ultimately, the combined signals emanating from the TCR/CD3 and costimulatory receptors lead to quantitative and qualitative changes that culminate in increased T cell proliferation, survival, metabolic fitness, and differentiation into effector cells (1, 2).

Costimulatory receptors primarily belong to either the immunoglobulin superfamily or the TNF receptor superfamily (TNFRSF), with both types of receptors contributing to the regulation of T cell immunity in a cell and infection specific manner (2). CD27 is a member of the TNFRSF expressed on almost all T cells, germinal centre and memory B cells, as well as a subset of NK cells (3). Earlier studies established an important role for CD27 in augmenting T cell responses in both humans and mice. Costimulation via CD27 was shown to play a complementary role to CD28 during the primary and secondary activation of CD8 T cells (4–7). In addition, CD27 costimulation was found to promote the development of CD4 Th1 T cells (8) and subsequently shown to exert suppressive effects on the function of Th17 cells (9). CD70, the CD27 ligand, is a homotrimeric type II transmembrane protein transiently upregulated on activated dendritic cells, B cells and T cells in response to CD40, Toll-like receptor or antigen receptor stimulation (6, 10–12).

The importance of the CD27-CD70 axis in human immunity was established with the discovery that individuals with inherited deficiency of either CD27 or CD70 have impaired CD8 T cell responses to Epstein-Barr virus (EBV) resulting in EBV-driven lymphoproliferation, hypogammaglobulinemia and lymphoma development (13–16). The non-redundant role of the CD27-CD70 axis in providing protection against EBV driven B cell malignancy suggests that enforced CD27 costimulation could potentially restore defective CD8 T cell-mediated immune surveillance of B cell tumours. To date, both agonist anti-CD27 antibodies and soluble forms of CD70 have been used to investigate the effect of enforced CD27 stimulation on T cell responses and anti-tumour activity (5, 17–21). However, there is no consensus on which of these agents represent the therapeutic of choice for delivering optimal CD27 costimulation. A wide range of agonistic activity has been reported for different anti-CD27 mAbs (21, 22). Furthermore, the activity of anti-CD27 mAbs may depend on antibody isotype which affects binding to the inhibitory and activatory Fcγ receptors (FcγRs) (18, 21). In contrast to anti-CD27 antibodies, soluble CD70 potentially offers an approach to deliver agonism without the need for FcγR mediated crosslinking. However, as soluble trimeric CD70 lacks biological activity, different approaches have been proposed to generate bioactive forms that form higher order oligomeric structures. One approach involved the attachment of the extracellular domain of CD70 to the C-terminus of human IgG1 Fc (5, 23). An alternative design to generate a more uniform hexameric protein comprising two adjacent trimeric CD70 proteins involved the attachment of three CD70 extracellular (ECD) domain fragments in a single chain format to the N-terminus of the Fc domain (20). Although these Fc fusion proteins were demonstrated to be functional, it was unclear if FcγR mediated hyper-crosslinking could further potentiate their stimulatory effects and therefore necessary for optimal activity. A soluble CD70 protein with a predictable activity profile could overcome the limitations of agonist anti-CD27 mAbs, but to date direct comparisons of the activity of soluble CD70 and anti-CD27 mAbs have not been reported.

Here we evaluate the *in vitro* and *in vivo* biological activity of soluble CD70 fusion proteins comparing them to agonist anti-CD27 mAb and identify key features that are required for optimal activity. Our data highlight the potential of CD70-based therapeutics as an alternative to agonist anti-CD27 mAbs.

Materials and methods

Generation of soluble CD70 fusion proteins and recombinant anti-CD27 antibody

Soluble trimeric CD70 (tCD70) was produced by fusing domains 3 and 4 of mouse CD4 to the CD4 to the ECD (S41-P195) of murine CD70. Briefly a DNA construct encoding a leader peptide (MEWSWVFLFFLSVTTGVHSEVQAHS), domains 3 and 4 of mouse CD4, a short linker (G3S) and the ECD of mouse CD70 was ordered commercially and supplied in the pcDNA3.1 expression plasmid. tCD70 was produced by transient transfection of 293F cells and purified from spent tissue culture supernatant by anti-CD4 affinity

column chromatography 7 days after transfection (24). Soluble single-chain trimeric CD70 (sctCD70) was produced by fusing domains 3 and 4 of mouse CD4 via a G3S linker to three CD70 ECD (S41-P195) fragments separated by flexible linkers $(G3S)_3$. The DNA construct was ordered commercially and supplied in pcDNA3.1. We also generated a dimer of trimer CD70-Fc fusion protein (dtCD70-Fc) by assembling three fragments encoding the ECD of mouse CD70 (S41-P195) separated by $(G3S)_3$ linkers followed by the hinge and CH_2/CH_3 domains of mouse IgG1. The DNA fragment was excised from pcDNA3.1 with HindIII and EcoRI and subcloned into the expression vector pEE14 (Lonza), which was then transfected into suspension adapted Chinese hamster ovary cells (CHO-K1S) to generate stable lines. CHO-K1S cells expressing dtCD70-Fc were grown in a shaking incubator at 37°C and 8% CO_2 in FortiCHO medium (Thermo Fisher) supplemented with methionine sulfoxamine, hypoxanthine and thymidine. The dtCD70-Fc protein was purified from 2-4 week spent tissue culture media by protein A column chromatography followed by preparative size exclusion chromatography (Superdex 200 26/950).

To produce anti-CD27 mouse IgG1, total RNA was extracted from the anti-CD27 hybridoma AT124-1 (17) and converted into cDNA using the SuperScript™ IV First-Strand Synthesis System (Thermo Fisher). Anti-mouse CD27 V_H and V_L sequences were amplified by PCR using degenerate 5' primers and constant region specific 3' primers. After verification by DNA sequencing, the V_H and V_L encoding DNA fragments were cloned in frame with the constant mouse heavy (IgG1) and light (kappa) chains, respectively, in pEE6.4 (Lonza). To generate stable CHO-K1S cell lines, the heavy and light chain expression cassettes in pEE6.4 were subcloned into a single expression plasmid (pEE12.4; Lonza) which was then transfected into CHO-K1S cells using GenePorter (Thermo Fisher).

Affinity measurements by surface plasmon resonance

A Biacore T200 instrument and HBS-EP+ running buffer was used throughout (GE healthcare). Anti-human IgG was first attached to the CM5 chip by amine coupling following the manufacturer protocol (GE healthcare). Recombinant mouse CD27-human Fc (R&D systems) was then captured for 1 min at a flow rate of 10 µl/min. The flow rate was then increased to 30 µl/min before injection of serially diluted CD70 fusion proteins. The chip was regenerated with injection of $MgCl_2$ (3 M) for 1 min at flow rate 20 µl/min. The k_a and k_d were determined using the Biacore Bioevaluation software and the K_D values were calculated as k_a/k_d.

To examine the binding of dtCD70-Fc and anti-CD27 mAb to FcRn, ~2000 response units of dtCD70-Fc or anti-CD27 were immobilized onto a CM5 chip via amine coupling. Serially diluted recombinant mouse FcRn (R&D systems) was injected for 3 min at a flow rate of 30 µl/min in HBS-EP+ buffer adjusted to pH 6. The chip was regenerated with injection of HBS-EP+ (pH 7.4). The K_D values were calculated using steady-state binding levels at different concentrations of FcRn.

Glycosylation analysis

Glycoproteins (50 µg) were subjected to proteolytic digestion with trypsin. Before digestion, samples were denatured, reduced and alkylated by incubation for 1 h at room temperature (RT) in a 50 mM Tris/HCl, pH 8.0 buffer containing 6 M urea and 5 mM dithiothreitol, followed by addition of 20 mM iodacetamide for a further 1 hr at RT in the dark, and then additional dithiothreitol (20 mM) for another 1 hr, to eliminate any residual iodoacetamide. The alkylated samples were buffer exchanged into 50 mM Tris/HCl, pH 8.0 using Vivaspin columns (GE healthcare). Trypsin (1.7 µg) was added to glycoproteins (50 µg) and the mixture incubated at 37°C for 16 h. Trypsin was heat inactivated and glycopeptides were extracted using C18 Zip-tip (Merck Millipore) following the manufacturers protocol.

The peptides were dried, re-suspended in 0.1% formic acid and analyzed by nanoLC-ESI MS with an Easy-nLC 1200 (Thermo Fisher Scientific) system coupled to a Fusion mass spectrometer (Thermo Fisher Scientific) using higher energy collision-induced dissociation (HCD) fragmentation. Peptides were separated using an EasySpray PepMap RSLC C18 column (75 µm × 75 cm). A trapping column (PepMap 100 C18 3µM 75µM x 2cm) was used in line with the LC prior to separation with the analytical column. The LC conditions were as follows: 275 min linear gradient consisting of 0-32% acetonitrile in 0.1% formic acid over 240 minutes followed by 35 minutes of 80% acetonitrile in 0.1% formic acid. The flow rate was set to 300 nl/min. The spray voltage was set to 2.7 kV and the temperature of the heated capillary was set to 40°C. The ion transfer tube temperature was set to 275°C. The scan range was 400–1600 m/z. The HCD collision energy was set to 50%, appropriate for fragmentation of glycopeptide ions. Precursor and fragment detection were performed using an Orbitrap at a resolution MS1 = 100,000. MS2 = 30,000. The AGC target for MS1 = 4e5 and MS2 = 5e4 and injection time: MS1 = 50ms MS2 = 54ms.

Data analysis and glycopeptide identification were performed using Byonic (Version 2.7) and Byologic software (Version 2.3; Protein Metrics Inc.). The glycopeptide fragmentation data were evaluated manually for each glycopeptide; the peptide was scored as true-positive when the correct b and y fragment ions were observed along with oxonium ions corresponding to the glycan identified. The MS data were searched using the Protein Metrics 305 N-glycan library with sulfated glycans added manually. The relative amounts of each glycan at each site as well as the unoccupied proportion were determined by comparing the extracted chromatographic areas for different glycotypes with an identical peptide sequence. All charge states for a single glycopeptide were summed. The precursor mass tolerance was set at 4 ppm and 10 ppm for fragments. A 1% false discovery rate (FDR) was applied. The relative amounts of each glycan at each site as well as the unoccupied proportion were determined by comparing the extracted ion chromatographic areas for different glycopeptides with an identical peptide sequence. Glycans were categorized according to the composition detected. Any composition containing HexNAc(2)Hex (>3) was classified as oligomannose-type, those containing at least one fucose and/or sialic acid were classified as Fucose or NeuAc respectively. Any composition containing Hex(3) was classified as

'Hex(3), no galactose'. GlcNAc(1)/GlcNAc(1)Fuc(1) is included as a separate category to highlight the remnant monosaccharide resulting from endoglycosidase H (Endo H) treatment.

Endo H treatment

Typically, 20 mg of dtCD70-Fc were incubated with 100000 units of Endo H in acetate buffer (0.1M, pH 5.2) at 37°C for 4 h. The optimal enzyme/substrate ratio determined by digestion trials. dtCD70-Fc was then dialysed against phosphate buffered saline and re-purified by size-exclusion chromatography.

T cell proliferation assay

Single cell suspensions were prepared from the spleens of C57BL/6 mice. Following lysis of red blood cells, splenocytes (2 x 10^5) in U-bottom shaped 96-well plates were stimulated with soluble anti-CD3 mAb (clone 145-2C11, prepared in-house) and additionally with CD70 proteins, anti-CD27 mAb or control mouse IgG1 (anti-human CD16 clone 3G8, prepared in-house) at the concentrations indicated in the Figure legends. Cells were incubated in a final volume of 200 µl at 37°C and 5% CO_2 in a humidified incubator for 48 h and then 1 µCi/well of ^{3}H-thymidine was added for an additional 17 h before harvesting. The cells were then lysed using a harvesting system and lysates transferred to filter plates (Opti-plate-96, Perkin Elmer). Scintillant fluid (40 µl/well) (Perkin Elmer) was added and incorporation of ^{3}H-thymidine into proliferating cells was measured using a β-emission counter.

NFκB reporter assay

The Jurkat NFκB GFP reporter cell line (System Biosciences) was transfected using Lipofectamine 2000 (Thermo Fisher Scientific) with pcDNA3.1 encoding mouse CD27 cDNA and stable clones were then selected using 1 mg/ml geneticin. To study NFκB activation, cells were incubated with fusion proteins or anti-CD27 mAb for 6 h at 37°C and the magnitude of NFκB activation was measured by detection of GFP production using flow cytometry. In some experiments Jurkat cells were co-cultured with CHO-K1 cells stably expressing mouse FcγRIIB (provided by Dr Hannah Smith and Prof Mark Cragg, University of Southampton).

Endotoxin detection

Recombinant proteins were regularly assessed for endotoxin levels using the Endosafe-PTS portable test system (Charles River, Massachusetts, USA) and found to contain < 5 EU per mg protein.

In vivo experiments

Mice (C57BL/6, BALB/c, OT-I and FcγR1,2,3,4 null) were maintained in the Biomedical Research Facility unit of University of Southampton. Mice were kept on a 12 h light/dark cycle, provided with environmental enrichment and the temperature was maintained between 20-24°C. OT-I TCR transgenic mice specific for the ovalbumin (OVA)-derived peptide 257-264 ($OVA_{257-264}$) (25) and FcγR1,2,3,4 deficient mice (generated by Dr Sjef Verbeek (26)) have been established previously. All experiments were conducted under UK Home Office licence numbers PA4C79999 and IE7C34E6C and following approval by the local ethics committee, reporting to the Home Office Animal Welfare Ethical Review Board (AWERB) at the University of Southampton. Age (8-12 weeks) and sex matched experimental animals were maintained in individually ventilated cages and food and water was available *ad libitum*. Mice were visually checked daily if adverse effects were anticipated or if mice were nearing a humane end point.

To determine the effect of CD27 agonists on T-cell activation *in vivo*, total leukocytes prepared from the spleens of OT-I mice were adoptively transferred into C57BL/6 recipients. In some experiments congenic OT-I mice expressing the CD45.1 allele were utilised. Mice were rested for 24 h before challenge with $OVA_{257-264}$ peptide (30 nmol) in combination with 250 µg control mouse IgG1, dtCD70-Fc variants or anti-CD27 mAb as described in the Figure legends. The number of transferred T cells was determined by PE-labelled H-$2K^b$ $OVA_{257-264}$ tetramers and then adjusted to achieve the desired numbers. When assessing the role of FcγRs *in vivo*, $CD8^+$ T cells from OT-I mice were first purified using CD8α MicroBeads to remove FcγR expressing accessory cells (Miltenyi Biotec) prior to adoptive transfer into FcγR1,2,3,4 null mice. OT-I T cell expansion in recipient mice was monitored by peripheral blood sampling and flow cytometry. To assess the endogenous $OVA_{257-264}$ $CD8^+$ T cell response, mice were injected i.v. with OVA protein (Sigma-Aldrich) in combination with antibodies or dtCD70-Fc and subsequently received 2 further i.v. injections of antibodies or dtCD70-Fc as described in the Figure legends.

For tumour immunotherapy experiments, groups of age-matched mice were injected i.v. with $5x10^6$ BCL_1 B cell lymphoma (17, 27, 28) on day 0 followed by CD27 agonist proteins on days 5, 6, 7 and 8 post tumour inoculation (200 µg/d). Survival period to the humane end point was plotted using the Kaplan-Meier method with analysis for significance by the log-rank (Mantle-Cox) test.

Serum concentrations of dtCD70-Fc proteins and anti-CD27 mAb after i.v. injection were measured by ELISA. For dtCD70-Fc, we used an anti-CD70 mAb (6) for capture and horseradish peroxidase-conjugated rat anti-mouse IgG (Jackson Immunoresearch) for detection. To determine the concentration of anti-CD27, we used CD27-Fc (R&D Systems) as a capture reagent and horseradish peroxidase-conjugated goat anti-mouse IgG for detection.

Flow cytometry

Antibodies used for staining were purchased from eBioscience: anti-CD8α-APC (53-6.7), anti-CD62L-eFluor450 (MEL-14), anti-

CD45.1-eFluor450 (A20) and anti-CD44-FITC (IM7). The numbers of adoptively transferred OT-I T cells were checked by staining with PE-labelled H-2K^b OVA$_{257-264}$ tetramers and their naïve phenotype confirmed by CD62L/CD44 staining (~95% CD62L high and CD44 low). Throughout, a blocking anti-FcγRII/III antibody (2.4G2; 10 µg/ml) was added to cells for 15 minutes at 4°C prior to incubation with surface staining antibodies for 30 minutes at 4°C. Red blood cells were then lysed and cells were washed prior to analysis on a BD FACS Canto II using the BD FACSDiva software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (9.4.1). Statistical analyses of pairwise comparisons are by two-tailed, non-paired Students t test and for multiple comparisons by one-way or two-way ANOVA with Tukey's *post hoc* multiple comparisons test, as appropriate. $p < 0.05$ is considered significant throughout. N numbers are defined in the relevant legends. Statistical comparisons between survival to the humane end point are by Log-rank test, and again statistical significance is

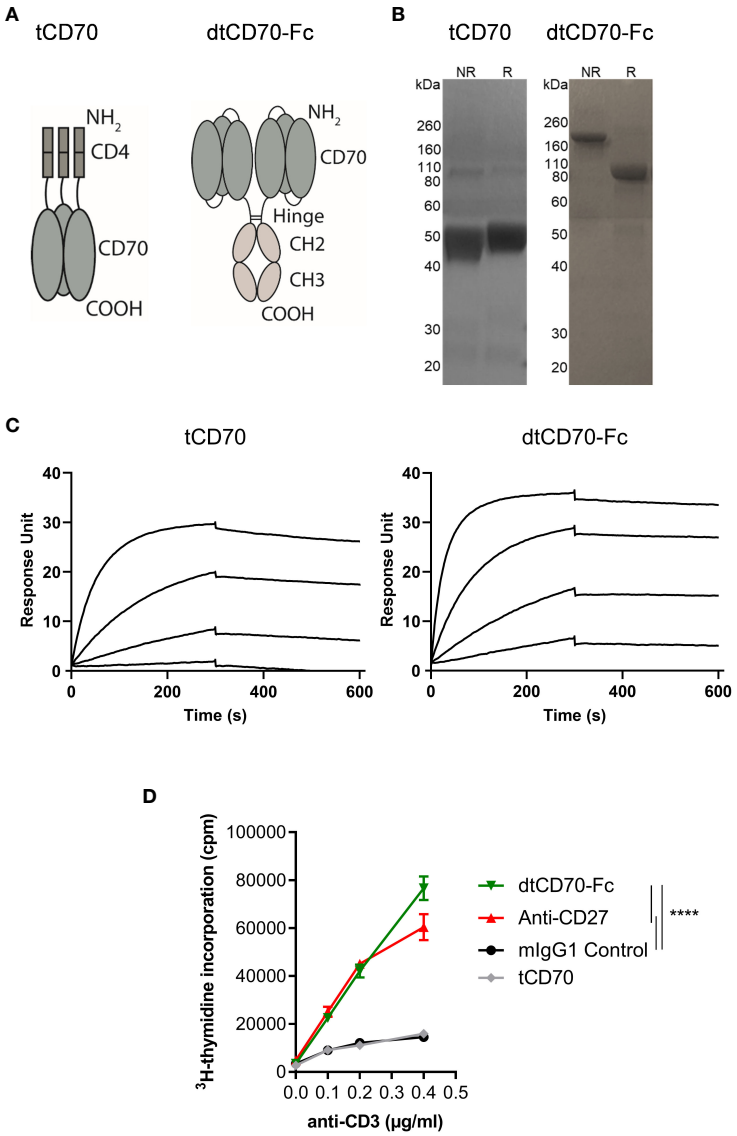

FIGURE 1
Structure, receptor binding profile, and *in vitro* T cell costimulatory effects of CD70 fusion proteins. **(A)** Schematic representation of tCD70 and dtCD70-Fc fusion proteins. **(B)** Purified tCD70 and dtCD70-Fc proteins (5 µg) were analysed using a 10% SDS-polyacrylamide gel under non-reducing (NR) or reducing (R) conditions. The gel was stained with Coomassie blue. **(C)** Overlay of SPR sensograms demonstrating binding of CD70 fusion proteins (1.56, 6.25, 25 and 100 nM) to captured recombinant mouse CD27 and their subsequent dissociation. **(D)** Splenocytes were stimulated for 72 h with various concentrations of soluble anti-CD3 and the indicated proteins (10 µg/ml). Proliferation of T cells as assessed by measurement of [^{3}H]-thymidine incorporation. Data points are the mean of triplicate measurements +/- SE and the data are representative of two independent experiments. Statistical comparisons at the highest anti-CD3 concentration are indicated. **** $P < 0.0001$, two-way ANOVA with Tukey's multiple comparison test.

considered at $p < 0.05$. P values are indicated in the figure legends; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Results

Generation and *in vitro* activity of soluble CD70 proteins

tCD70 was produced by fusing the murine CD70 ECD, which naturally trimerises, to a monomeric tag that consists of domains 3 and 4 of mouse CD4 (Figure 1A). A similar approach was used previously to produce soluble trimeric OX40 and CD30 ligands (24, 29). dtCD70-Fc was produced by fusing three copies of the murine CD70 ECD in a single chain format to the hinge-CH_2-CH_3 domains of mouse IgG1 (Figure 1A). Proteins were purified by mAb affinity (tCD70) or protein A (dtCD70-Fc) column chromatography and subsequently by size-exclusion chromatography. The observed molecular weights (MW) of the single polypeptide chains under reducing conditions were consistent with the predicted MW of 51.5 kDa and104.3 kDa for the CD4 and Fc fusion proteins, respectively (Figure 1B). A higher MW band for dtCD70-Fc was observed under non-reducing conditions verifying the presence of a disulfide-linked Fc dimer (Figure 1B). Furthermore, protein purity and the absence of protein aggregates were confirmed by analytical size-exclusion chromatography (Supplementary Figure 1). To further confirm the integrity of the CD70 proteins we used SPR analysis to assess the binding to CD27. The CD70 proteins showed similar association and dissociation profiles and bound to immobilized mouse CD27 protein with an apparent affinity of 1.2 nM and 0.6 nM, respectively (Figure 1C). To assess the immune stimulatory activity of the soluble CD70 proteins, we examined their effects on T cell proliferation by measurement of cellular [^{3}H]-thymidine incorporation. Stimulation of splenic cells with sub-optimal concentrations of anti-CD3 resulted in limited cell proliferation, which was not enhanced by the addition of tCD70, consistent with previous findings (23). In contrast, the addition of either agonist anti-CD27 mAb (17, 30) or dtCD70-Fc resulted in 3-4-fold increase in T cell proliferation (Figure 1D). To examine if crosslinking could potentiate the activity of tCD70, we developed an assay that utilises human Jurkat cells engineered to express mouse CD27 and a GFP reporter of NFκB activation. Crosslinking of tCD70 was then attempted using an anti-mouse CD4 mAb that binds to the CD4 tag on the tCD70 protein, but this did not result in increased NFκB activity (Supplementary Figure 2A). We reasoned that the presence of 3 copies of the CD4 tag in tCD70 interfered with the ability of the anti-CD4 mAb to crosslink tCD70 and therefore generated a single-chain tCD70 protein (sctCD70) with a single CD4 tag (Supplementary Figures 2B-D). Although crosslinking did enhance the activity of sctCD70, the magnitude of NFκB activation as determined by GFP expression was substantially lower than that achieved using dtCD70-Fc (Supplementary

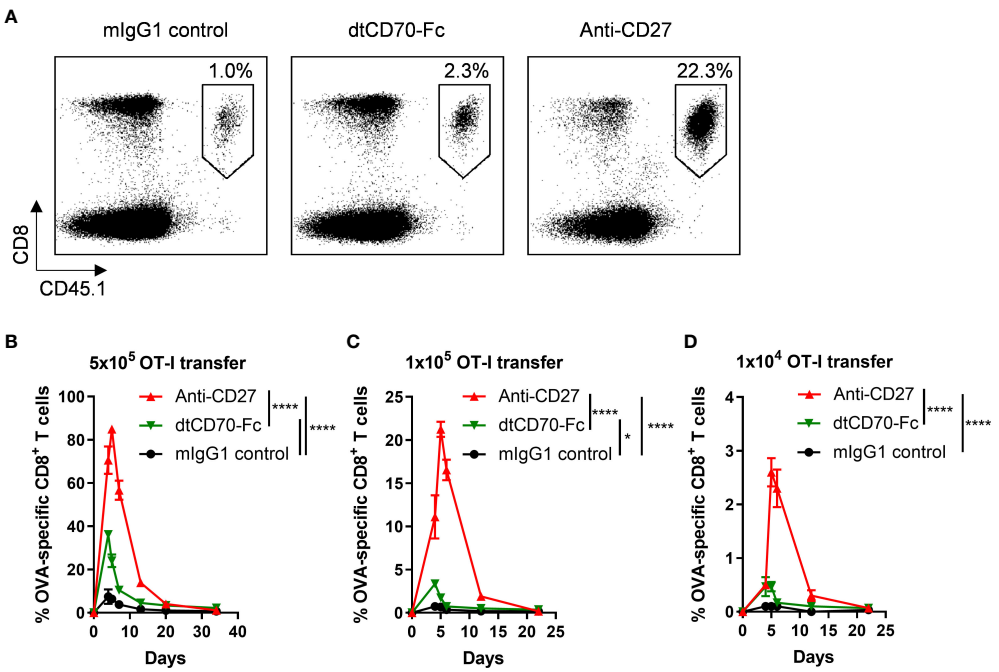

FIGURE 2
Effects of anti-CD27 mAb and dtCD70-Fc on OT-I T cell expansion *in vivo*. OT-I TCR transgenic T cells were adoptively transferred into C57BL/6 recipients. Mice were then immunised by i.v. injection of $OVA_{257-264}$ in combination with control mouse IgG1 (mIgG1), dtCD70-Fc or anti-CD27. The next day mice received an additional dose of mIgG1, dtCD70-Fc or anti-CD27. Antigen specific $CD8^+$ T cells in peripheral blood were enumerated at the indicated time points by staining with anti-CD8α and anti-CD45.1 **(A, C, D)** or anti-CD8α and $OVA_{257-264}$ tetramer **(B)**. **(A)** Representative dot plots showing the percentage of OVA specific $CD8^+$ T cells out of lymphocytes at the peak of the response (day 5). **(B–D)** Expansion of OVA specific $CD8^+$ T cells after adoptive transfer of different numbers of OT-I T cells plotted as percentage out of lymphocytes. Data points represent the mean +/- SE (n = 3 mice/group). * $P < 0.05$, **** $P < 0.0001$, two-way ANOVA with Tukey's multiple comparison test.

Figure 2E). Given that soluble CD70 in its trimeric form lacks bioactivity and that our study is mainly concerned with developing agents suitable for *in vivo* applications, we decided to focus on the dtCD70-Fc protein and compare its activity with anti-CD27 mAb.

dtCD70-Fc induces expansion of T cells *in vivo*

We assessed the activity of the dtCD70-Fc protein in the OT-I adoptive transfer model. We adoptively transferred different numbers of OT-I CD8 T cells into recipient mice and then challenged them with $OVA_{257-264}$ peptide. Three groups of mice were then given either irrelevant mouse IgG1 as a control, agonist anti-CD27 mAb or dtCD70-Fc. Although agonist anti-CD27 mAb and dtCD70-Fc were both able to boost OT-I T cell expansion when compared with the IgG1 control, the magnitude of OT-I T cell expansion in the dtCD70-Fc group was significantly lower than that in the agonist anti-CD27 mAb group (Figure 2). Furthermore, when the frequency of adoptively transferred OT-I T cells was reduced, the difference in activity between the agonist mAb and dtCD70-Fc became more pronounced (Figure 2). Thus, at OT-I frequencies approaching physiological levels, dtCD70-Fc induced ~6-7 fold less T cell expansion compared with the anti-CD27 mAb. Since dtCD70-Fc and agonist anti-CD27 mAb were similarly able to stimulate T cell proliferation in cultures of splenocytes, we reasoned that the differences in the observed activity *in vivo* might be due to faster plasma clearance of the dtCD70-Fc protein.

Oligomannose-type glycans contribute to reduced persistence of dtCD70-Fc in the circulation

We measured the serum concentrations of anti-CD27 mAb and dtCD70-Fc in the circulation over a period of 7 days and found that in contrast with anti-CD27 mAb, dtCD70-Fc was rapidly cleared from the circulation (Figure 3A). The serum concentration of dtCD70-Fc 1 h after i.v. administration was approximately one tenth that of the anti-CD27 mAb and was below 0.5 µg/ml by 6 h (Figure 3A). The neonatal Fc receptor (FcRn) within the acidic endosomal compartment binds to the Fc domain and facilitates recycling to the cell surface leading to the observed long circulatory half-lives of antibodies (31). Given the poor persistence of the dtCD70-Fc protein, we speculated that attachment of the CD70 ECD to the Fc domain could have reduced binding to FcRn. However, assessment of the dtCD70-Fc interaction with mouse FcRn by SPR showed that binding remained intact (Figure 3B), and the affinity of the interaction was similar to that of anti-CD27 mouse IgG1 binding to mouse FcRn ($K_{D(dtCD70-Fc)}$ = 4.6 x 10^{-8} M; $K_{D(anti-CD27)}$ = 4.9 x 10^{-8} M).

The dtCD70-Fc protein is predicted to be heavily glycosylated due to the presence of 10 potential N-linked glycan sites in each of its polypeptide chains. Nine of the N-linked glycosylation sites are found in the CD70 part (3 in each of the CD70 ECDs) with the remaining site present in the CH_2 domain of the Fc. In contrast, the anti-CD27 mAb contains the canonical N297 glycosylation site in the Fc region as well as an additional site in the variable domain of the heavy chain (N59). Given that the type of N-glycan can have a major impact on the plasma half-life of glycoproteins (32–34), we performed site-specific glycan analysis of dtCD70-Fc and anti-CD27 mAb by liquid chromatography-mass spectrometry. This analysis revealed that on average 74% of the total glycans present in dtCD70-Fc were oligomannose-type with Man5-9 representing the major (95%) forms, whereas the figure for anti-CD27 mAb was 10% (Figure 3C). As the presence of oligomannose-type glycans is known to accelerate the clearance of glycoproteins, including antibodies, via uptake by the mannose receptor (32–35), we investigated if enzymatic removal of oligomannose-type glycans with Endo H could improve the persistence of dtCD70-Fc. Reduction of oligomannose-type glycans following Endo H treatment of dtCD70-Fc was confirmed by glycan analysis using liquid chromatography-mass spectrometry (Figure 3C, Supplementary Figure 3) as well as by SDS-PAGE which demonstrated increased mobility of the partially deglycosylated dtCD70-Fc (Figure 3D). Concurrent with the reduction in oligomannose-type glycans, there was an increase in N-acetylglucosamine (GlcNAc) and/or GlcNAc-fucose (Figure 3C), consistent with Endo H mediated cleavage between the two GlcNAc residues in the core region. Overall, although Endo H treatment of dtCD70-Fc reduced the number of N-linked glycans that contain oligomannose, there were still more oligomannose-containing glycans per dtCD70-Fc molecule after Endo H treatment compared with the anti-CD27 mAb (Supplementary Figure 3). To assess the effect of the reduction in the abundance of oligomannose-type glycans on the persistence of dtCD70-Fc *in vivo*, we compared the plasma half-lives of the two dtCD70-Fc proteins and found that removal of oligomannose-type glycans resulted in delayed clearance (Figure 3E). These results identify oligomannose-type glycans as important mediators of the rapid *in vivo* clearance of dtCD70-Fc and highlight a potential approach to improve bioactivity.

Glycan trimming converts dtCD70-Fc into a potent agonist *in vivo*

An initial assessment of the costimulatory effects of dtCD70-Fc demonstrated that Endo H treatment did not significantly alter its ability to enhance T cell proliferation *in vitro* (Supplementary Figure 4). Next, we investigated if the improved half-life of Endo H treated dtCD70-Fc would translate into improved bioactivity *in vivo*. We used a vaccination model wherein adoptive transfer of low numbers of OT-I T cells and injection of unmanipulated dtCD70-Fc gave a minimal T cell response. Figure 4A shows that OT-I expansion was markedly enhanced following injection of Endo H treated dtCD70-Fc, leading to levels of T cell expansion that surpassed that observed with agonistic anti-CD27 mAb. Similarly, the endogenous $OVA_{257-264}$ specific CD8 T cell response was significantly enhanced after administration of dtCD70-Fc compared to anti-CD27 mAb (Supplementary Figure 5).

Several studies have shown that the agonistic activity of antibodies targeting various members of the TNFRSF are

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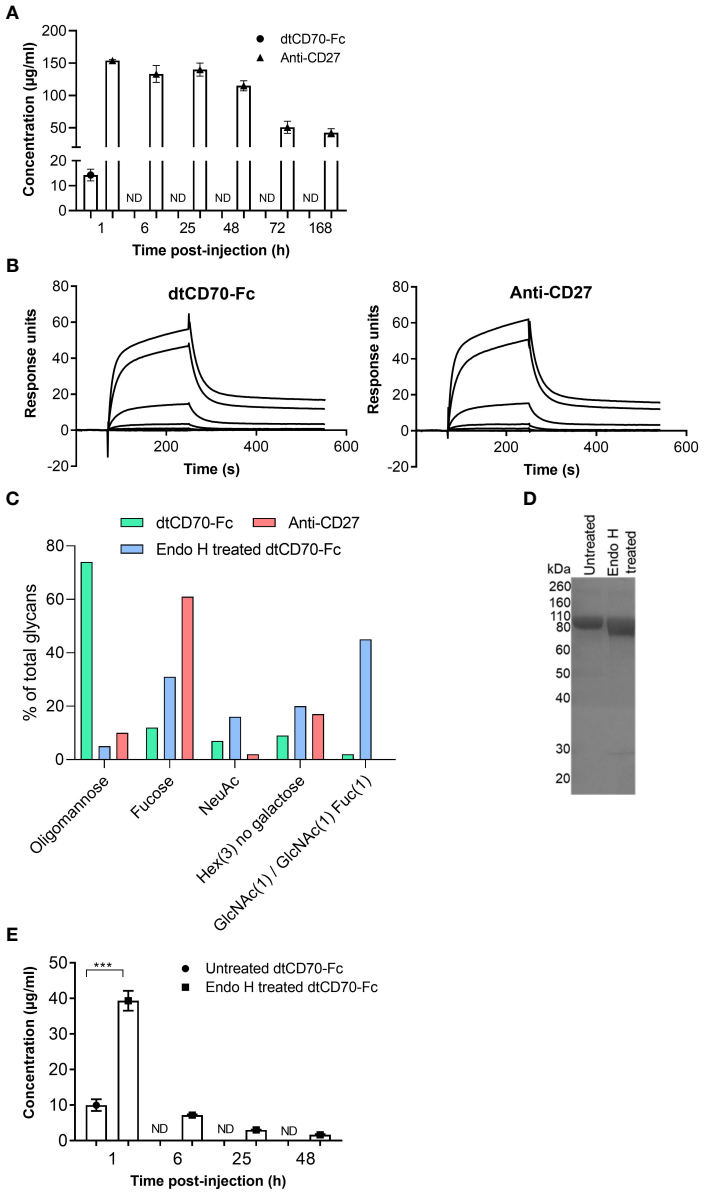

FIGURE 3
Oligomannose-type glycans in dtCD70-Fc contribute to its short half-life *in vivo*. **(A)** The concentrations of dtCD70-Fc and anti-CD27 mAb were determined in serum samples by ELISA at the indicated intervals following i.v. injection of proteins (250 µg). **(B)** Overlay of SPR sensograms demonstrating binding of FcRn at different concentrations (0, 0.8, 4, 20, 100, 500 nM) to dtCD70-Fc or antiCD27 immobilized directly onto a CM5 sensor chip. **(C)** Site-specific glycan analysis of dtCD70-Fc with and without Endo H treatment and anti-CD27 mAb. Bar graphs represent the average relative abundance of glycans detected across all sites on the molecule. Any composition containing HexNAc(2)Hex(>3) was classified as oligomannose-type, those containing at least one fucose and/or sialic acid were classified as Fucose or NeuAc respectively. Any composition containing Hex(3) was classified as "Hex(3), no galactose". GlcNAc(1)/GlcNAc(1)Fuc(1) is included as a separate category to highlight the remnant saccharides resulting from Endo H treatment. **(D)** Analysis of oligomannose digestion by SDS-PAGE. Untreated dtCD70-Fc or an aliquot of the Endo H reaction (~ 5 µg protein) was run on a 10% SDS-polyacrylamide gel under reducing conditions. Proteins were revealed by Coomassie blue staining. **(E)** The concentrations of untreated or Endo H treated dtCD70-Fc were determined in serum samples by ELISA at the indicated intervals after i.v. injection of proteins (250 µg). Data points represent the mean +/- SE (n = 3 mice/group) and are representative of two independent experiments. *** $P < 0.001$, unpaired two-tailed t test.

dependent on antibody hyper-crosslinking mediated by antibody binding to FcγRs, especially inhibitory FcγRIIB (36–38). Consistent with previous findings, the agonistic activity of the anti-CD27 mAb (mouse IgG1) was significantly diminished when OT-I T cells were adoptively transferred into FcγR deficient recipient mice (Figure 4B). In contrast, Endo H treated dtCD70-Fc was still able to induce OT-I T cell expansion in the absence of FcγRs, suggesting that FcγR-mediated dtCD70-Fc hyper-crosslinking is not essential for its activity (Figure 4B). Although dtCD70-Fc was clearly active in FcγR deficient mice, the magnitude of the OT-I T cell response was lower than that reached in the FcγR sufficient mice (Figures 4A, B). To further explore the possibility that the activity of dtCD70-Fc may have been potentiated by binding to FcγRs, we first confirmed that Endo H treated dtCD70-Fc is capable of binding to FcγRIIB

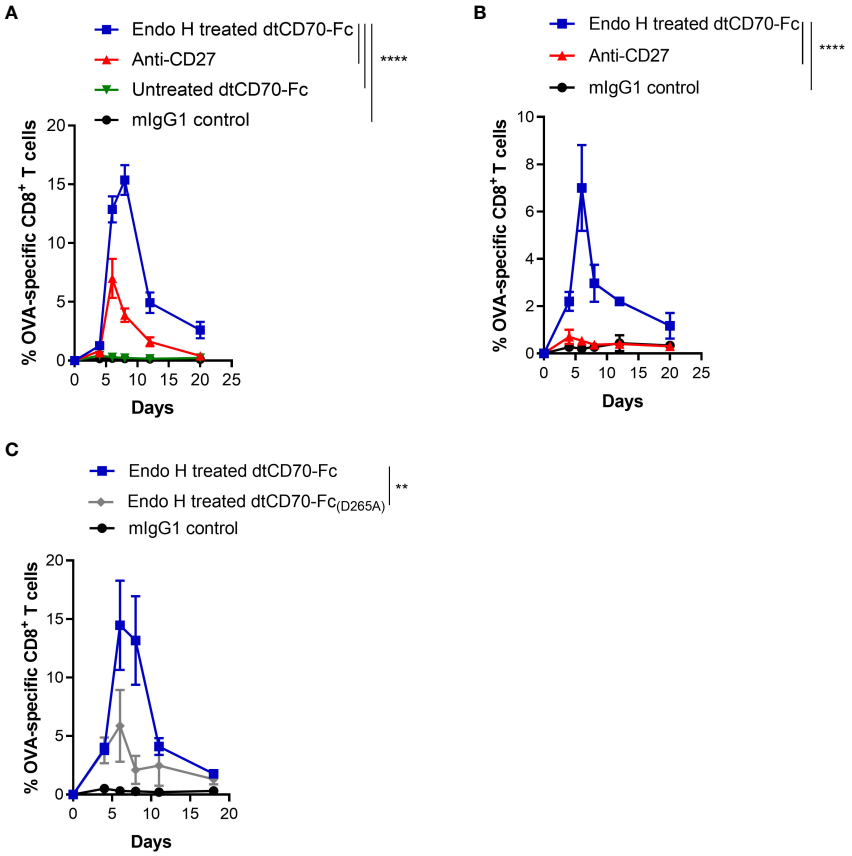

FIGURE 4
Glycan trimming and FcγR binding potentiate the immunostimulatory activity of dtCD70-Fc *in vivo*. **(A)** Purified OT-I CD45.1$^+$ congenic CD8$^+$ T cells (1 x 10^4) were adoptively transferred into C57BL/6 recipients. Mice were then immunised with $OVA_{257-264}$ in combination with control mIgG1, dtCD70-Fc, Endo H treated dtCD70-Fc or anti-CD27. The next day mice received an additional dose of mIgG1, dtCD70-Fc, Endo H treated dtCD70-Fc or anti-CD27. Antigen specific CD8$^+$ T cells in peripheral blood were enumerated at the indicated time points and data are presented as percentage OVA-specific CD8$^+$ T cells out of total CD8$^+$ T cells. **(B)** *In vivo* agonistic activity of Endo H treated dtCD70-Fc in FcγR null mice. Adoptive transfer of OT-I T cells and immunisation was carried out as in **(A)** except that the recipient mice were FcγR null. **(C)** Comparison of the agonistic activity of Endo H treated dtCD70-Fc and dtCD70-$Fc_{(D265A)}$ proteins. Purified OT-I T cells were adoptively transferred into C57BL/6 recipients and mice were immunised as indicated in **(A)**. Data points represent the mean +/- SE (n = 3 mice/group) and are representative of two independent experiments. ** P < 0.01, **** P < 0.0001, two-way ANOVA with Tukey's multiple comparison test.

and FcγRIII (Supplementary Figure 6), consistent with the binding specificity of mouse IgG1 Fc to murine FcγRs (36). Next, we introduced a mutation (D265A) in the CH_2 domain known to abolish binding to mouse FcγRs without affecting half-life (39) and then compared the activity of Endo H treated dtCD70-$Fc_{(D265A)}$ with the FcγR competent dtCD70-Fc. Figure 4C shows that while both dtCD70-Fc and dtCD70-$Fc_{(D265A)}$ were able to stimulate OT-I T cell expansion, the presence of the wild-type Fc domain resulted in a 3-fold higher OT-I T cell expansion during the primary response. Further, we confirmed that introduction of the D265A mutation did not have a detrimental effect on the half-life as both Endo H treated dtCD70-Fc and dtCD70-$Fc_{(D265A)}$ were similarly cleared from the circulation (Supplementary Figure 7, Figure 3E). Lastly, we confirmed that although functional as a soluble protein, the activity of dtCD70-Fc was enhanced when Jurkat NFκB-GFP reporter cells expressing mouse CD27 were co-cultured with FcγRIIB expressing cells (Supplementary Figure 8). Thus, taken together these findings support the notion that although not essential for activity, the interaction with FcγRs may be desirable for maximising the potency of dtCD70-Fc.

Finally, we evaluated the therapeutic activity of dtCD70-Fc against the BCL_1 lymphoma, a transplantable B cell tumour that originally arose spontaneously in a BALB/c mouse (27, 28). BCL_1 lymphoma, which primarily develops in the spleen of recipient mice, is supressed by anti-CD27 mouse IgG1, an isotype that lacks effector function (ADCC and ADCP), consistent with the CD8 T cell stimulatory effects delivered by this isotype (18). Administration of anti-CD27 mAb, Endo H treated dtCD70-Fc or Endo H treated dtCD70-$Fc_{(D265A)}$ significantly prolonged the survival of BCL1-bearing mice when compared to the mouse IgG1 control group (Figure 5). In contrast, administration of dtCD70-Fc with a large population of oligomannose-type glycosylation (untreated with Endo H) did not lead to statistically significant improvement in survival, consistent with lesser ability of this protein to stimulate expansion of OT-I T cells *in vivo* (Figure 4A). The median survival of mice given anti-CD27 mAb,

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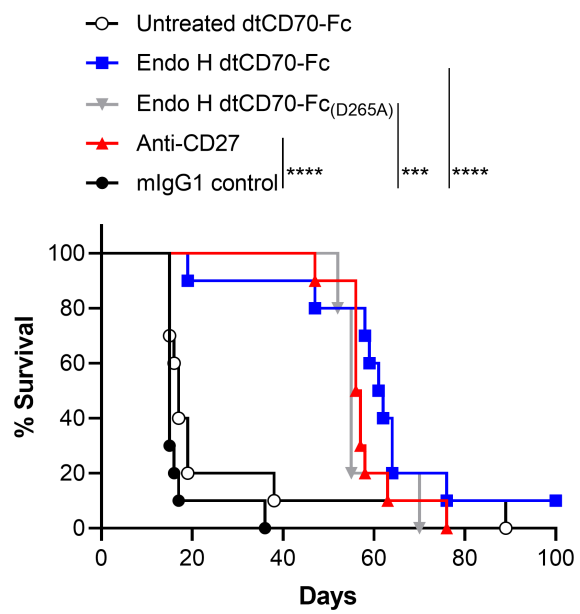

FIGURE 5
Therapeutic activity of dtCD70-Fc against BCL_1 lymphoma. Groups of mice received 5 x 10^6 BCL_1 cells i.v. on day 0 and then mIgG1 control, dtCD70-Fc, Endo H treated dtCD70-Fc, Endo H treated CD70-$Fc_{(D265A)}$ or anti-CD27 on days 5, 6, 7 and 8 (200 µg/d). Mice were monitored for tumour development and survival to the humane end point was plotted using the Kaplan-Meier method. *** P < 0.001, **** P < 0.0001, log-rank (Mantle-Cox) test (n = 5 - 10 mice/group).

Endo H treated dtCD70-Fc and Endo H treated dtCD70-$Fc_{(D265A)}$ was 56.5, 61.5 and 55 days, respectively, which compared favourably with a median survival of 15 days in the control group. Overall, the data demonstrate that a substantial part of the dtCD70-Fc activity is retained in the absence of FcγR binding.

Discussion

The overarching aim of the current study was to develop a potent CD27 agonist suitable for *in vivo* application. Current efforts to develop agonist antibodies targeting CD27 as well as other members of TNFRSF have been fraught with difficulties due to the vastly different immunostimulatory activities displayed by agents targeting the same receptor (21, 22, 40). Agonism is known to correlate with the ability of antibodies to induce receptor clustering, an attribute that is affected by epitope, antibody hinge flexibility, interaction with FcγRs, and affinity (18, 21, 36, 40–42). Furthermore, although co-engagement of FcγRIIB by anti-TNFRSF antibodies has been shown to promote agonism *in vivo* (36–38), this approach is highly sensitive to levels of FcγRIIB expression which vary depending on the tissue and cellular source (43).

Here we have evaluated an alternative approach that could overcome some of the limitations associated with antibody-based agonists. Given that soluble tCD70 fails to costimulate T cells despite high affinity binding to CD27 (Figure 1 and (23)), we opted to generate a protein with two adjacent trimeric CD70 units, wherein 3 extracellular CD70 fragments were fused to the hinge-CH_2CH_3 domains of mouse IgG1 in a single chain format (dtCD70-Fc). dtCD70-Fc provided potent T cell costimulation signals culminating in increased T cell proliferation, demonstrating that forced dimerization of CD70 trimers is required for activating CD27 signalling (Figure 1). Members of the TNFRSF can be subdivided into those that are effectively activated by trimeric ligands (category I) and others that require further oligomerisation (category II) to facilitate downstream assembly and activation of the signalosome (44). Our findings showing that dtCD70-Fc, but not tCD70, was able to costimulate T cells, together with the knowledge that the natural form of CD70 is a membrane-bound protein, firmly place CD27 in the TNFRSF category II group.

Despite having equivalent costimulatory activity to agonist anti-CD27 mAb *in vitro*, our initial evaluation of dtCD70-Fc *in vivo* suggested that the CD70 protein was less effective than anti-CD27 mAb in stimulating antigen-specific CD8 T cells (Figure 2). The difference in the activity between the two agents was particularly striking when the number of transferred OT-I T cells approximated the endogenous antigen-specific T cells (Figure 2D). Fc-fusion proteins are often cleared from the circulation more rapidly than antibodies due to several factors, including reduced affinity to FcRn and alterations in glycosylation (33). Although we did not detect differences in FcRn binding between dtCD70-Fc and anti-CD27 mAb (Figure 3B), glycan analysis demonstrated enrichment of oligomannose-type glycans in the dtCD70-Fc (Figure 3C, Supplementary Figure 3), which upon enzymatic removal improved its half-life (Figure 3E). As a result, the *in vivo* stimulatory activity of dtCD70-Fc was substantially enhanced and exceeded that of anti-CD27 mAb (Figure 4A, Supplementary Figure 5). We do not fully understand why dtCD70-Fc retains a high content of oligomannose-type glycans, but it is plausible that the presence of a large number (10) of N-linked glycans per polypeptide chain impacts on the efficiency of mannose trimming in the endoplasmic reticulum (45). Our data highlight the importance of glycan analysis when evaluating the *in vivo* behaviour of therapeutic glycoproteins and are consistent with previous studies on the role of oligomannose-type glycosylation in antibody and Fc-fusion protein clearance (32–34). Although our study did not reveal the identity of the receptor responsible for the rapid clearance of dtCD70-Fc, we speculate that this is largely mediated through uptake by the endocytic mannose receptor which is expressed on subpopulations of macrophages, dendritic cells and the hepatic sinusoidal endothelium. Previous work by Lee and colleagues (35) demonstrated that mannose receptor deficient mice exhibited a defect in the clearance of proteins bearing mannose or N-acetylglucosamine residues, highlighting the non-redundant role for this receptor in regulating glycoprotein half-life *in vivo*. Since two of the three N-glycosylation sites in the ECD of murine CD70 are conserved in human CD70, our findings will likely have relevance for the generation and use of human dtCD70-Fc.

Whilst the role of FcγRs in enhancing agonism by anti-TNFRSF antibodies is well established (36–38), to our knowledge this is the first demonstration that this phenomenon applies to soluble oligomeric CD70 (Figure 4, Supplementary Figure 8). However, unlike anti-CD27 mAb, dtCD70-Fc retained a significant proportion of its T cell stimulatory effects without the requirement of FcγR binding

(Figures 4, 5, Supplementary Figure 8). Previous studies have suggested that forced dimerization of soluble trimeric TNFSF ligands is required for activation of category II receptors (23, 46). Our data is consistent with this notion and additionally suggest that membrane association is required for maximal activity. In the current study, the Fc domain in dtCD70-Fc performed a dual function enforcing dimerization of CD70 trimers and tethering the protein to the plasma membrane of FcγR expressing cells. Further studies are required to assess if modulation of dtCD70-Fc binding to FcγR can be harnessed to tailor the magnitude of immune stimulation to the desired level and thus avoid a scenario whereby immune activation leads to an overt inflammatory response. In addition, it will be important in the future to understand how CD27 stimulation with or without engagement of FcγRs impact the differentiation and longevity of effector and memory T cell subsets.

In summary, we provide a method for the generation of a CD27 agonist with a tunable activity profile. The approach described here may encourage further exploration of TNFSF proteins in vaccine development and immunotherapy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by UK Home Office licence numbers PA4C79999 and IE7C34E6C and following approval by the local ethics committee, reporting to the Home Office Animal Welfare Ethical Review Board (AWERB) at the University of Southampton. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

OD and CIM performed the experiments with the help of JK, HTCC, PJD, SLB and AR. JDA performed the site-specific glycan analysis. OD, JDA, SLB, CIM, AR, MC and AA-S analysed and interpreted the data. AA-S conceived the project. OD, JDA and AA-S wrote the manuscript with feedbacks from MC, SLB, AR and HTCC. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AA-S is an inventor on patents pertaining to the generation and therapeutic use of agonist anti-CD27 antibodies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1252274/full#supplementary-material

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