Discovery and Development of Agonist Antibodies for T cell Receptors

By

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biomedical Engineering)
in the University of Michigan
2022

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Dedication

To my partner, Rose
To my parents and sisters

Acknowledgements

I feel very honored to be part of the University of Michigan graduate community and my PhD program in Biomedical Engineering. I faced multiple challenges in my early years in the graduate program, both in terms of personal growth and dealing with extraneous circumstances, including the COVID-19 pandemic. My yearning to persevere through difficult times no matter the circumstances and remembering my family's sacrifice to leave their homeland to find better opportunities in the United States kept me going. My desire to improve my scientific knowledge and professional growth would not have been possible without the support of Professor Peter Tessier who has been an excellent mentor for my graduate studies for the past five years. I learned how to design compelling research experiments, improve my writing skills, and effectively communicate science to my peers. I am greatly thankful to Dr. Tessier and feel honored to learn from an excellent scientist.

I have had the opportunity to work with excellent lab colleagues who have helped me improve my scientific skills to become a better scientist. John has worked alongside me collaboratively on the research project and has guided me through difficult times when I faced roadblocks in my experiments. I would like to thank all the other lab members for their generous support as well. Finally, my exceptional undergraduate students, Timon and Emily, have inspired me to improve my research skills and allowed me to hone critical thinking skills. The countless zoom meetings on Sundays to discuss research progress each week have inspired me to think of

new questions to address scientific problems. My experiences in mentoring have solidified my desire to pursue further research positions in the future.

My thesis committee has provided critical feedback on my research progress every year and I am greatly thankful to my committee members: Profs. Lonnie Shea, James Moon, Thomas Gardner and Deepak Nagrath for their support and encouragement. My coursework at Michigan has allowed me to improve my fundamental knowledge in my research area and encouraged me to improve my ability to ask the right scientific questions. In addition, my journey to graduate school would not have been possible without my mentors before entering graduate school. My research experience under the mentorship of Dr. Farouc Jaffer at the Massachusetts General Hospital and Dr. Markus Bitzer at the University of Michigan instilled my desire to pursue graduate school.

Finally, attending the University of Michigan has allowed me to be close to family and I am greatly thankful for their support. My mom and dad believed in me when I doubted myself and they have inspired me to persevere. Just watching them navigate their lives in a new country has inspired me to build courage to pursue my dreams and make them proud. My sisters, Kamal and Nicky, are the best sisters I could ask for and their unconditional love and support have made my graduate school experience a lot easier to navigate. Finally, to my partner, Rose, who is one of the smartest women I know. Her tenacity to succeed even during difficult times has inspired me to follow my career endeavors and made me a better human being. My family gives meaning to my life and inspires me to improve myself every day.

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Abstract

Agonist antibodies that activate co-stimulatory immune receptors, such as the tumor necrosis factor (TNF) receptors OX40 and CD137, are an important class of emerging therapeutics due to their ability to regulate immune cell activity. Despite their promise, there are no approved agonist antibodies for treating cancer as demonstrated by previous unsuccessful clinical trials. Although multiple factors are responsible for poor clinical efficacy, one major bottleneck is the reliance on FcγR-mediated crosslinking for sufficient receptor activation. This is inherently problematic because FcγR expression varies greatly on different immune cells, leading to a wide range of receptor agonism. Emerging research suggests that antibodies engaging two different epitopes on the same immune receptor mediate receptor superclustering and enable robust antibody agonism without extrinsic Fc crosslinking. However, there are no systematic methods for identifying such biepitopic (also known as biparatopic) agonist antibodies. Therefore, the objective of this research work is to develop facile methods for reliably identifying biepitopic antibodies to activate immune receptors for immunotherapeutic applications.

Biepitopic antibodies have been shown to mediate potent receptor activation for a variety of immune receptors. Traditionally, the generation of these antibodies requires four key steps including animal immunization, epitope binning to identify unique antibody pairs, and combining antibody pairs to engineer biepitopic antibodies. While this approach has been used to successfully discover biepitopic antibodies, it suffers from key limitations. Notably, animal immunization and subsequent antibody isolation is an arduous and unpredictable process. Even when successful clones are discovered from these processes, further epitope binning experiments are needed to

select antibody pairs to discover potent immune therapeutics. To overcome these limitations, we developed an antibody screening strategy that greatly simplifies the discovery of biepitopic antibodies. Our approach eliminates the need for animal immunization by using existing, off-the-shelf IgG antibodies specific to the target receptor. Next, we perform *in vitro* selections by blocking the receptor epitope of the existing antibody and conducting subsequent sorts to identify single-chain antibodies with orthogonal binding sites. Our work has shown that the antibody screening strategy can be used to discover antibodies for a variety of TNF receptors including OX40 and CD137.

Given that receptor clustering of three or more receptors is critical for activating TNF receptors, we first generated biepitopic tetravalent OX40 antibodies by attaching novel single-chain antibodies to the C-termini of the light chain of existing clinical-stage antibodies. These tetravalent biepitopic antibodies showed strong T cell proliferation and cytokine secretion for biepitopic antibodies compared to their monoepitopic counterparts. Next, we sought to improve additional clinical-stage OX40 IgGs which we engineered as biepitopic antibodies to demonstrate the generality of our findings that biepitopic antibodies can mediate superior and FcγR-independent activities. Beyond OX40 IgGs, we also show that biepitopic antibodies can be used to mediate superior T cell proliferation for a second TNF receptor (CD137). Looking forward, we anticipate that these research advancements will accelerate the discovery and development of the next generation of immunotherapeutics.

Chapter 1: Introduction

Abstract

Immunomodulatory receptors play a critical role in regulating immune cell activity. These immune receptors are known as co-stimulatory immune checkpoints because the activation of these receptors has been shown to modulate the immune response to treat various diseases, including cancer, autoimmune diseases, and inflammatory syndromes. One group of immune checkpoints, known as the tumor necrosis factor (TNF) receptor superfamily, is a key focus for agonist antibody development and several agents targeting these receptors are making their way into the clinical settings for therapeutic use. Specifically, antibodies that target TNF receptors have been shown to impart potent anti-tumor responses to improve disease outcomes. Although this is a remarkable success story, the clinical translation of these antibodies has faced multiple challenges including safety and low efficacy as demonstrated by previous unsuccessful clinical trials. To overcome these challenges, my research work aims to develop novel antibody discovery and development strategies to engineer the next generation of improved cancer immunotherapeutic for preclinical and clinical settings. This chapter will discuss the recent advancements in the discovery and development of agonist antibodies with a particular focus on TNF receptors for T-cell mediated immunity.

1.1 Antibody classification based on their function

Antibodies are glycoproteins that regulate a wide range of body functions, from immune system regulation to cellular signaling(1,2). Although they exist in many different forms, immunoglobulin G (IgG) is the most common type, representing 75% of the serum antibodies in humans. The unique Y-shaped structure of IgG consists of six domains where (1) four domains encompass antigen-binding sites (also known as fragment antigen-binding domains, Fab) and (2) two domains known as fragment crystallizable (Fc) regions that are responsible for effector function **(Figure 1-1).** The advantage of these molecules lies in their biophysical properties including high specificity, stability, developability, and strong pharmacokinetics that are unmatched in the molecular therapeutic world (3). As a result, antibodies have been used to treat a variety of diseases and their success is evident by the approval of over 100 antibody-based drugs by the Food and Drug Administration (FDA) (4). Today, this class of therapeutics represents nearly one-fifth of the FDA's new drug approvals each year.

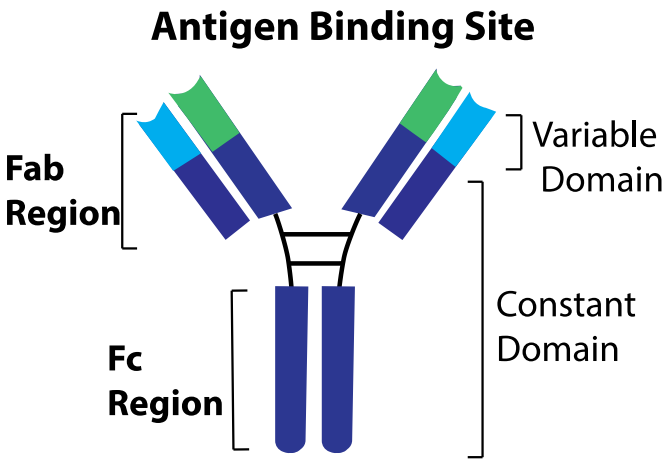

Figure 1-1: Antibody structure. The antibody structure consists of the fragment antigen binding domain (Fab) and the crystallizable fragment (Fc) domain. The Fab domain is responsible for binding to the antigen (i.e., receptors) and Fc is responsible for effector function (i.e., engaging Fcɣ receptors).

The majority of the FDA-approved therapeutic antibodies are classified as receptor/ligand blockades or neutralizing antibodies which neither activate nor inhibit the receptor signaling associated with their targets (5). The primary objective of these antibodies is to prevent the receptor/ligand binding or neutralize invading pathogens to prevent the infection from spreading throughout the body. In contrast, functional antibodies that directly inhibit (antagonist) or activate (agonist) the receptor signaling account for only a small subset of approved drugs (6). These antibodies represent a burgeoning area for therapeutic antibodies because they can fix aberrant cell signaling to restore body homeostasis. However, such functional antibodies are difficult to discover and develop because most antibodies that bind to their receptor fail to act as agonists (or even antagonists). This is because receptor activation or inhibition necessitates protein-protein interactions that are favorable for activating the downstream cellular signaling pathways in addition to receptor binding. To overcome these challenges, novel antibody discovery and

development strategies are expected to improve drug discovery and pave the way for the next generation of therapeutics (6).

Encouragingly, several immunotherapies have been developed that utilize antagonist antibodies that inhibit receptor signaling (7,8). For example, antibodies that inhibit receptors on T cells such as PD-1 and CTLA-4 are a key focus in this area and a number of these antibodies have been FDA-approved for clinical use. This includes antibodies against PD-1 (*e.g.*, pembrolizumab, nivolumab, and cemiplimab), PD-L1 (*e.g.*, atezolizumab, durvalumab, and avelumab) and CTLA-4 (*e.g.*, ipilimumab). While these therapies have shown remarkable promise, the benefit of these agents is limited to a subset of patients and additional therapies are urgently needed for alternative or complementary immunotherapy.

Agonist antibodies that activate critical immune receptors have been shown to be equally important in mediating potent immune responses against a variety of therapeutic targets (**Figure 1-2**) (9). However, their success has lagged antagonists due to the challenges associated with the discovery and development of these molecules. One major reason is that conventional bivalent IgG antibodies have shown a poor safety profile and efficacy due to their lackluster ability to mediate receptor activation (10). This phenomenon is especially evident in the development of agonist antibodies against the TNF receptors where receptor clustering on the cell surface has been shown to be critical for receptor signaling. Therefore, the objective of this chapter is to highlight the recent advancements in the discovery and development of agonist antibodies and the implications of my dissertation's contribution to this burgeoning area.

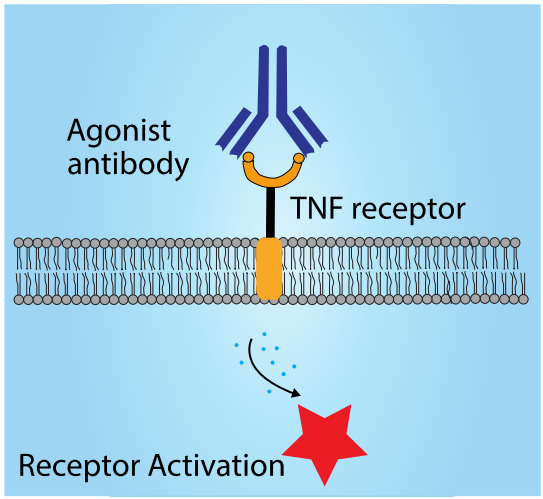

Figure 1-2: The biological function of agonist antibodies. Agonist antibodies that target TNF receptors bind to the receptor on the cell surface. This interaction leads to the activation of the NF-kB signaling pathway intracellularly to induce a potent pro-inflammatory response.

1.2 The promise of antibodies in modulating immune function

The immune system plays a key role in eliminating pathogens or diseases that seek to wreak havoc on an organism. This extraordinarily complex biological system consists of a variety of immune cells, cytokines, and antibody-mediated responses that allows for eradicating unwanted pathogens (11). Broadly, the immune system can be simplified into two major categories: the innate (non-specific) and the adaptative (specific) immune responses (12). For example, when an individual is exposed to an external pathogen, the innate immune response mounts the first line of defense, which includes recruitment of macrophages, neutrophils, etc. That is followed by the recruitment of adaptive immune cells, such as B and T cells, at a later stage to store long-term memory of the event for preventing repeated infections. In the majority of cases, the immune system works as expected where the pathogen/infection is eliminated without causing harm to a cell or an organism. However, in a small number of cases, abnormal upregulation and

downregulation of these immune cells give rise to a variety of diseases such as cancer and autoimmune diseases.

In the context of cancer, tumor cells can hijack critical immunomodulatory receptors to evade the immune response and promote its survival (13,14). Over the last two decades, there has been a great interest in developing antibodies that can reverse the onset of tumor resistance and restore unwanted immune manipulation. For example, antagonist antibodies that block or dampen immune receptor signaling can reverse immune resistance by promoting T cell survival and differentiation. This is elegantly highlighted by the development of antagonist antibodies against programmed cell death (PD-1) and cytotoxic T lymphocyte protein 4 (CTLA4) targets that have shown great promise in combating immune resistance and a number of these agents are approved by the U.S. Food and Drug Administration (FDA).

Agonist antibodies that activate immune receptor signaling are equally as important in mediating strong immune responses. For example, agonist antibodies that target T cell receptors have been shown to impart a strong anti-tumor response in preclinical tumor models. This exciting result has led to the development of therapeutic antibodies for treating a variety of diseases, including cancer and autoimmune diseases. One of the main targets for these clinical antibodies includes the tumor necrosis factor (TNF) receptor superfamily, which compose key signaling pathways involved in cell survival and differentiation in T cell biology (15,16). Briefly, the TNF receptor superfamily consists of 29 members, out of which six have shown immunotherapeutic potential: (TNFRSF4 (OX40) , TNFRSF5 (CD40), TNFRSF9 (CD137) TNFRSF7 (CD27), TNFRSF18 (glucocorticoid-induced TNFR-related protein, GITR) and TNFRSF8 (CD30). . For example, antibodies that activate OX40 and CD137 receptor pathways, which include nuclear Factor-kappa B (NF-κB), have been shown to promote the differentiation and survival of CD4 and

CD8 T cells in preclinical and clinical trials. As a result, therapeutic antibodies that target TNF receptors have been used to restore the proper immune response in ameliorating the disease outcome.

1.3 The promise and challenges of agonist antibody discovery

The traditional antibody discovery platforms that rely on binding-based screening have facilitated great progress in the discovery of therapeutic antibodies for clinical use (6). This is particularly true for antagonist antibodies, where antibody binding to its target receptor is a good predictor of their ability to inhibit cellular signaling. Unfortunately, discovery platforms and optimization strategies for antagonist antibodies are inadequate for the discovery of useful agonist antibodies (9). This is because binding is a poor predictor of agonist antibody ability to mediate potent receptor signaling which can varies greatly based on the receptor of interest and the corresponding intracellular signaling. This critical pharmacologic hurdle has slowed the progress of agonist antibody discovery and more work is needed to overcome these challenges. Although binding-based assay have been used to discover agonist antibodies. In these rare cases, common characteristics of a particular receptor family must be critical considered to increase the likelihood of discovering agonist antibodies with binding-based methods. In this section, I seek to review the literature regarding recent advancements in agonist antibody discovery platforms for the purpose of generating potent agonist antibodies **(Figure 1-3)**.

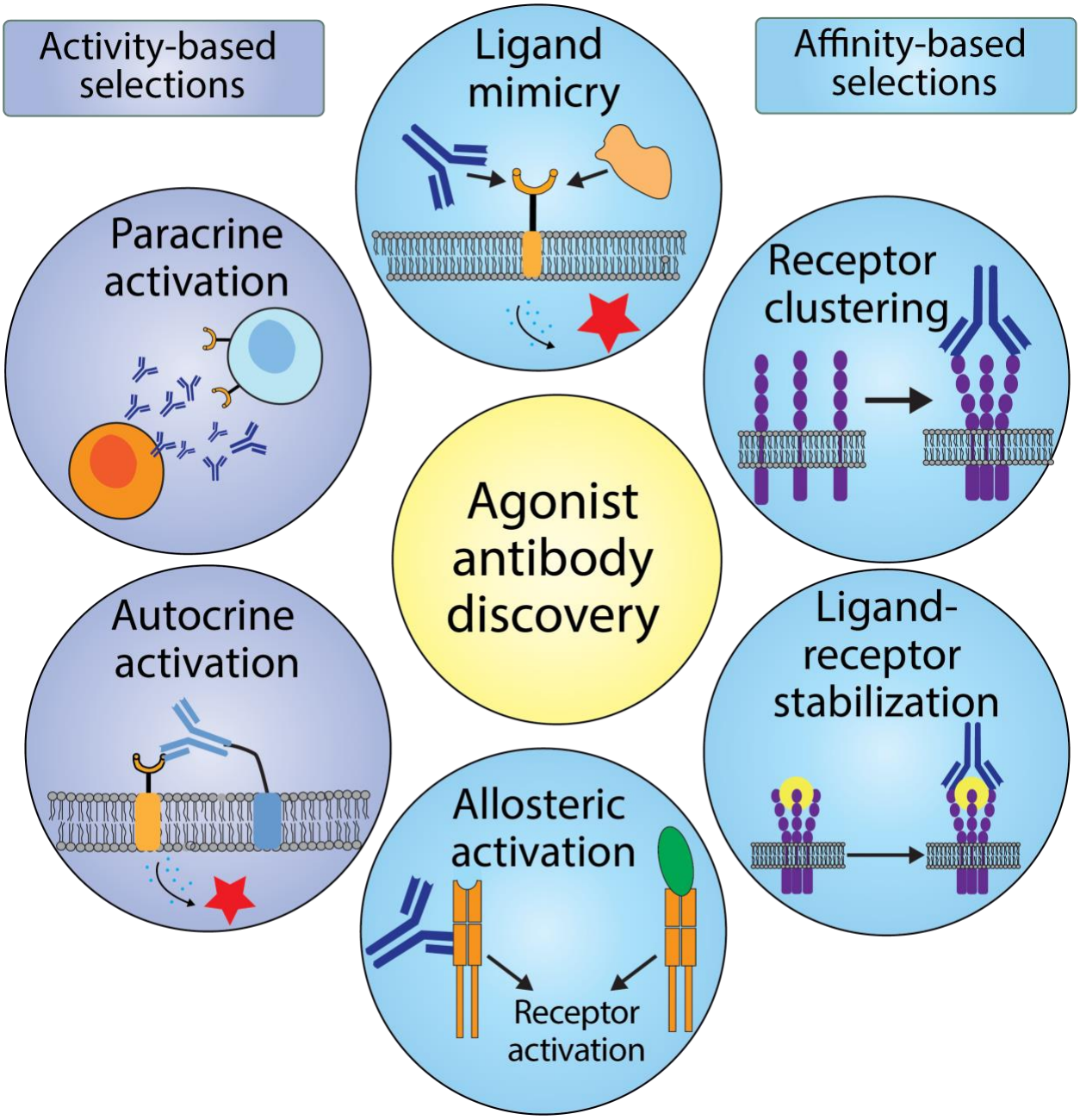

Figure 1-3: Schematic illustration of the agonist discovery methods. Agonist antibodies that target TNF receptors have been discovered using affinity-based assays as well as activity-based selections. To apply the affinity/binding-based assay, the common characteristics of the receptor biology must be at the forefront of antibody discovery techniques. On the other hand, activity-based selection methods use receptor activation as a primary tool in the antibody screening process.

1.3.A Agonist antibody discovery using binding-based selection: Given that antibody binding to its target receptor is not a good predictor of agonist function, affinity-based selection methods that

incorporate receptor clustering as a key component in their discovery platforms have led to improved antibody discovery platforms. For example, activation of TNF receptors such as OX40, CD40, and CD137 necessitates higher order receptor clustering for signal transduction (6,9). In addition, targeting certain receptor domains has shown to impart improved receptor clustering and agonist activity. In one recent study, investigators sought to discover antibodies against the OX40 receptor to elucidate the relationship between agonist function and epitope binding (17). To do this, the authors first discovered a panel of mouse OX40 antibodies through hybridoma technology with the use of the OX40 ectodomain (ECD). Next, the antibodies were characterized based on their binding domain to one of the four cysteine-rich domains (CRD 1-4, CRD1: membrane distal, CRD 4: membrane-proximal) and evaluated for their biological function using *in vitro* and *in vivo* assays. The results showed that antibodies bound to CRD 2 and 4 were able to mediate superior receptor agonism compared to antibodies targeting other CRD domains. Additionally, a similar study on CD40 receptors also showed that antibodies that target certain receptor domains such as the membrane distal CRD1 was able to mediate superior receptor agonism (18). In the case of CD40, the membrane distal domain allows ample access to Fcγ receptors in a less steric hindered environment. Collectively, an affinity-based detection platform that focuses on targeting certain CRD domains on TNF receptors allows for a higher chance of developing potent agonists. It is important to take receptor biology into account when developing these antibodies given that there are no easy rules that can be generalized across receptor families. Further work is still needed to explore the relationship between antibody binding and their impact on agonist function.

1.3.B agonist antibodies discovery based on stabilizing ligand/receptor complexes: Another unique approach to improve receptor activation is to discover antibodies that bind to the receptor away from the ligand-binding site. This approach is useful because concomitant binding of ligand and

antibody can lead to synergistic effects to improve receptor signaling. To discover such antibodies, antibody screening must be conducted in the presence of ligand-bound receptor complexes that allow the discovery of non-overlapping antibodies. In a recent study, investigators sought to discover nanobodies against the IL-6-gp80 (ligand-receptor) complex using hybridoma technology (19). To do this, camels were immunized with the ligand-receptor complex and several nanobodies were identified that bound strongly to the antigen. Structure-based studies showed that nanobodies that bind away from the ligand-binding site led to improved agonist activity. These findings were further corroborated by other studies for IL-4 receptor which showed that antibodies possessing orthogonal binding sites away from the ligand led to improved receptor activation. Collectively, this strategy allows for discovering unique antibodies that can be used in conjunction with the ligand to mediate amplified receptor activation.

1.3.C Agonist antibodies discovery based on functional-based selection: Although binding-based assays are useful for discovering agonist antibodies, further protein engineering is often warranted to ensure that the antibody is indeed capable of activating the target receptor in a biological system. Binding-based approaches often require low-throughput methods to evaluate antibody candidates which is often arduous and time-consuming. To overcome these challenges, recent studies have focused on developing agonist antibodies based on their biological activity rather than binding (20). In addition, activity-based approaches that are high-throughput in nature are expected to accelerate the development of these antibodies. In one study, researchers developed an antibody screening strategy using mammalian cells that allows the discovery of single-chain antibodies based on receptor activation. These approaches often necessitate a link between genotype (i.e., sequence of the desired molecule) and phenotype (i.e., biological activity) within the same cell to isolate desired clones. Specifically, in an autocrine screening system, the antibody library is

typically encoded into a mammalian cell genome using a lentivirus delivery system. This allows the membrane-tethered antibody to display on the cell surface to activate receptors on the same cell (autocrine system) or neighboring cells (paracrine system). Next, the receptor activation can be linked to a fluorescence marker such as a beta-lactamase reporter which can be easily detected by fluorescence-based approaches including fluorescence-activated cell sorting (FACS).

An autocrine antibody display system was highlighted in a recent paper where investigators sought to discover agonist antibodies against the tyrosine kinase receptors (TrkB) using membrane-tethered antibodies (21). This library screening strategy involved two steps where a large antibody library ($>10^{10}$) is first enriched using a phage display system to enrich clones that bind to the TrkB receptor. Next, the enriched library is subcloned into the lentivirus plasmid for a mammalian surface platform to screen for activity using FACS. Encouragingly, the authors discovered several single-chain antibodies that mediate strong activation of the TrkB receptor. Furthermore, *in vitro* assays showed that full-length antibodies as soluble proteins mediated strong TrkB receptor activation where some clones had similar activity to the natural ligand. Since then, this platform has been used to discover antibodies for a variety of therapeutic targets including G-protein coupled receptors (22).

The paracrine-display system typically involves two cell types where one cell type, such as yeast or phage, expresses the library of interest and another cell produces a biological signal to select for activity-based screening. The main issue with this technique is that the genotype and phenotype are not linked within the same cell, which creates complexity with co-culturing different cell lines. On the other hand, this system offers advantages for screening large antibody libraries and provides iterative sorting for enriching against desired clones. In one study, investigators sought to discover TrkB antibodies by combining phage display and activity-based sorting in

which phage-producing *E. coli* were co-encapsulated with a mammalian reporter system in microdroplets (23). As a proof of concept, the authors showed that microdroplets containing both phage and mammalian cell reporters led to higher receptor cell activation as demonstrated by FACS. This approach is expected to improve drug discovery for target proteins where large library size is desired for therapeutic applications.

1.4 The development of agonist antibodies against tumor necrosis factor (TNF) receptor superfamily

To develop agonist antibodies against the TNF receptors, it is critical to understand receptor biology to identify key features that can guide the discovery process. First, this receptor family mainly signals via the NF-κB pathway that is involved in pro-inflammatory processes. For example, agonist antibodies that upregulate the NF-κB pathway have been shown to improve anti-tumor responses against a variety of tumors. Second, the receptor activation requires the unique configuration of trimeric ligand and receptor interactions where monomeric receptors are brought together in 3: 3 configurations. Towards this goal, antibodies possessing multiple arms have been shown to impart improved receptor activation due to their ability to cause efficient receptor clustering. Third, the antibody-mediated receptor activation can be significantly improved with the interactions between the Fc region and Fcγ receptors present on antigen-presenting cells to cause strong receptor superclusters. Using these basic principles can often provide foundational knowledge to develop agonist antibodies for these TNF receptors. In this section, I seek to highlight important protein engineering strategies that have been developed to improve the efficacy of antibodies that target TNF receptors **(Figure 1-4)**. Furthermore, I will also explore the impact of these strategies on improved T cell immunity for pre-clinical and clinical use.

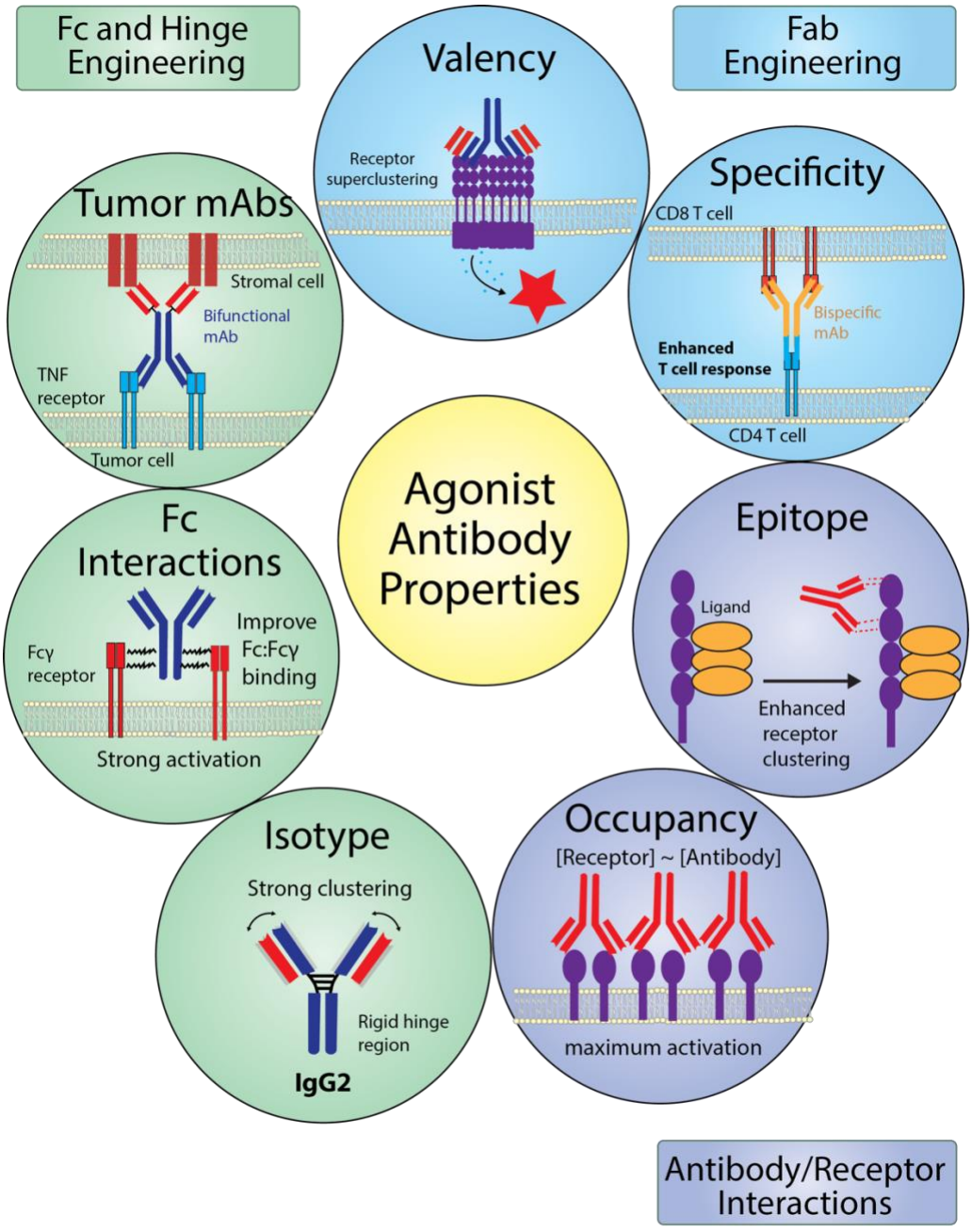

Figure 1-4. Schematic illustration of approaches for optimizing agonist antibodies for therapeutic applications. Designing antibodies with novel binding epitopes, valency, specificity, Fc-mediated interactions, and isotypes can improve their clinical use as a cancer therapy.

1.4.A. Antibody valency**:** Receptor clustering is a critical aspect of immune cell activation where monomeric units are brought together to form trimeric complexes to transduce intracellular signaling (24–26). One way to achieve clustering is by developing multivalent antibodies capable

of targeting multiple receptors to enhance agonist activity. Thus, there is great interest in leveraging antibody valency to generate potent immunotherapeutics (**Figure 1-5a**).

Mounting evidence has demonstrated that multivalent mAbs elicit improved agonistic function compared to their conventional bivalent immunoglobins (IgG). In one study, investigators engineered a novel hexameric Fc protein (MEDI1873) that targets glucocorticoid-induced TNFR-related protein (GITR, TNFR18) to improve anti-tumor immunity (27). The novel antibody format was constructed by attaching an IgG Fc region to a trimeric motif and human GITR ligand ectodomain. *In vitro* studies using this protein demonstrated a >2.5-fold increase in T cell proliferation. **(Figure 1-5b**) Next, MEDI1873 was evaluated in a primate model where it induced increased T cell proliferation and elevated levels of IgG circulating antibodies, indicating both a strong cellular and humoral immune response **(Figure 1-5c)**. Given GITR is highly expressed on both human effector and regulatory T cells (Tregs), it represents an important target for developing effective therapeutics (27,28).

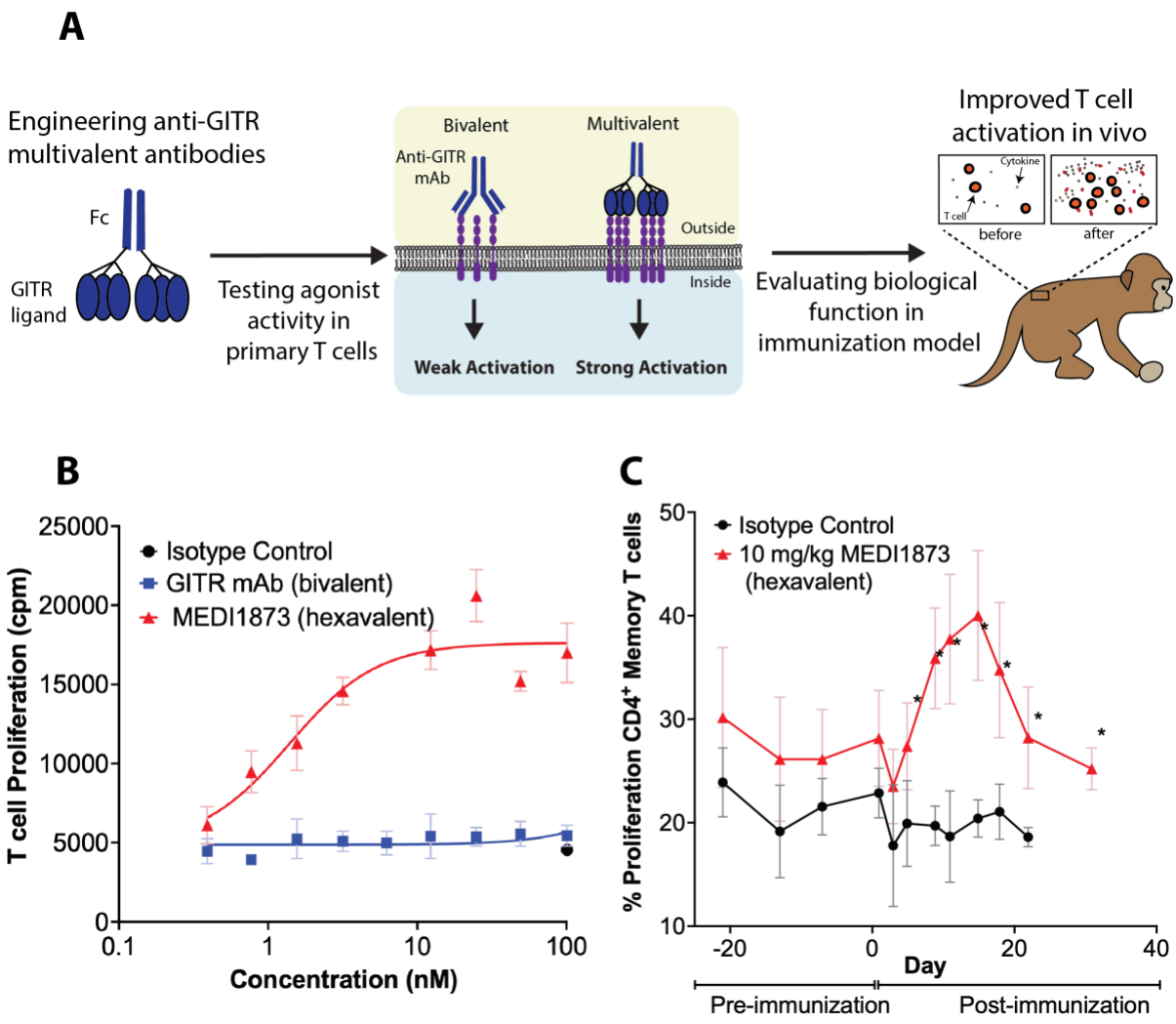

Figure 1-5. Multivalent Antibodies show potent agonist function compared to their bivalent IgGs. (**A**) Schematic illustration of an engineered anti-GITR multivalent antibody that shows potent receptor clustering and improved T cell activation. (**B**) The hexameric anti-GITR mAb (MEDI1873) antibody induces >3-fold improvement in thymine-incorporation with plate bound anti-CD3/CD28 antibodies suggesting its ability to enhance T cell proliferation. (**C**) The in vivo cynomolgus monkey model demonstrates that MEDI1873 significantly ($p<0.05$) augments proliferative (Ki67+) memory T cells from day 5 onward compared to the negative control. Adapted from (27).

The role of valency is further bolstered by a similar study where multivalent nanobodies were designed to improve DR-5 receptor signaling (29). To do this, nanobodies were fused together with a flexible glycine-serine linker to create trivalent and tetravalent constructs. These formats significantly reduced tumor cell viability by 40%, demonstrating a greater apoptotic

response compared to the conventional bivalent IgGs. Interestingly, the agonist activity of the trivalent nanobody was comparable to the natural ligand as measured by their caspase activity. Other studies on TNF receptors including OX40 further support the claim that higher-order valency mediates strong receptor activation (10,30).

The success of multivalent antibodies in preclinical settings has garnered great interest for their use in early-stage clinical trials. This is exemplified by IGM-8444, an anti-DR5 multivalent mAb, which is currently undergoing a phase 1 study (NCT04553692) for relapsed and refractory cancer, including colorectal cancer, sarcoma, and chronic lymphocytic leukemia (31). The objective of this study is to investigate the efficacy of IGM-8444 monotherapy with dose escalation. Additionally, this multivalent mAb is also being tested in combination therapies with chemotherapy drugs, targeted therapy, and second mitochondrial-derived activators of caspase (SMAC) mimetic drugs. Given it is in an early clinical stage, the efficacy of the drug remains to be seen. Another multivalent mAb against OX40 known as MEDI6368 has recently completed phase 1 clinical testing. In this trial, the OX40 mAb is being assessed as a monotherapy and combination therapy with PD-1 antagonist MEDI4736, but study results have not yet been published (NCT02221960) (32). With an ever-increasing interest in the potency of multivalent mAbs, we expect to see considerable progress in designing these antibodies for therapeutic use.

1.4.B. Antibody Specificity: Antibody specificity is an important property to consider for optimizing agonist function for TNF receptors. In the case of bispecific and biepitopic antibodies, studies have shown that these antibodies can bridge together multiple receptors to induce receptor superclustering without the need for FcyR-crosslinking. This section highlights the role of specificity in designing more effective mAb to improve their agonist activity.

Biepitopic antibodies that target two different epitopes on the same receptor provide a useful avenue to induce Fc-independent receptor clustering (**Figure 1-6a**). In a recent study, investigators engineered biepitopic tetravalent antibodies targeting the OX40 receptor to understand their effect on T-cell proliferation (10). These unique antibodies demonstrated a >3-fold improvement in CD4+ T cell proliferation compared to their bivalent IgGs (**Figure 1-6b**). These findings were further corroborated by murine studies that showed comparable results of >2-fold proliferation of effector CD4+ T cells and enhanced IFN-γ production (**Figure 1-6c**). To understand the mechanism, the investigators conducted structural-based studies on the biepitopic antibodies which revealed their ability to cluster nearby receptors through daisy-chaining manner. Taken together, designing biepitopic antibodies provides a novel approach to mediate receptor clustering for improving agonist function.

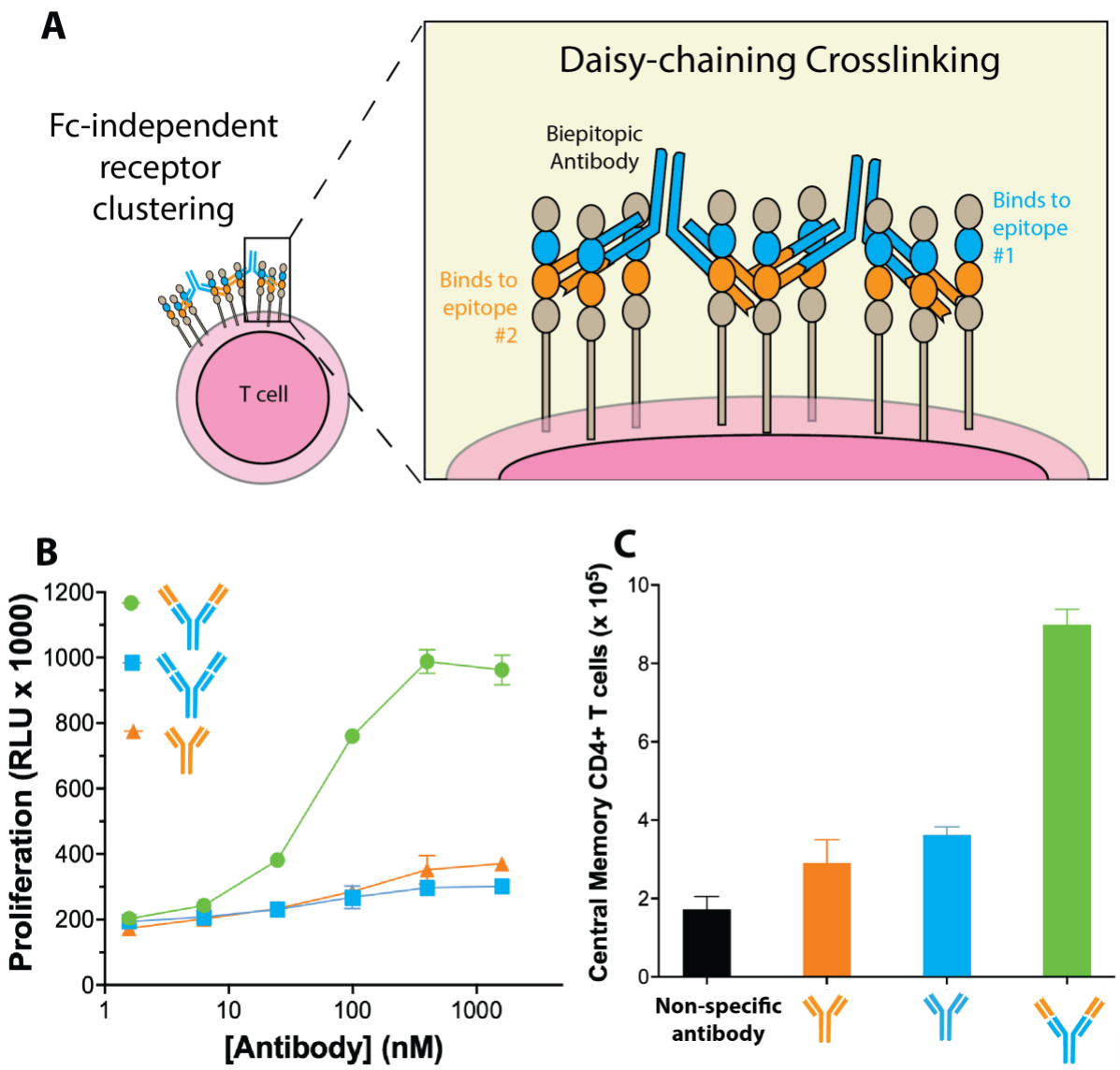

Figure 1-6. Biepitopic antibodies that target two binding epitopes on the same receptor impart superior receptor agonism in an Fc-independent manner (**A**) Schematic illustration of biepitopic tetravalent antibodies targeting an OX40 receptor undergoing receptor clustering through daisy-chaining crosslinking. (**B**) Biepitopic tetravalent antibodies display superior CD4+ T cell proliferation compared to their monoepitopic tetravalent and bivalent counterparts in the absence of Fc-crosslinking and CD28 co-stimulation. (**C**) Pharmacodynamic analysis of tetravalent biepitopic antibody, parent OX40 antibodies, and non-specific antibody administered to keyhole limpet hemocyanin (KLH)-immunized human OX40-expressing mice. As shown,the biepitopic tetravalent construct significantly increased central memory CD4+ effector T cells ($p<0.001$) compared to parental bivalent antibodies. Adapted from (10).

Bispecific antibodies provide another tool for inducing enhanced receptor clustering by targeting two different antigens on the cell surface. In one study, investigators constructed a unique antibody that targets two different TNF receptors, namely CD137 and OX40, to improve antitumor immunity (33). To do this, the bispecific antibody (FS120) was engineered to bind CD137 via the Fab region and the OX40 receptor via the Fc region. *In vitro* studies showed that FS120 mediates strong IL-2 production with >20 fold-improvement compared to monospecific antibodies. Furthermore, murine studies demonstrated that FS120 elicited > 1.5-fold reduction in tumor volume and >3-fold greater $CD4^+$ T cell proliferation. This phenomenon is also supported by other studies including an antibody targeting the EGFR and CD137 receptors which led to an improved agonist function (34–36). Collectively, these studies highlight how bispecific antibodies can be used to achieve potent agonist activity without the need for Fc-mediated clustering.

In terms of clinical applications, bispecific antibodies have been assessed for their ability to mediate anti-tumor efficacy in early-stage clinical trials. This is exemplified by a novel bispecific antibody, FS120, which has successfully transitioned to phase 1 testing for patients with advanced or metastatic cancer (NCT04648202). The objective of the trial is to assess key parameters including safety, dosing, and efficacy of the bispecific antibody in mediating anti-tumor response. In addition, another bispecific antibody (ATOR-1015) that targets CTLA-4 and OX40 has been shown to improve anti-tumor immunity (37). It is currently under evaluation in phase 1 clinical trial for advanced solid malignancies (NCT03782467). Thus far, the preliminary results indicate that the treatment is well-tolerated at doses of less than 200 mg and further dose escalation is currently being explored. Although these clinical trials are still in progress, there is great interest in developing bispecific antibodies for clinical use.

1.4.C. Antibody Isotype: Antibody isotype has been implicated as a major determinant for mediating receptor activation. Immunoglobulin G (IgG) is comprised of four major subclasses (IgG1-4) that are distinct in the number of cysteine bonds in their hinge region and impart diverse effector functions (38). Here, we highlight recent studies that highlight the use of isotype engineering approaches to develop potent cancer therapeutics against a variety of TNF receptors.

One major theme in isotype selection is the inverse relationship between hinge region flexibility and improved agonist function (**Figure 1-7a**). A recent study constructed a panel of CD40 antibodies with distinct constant domains (i.e., IgG1-4) to evaluate the impact of the CH1-hinge region on agonist function (39). Using a murine model, each isotype exhibited differential levels of $CD8^+$ T cell activation where IgG2 imparts a > 6-fold improved response compared to other isotypes (**Figure 1-7b**). Next, the researchers sought to investigate the biophysical properties of each isotype in mediating divergent agonist activity. Structural analysis revealed that the IgG2 isotype had the most rigid hinge region whereas IgG3 was the most flexible. This finding demonstrates that rigidity of the IgG2 hinge region led to improved clustering of CD40 receptors compared to other isotypes (40,41). This phenomenon has been observed for other TNF receptors including CD137 which highlights that IgG2 antibodies, specifically the more rigid B isoform, impart superior agonism in T cells (42).

The murine tumor models show improved agonist activity with IgG2 compared to other isotypes. This is exemplified in a CD40 study where IgG2 mAb significantly inhibited MC38 colon cancer cell growth compared to its other IgG counterparts (**Figure 1-7c**).(41) Similarly, IgG2 antibodies targeting CD137 receptor also displayed improved anti-tumor efficacy (43). One possible explanation for the superior activity is its ability to mediate both regulatory T cell (Tregs) depletion and effector CD8+ T cell function. Unlike other IgG isotypes, IgG2 can interact with activating

Fcγ receptors which play a key role in depleting Treg population to improve anti-tumor immunity. Other TNF receptors such as OX40 and GITR have also demonstrated IgG2's ability to mediate potent Treg depletion and enhanced anti-tumor response (44,45).

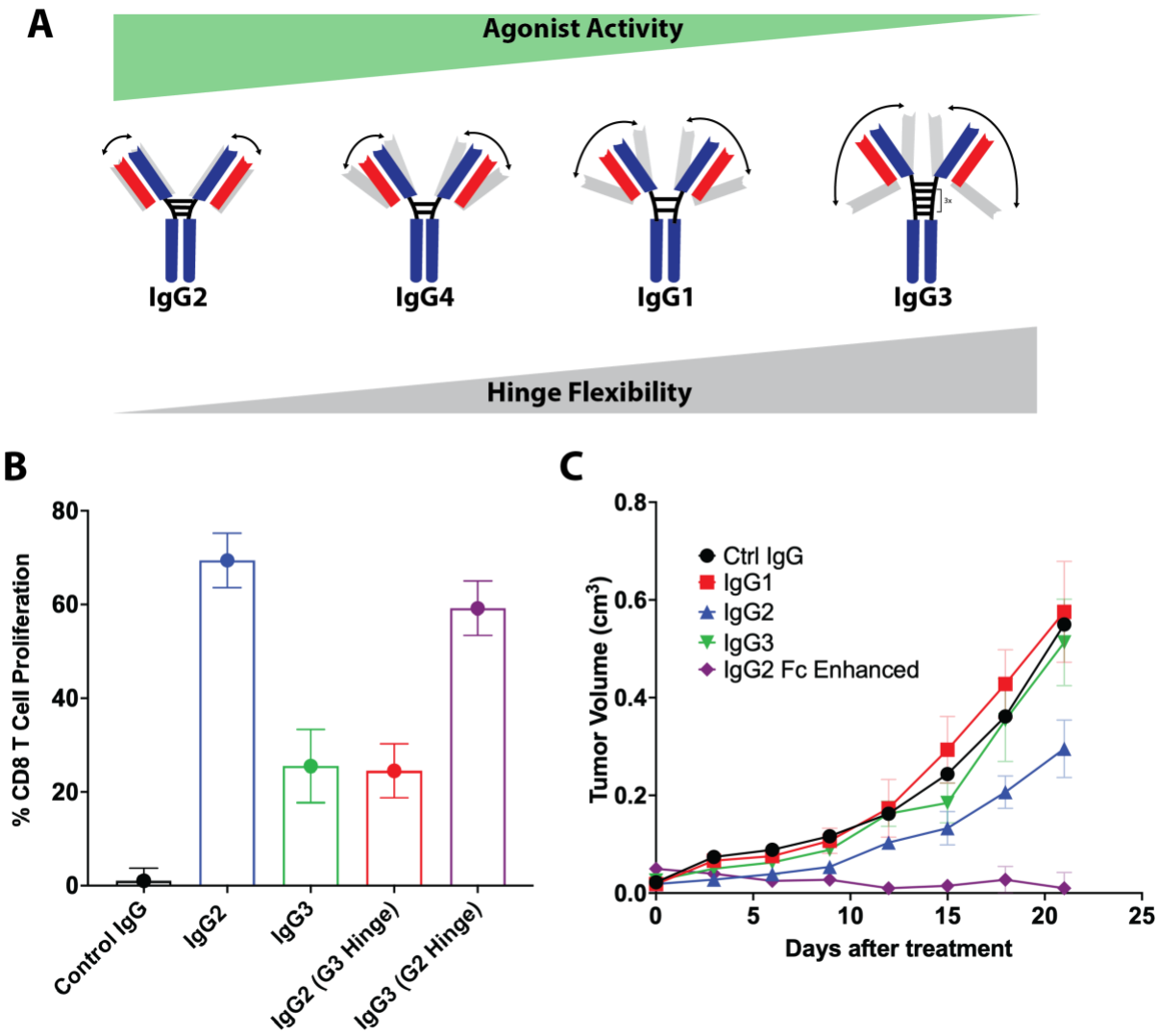

Figure 1-7: The inverse relationship between hinge region flexibility of varying isotype and antibody agonism. (**A**) Schematic illustration of negative correlation between agonist activity of CD40 receptor and CH1-hinge region flexibility. (**B**) The IgG2 isotype of CD40 antibodies display superior CD8+ T cell response ($p<0.0001$) compared to their IgG3 counterparts. Swapping the CH1-hinge regions of IgG2 and IgG3 exchanges their respective activity, demonstrating that receptor activation is dependent on hinge region flexibility. (**C**) Anti-tumor studies of CD40 antibody in varying isotypes were conducted using FcγR-humanized mice inoculated with MC38 tumor cells. The IgG2 isotype and Fc enhanced mAb significantly diminished tumor growth ($p<0.01$) while the IgG1 and IgG3 isotype had insignificant effect. Adapted from (39).

A unique phenomenon of isotype switching has been observed in a recent study where the CD40 IgG4 antagonist antibody (bleselumab) was engineered to express as IgG1 and IgG2 isotype (40) . Interestingly, the IgG1 isotype retained its antagonism as expected whereas the IgG2 isotype was converted to a super-agonist. *In vitro* studies illustrated that IgG2 isotype induced >3-fold improvement in B cell proliferation compared to the most effective clinical CD40 antibody, CP-870,893. These findings were further corroborated in *in vivo* model, where switching to IgG2 isotype induced > 4-fold CD8+ T cell expansion. This phenomenon was also generalized to other CD40 antagonists and showed improved agonistic activity with isotype switching. Although the exact mechanism of the super-agonist properties of IgG2 remains unknown, it is possible that the rigidity of IgG2 overrides the antagonist nature of the molecule through enhanced receptor clustering.

Given their importance, IgG2 antibodies that target TNF receptors including CD137 and CD40 are currently undergoing early phase clinical trials. In a recent trial a CD137 antibody known as utomilumab has shown to be well tolerated in patients with advanced solid tumors (NCT01307267).(46) In terms of therapeutic efficacy, monotherapy treatment only showed a mild improvement in mediating antitumor immunity. This led the investigators to use this antibody with other immune targeting drugs such as rituximab in a combination trial that yielded positive clinical outcomes (47). For instance, 20% of patients with advanced solid tumors including colorectal and pancreatic cancer demonstrated complete or partial tumor reduction while > 42% maintained stable disease. The tumor biopsy studies showed amplified T cell activation and tumor cytotoxicity which is consistent with an improved antitumor response. Finally, antibodies targeting CD40 in a combination trial have yielded comparable results in early phase clinical trials (48,49). Despite

IgG2 displaying improved disease outcome in pre-clinical models, it remains to be seen whether IgG2 is indeed clinically superior to other IgG isotypes.

1.3.D Fc Interactions: The interaction between IgG Fc regions and Fcγ receptors is a crucial factor that influences agonist activity. Briefly, Fcγ receptors are distinguished into five activatory (i.e., FcγRIIA) and one inhibitory (FcγRIIB) receptor characterized by their unique affinity for different IgG antibodies (50). Recent studies have highlighted the importance of Fc engineering methods to improve Fc:FcγR interactions and develop potent immunotherapeutics.

One theme is the introduction of favorable mutations in the Fc regions that selectively increase affinity to inhibitory receptor FcyRIIB (**Figure 1-8a**). This is effective because FcyRIIB is solely used as a scaffold for clustering monomeric receptors to meditate strong agonist activity (51).To demonstrate this point, researchers constructed CD40 mAbs with Fc mutations such as S267E (SE) and S267E/L328F (SELF) which increased binding to both inhibitory and activatory Fcγ receptors (52). Compared to wildtype, these mutants demonstrated >2-fold improvement in CD8 T cell activation, suggesting improved T cell immunity. Given SE and SELF mutations increase affinity to both types of receptors, the investigators reasoned that selective engagement to FcyRIIB could further improve agonist function. To test this hypothesis, additional Fc mutants were developed to selectively optimize Fc engagement of FcyRIIB over FcγRIIA. The results demonstrated that the V11 mutant (contain 5 mutations) resulted in a >97-fold improvement in affinity to FcyRIIB while FcγRIIA binding was reduced by 3-fold. This mutant displayed significant improvement in CD8+ T cell activation compared to the wild-type antibody and >5-fold improvement over the SELF variant (**Figure 1-8b**). Next, *in vivo* tumor studies demonstrated that while the SELF variant reduced tumor volume by 65%, the V11 variant completely abrogated tumor growth (**Figure 1-8c**). These findings indicated that high FcγRIIB/FcγRIIA ratio of binding

affinity presents a unique approach to improve the efficacy of agonist antibodies. Other studies on CD137 and OX40 antibodies highlight comparable results where selective point mutations in the Fc region can dramatically improve agonist activity (53,54).

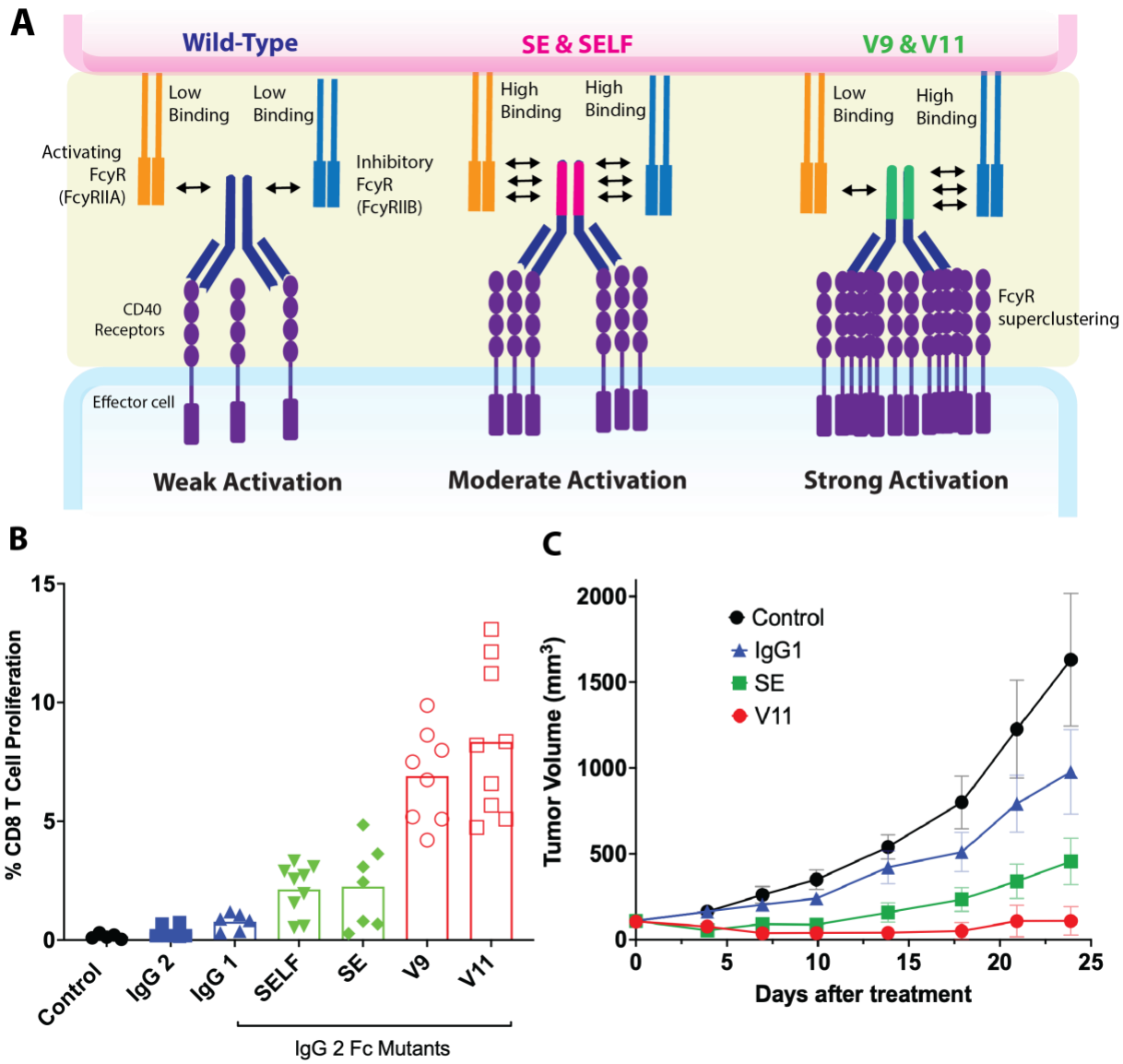

Figure 1-8. Selective FcyR engagement is required for optimal antibody agonism and anti-tumor activity. (**A**) Schematic illustration of the impact of activating and inhibitory FcyRs on CD40 receptor agonism. As shown, the selective binding to the inhibitory receptor FcyRIIB is positively correlated with optimal receptor activation. (**B**) Administration of the IgG 2 CD40 antibody (CP-870,893) with varying Fc mutations in humanized FcyR/CD40 mice. Antibody variants with selective affinity for the FcyRIIB receptor (V9/V11) have improved CD8 T cell activation ($p<0.01$) compared to antibodies with increased affinity for all Fcy receptors (SE/SELF). (**C**) Humanized CD40/FcyR mice inoculated with MC38 colon

tumors were treated with CD40 antibodies. Adding the FcyRIIB-selective V11 mutation significantly improves anti-tumor activity ($p<0.1$) compared to control antibodies. Adapted from (51).

Although improving affinity to Fcγ receptors is a viable option, this approach inherently depends on the availability of Fc receptors on antigen-presenting cells (APCs). Fc mutations that promote Fc-Fc interactions provide a unique method for improving agonist activity in a FcyR-independent manner. This is highlighted in an OX40 study where a set of Fc mutations (E345R, E430G, S440Y) promote hexamerization of IgG mAbs (55). Compared to wildtype, the double mutant (E345R/E430G) showed the highest multimerization of receptor clusters in solution followed by the triple and single mutations. Next, *in vitro* NF-kB assay was used to assess their biological response where single mutation (E345R) induced the highest dose-dependent response compared to the double and triple mutants. The authors hypothesized that the discrepancy between receptor multimerization in solution and agonist activity was partly due to the E345R mutation forming a favorable hexameric configuration upon antigen binding. Similar studies in other TNF receptors including DR-5 demonstrate these favorable Fc mutations led to enhanced receptor agonism (56,57).

While the function of the inhibitory Fcγ receptor is well understood, activating Fcγ receptors are equally as important in mediating anti-tumor immunity. This is because activating FcγRs can induce antibody dependent cellular cytotoxicity (ADCC) to deplete Tregs which is a critical component of the anti-tumor response. To demonstrate this point, investigators highlighted the role of activating FcγRs on OX40-mediated Treg depletion (58). The engagement of an OX40 mAb with activating FcγRs significantly depleted tumor infiltrating Treg cells while maintaining the CD8+ T cell subpopulation in a murine model, indicating the importance of activating receptors

for mediating effective anti-tumor response. This observation is further supported by CD137 studies where binding to activating receptors resulted in amplified anti-tumor response(42).

Several Fc-enhanced antibodies have entered early-stage clinical trials to determine their practicality as a cancer treatment. A CD40 agonist mAb known as CP-870,893 (selicrelumab) engineered with the V11 Fc mutation is currently being evaluated in a phase I combination trial with carboplatin and paclitaxel (NCT00607048) (59). This treatment has shown promise against melanoma cancer, where two patients displayed an increase in T-cell response. In terms of advanced solid tumors, 20% of patients exhibited a moderate decrease in target lesions, suggesting mild improvement in anti-tumor efficacy. Additionally, a CD137 antibody (LVGN6051) containing Fc mutations is also undergoing phase I clinical trials for advanced/metastatic cancer as a single agent and in combination with Keytruda, a humanized PD-1 inhibitor (NCT04130542) (60). Thus far, preliminary data indicates no adverse effects in monotherapy and only mild effects in combination therapy. In particular, a patient with metastatic head and neck squamous cell carcinoma has experienced 50% tumor reduction lasting for more than 6 months in combination therapy (61). Given the early positive outcome of this combination therapy; investigators are optimistic regarding the success of this antibody in early phase clinical trials. Finally, a DR-5 antibody GEN1029 containing Fc hexamerization mutations is currently being evaluated in a phase I clinical trial (NCT03576131) (62). Thus, Fc-engineered antibodies have shown great promise in preclinical and clinical settings, strengthening their potential as cancer immunotherapeutics.

1.4.E. Receptor Epitope and Occupancy: Receptor epitope selection and occupancy are critical considerations for optimizing agonist activity of therapeutic antibodies. The structure of TNF receptors (i.e., OX40, CD40, and CD137) consists of cystine rich domain (CRD) subunits that can be targeted by antibodies to impart superior agonist activity.

Mounting evidence shows that antibodies that target CRD regions outside the ligand-binding domain are correlated with improved agonist function. **(Figure 1-9a).** In the case of CD40 and CD137 receptors, antibodies that target membrane distal CRD1 domain have significantly higher agonist activity compared to ligand-blocking antibodies that bind to CRD 3 and 4 (63,64) . This is because antibodies that target distal domains allow for access to Fcγ receptors with reduced steric hindrance compared to antibodies targeting proximal domains. In a recent CD137 study, the epitope selection of clinical antibodies, urelumab and utomilumab, proved influential in mediating potent agonist activity. The structural analysis revealed that urelumab binds to CRD1 while utomilumab binds near the ligand site on CRD3 and 4. Next, the functional studies in CD8+ T cells showed that the non-blocking antibody urelumab elicited >2 fold greater IL-2 and IFN-ɣ cytokine secretion than ligand-blocking utomilumab **(Figure 1-9b).** Finally, the synergistic impact of native ligand and clinical antibodies were evaluated for their ability to mediate clustering where urelumab resulted in >4 times more receptor clusters compared to utomilumab, suggesting a greater enhancement in agonist function **(Figure 1-9c)** (64). Further studies on other TNF receptors such as OX40 showed that non-blocking antibodies exhibit potent anti-tumor response (44,65).These findings demonstrate the importance of receptor epitope selection as a key consideration for engineering potent therapeutic antibodies.

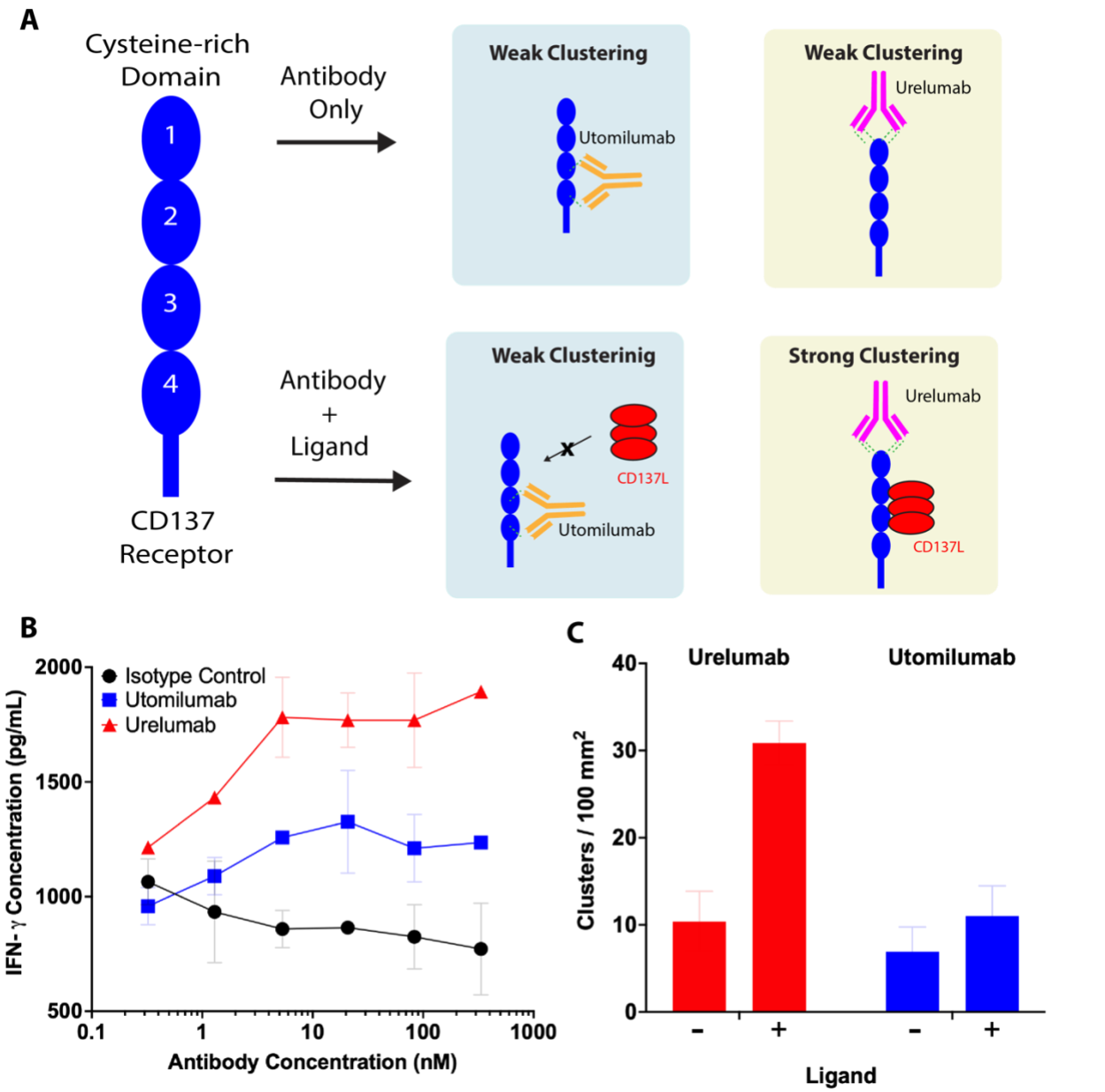

Figure 1-9. Domain binding influences the agonist activity of TNF receptors. (**A**) Schematic illustration of the impact of receptor epitopes on the agonist activity of two CD137 antibodies urelumab and utomilumab. As shown, a non-ligand-blocking urelumab (pink) targets membrane-distal CRD1 which allows greater access to Fcy receptors and can cluster ligand-bound receptors, resulting in enhanced agonist activity. In contrast, the ligand-blocking utomilumab (orange) binds to CRD2-3 which causes steric hindrance and reduces agonist function. (**B**) Urelumab induces higher IFN-γ cytokine secretion than utomilumab in primary CD8+ T cell assay. (**C**) The impact of ligands on antibody-bound receptors is quantified by measuring the number of receptor clusters on Jurkat cells facilitated by urelumab and utomilumab. The addition of the CD137 ligand significantly ($p<0.001$) improves the clustering potential of urelumab compared to urelumab alone. Adapted from (64).

The co-stimulation between two different TNF receptors has also been explored to evaluate their impact on agonist function. Towards this goal, a study on OX40 and CD137 receptors examined the influence heterodimers in mediating T cell proliferation (66). The results showed that heterodimer complexes of OX40/CD137 provide distinct intracellular signal compared to OX40 or CD137 homodimers. Unexpectedly, the combination dosing of OX40 and CD137 antibodies exhibited a reduction in NF-kB signaling by 2.5-fold compared to the signal induced by OX40 or CD137 antibodies alone. To understand the mechanism, the investigators demonstrated that OX40/CD137 heterodimers are weakly associated with TRAF1, which is critical for potent NF-kB activation. Interestingly, the concomitant stimulation with OX40 and CD137 antibodies led to an inhibitory signal in CD4+ and CD8+ T cells suggesting an apoptosis response mediated by heterodimer clustering; however, the mechanism remains poorly understood. Other studies highlight that heterodimerization leads to pleiotropic effects on TNF receptor signaling and further work is still needed to determine their role on agonist function (67).

Receptor occupancy is also an important criterion for establishing optimal agonist function. Unlike antagonist antibodies that show sigmoidal dose-response, agonist antibodies exhibit a bell-shaped dose-response curve, suggesting maximum activity occurs at intermediate concentrations (68–70)To highlight this phenomenon, the investigators demonstrated that OX40 antibodies mediated potent agonist activity at intermediate concentrations using a murine model (71). To further test this hypothesis, the researchers examined antibody-induced proliferation of human T cells and found that approximately 40% receptor occupancy led to maximum CD4+ T cell proliferation suggesting a non-linear relationship between antibody dose and agonist activity.

Similarly, this phenomenon has been observed for other T cell receptors including CD28 where occupying half of the receptors on the cell surface leads to sufficient receptor activation (72,73).

Receptor epitope selection is an important consideration when designing effective antibodies for cancer therapeutics in clinics. One of the most compelling cases is the CD137 agonist antibody urelumab (BMS-663513) that binds to CRD1 and has been the focus of multiple phase 1 clinical trials. In a combination trial (NCT01471210 & NCT01775631) with rituximab (CD20 mAb), participants with refractory B-cell lymphoma and follicular lymphoma were treated with urelumab monotherapy and combination therapy. The combination trials demonstrated promising results where 35% of follicular lymphoma patients experienced a >30% tumor decrease, and 71% had stable disease, suggesting improved disease outcome. As a result, urelumab is proceeding to further clinical testing in combination with other drugs. The most notable example is an ongoing combination trial (NCT03792724) with PD-1 inhibitor nivolumab for patients with B-cell lymphoma. In the future, more clinical studies may be necessary to explore the relationship between epitope selection for designing effective clinical antibodies.

Given receptor occupancy is important, dosing regimen must be critically considered for therapeutic antibodies. For example, an OX40 antibody, BMS-986178 that has shown peak agonist function at ~ 40% receptor occupancy in preclinical settings is the subject of multiple clinical trials (74). In a phase I trial (NCT02737475), BMS-986178 has been tested as a monotherapy and combination therapy with nivolumab and ipilimumab (CTLA-4 antagonist) in 165 patients with advanced solid tumors. Amongst all treatment cohorts, only a small percentage (<15%) of participants experienced moderate to high-grade adverse effects, and no toxicity was served even at the highest dose of 320 mg BMS-986178, indicating that the maximum tolerable dosage was not reached in this study. In terms of treatment efficacy, 12% of patients experienced a >30%

tumor shrinkage, and one bladder cancer patient achieved complete eradication of cancerous tumors in nivolumab combination therapy. In combination cohorts with nivolumab and ipilimumab, the investigators also observed an increase in proliferating CD8+ T cells along with a decreasing percentage of Fox3+ regulatory T cells associated with an anti-tumor response. Although the biological effects of the treatment were moderate, there are many easily adjustable parameters that could positively influence the outcome of future BMS-986178 studies. First, researchers must determine the maximum tolerable dose to accurately evaluate the clinical efficacy of BMS-986178 therapy. Additionally, tumor samples revealed that over 80% of tumors expressed <1% of OX40 receptors, suggesting a more personalized approach to treating patients may augment differential OX40 receptor expression. To overcome these challenges, preclinical studies indicate receptor expression can be boosted with vaccines, toll-like receptor (TLR) agents, oncolytic viruses, and radiation. These methods to optimize receptor expression can be applied to future clinical trials to improve clinical efficacy.

1.4.F. Tumor-targeting Bifunctional Antibodies: Targeting tumor-associated antigens (TAAs) provides a useful approach for improving the function of agonist antibodies by directing therapeutics to the tumor microenvironment. To engineer such antibodies, TAA-specific proteins can be conjugated to the desired antibody to enhance tumor site localization and payload delivery (75,76). These approaches have been utilized to design antibodies that selectively target TAAs and activate TNF receptors to increase their therapeutic potential for cancer immunotherapy.

One key advantage of engineering TAA-specific antibodies is their improved ability to target tumor sites while minimizing off-target effects. In one such study, investigators engineered antibodies that target both DR5 and fibroblast-activation protein (FAP) to improve the agonist function of DR5 antibodies (**Figure 1-10a**) (77). Given FAP is highly expressed in the tumor

microenvironment, the researchers reasoned that FAP binding would lead to avidity-driven receptor clustering to induce strong antitumor activity. This simultaneous targeting also limits the dependency of Fc-mediated cross-linking which is a critical limitation of agonist antibodies. In an *in vitro* assay, the engineered antibody elicited a >2-fold improved apoptotic response compared to its parent DR5-targeting antibody (**Figure 1-10b**). Additionally, human xenograft mouse models showed that the antibody induced >4-fold decrease in tumor volume relative to a clinical antibody in an Fc-independent manner (**Figure 1-10c**). Using a patient-derived colorectal tumor model, this antibody demonstrated a significant decrease in tumor burden which suggests its heightened applicability in clinical settings. Taken together, these results support the hypothesis that TAA-targeting antibodies provide a unique approach to improve tumor selectivity and agonist function for therapeutic antibodies.

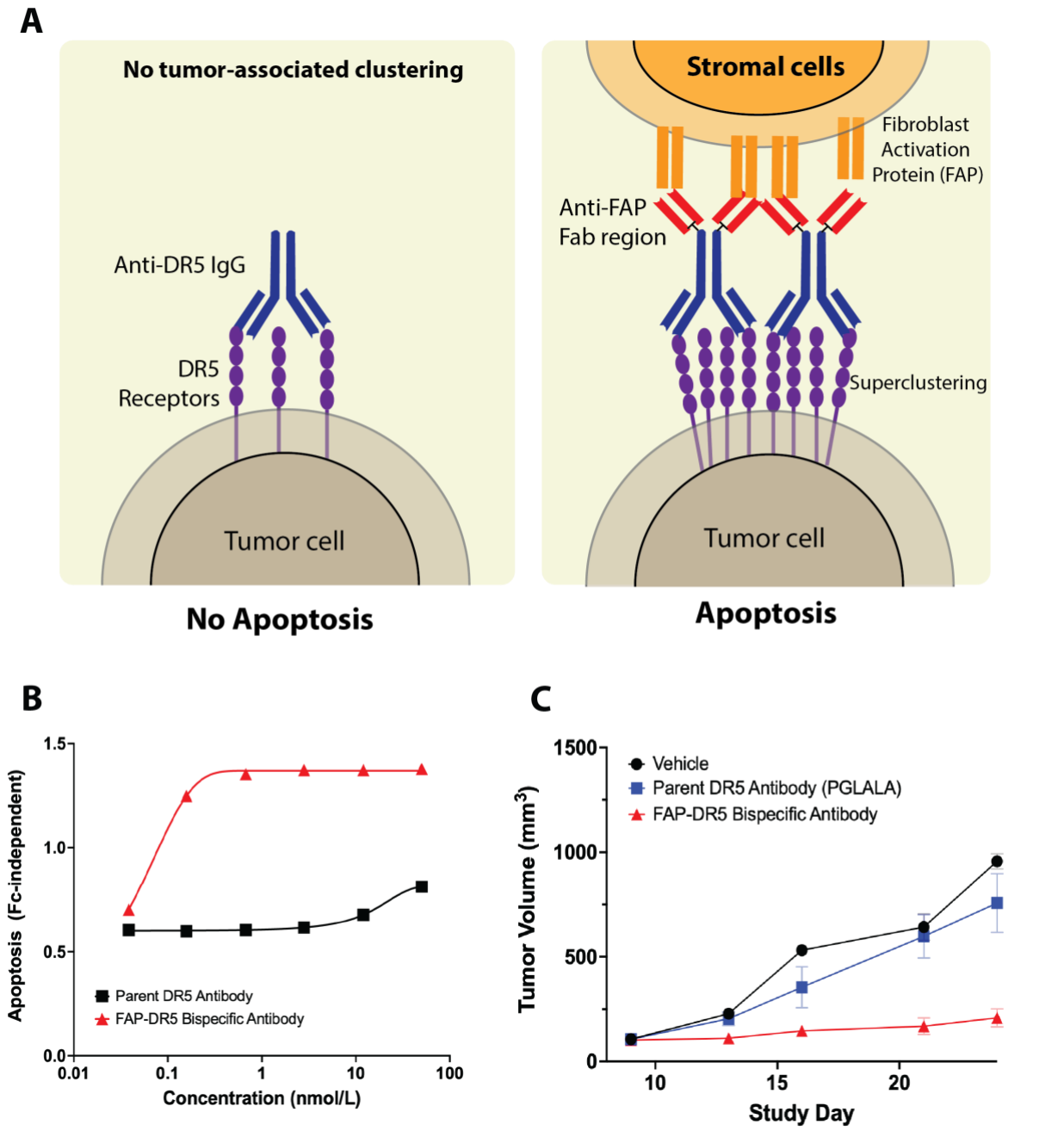

Figure 1-10. Bispecific antibodies that bind to multiple targets allows for Fc-independent receptor clustering. (**A**) Schematic illustration of tumor-specific antibodies targeting the DR5 receptor and binding to fibroblast activation protein (FAP) expressed on surrounding stromal cells for enhanced receptor clustering. (**B**) Novel FAP-DR5 bispecific antibody (RG7386) displays superior Fc-independent apoptotic activity compared to parent DR5 antibody in MDA-MB-231 breast cancer and GM05389 fibroblast coculture. (**C**) Anti-tumor studies of FAP-DR5 bispecific antibody RG7386 and FcyR-abrogated DR5 antibody (drozitumab) were conducted using mouse xenograft model inoculated with LOX-IMVI desmoplastic melanoma cells. RG7386 significantly reduced tumor volume compared to the DR5 antibody. Adapted from (77).

Another method that has shown great promise is the conjugation of antibodies with extracellular matrix (ECM) peptides to improve drug localization at the tumor site. Previously, the heparin-binding domain of placenta growth factor-2 (PIGF-2) that binds to ECM proteins have been conjugated to antibodies that target immune checkpoint inhibitors (78) . To test this approach for agonist antibodies, the investigators engineered a CD40 antibody with the super-affinity PIGF-2 peptide to improve antitumor potency and reduce adverse effects.(79) Using a murine model, the antibody-peptide conjugate demonstrated >4-fold greater retention at the injection site compared to the unconjugated antibody. Next, the antitumor profile was measured using multiple tumor models including B16F10 melanoma tumors, which demonstrated that the antibody reduced tumor growth by >3-fold. In terms of T-cell mediated response, the antibody increased the ratio of CD8+/Treg cells in the tumor microenvironment which is indicative of an improved anti-tumor response. These findings point to a novel method of designing optimized agonist antibodies with improved tumor localization.

Antibodies that target both tumor antigens and TNF receptors such as CD40 and CD137 have shown clinical significance as cancer therapeutics. One such antibody that is currently being evaluated in phase 1 clinical trials is RG7827(also known as RO7122290) which binds to the CD137 receptor and FAP (80). Preclinical studies have demonstrated that RG7827 is able to decrease off-target adverse effects and improve agonist function. The safety, dosage, and anti-tumor efficacy of RG7827 is currently being evaluated in a combination trial with atezolizumab (PD-L1 inhibitor) for advanced solid tumors (EUDRACT Number: 2017-003961-83; Protocol Number: BP40087). The preliminary results showed an increase in soluble CD137 expression in patients' blood which is a primary indicator of T cell activation. Furthermore, tumor biopsies

revealed an increased level of proliferative CD8+ T-cells as measured by the Ki67+ proliferative marker. Due to the treatment having a tolerable safety profile with <10% incidence of high-grade adverse events, the maximum tolerated dose was not reached which warrants further dose-escalation studies. RO7300490, which targets CD40 and FAP, is another antibody that is currently being evaluated for its efficacy in ongoing phase 1 clinical trials (NCT04857138). The successful translation of these bispecific antibodies represents a novel approach to ameliorate off-target effects while improving the efficacy of current cancer therapeutics.

Conclusion

Agonist antibodies have shown great promise in mediating anti-tumor efficacy as cancer therapeutics. Despite their potential, these antibodies have faced many roadblocks that have hindered their progress in clinical settings. Recently, designing antibodies with unique binding epitopes, valency, specificity, Fc-mediated interactions, and isotypes has enabled the optimization of their biological function. These approaches have served a critical role in improving receptor activation while mitigating the current limitations of IgG mAb therapy. Their success in a preclinical setting has led to the evaluation of these antibodies in numerous early phase clinical trials. Further research and development are needed to fully uncover their potential as cancer therapeutics. Altogether, agonist antibodies provide a promising approach to treat a variety of pathological afflictions.

Acknowledgement

The introduction entails information from my first author review paper (to be submitted) titled "Unlocking the potential of agonist antibodies for treating cancer using antibody engineering". My undergraduate student, Timon and Emily, made substantial contributions in preparing the manuscript. Additional, Dr. Peter Tessier made significant contributions in revising the manuscript.

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Chapter 2: Facile Generation of Biepitopic Antibodies with Intrinsic Agonism for Activating Receptors in the Tumor Necrosis Factor Superfamily

Abstract

Agonist antibodies that activate cellular receptors are being pursued for therapeutic applications ranging from neurodegenerative diseases to cancer. For the TNF receptor superfamily, higher-order clustering of three or more receptors is key to their potent activation. This can be achieved using antibodies that recognize two unique epitopes on the same receptor and mediate receptor superclustering. However, identifying compatible pairs of antibodies to generate biepitopic antibodies (also known as biparatopic antibodies) for activating TNF receptors typically requires animal immunization and is a laborious and unpredictable process. Here we report a simple method for predictably identifying biepitopic antibodies that potently activate TNF receptors without the need for additional animal immunization. Our approach uses off-the-shelf, receptor-specific IgG antibodies, which lack intrinsic (FcγR-independent) agonist activity, to first block their corresponding epitope. Next, we perform selections for single-chain antibodies from human nonimmune libraries that bind accessible epitopes on the same ectodomains using yeast surface display and fluorescence-activated cell sorting. The selected single-chain antibodies are finally fused to IgGs to generate human tetravalent antibodies that engage two different receptor epitopes and mediate potent receptor activation. We highlight the broad utility of this approach by converting several existing clinical-stage antibodies against TNF receptors, including ivuxolimab

and pogalizumab against OX40 and utomilumab against CD137, into biepitopic antibodies with highly potent agonist activity in primary T cell cultures. We expect that this widely accessible methodology can be used to predictably generate biepitopic antibodies for activating other receptors in the TNF receptor superfamily and many other receptors whose activation is dependent on strong receptor clustering.

Introduction

Antibodies that target immune checkpoints are an important class of emerging therapeutics due to their ability to regulate immune cell function (1). In the context of cancer, the tumor microenvironment is often plagued by immunosuppression, which can be reversed by targeting co-inhibitory and co-stimulatory receptors on T cells (2, 3). Tumor cells have been shown to hijack these immune checkpoints to evade clearance by the host immune response (4). Specifically, monoclonal antibodies that block the immunosuppressive interactions on co-inhibitory receptors have shown potent anti-tumor responses. Encouragingly, numerous immunotherapies have been developed for these inhibitory checkpoints and many have received FDA approval for treating a variety of cancers, including those against PD-1 (*e.g.*, pembrolizumab, nivolumab, and cemiplimab), PD-L1 (*e.g.*, atezolizumab, durvalumab, and avelumab) and CTLA-4 (*e.g.*, ipilimumab) (5). While these therapies have demonstrated impressive efficacies, the benefits are limited to only a subset of patients and there remains an urgent clinical need for alternative or complementary immunotherapy approaches (6, 7).

The development of agonist antibodies that activate co-stimulatory checkpoints, such as the tumor necrosis factor (TNF) receptor superfamily (*e.g.*, OX40, CD137, CD40), has garnered intense interest. This is evident in the myriad of ongoing, early-stage clinical trials evaluating TNF receptor agonist monotherapy and combination immunotherapies in oncologic applications (8, 9).

Despite their promise, clinical translation has been muddled by challenges concerning safety and efficacy (10). In particular, OX40 and CD137 agonist antibodies (*i.e.*, bivalent IgGs) have been limited by poor-to-modest clinical activity, which is not surprising given that complete activation of OX40 and CD137 (like other TNF receptor superfamily members) requires trimerization and subsequent higher-order receptor superclustering (11, 12). Bivalent IgGs typically rely on engagement with Fcγ receptors (FcγRs) on antigen-presenting cells to achieve such higher-order receptor clustering (13, 14). However, FcγR expression is observed to vary greatly between different immune cells and is difficult to predict and control *in vivo* (15, 16). Overall, novel strategies to activate immune co-stimulatory receptors predictably and reliably in an FcγR-independent manner are anticipated to improve therapeutic limitations associated with modest clinical activity.

Recently, biepitopic antibodies targeting two non-overlapping receptor regions have emerged as a robust strategy to activate immune co-stimulatory receptors via the induction of extensive daisy-chain-like receptor superclusters (17). Encouragingly, potent agonist activity has been observed *in vivo* for tetravalent biepitopic OX40 antibodies lacking affinity for FcγRs. Conventional methods of generating such antibodies generally involve four key steps: i) immunizing animals; ii) isolating individual antibodies; iii) epitope binning antibodies to identify pairs with unique epitopes; and iv) combining pairs of antibodies into non-conventional (non-IgG) formats that present two unique antigen-binding sites in a single antibody with a total of three or more antigen binding sites (18–20).

Despite previous success using this approach (21, 22), it suffers from four main problems. First, animal immunization and subsequent monoclonal antibody identification are slow and unpredictable processes. Second, it is challenging to reliably obtain sufficient epitope coverage,

especially due to immunodominant epitopes. Third, the resulting antibodies typically need to be humanized for therapeutic applications. Even in cases where animals with humanized immune systems are used, the benefits of not needing to humanize are often diminished by reduced antibody diversity and epitope coverage. Fourth, the need to combine pairs of IgGs into non-native antibody formats, such as IgGs fused to single-chain antibodies, to achieve at a total of at least three antigen-binding sites can result in partial or complete loss of binding of the reformatted antibodies.

Therefore, we have sought to develop an approach that addresses each of these challenges and greatly simplifies the generation of biepitopic antibodies. First, we eliminate the need for additional animal immunization by using existing, off-the-shelf IgGs specific for the target receptor. While such antibodies are typically generated by immunization, there are many existing IgGs specific for most receptors of therapeutic interest and the key problem is to convert them into biepitopic antibodies in a simple and predictable manner. Second, we perform *in vitro* selections of single-chain antibodies after blocking the receptor epitope of the existing IgG, which results in simple and predictable isolation of pairs of antibodies with unique epitopes. Third, the *in vitro* antibody selections are performed using human non-immune libraries, which are the same libraries for every antigen and do not require humanization. Fourth, we perform *in vitro* selections for single-chain antibodies in the final antibody format that will be used to generate biepitopic antibodies, such as the IgG-single chain antibody format used in this work, which greatly reduces the risk of loss of binding due to reformatting. Herein, we demonstrate the utility of this approach using existing clinical-stage antibodies specific for multiple receptors in the TNF receptor superfamily that lack intrinsic (FcγR-independent) agonist activity and demonstrate how they can be predictably converted into biepitopic antibodies with potent activity to activate human T cells.

Experimental Method Section

Antibody discovery

The human non-immune library (library #1) (24) and synthetic library (library #2) (26) were expressed as Aga2-scFv fusion proteins on the surface of yeast using a standard yeast plasmid (pCTCON). Library #1 was used for OX40 and library #2 was used for CD137. For library sorting, two initial MACS selections were conducted using human OX40-Fc or CD137-Fc (produced in-house). 10^9 yeast cells were incubated with 300 nM of the antigen (OX40-Fc or CD137-Fc) in PBS supplemented with 1 g/L BSA (PBSB) and 1% milk at room temperature for 3 h. Post-incubation, the yeast cells were washed once with PBSB and incubated with Protein A microbeads (Miltenyi Biotec, Cat. #130071001) gently rocking for 30 min at 4 °C. Next, the cells were rinsed with PBSB and passed through a MACS column to isolate cells bound to beads under a magnetic field. The captured beads were washed once with PBSB under the magnetic field and subsequently eluted into SDCAA liquid media (2% glucose, 0.67% yeast nitrogen base, 0.54% Na_2HPO_4, 0.86% $NaH_2PO_4H_2O$, 0.5% casein amino acids) at 30 °C. Subsequent sorts were performed by FACS at antigen concentrations of 300 nM for FACS rounds 1-3 against biotinylated OX40 ectodomain (OX40: Acro Biosystems TN4-H82E4) and rounds 1-4 against biotinylated CD137 ectodomain (CD137: Acro Biosystems 41B-H82E6). Following the 3 h incubation (as described above), yeast cells (10-fold excess number of cells as the remaining library diversity) were washed with PBSB and labeled with

streptavidin-Alexa647 (Jackson laboratories, 016-600-084) at 1:300 dilution for OX40 and neutravidin-PE (Invitrogen, Cat. #A2660) for CD137 in terms of antigen binding and anti-myc (Invitrogen, Cat. #2276S 1:1000, Cat. #A11001 1:200) for antibody display. For FACS rounds 4-5 (OX40) and rounds 5-6 (CD137), 11D4 IgG or utomilumab IgG were prepared at equimolar

concentrations relative to the receptor ectodomain for the first round of competition sorting, and 10-fold excess for the terminal round of sorting. Finally, the cells were incubated with the ectodomain/IgG complexes for 3 h prior to labeling with goat anti-mouse AlexaFluor 488 (Invitrogen, A11001) at 1:200 dilution and neutravidin-PE (Invitrogen, Cat. #A2660, 1:300) or streptavidin-Alexa647 (Jackson laboratories, 016-600-084). Finally, the library was sorted using FACS and collected into SDCAA media. The cells were regrown, and Sanger sequenced.

Competitive binding analysis

Yeast cells expressing OX40 single-chain antibodies were prepared at 10^5 cells per well and washed twice with PBSB. They were then combined with different concentrations of biotinylated human OX40 ectodomain (Acro Biosystems TN4-H82E4) with or without a sixfold molar excess of soluble 11D4 IgG in PBSB containing 1% milk. Afterward, anti-myc antibody (Cell Signaling, #2276S) at 1:1000 dilution was added for 3 h at room temperature under gentle agitation. Following the antigen incubation step, yeast cells were centrifuged, washed once in ice-cold PBSB and incubated with labeling reagents. These reagents were goat anti-mouse AlexaFluor 488 (Invitrogen, A11001) at 1:200 dilution and streptavidin-Alexa647 (Jackson laboratories, 016-600-084) at 1:300 dilution, which were added on ice for 5 min. Afterward, the cells were washed once with ice-cold PBSB and analyzed by flow cytometry.

For the bead-based competition assay, antibodies were immobilized on Protein A magnetic beads (Pierce, 88846) following the manufacturers' protocol. The beads were washed twice with PBSB, blocked with 10% milk in PBSB, mixed end-over-end at room temperature for 1 h, and washed

once more with PBSB. In a 96-well plate, the beads (10^5 beads/well) were incubated with 100 nM biotinylated OX40 extracellular domain (Acro Biosystems, TN4-H82E4) in the presence or absence of a competition antibody (fivefold molar excess) in PBSB with 1% milk at room temperature for 3 h. Following the incubation, the beads were washed once with ice-cold PBSB and then incubated with streptavidin-Alexa647 (Jackson laboratories, 016-600-084) at 1:300 dilution on ice for 5 min. After labeling, the beads were washed once with ice-cold PBSB and evaluated by flow cytometry.

Isolation and sequencing of single-chain antibodies

The terminal sorts of the yeast-displayed libraries were miniprepped using the Zymo Research kit (D2004) to recover enriched yeast plasmids. To recover the scFv library genes, plasmids were first transformed into DH5α bacterial cells and plated overnight at 37 °C on LB agar plates with 100 μg/ml ampicillin. After incubation, individual bacterial colonies were picked and grown overnight in LB media supplemented with ampicillin, and then miniprepped using Qiagen Kit (Cat. #29106) for Sanger sequencing.

Soluble antibody expression and purification

Following scFv sequence identification, gene fragments (gBlocks Integrated DNA Technology) were designed to incorporate enriched scFv sequences at the C-termini of the light chains of the IgGs. To express these tetravalent antibodies, HEK 293-6E cells were grown and passaged in F17 media (Fisher Scientific, A1383502) supplemented with glutamine (Invitrogen, 2530081), Kollipher (Fisher Scientific, NC0917244) and G418 at 1.5-2 million cells/ml. Next, 15 μg of vector

plasmid (7.5 μg of variable light chain plasmid and 7.5 μg of variable heavy chain plasmid) and PEI (3-fold excess, 45 μg) were mixed in F17 media for 10-15 min at room temperature and added to cells at 1.5-1.8 million cells/ml. 24-48 h after transfection, cells were fed using 20% Yeastolate followed by another 2-4 d growth period at 37 °C. After protein expression, cells were collected via centrifugation at 2500 x g for 45 mins and transferred to new tubes. Next, 0.5-1 ml of Protein A beads (Thermo Fisher Scientific 89898) were added and gently rocked overnight at 4 °C. Protein A beads were collected from the media with vacuum filtration and washed with 50-100 ml of PBS. Glycine buffer (0.1 M, pH 3) was added followed by 1X buffer exchange into 20 mM pH 5 acetate using Zeba desalting columns to elute the desired protein from Protein A beads. Finally, the protein was filtered with 0.2 μm filters, the concentration was determined using nanodrop at A280, and the purity was analyzed using SDS-PAGE.

OX40 and CD137 HEK-293T binding analysis

HEK-293T cell lines were first engineered to express a stable doxycycline-inducible human OX40 or CD137 receptor (developed in-house). In 96-well plates, 50,000 OX40 or CD137 cells were plated with 100 ng/ml doxycycline for 2 d at 37 °C (5% CO_2). Afterward, antibodies were added on ice for a final volume of 200 μl. One hour later, the cells were spun down and washed with PBS at 300 x g for 3 min to pellet. After removal of excess media, the wells were incubated with goat anti-human Fc Alexa 647 at 1:300 dilution (Jackson ImmunoResearch Laboratories, 109-605-098) for 4 min on ice. Cells were then spun and washed with 150 μl, as described above, and then analyzed via flow cytometry.

OX40 Jurkat T cell assay

The OX40 bioassay (Promega, CS19770) was conducted following the manufacturer's protocol. Briefly, thaw-and-use NFkB-*luc2*/OX40 Jurkat cells (CS197702) were seeded in 96-well plates (Costar, 3917) and incubated overnight under 5% CO_2 at 37 °C. Serial diluted antibodies or control treatments were applied to NFkB-*luc2*/OX40 Jurkat cells and incubated for 5 hours at 37 °C under 5% CO_2. The assay plates were then equilibrated to room temperature for 10 min and 80 µL of Bio-Glo reagent (Promega, G7941) was added to each well. The assay plates were incubated at room temperature for 5 min, and chemiluminescence was measured using a Molecular Devices SpectraMax microplate reader set for 500 ms integration/well.

CD4+ T cell Proliferation Assay

Human peripheral blood CD4+ T cells were purchased from Stem Cell Technologies (Cat. #200-0165) and cultured with ImmunoCult-XF T cell Expansion media (Stem Cell Technologies, Cat. #10981) in an incubator at 37 °C with 5% CO_2. For the experimental assay, T cells were seeded in duplicate using 96-well plates with each well containing 50,000 T cells per 50 µl of T cell media. ImmunoCult Human CD3/CD28 T Cell Activator (Stem Cell Technologies, Cat. #10971) reagent was prepared at 5 µl/ml in T cell media, and then 100 µl was added per well. Antibodies were added (50 µL per well), which resulted in a total volume of 200 µl per well. Finally, the plates were incubated for 6 d before 100 µl of CellTiterGlo (Promega Cat. #G7573) was added per well and left to incubate at room temperature for 10 min. Luminescence was measured on a SpectraMax M3 microplate reader (Molecular Devices).

CD8+ T cell Proliferation Assay

Human peripheral blood CD8+ T cells were purchased from Stem Cell Technologies (Cat. #200-0164) and cultured with T cell Expansion media (Stem Cell Technologies, Cat. #10981) in an incubator at 37 °C with 5% CO_2. Antibodies were immobilized on Dynabeads (M-280 tosylactivated, Invitrogen), as described previously (46). Briefly, a total of 10 μg of antibody consisting of 2 μg of anti-human CD3 antibody (BE0001-2, Bio X cell) and 8 μg of experimental antibody in 1 ml of 1X PBS solution was incubated at room temperature for 2 d. The beads were subsequently blocked with a 10 mM glycine buffer for 1 h, followed by washing twice with PBS containing 0.1 % BSA. Next, the CD8+ T cells were plated at the density of 50,000 cells per well in a 96-well plate with a total volume of 150 μl of media solution and 50 μl of beads solution consisting of a 1:1 ratio of conjugated beads:T cells. After 96 h, the cells were stained with fluorescent-labeled anti-human CD8 BV510 (Biolegend Cat. #344732) and anti-CD25 APC (Biolegend Cat. #302610) antibodies at a dilution of 1:200, and analyzed by flow cytometry.

IL-2 ELISA

Human peripheral blood $CD4^+$ T-cells were incubated with OX40 antibodies in 96-well microplates (200 μL per well). After 48 h of incubation at 37 °C (5% CO_2), the supernatants were collected, and cytokine production was detected via ELISA using the manufacturer's protocol (R&D Cat #D2050). The absorbance was detected at 450 and 570 nm for signal and background readings using a SpectraMax M3 microplate reader (Molecular Devices). Finally, the concentration of IL-2 (pg/ml) was calculated using a standard curve.

Results

Competition-based selection of OX40 single-chain antibodies

To develop biepitopic OX40 antibodies, we sought to identify single-chain antibodies (scFvs) to pair with a clinical-stage OX40 agonist antibody that lacks intrinsic agonist activity, namely 11D4 (also known as ivuxolimab, PF-04518600, PF-8600 and B110) (23). To establish proof-of-principle results for our competition-based discovery approach, we first evaluated the expression and antigen-binding activity of 11D4 as a single-chain Fab (scFab) fragment on the surface of yeast (**Figure 2-1**). Encouragingly, the 11D4 scFab expressed well and bound the OX40 extracellular domain. Moreover, we confirmed that pre-incubation of 11D4 IgG with OX40 effectively blocked binding to the yeast-displayed 11D4 scFab. This demonstrates our ability to specifically block the 11D4 epitope on OX40, which we aim to use for selecting antibodies that recognize non-overlapping OX40 epitopes.

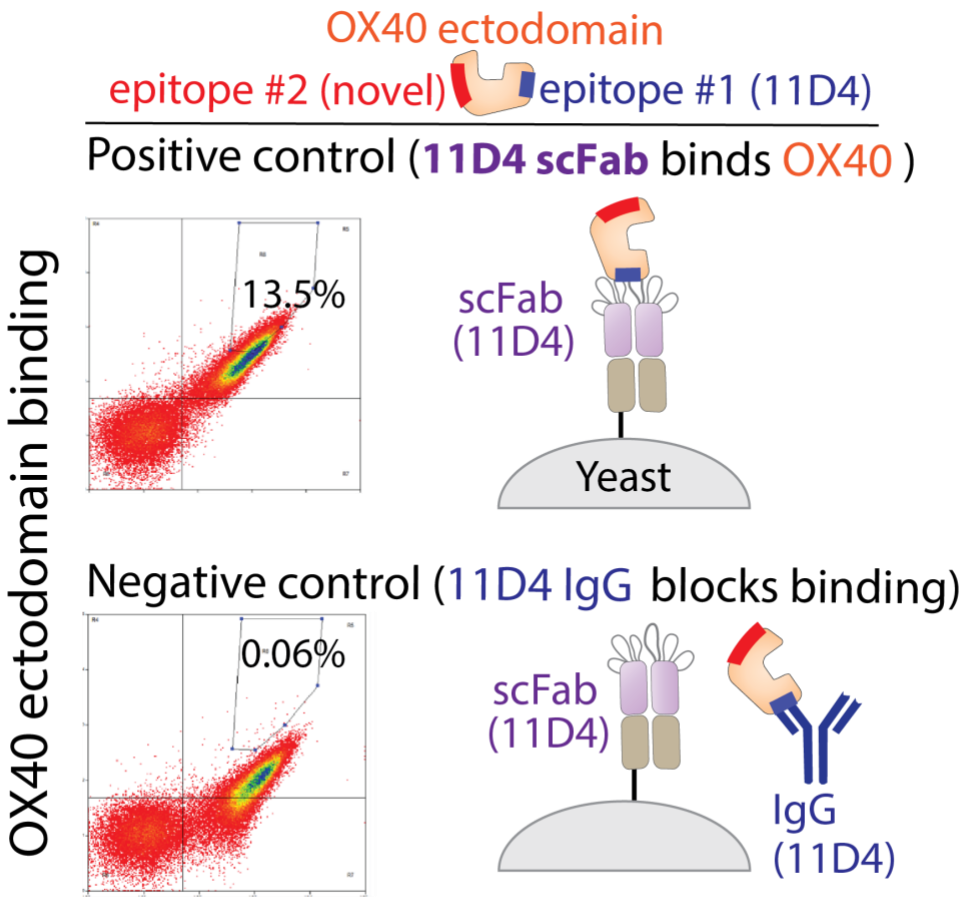

Figure 2-1. Flow cytometry analysis of the 11D4 single-chain antibody displayed on yeast binding to human OX40 in the absence and presence of 11D4 IgG. (Top) The parental 11D4 IgG was reformatted as a single-chain Fab (scFab), displayed on yeast, and confirmed to bind strongly to the OX40 ectodomain. (Bottom) The OX40 ectodomain was pre-blocked with 11D4 IgG and confirmed to bind weakly to the OX40 scFab on yeast due to epitope blocking.

Next, we performed a series of *in vitro* selections to identify scFvs that recognize unique OX40 epitopes from a nonimmune human library (**Figure 2-2**) (24). First, the library was enriched for OX40 binding via an initial round of magnetic-activated cell sorting (MACS) against the bivalent OX40-Fc. Following one round of MACS, the yeast population was propagated and evaluated via fluorescence-activated cells sorting (FACS) for binding to biotinylated monovalent OX40.

Significant enrichment of the antigen-binding population was observed after each sort (FACS rounds 1-3), as shown in **Figure 2-2**.

We next sought to identify scFvs that engaged non-overlapping OX40 epitopes relative to the 11D4 epitope. We applied a competition-based strategy by screening for antigen binding in the presence of soluble 11D4 IgG. Consecutive rounds of screening were conducted, first at an equimolar concentration of OX40:11D4 IgG followed by a 10-fold molar excess of 11D4 IgG relative to OX40. Encouragingly, we retained a clear binding population after pre-blocking the 11D4 epitope on OX40 (**Figure 2-2**, FACS sort 4) and subsequently observed significant library enrichment (FACS sort 5), suggesting an enrichment for antibodies with non-overlapping epitopes had been achieved. Finally, we Sanger sequenced antibodies from the enriched libraries and identified three unique variants (OX.F1, OX.F2 and OX.F3) for continued evaluation (**Table 2-1**)

Table 2-1: Amino acid sequences of single-chain OX40 antibodies. (A) The scFv amino acid sequences presented in the following format: variable heavy region - linker (G4S)x3 - variable light chain region.

OX.F1
QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSVSWDWIRQSPSRGLEWLGRTYYRSKWYNEYAVSVESRI
TINPDTSKNQFSLQLNSVTPEDTAIYFCVRNNYFFDLWGRGTLVTVSSGILGSGGGGSGGGGSGGGGSEIV
LTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASDRATGIPARFSGSGSGTDFTLT
ISSLEPEDFAVYYCQLRSNWPPGYTFGQGTKVEIK

OX.F2
QVQLQQSGPGLVKPSQTLSLTCGISGDSVSSNSVSWDWIRQSPSRGLEWLGRTYYRSKWYNEYAVSVESRI
TINPDTSKNQFSLQLNSVTPEDTAIYFCVRNNYFFDLWGRGTLVTVSSGILGSGGGGSGGGGSGGGGSEIV
LTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLT
ISSLEPEDFAVYYCQQRSNWPPMYTFGQGTKLEIK

OX.F3
QVQLQQSGPGLVKPWQTLSLTCGISGDSVSSNSVSWDWIRQSPSRGLEWLGRTYYRSKWYNEYAVSVESRI
TINPDTSKNQFSLQLNSVTPEDTAIYFCVRNNYFFDLWGRGTLVTVSSGILGSGGGGSGGGGSGGGGSEIV
LTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLT
ISSLEPEDFAVYYCQQRSNWPPMYTFGQGTKLEIK

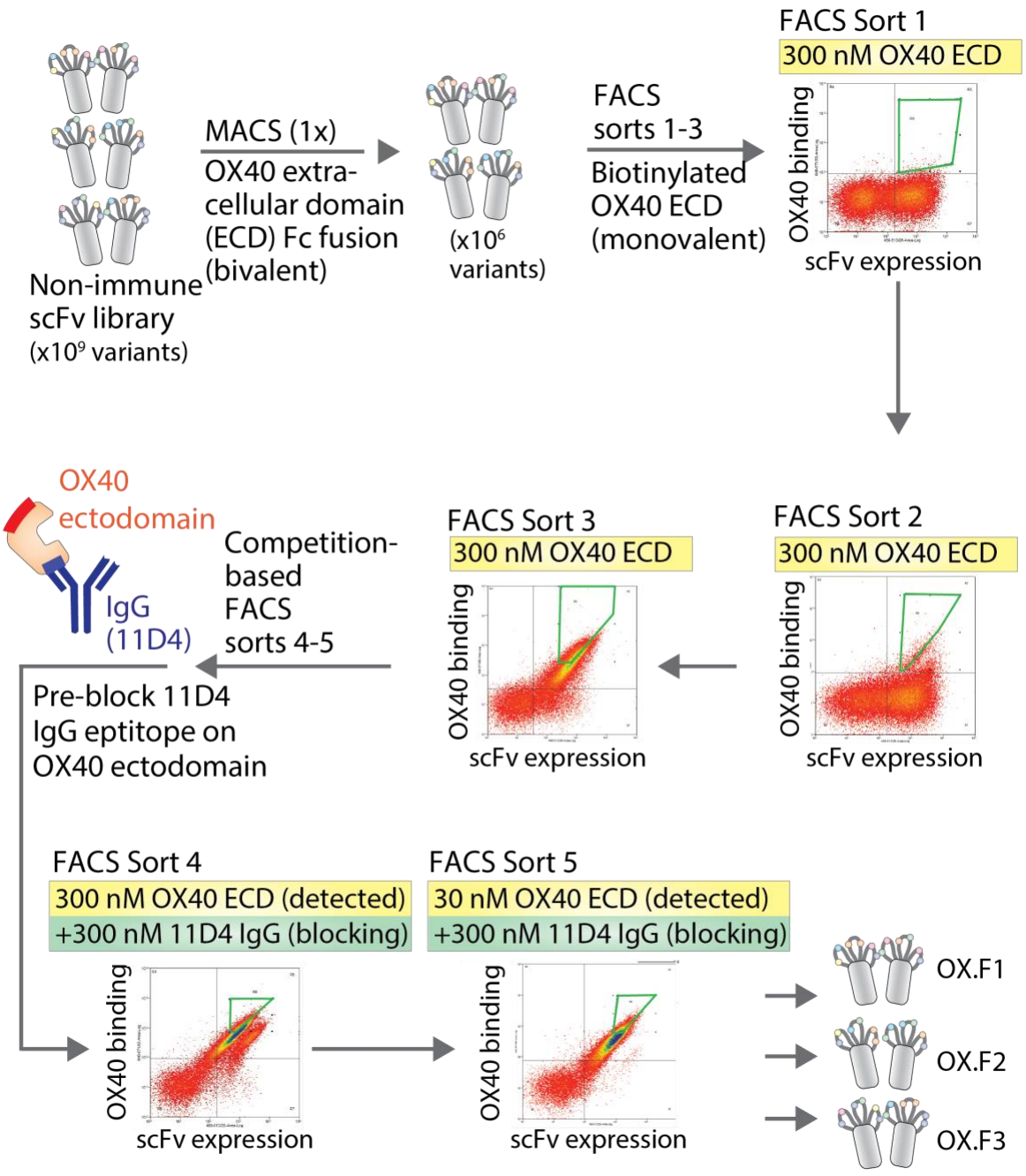

Figure 2-2: Overview of approach for isolating single-chain antibodies from human libraries with unique receptor (OX40) epitopes relative to a clinical-stage OX40 antibody. A human single-chain (scFv) library displayed on yeast was enriched for binding to the human OX40 ectodomain via magnetic-activated cell sorting (MACS, one sort) and fluorescence-activated cell sorting (three sorts). Next, a clinical-stage OX40 IgG (11D4, also known as ivuxolimab) was used to block its OX40 epitope on the soluble OX40 ectodomain, and the library was further enriched against the OX40/IgG complex to identify scFvs with unique OX40 epitopes. Three unique scFvs (OX.F1, OX.F2, and OX.F3) were discovered using this approach.

Selected single-chain antibodies recognize unique epitopes relative to existing OX40 IgG

To evaluate the OX40 binding of the identified scFvs, affinity analysis on yeast was conducted in the presence or absence of 11D4 IgG pre-blocking (**Figure 2-3A)**. Encouragingly, each of the three scFvs (OX.F1, OX.F2 and OX.F3) retained binding to OX40 in the presence and absence of 11D4 IgG, suggesting that the scFvs engage distinct epitopes (**Figure 2-3B-D**). Interestingly, the scFvs exhibited stronger OX40 binding after 11D4 IgG pre-blocking compared to the control without pre-blocking. Importantly, we confirmed that the three scFvs did not bind 11D4 IgG (**Figure 2-4**). As expected, 11D4 pre-blocking significantly reduced 11D4 scFab binding to OX40, confirming that antibodies with overlapping epitopes reduce OX40 binding (**Figure 2-3E**). Overall, these results demonstrate that the selected single-chain antibodies engage unique OX40 epitopes compared to 11D4 IgG.

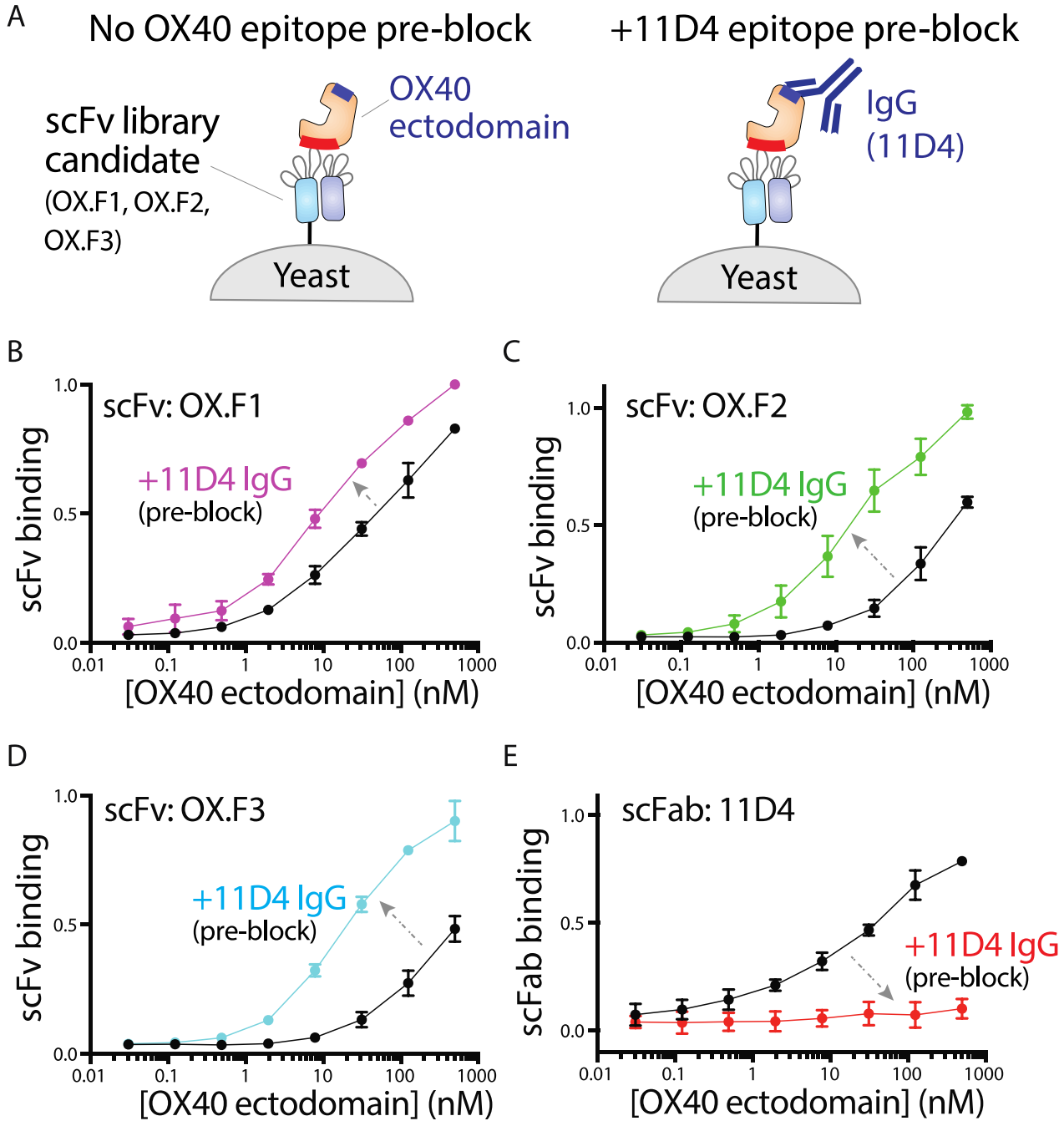

Figure 2-3. Selected single-chain antibodies possess unique OX40 epitopes relative to 11D4 IgG. (A) The candidate single-chain antibodies were displayed on yeast and evaluated if they can bind to OX40 ectodomain when the 11D4 epitope is pre-blocked. (B-D) Selected OX40 scFvs (OX.F1, OX.F2, OX.F3) show strong binding to OX40 in the presence and absence of 11D4 IgG pre-blocking. € The wild-type 11D4 single-chain Fab (scFab) on yeast loses binding to OX40 when the ectodomain is pre-incubated with 11D4

IgG, as expected. The results are averages of three independent experiments and the error bars are standard deviations.

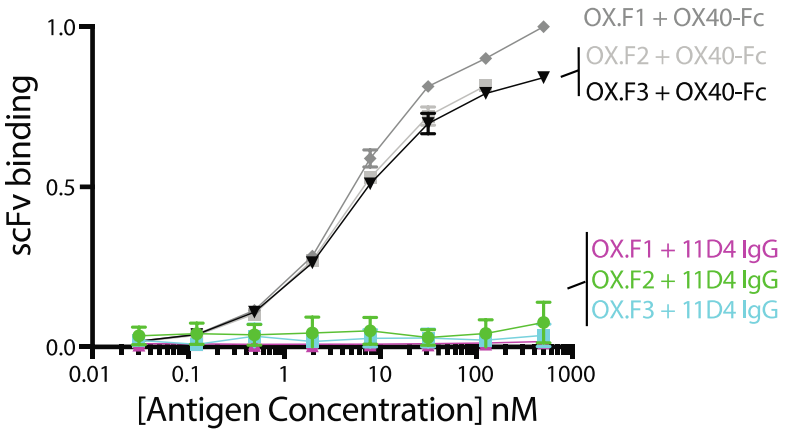

Figure 2-4. OX40 single-chain antibodies lack affinity for 11D4 IgG. Selected OX40 scFvs were displayed on the surface of yeast and tested for binding to 11D4 IgG using flow cytometry. As a control, the OX40 scFvs were confirmed to bind OX40 (as a Fc fusion protein). The results are averages of two independent experiments and the error bars are standard deviations.

Next, we investigated the binding of the selected scFvs as Fc-fusion proteins to OX40 on HEK cells to ensure that the clones bound the native OX40 receptor in addition to the recombinant extracellular domain. Encouragingly, the scFv-Fc fusion proteins showed dose-dependent binding to the OX40 receptor (**Figure 2-5A**). Collectively, these results indicate that the selected scFvs bind to unique OX40 epitopes relative to the 11D4 IgG and recognize authentic OX40 receptor, which motivates their combination into biepitopic antibodies and evaluation as potent OX40 receptor agonists.

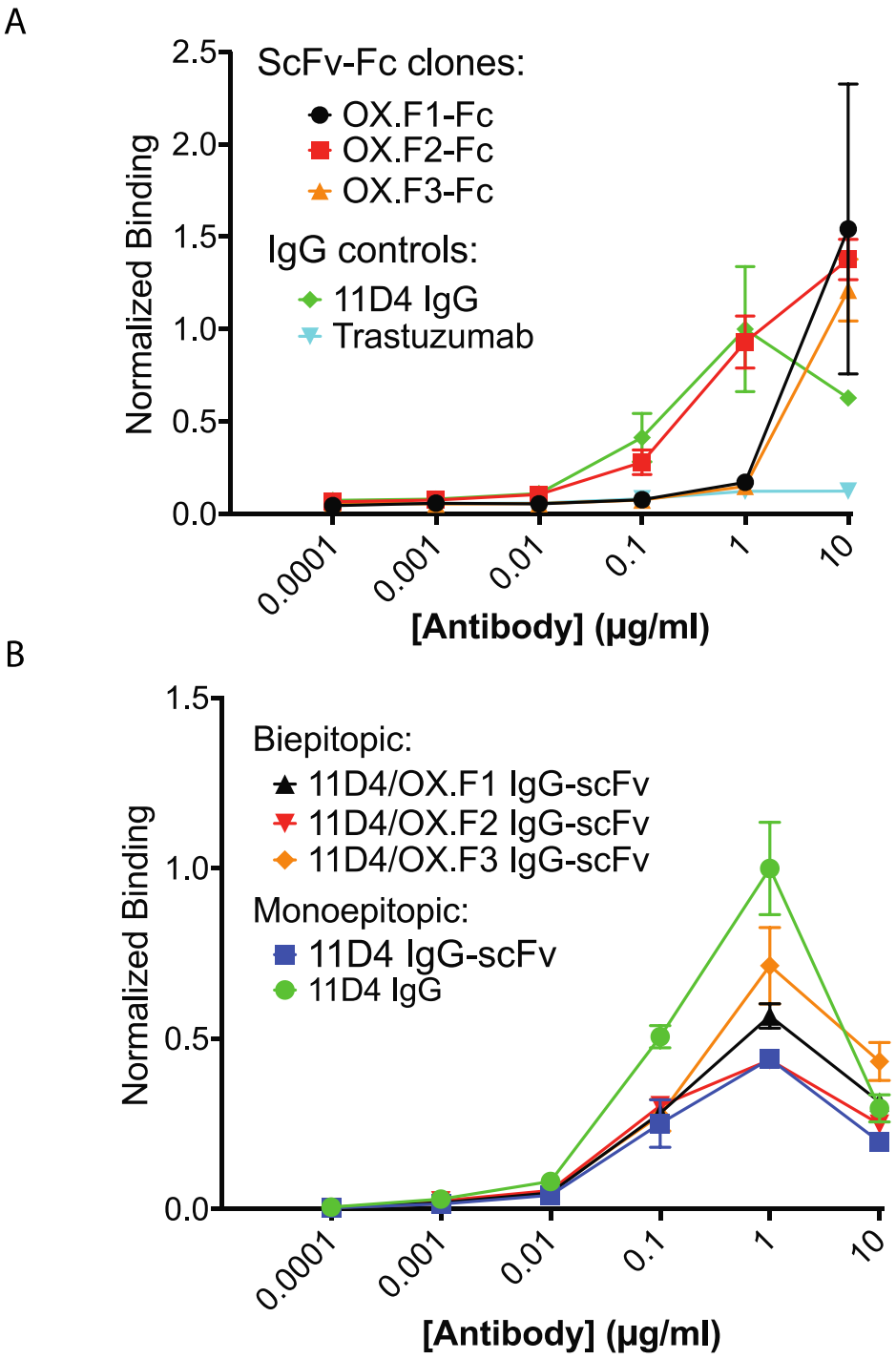

Figure 2-5. scFv-Fc and IgG-scFv binding to OX40 on HEK293 cells. (A) Selected scFvs were generated as scFv-Fc fusion proteins and their binding to OX40 on HEK293 cells was evaluated. (B) The binding of biepitopic (tetravalent) and monoepitopic (tetravalent and bivalent) antibodies to OX40 on HEK293 cells

was evaluated. The results are averages of three independent experiments and the error bars are standard deviations.

Biepitopic OX40 antibodies show potent, FcγR-independent bioactivities in CD4+ primary T cells

We next generated biepitopic antibodies – without any molecular reformatting of the 11D4 IgG or scFvs – by simply fusing the selected scFvs to the C-termini of the light chains of 11D4 IgG (**Figure 2-8A**). The resulting tetravalent antibodies are referred to as IgG-scFv antibodies. They could be easily expressed and isolated at high purity (**Figure 2-6**). The biepitopic IgG-scFv antibodies displayed generally similar binding profiles to OX40 HEK293 cells as the 11D4 IgG with maximal binding at 1 μg/mL and reduced binding at 10 μg/mL (**Figure 2-5B**), which is likely due to saturation of OX40 receptor and reduced available binding sites per antibody.

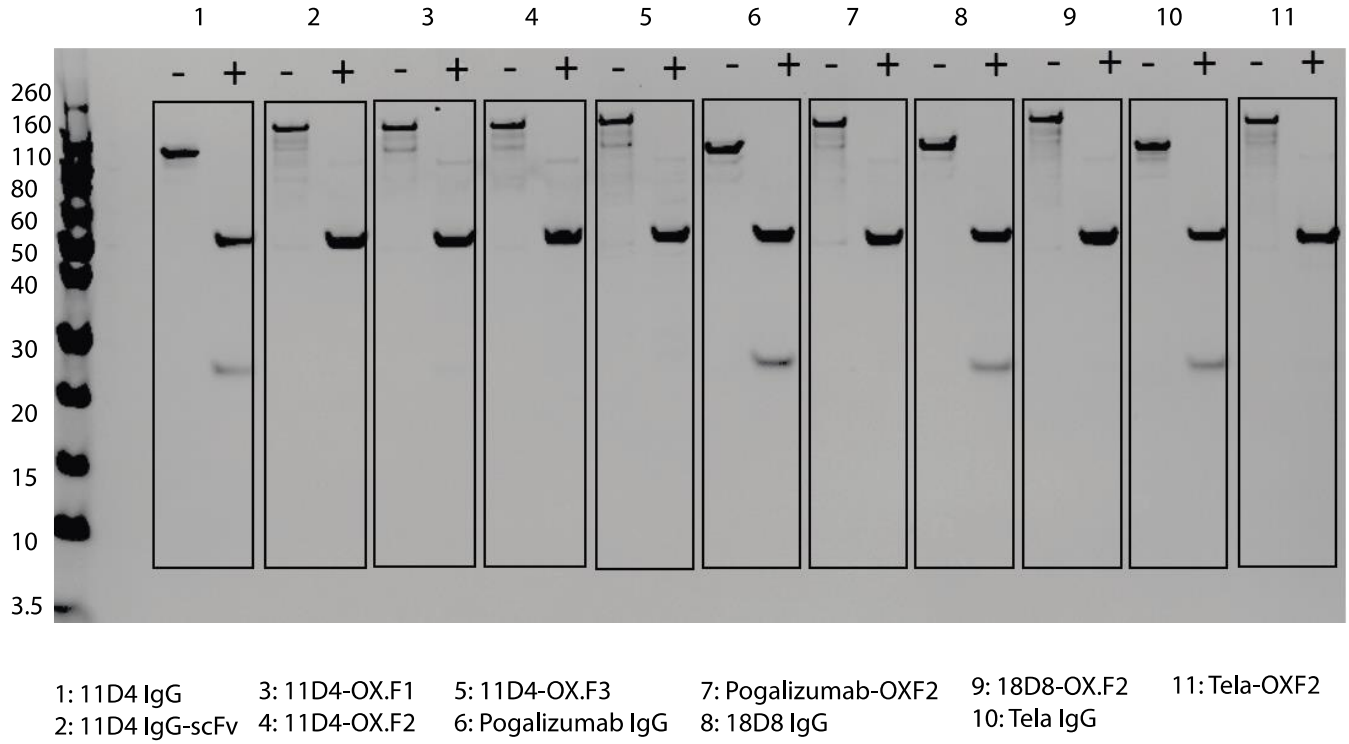

Figure 2-6. SDS-PAGE analysis of OX40 monoepitopic and biepitopic antibodies. The antibodies were evaluated in reducing (+) and non-reducing conditions (-).

The ability of the biepitopic IgG-scFv antibodies to promote receptor activation was first evaluated using engineered OX40 Jurkat cells that report intracellular signaling (NF-κB activation) due to receptor activation via luciferase expression (**Figure 2-7**). In the absence of FcγR-mediated crosslinking, the tetravalent antibodies induced strong NF-κB activity in a dose-dependent manner. In comparison, the bivalent 11D4 IgG was unable to induce receptor activation due to the lack of FcγR-mediated crosslinking.

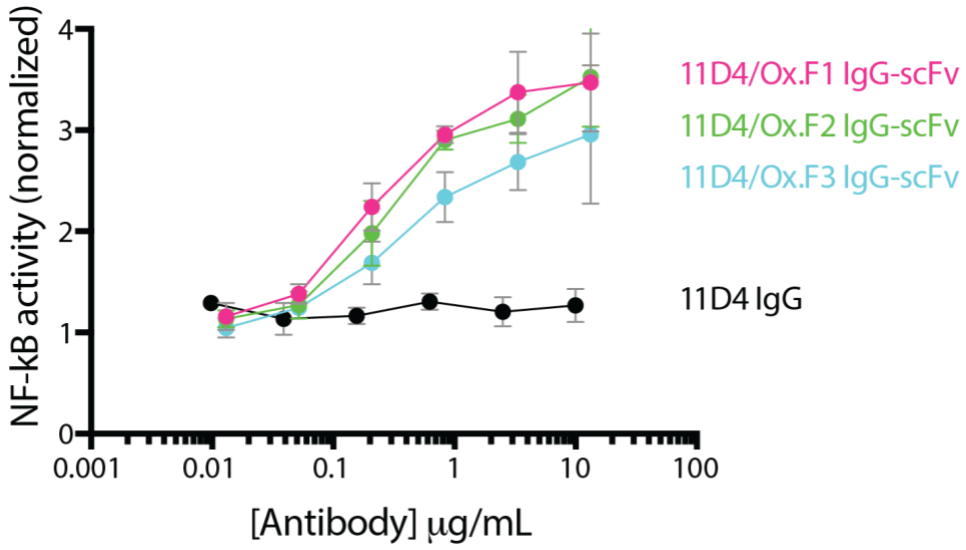

Figure 2-7. Evaluation of FcγR-independent agonism of human OX40 using a Jurkat T cell assay. The antibodies were evaluated for their ability to promote NF- B activation in an OX40+ reporter Jurkat T cell line without Fc-mediated crosslinking. The results are averages of three independent experiments and the error bars are standard deviations.

Next, we sought to examine the relative activities of the biepitopic IgG-scFv antibodies for their ability to induce human CD4+ T cell proliferation (**Figure 2-8A)**. To evaluate their dose-dependent responses, CD4+ T cells were incubated with a wide range of concentrations of the OX40 antibodies along with anti-CD3/CD28 antibodies (primary/secondary T cell activators) (**Figure 2-8B**). The results demonstrated that the biepitopic IgG-scFv antibodies induced elevated T-cell proliferation compared to the monoepitopic tetravalent control treatment. In contrast, 11D4 IgG showed no T cell proliferation response, as expected as a result of its lack of intrinsic receptor agonism due to its bivalent nature. Overall, these data suggest the importance of multivalent antibodies in promoting strong receptor activation and improving agonist activity compared to the bivalent IgGs.

Given the important role of IL-2 production in mediating T cell proliferation, we also sought to measure the IL-2 secretion for human primary CD4+ T cells **(Figure 2-8C)** (25). Consistent with our observations on T cell proliferation, biepitopic IgG-scFv antibodies mediated elevated levels of IL-2 secretion relative to the monoepitopic tetravalent control antibody, whereas the 11D4 IgG showed limited activity. Interestingly, we observed a bell-shaped dose-dependent response for all antibodies tested, with IL-2 expression reaching a maximum at antibody concentrations of 0.1-1 μg/ml. Collectively, our data indicate that the biepitopic IgG-scFv antibodies exhibit improved FcγR-independent T cell activation compared to monoepitopic antibodies.

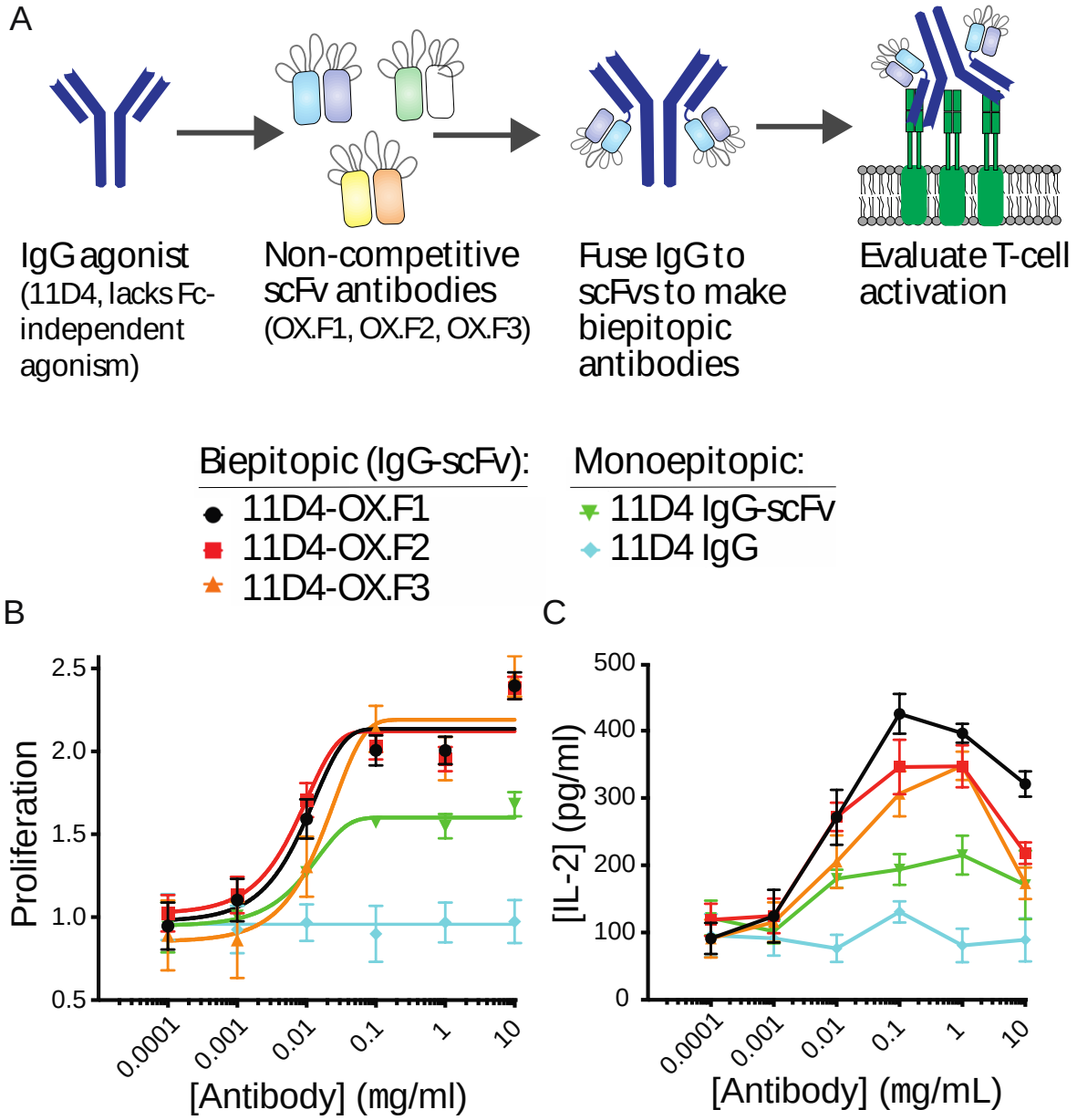

Figure 2-8. Biepitopic OX40 antibodies induce strong human CD4+ T cell activation in a Fc R-independent manner. (**A**) Schematic illustration of the approach used to convert existing clinical-stage antibodies into potent OX40 antibodies with intrinsic agonist activity. (B, C) The biepitopic antibodies induced strong human CD4+ T cell (**B**) proliferation and (C) IL-2 secretion. Proliferation was evaluated at day 6 while IL-2 secretion was evaluated at day 2. The results are averages of 6-8 independent experiments for proliferation assay and three independent experiments for IL-2 analysis. The error bars are standard errors. In (B), the biepitopic (IgG-scFv) antibodies displayed higher levels of proliferation than the monoepitopic IgG (p-values <0.0001 at 0.1-10 mg/mL) and IgG-scFv (p-values <0.01 at 0.1-10 mg/mL). In (C), the biepitopic (IgG-scFv) antibodies displayed higher levels of IL-2 secretion than the monoepitopic IgG (p-values <0.05 at 0.01-1 mg/mL) and IgG-scFv (p-values <0.05 at 0.1-1 mg/mL).

Additional clinical-stage OX40 IgGs engineered as biepitopic antibodies demonstrate predictable ability to activate human CD4+ primary T cells

Next, we investigated the generality of our findings—that biepitopic IgG-scFv antibodies induce superior and FcγR-independent activities relative to their monoepitopic counterparts—by identifying other clinical-stage antibodies to pair with OX.F2 and evaluating them for bioactivity in primary T cell models (**Figure 2-10A**). Toward this goal, we first conducted competitive binding analysis to compare the epitope of other clinical-stage OX40 IgGs (pogalizumab, 18D8 and telazorlimab) relative to that of 11D4 and OX.F2. All three clinical-stage antibodies did not compete with 11D4 for OX40 binding (**Figure 2-10B**), revealing that they possess unique epitopes. Interestingly, pogalizumab and 18D8 engage a unique epitope relative to OX.F2, while telazorlimab engages an overlapping epitope (**Figure 2-10C**). These findings were further corroborated by a second competitive binding assay (**Figure 2-9**). Altogether, these results demonstrate that the OX.F2 engages a unique OX40 epitope relative to the additional clinical IgGs (pogalizumab and 18D8) and motivates combining them as IgG-scFv biepitopic antibodies and evaluating their function to judge the generality of our approach.

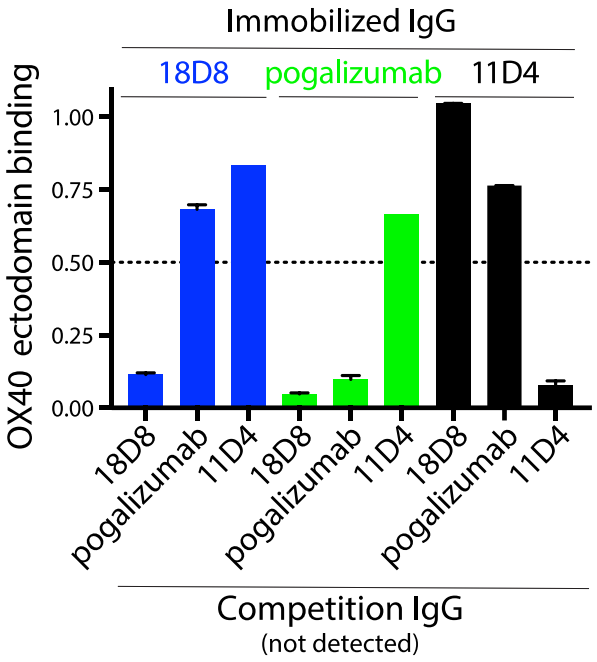

Figure 2-9. Competitive binding analysis of OX40 IgGs. Three OX40 IgGs were separately immobilized on Protein A beads, and their binding to OX40 was evaluated in the presence of different OX40 IgGs via flow cytometry. The results are averages of three independent experiments and the error bars are standard deviations.

Therefore, we evaluated the ability of the OX.F2 scFv paired with the panel of clinical-stage IgGs (pogalizumab, 18D8, and telazorlimab) to activate OX40 on human CD4+ T cells (**Figure 2-10D**). We observed that the biepitopic antibodies, namely pogalizumab-OX.F2 and 18D8-OX.F2, mediated strong CD4+ T cell proliferation in a similar manner similar as 11D4-OX.F2. Interestingly, telazorlimab-OX.F2 elicited significantly weaker T cell proliferation response, which was predictable based on the competitive binding analysis that revealed telazorlimab and OX.F2 recognize overlapping epitopes (**Figure 2-10C**).

To further evaluate these findings, we also conducted subsequent analysis of IL-2 secretion in human primary CD4+ T cells. The biepitopic antibodies induced superior IL-2 secretion response compared to the OX40 ligand and telazorlimab-OX.F2 (**Figure 2-10E**), as expected based on the proliferation results (**Figure 2-9D**). Interestingly, pogalizumab-OX.F2 led to a twofold increase in the maximum IL-2 secretion levels relative to 11D4-OX.F2 (**Figure 2-10E**), suggesting that the different biepitopic antibodies possess unique abilities to induce high levels of IL-2 secretion. Altogether, these data demonstrate that the OX.F2 single-chain antibody can be paired with existing clinical-stage OX40 IgGs – without any molecular reformatting – to generate biepitopic antibodies with predictable and strong ability to activate human T cells.

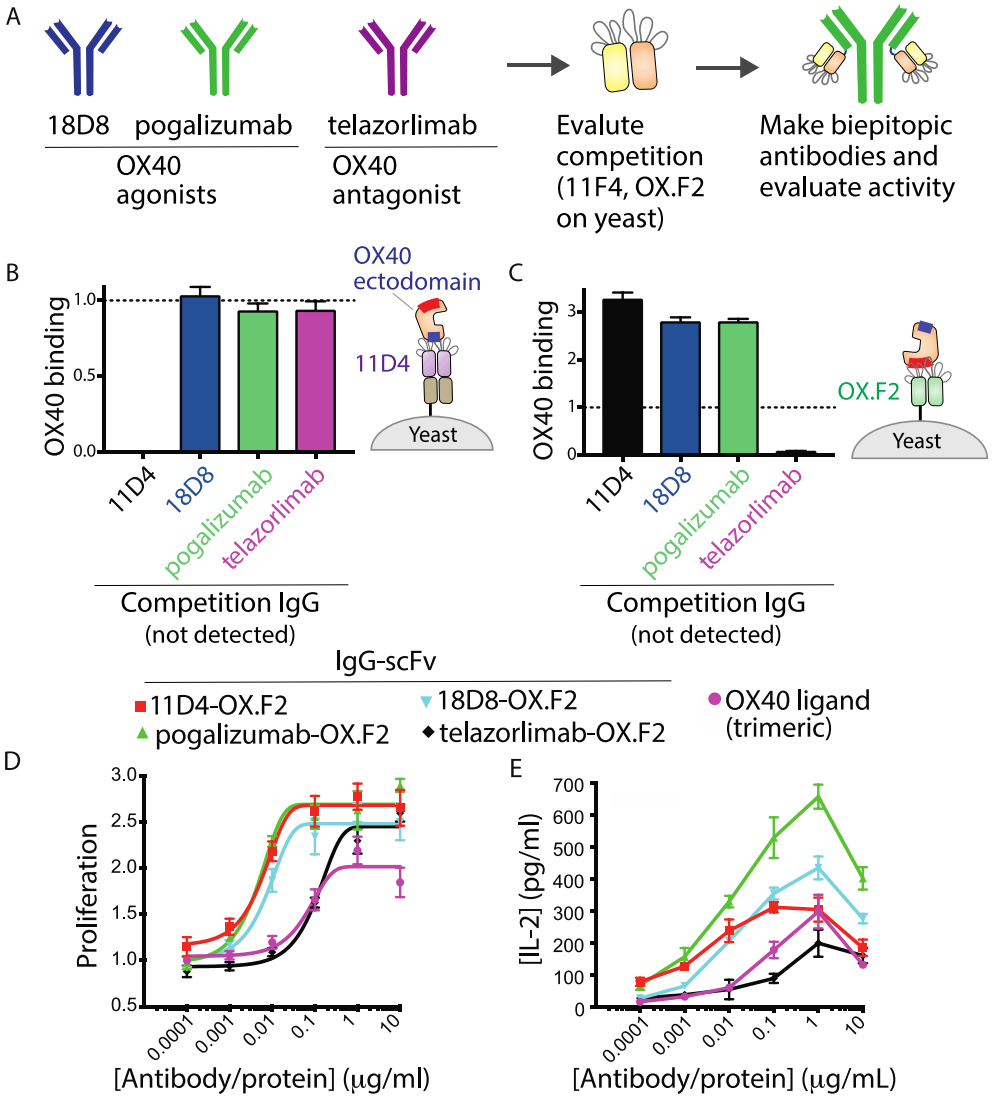

Figure 2-10. Generalization of the biepitopic antibody approach to additional OX40 clinical-stage antibodies results in predictable and potent human CD4+ T cell activation. (**A**) Schematic of the process of competition-based analysis of antibody epitopes for clinical-stage OX40 agonists and antagonists, the resulting combination of IgGs with the OX.F2 scFv into biepitopic formats, and their evaluation for T cell activation. (**B-C**) Competition-based analysis of (**B**) 11D4 and (C) OX.F2 single-chain antibodies displayed on yeast binding to OX40 after pre-blocking OX40 with a clinical-stage OX40 antibody. (**D, E**) The biepitopic antibodies induced strong human CD4+ T cell (**B**) proliferation and (**C**) IL-2 secretion. In (**E**), the OX40 ligand was included as a control. Proliferation and IL-2 secretion were measured as described in Figure 3. The results are averages of three independent experiments and the error bars are standard errors. In (D), 11D4-OX.F2, 18D8-OX.F2 and pogalizumab-OX.F2 displayed higher levels of proliferation than telazorlimab-OX.F2 (p-values <0.01 at 0.01-0.1 mg/mL) and OX40 ligand (p-values <0.01 at 0.01-0.1 and p-values <0.05 at10 mg/mL). In (E), 11D4-OX.F2 displayed higher levels of IL-2 secretion than telazorlimab-OX.F2 and OX40 ligand (p-values <0.05 at 0.0001-0.1 mg/mL), 18D8-OX.F2 displayed higher levels of IL-2 secretion than telazorlimab-OX.F2 and OX40 ligand (p-values <0.01 at 0.1-1 mg/mL and 10 mg/mL), and pogalizumab-OX.F2 displayed higher levels of IL-2 secretion than telazorlimab-OX.F2 and OX40 ligand (p-values <0.001 at 0.001-10 mg/mL).

Competition-based discovery platform can be generalized to other TNF receptors

To evaluate the broad applicability of the antibody screening platform, we sought to discover scFvs that could be paired with a clinical-stage CD137 antibody that lacks intrinsic agonist activity, namely utomilumab (12). To do this, we used the same discovery approach we established for OX40 (**Figure 2-14A**) (24, 26). We first used MACS to enrich the antibody library against the CD137 ectodomain and then used FACS to further enrich the library against CD137 (sorts 1-4). Next, competition-based screening was performed by pre-blocking biotinylated CD137 with utomilumab IgG and selecting scFvs that maintained CD137 binding (sorts 5-6). This included FACS sorts that used equimolar concentrations of CD137 and utomilumab IgG (sort 5) followed by sorts that used excess utomilumab IgG relative to CD137 (sort 6). Finally, the enriched libraries were Sanger sequenced and one scFv was identified, namely CD.K2 (**Table 2-2**)

Table 2. Amino acid sequence of the isolated CD137 single-chain antibody (CD.K2). The scFv amino acid sequence is presented in the following format: variable light region - linker region - variable heavy chain region.

CD.K2
DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDF
TLTISSLQPEDFATYYCQQSYSTPLTFGQGTKVEIKSGILGTTAASGSSGGSSSGAEVQLVQSGAEVKKPG
ESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQGQVTISADKSISTAYLQWSSL
KASDTAVYYCARFTYYYYDYDYAGFDYWGQGTLVTVSS

The binding and functional analysis of CD.K2 scFv was performed in multiple steps (**Figure 2-14B**). First, we evaluated the binding of CD.K2 to recombinant CD137 in the presence and absence of utomilumab IgG pre-blocking and did not observe competition (**Figure 2-14C**), suggesting that the two antibodies recognize unique CD137 epitopes. As a positive control, we confirmed that utomilumab scFv lost binding in the presence of utomilumab IgG pre-blocking

(**Figure 2-11**). We also generated CD.K2 as a soluble Fc-fusion protein and confirmed it bound to CD137 on HEK293 cells (**Figure 2-12**). Finally, we generated an IgG-scFv antibody using the CD.K2 scFv (**Figures 2-14B** and **2-13**) and tested its ability to activate human primary CD8+ T cells (**Figure 2-14D**). Encouragingly, the biepitopic antibody (uto-CD.K2) displayed predictable T cell activation, as judged by increased CD25 levels, while the monoepitopic and control antibodies did not. Collectively, these results demonstrate that the competition-based antibody screening platform can be generalized to other members of the TNF receptor superfamily and used to greatly simplify the development of potent T cell agonist antibodies.

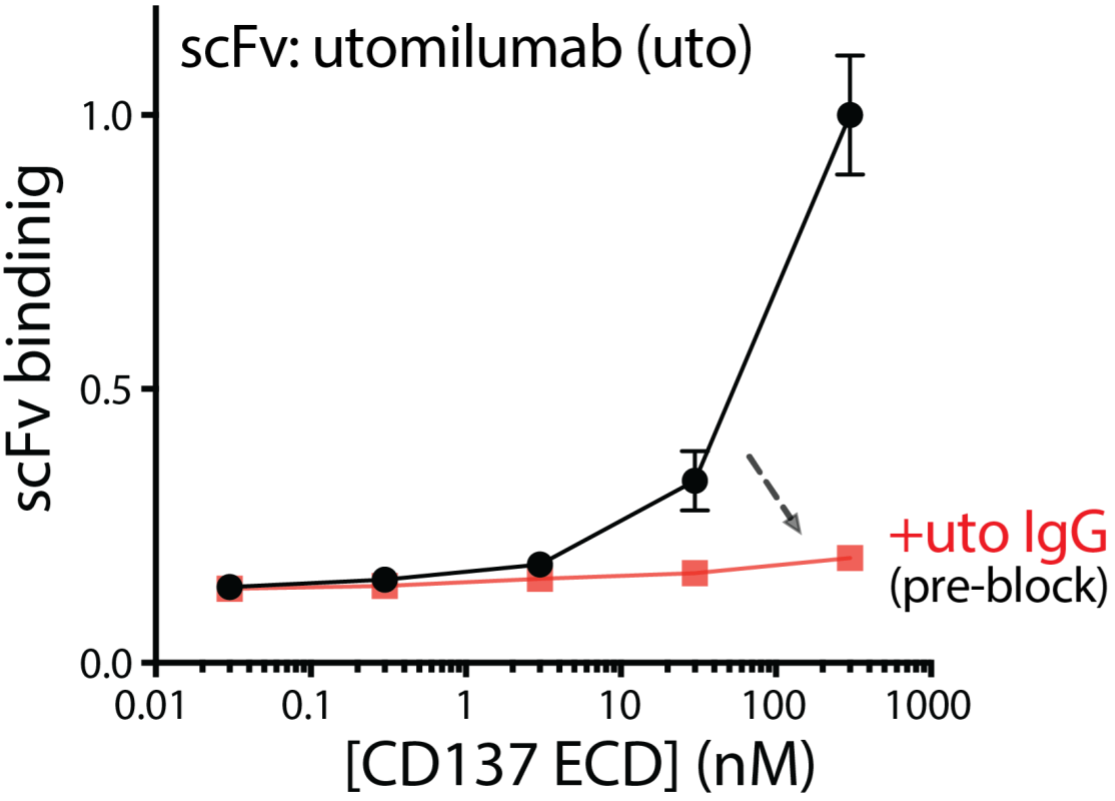

Figure 2-11. CD137 binding to the utomilumab single-chain antibody on yeast is inhibited by utomilumab IgG. The CD137 ectodomain was pre-incubated with utomilumab IgG (1:5 molar ratio) and then the binding of the CD137/utomilumab complex to utomilumab scFv was evaluated via flow cytometry.

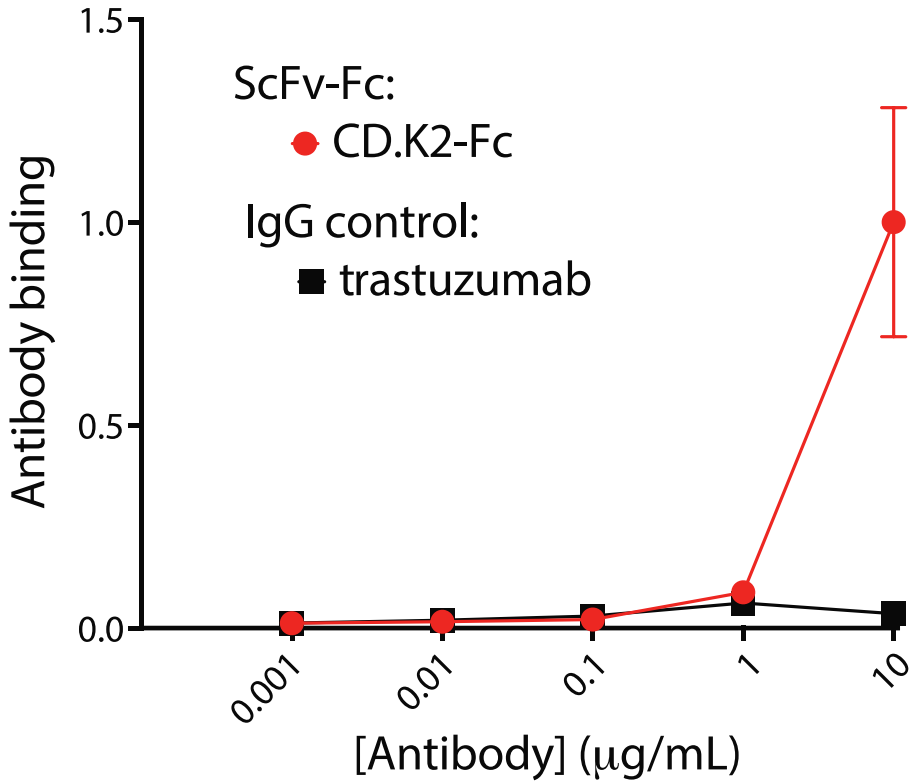

Figure 2-12. scFv-Fc binding to CD137 on HEK293 cells. (A) Select scFv (CD.K2) was generated as a scFv-Fc fusion protein and its binding to CD137 on HEK293 cells was evaluated. The results are averages of median values and the error bars are standard deviations.

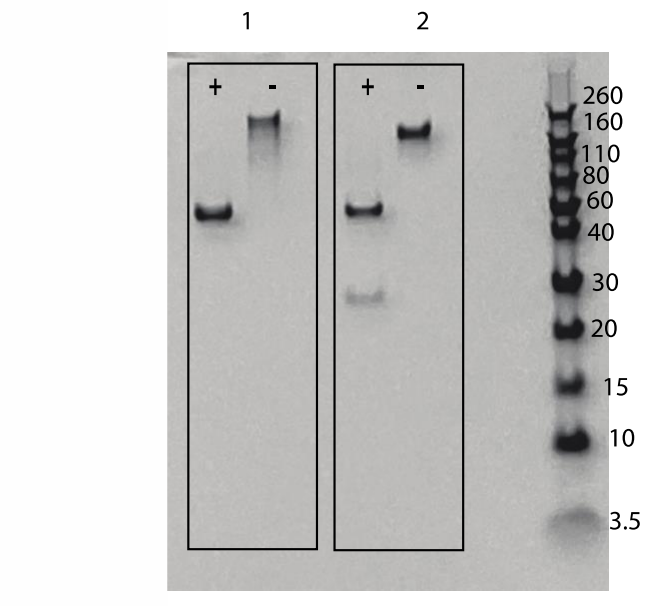

Figure 2-13. SDS-PAGE analysis of CD137 antibodies. The antibodies (1: utomilumab-CD.K2; 2: utomilumab) were evaluated in reducing (+) and non-reducing conditions (-).

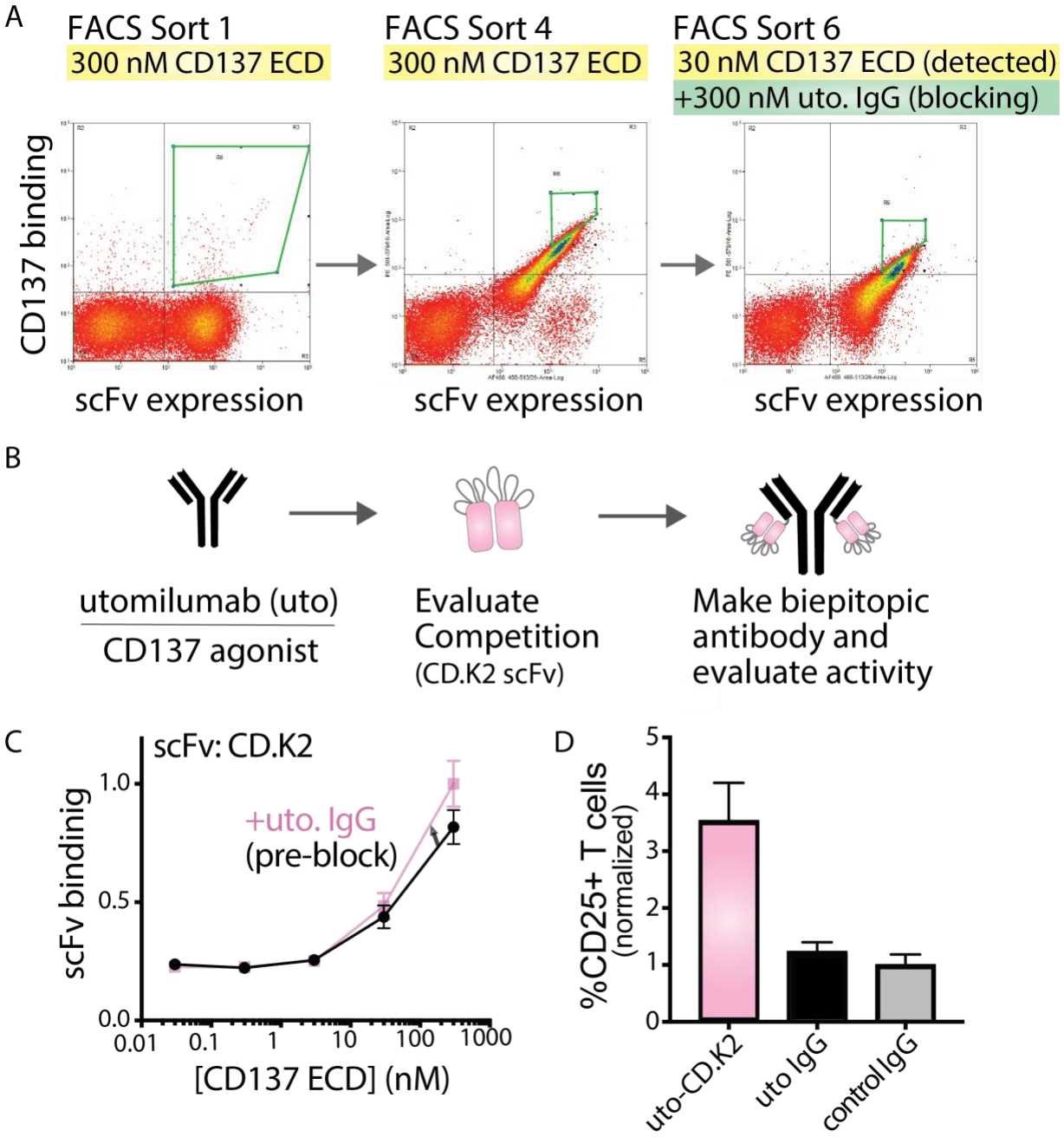

Figure 2-14. Generalization of the biepitopic antibody approach to an additional TNF receptor (CD137) result in predictable and potent human CD8+ T cell activation. (**A**) A human scFv library was enriched against the human CD137 ectodomain via MACS (one sort) and FACS (three sorts). Next, the library was enriched for CD137 binding in the presence of a clinical-stage CD137 IgG (utomilumab, uto; two sorts). (**B**) Schematic of the approach used to convert utomilumab into a biepitopic CD137 antibody with intrinsic agonist activity. (**C**) The isolated CD137 single-chain antibody (CD.K2) displayed on yeast bound CD137 ectodomain in the absence and presence of utomilumab IgG. (**D**) The biepitopic antibody (utomilumab-CD.K2; uto-CD.K2) induced strong human CD8+ T cell proliferation, as judged by CD25 expression, relative to utomilumab IgG and a control IgG (trastuzumab). The results are averages of four independent experiments and the error bars are standard errors. *** = p-value < 0.001.

Discussion

To generate potent agonists of OX40 and CD137, we developed a competition-based screening platform that is predictable, high-throughput, and easy to use. Our approach has several advantages relative to conventional methods that use a combination of animal immunization, primary antibody identification, and pair-wise screening of complementary antibodies based on epitope specificity (18–21). The latter process is especially challenging because, at least in some cases, most isolated antibodies target relatively few immunodominant epitopes. For example, one study sought to discover antibodies against unique epitopes against a target antibody (mesothelin protein) using hybridoma technology (27). Among the 7,680 hybridomas, only 232 clones were specific for the target antigen and 96% (223 clones) shared the same epitope. Even conventional *in vitro* selection strategies are typically challenging and time-consuming due to the need for several steps in this process, including generating immune libraries, selecting primary antibodies via display technologies, reformatting the selected antibodies as soluble antibodies, performing selections for additional antibodies with orthogonal epitopes, and finally reformatting into biepitopic antibodies (28). To overcome these challenges, our approach uses off-the-shelf IgGs that can be readily converted into biepitopic antibodies. We anticipate that this screening platform can be used for discovering a variety of novel biepitopic antibodies for a broad range of applications.

Our findings also highlight the importance of engineering multivalent antibodies to enhance OX40 and CD137 receptor activation, which is consistent with previous reports of using multivalency to improve agonist activity (17, 29, 30). In our work, we designed tetravalent antibodies that exhibited improved NF-κB activation and T cell proliferation. These results are

consistent with previous studies for the OX40 receptor where tetravalent antibody constructs, such as those in the dual-variable-domain (DVD) format, were shown to impart superior agonism due to their ability to promote high-order receptor clustering (17). Moreover, the DVD antibodies mediated significantly improved NF-κB activation compared to their bivalent counterparts. One explanation for enhanced agonist activity with higher valency is the requirement for trimerization of several receptors in the TNF receptor superfamily (31) which can be achieved using antibodies possessing at least three antigen-binding sites. To test this hypothesis, investigators engineered trivalent and tetravalent nanobody formats against death receptor 5 (DR-5). They found that these formats strongly decreased tumor viability and displayed an increase in apoptotic response compared to the corresponding bivalent nanobody (29). Antibodies with even higher valency, such as hexavalent antibodies, have also been explored to improve TNF receptor activation. In the case of the GITR receptor, a hexameric Fc-fusion protein (MEDI1873) exhibited significantly improved T cell proliferation relative to its bivalent IgG counterpart. These findings were further corroborated in a primate model where MEDI1873 showed enhanced T cell proliferation and elevated levels of IgG circulating antibodies, suggesting both strong cellular and humoral immune responses (30). Beyond TNF receptors, more work is needed to evaluate the potential of multivalent antibodies for strongly activating other T cell receptors.

In addition to valency, our results also highlight significant impacts of engaging multiple receptor epitopes, which builds on previous findings related to strong activation of multiple TNF receptors (17, 28, 32). Overall, biepitopic antibodies with superior bioactivities relative to their monoepitopic counterparts have been generated in a variety of molecular formats, including as tetravalent mAb-scFvs (as used in this work), tetravalent DVDs (17), bivalent IgGs (28), and Fc-engineered constructs that non-covalently trimerize upon antigen binding (32). A recent report has

shown that biepitopic antibodies in a bivalent IgG format induce receptor clustering of soluble and cell-bound epidermal growth factor receptors (EGFR) (28). The authors hypothesized that the binding kinetics of biepitopic antibodies are favorable for receptor clustering due to a mixture of low and high-affinity Fab arms that lead to continuous binding and releasing effects to induce receptor superclusters. This phenomenon is even more amplified by tetravalent biepitopic antibodies where multiple receptor clusters can be brought together to induce greater receptor activation.

The superior activity of biepitopic antibodies can also be explained by the theoretical extensive daisy-chain-like receptor superclustering made possible via intermolecular receptor engagement as opposed to terminating, intramolecular receptor engagement (17, 33, 35). Molecular geometry, linker length, and linker rigidity are expected to influence such molecular interactions, and by extension, molecular engineering can be employed to bias toward desired molecular interactions. Biochemical methods, such as size-exclusion chromatography and cell-based methods such as fluorescence microscopy, have been established for characterizing the influence of antibody molecular format on receptor clustering, which may be useful for rapidly identifying the most effective formats and epitopes that maximize receptor activation (17, 33).

Conversely, biepitopic antibodies have also been reported in which each antibody molecule is suggested to engage multiple epitopes on a single receptor molecule (intramolecular receptor engagement) (34). These biepitopic antibodies were found to possess enhanced affinity relative to their monoepitopic counterparts. These types of antibodies may be particularly useful for activating receptors that do not require clustering. Finally, given the potential liabilities of these complex (non-conventional) antibody formats, future investigations should thoroughly

characterize the influence of the formats of biepitopic antibodies on their biophysical properties and *in vivo* pharmacokinetics.

An important observation in our studies is that the biepitopic/tetravalent antibodies exhibit a bell-shaped dose-dependent response for receptor binding and agonist function. For example, the IL-2 cytokine secretion shows peak levels at intermediate antibody concentrations, suggesting a key role of receptor occupancy on receptor activation. A previous study of OX40 agonist antibodies showed that partial receptor occupancy is needed for achieving optimal CD4+ T cell proliferation (35). The investigators found that receptor levels were highly sensitive to receptor occupancy for the OX40 antibody (BMS-986178), as >40% occupancy led to antibody-mediated internalization and reduced OX40 receptor levels. These findings suggest that dosing regimens and administration schedules of therapeutic antibodies must be considered to achieve optimal receptor occupancy for clinical use. Furthermore, other T cell receptors such as CD28 show similar findings where maximum T cell activation and cytokine secretion was observed at intermediate antibody concentrations (*e.g.*, 5 μg/ml), and all activity was lost at higher concentrations (≥100 μg/ml) (36). A similar CD28 agonist antibody study found that only 50-80% receptor occupancy was necessary to achieve peak T cell activation (37).

One plausible reason for maximal receptor activation at intermediate receptor occupancies is that such levels enable maximal receptor supercluster formation via serial bridging between antibodies and receptors. At low antibody concentrations, the receptor engagement is insufficient to mediate higher-order clustering, while elevated levels of receptor engagement inhibit clustering due to the inability of the same antibody to engage multiple receptors. A second explanation for the bell-shaped curve is T cell overstimulation at high antibody concentrations, leading to T cell exhaustion (38). This over-activation leads to expression of inhibitory receptors, which causes the

downregulation of effector T cell function and cell death. Finally, a third possibility is simply increased receptor internalization at high antibody concentrations, which can also inhibit receptor activation (39). It is also possible that more than one of these mechanisms work in concert, resulting in the strong inhibition of receptor activation at high antibody concentrations, which will need to be further explored in the future. An important observation in our studies is that the biepitopic/tetravalent antibodies exhibit a bell-shaped dose-dependent response for receptor binding and agonist function. For example, the IL-2 cytokine secretion shows peak levels at intermediate antibody concentrations, suggesting a key role of receptor occupancy on receptor activation. A previous study of OX40 agonist antibodies showed that partial receptor occupancy is needed for achieving optimal CD4+ T cell proliferation (35). The investigators found that receptor levels were highly sensitive to receptor occupancy for the OX40 antibody (BMS-986178), as >40% occupancy led to antibody-mediated internalization and reduced OX40 receptor levels. These findings suggest that dosing regimens and administration schedules of therapeutic antibodies must be considered to achieve optimal receptor occupancy for clinical use. Furthermore, other T cell receptors such as CD28 show similar findings where maximum T cell activation and cytokine secretion was observed at intermediate antibody concentrations (*e.g.*, 5 μg/ml), and all activity was lost at higher concentrations (≥100 μg/ml) (36). A similar CD28 agonist antibody study found that only 50-80% receptor occupancy was necessary to achieve peak T cell activation (37). One plausible reason for maximal receptor activation at intermediate receptor occupancies is that such levels enable maximal receptor supercluster formation via serial bridging between antibodies and receptors. At low concentrations, the receptor engagement is insufficient to mediate higher-order clustering, while elevated levels of receptor engagement inhibit clustering due to the inability of the same antibody to engage multiple receptors. A second explanation for the bell-

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Previous reports indicate the promise of biepitopic antibodies for broad therapeutic applications (33, 40–45). For example, biepitopic antibodies have recently been pursued in chimeric antigen receptor T cell (CAR-T) therapy for the treatment of B cell malignancies (40, 41). Notably, the *in vivo* anti-tumor activity of CAR-T cells endowed with CD5 biepitopic targeting were much more effective relative to those that used monoepitopic targeting (41). Beyond applications relating to cellular activation, biepitopic targeting has been pursued for many applications involving inhibition of cellular processes, including for treating cancer and infectious disease (42, 43). Recent work highlights the utility of biepitopic targeting of tyrosine kinase receptors for anti-cancer applications via multiple mechanisms (33, 44, 45). For example, HER2-targeted biepitopic antibody-drug conjugates (ADCs) showed enhanced ADC internalization and improved bioactivity relative to their monoepitopic counterparts (33). Biepitopic antibodies have also shown promise for controlling receptor trafficking (44) and overcoming compensatory pro-mitogenic signaling in cancer models (45). We expect many more creative and impactful uses of biepitopic antibodies in the future, which can be generated in a simple and predictable manner using our competition-based discovery method.

Acknowledgements

We thank members of the Tessier lab for their helpful suggestions. This work was supported by the National Institutes of Health (R35GM136300 and RF1AG059723 to P.M.T. and F32GM137513 to J.S.S.), National Science Foundation (CBET 1813963, 1605266 and 1804313 to P.M.T.), and the Albert M. Mattocks Chair (to P.M.T).

Conclusions

Agonist antibodies that target the TNF receptor superfamily enhance a wide range of immune functions. However, their therapeutic use faces multiple challenges, including their typical lack of intrinsic ability to efficiently cluster receptors and mediate strong activation without the need for secondary antibody clustering via other immune cells. Biepitopic antibodies that target two distinct epitopes on the same receptor possess intrinsic ability to potently activate TNF receptors via receptor superclustering, but their generation requires laborious trial-and-error methods. Here we report a facile method for selecting antibodies with unique pairs of epitopes and demonstrate how they can be combined to develop biepitopic antibodies that strongly activate multiple clinically important TNF receptors.

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Chapter 3: Conclusion

Agonist antibodies that target TNF receptors hold great promise to treat a wide range of diseases including cancer, autoimmune diseases, and inflammatory syndromes. Despite their potential, the clinical translation of these antibodies has made limited progress due to low efficacy and toxicity profiles (1). One major challenge is that conventional bivalent IgGs are insufficient at mediating receptor clustering to mediate optimal receptor signaling **(Figure 3-1)** (2). Additionally, they rely on Fc-mediated clustering, which can lead to a wide range of receptor agonism, thus limiting their therapeutic potential (**Figure 3-2**). To develop the next generation of therapeutics, novel agonist antibody discovery and development approaches are needed to improve the therapeutic potential of existing antibodies to improve their clinical success (3).

A. B.

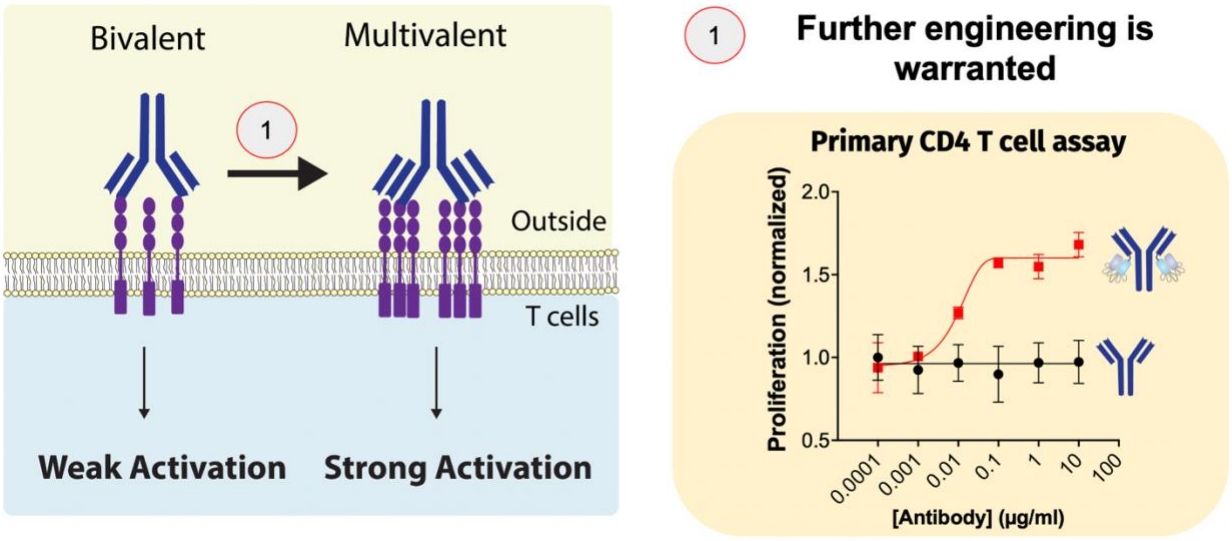

Figure 3-1: The impact of multivalency to mediate potent receptor activation. (A) schematic illustration of the impact of multivalency to improve receptor activation. (B) Typically, improving agonist activity requires further protein engineering to improve their therapeutic potential. The graph highlights the power of multivalency to improve receptor activation.

A. B.

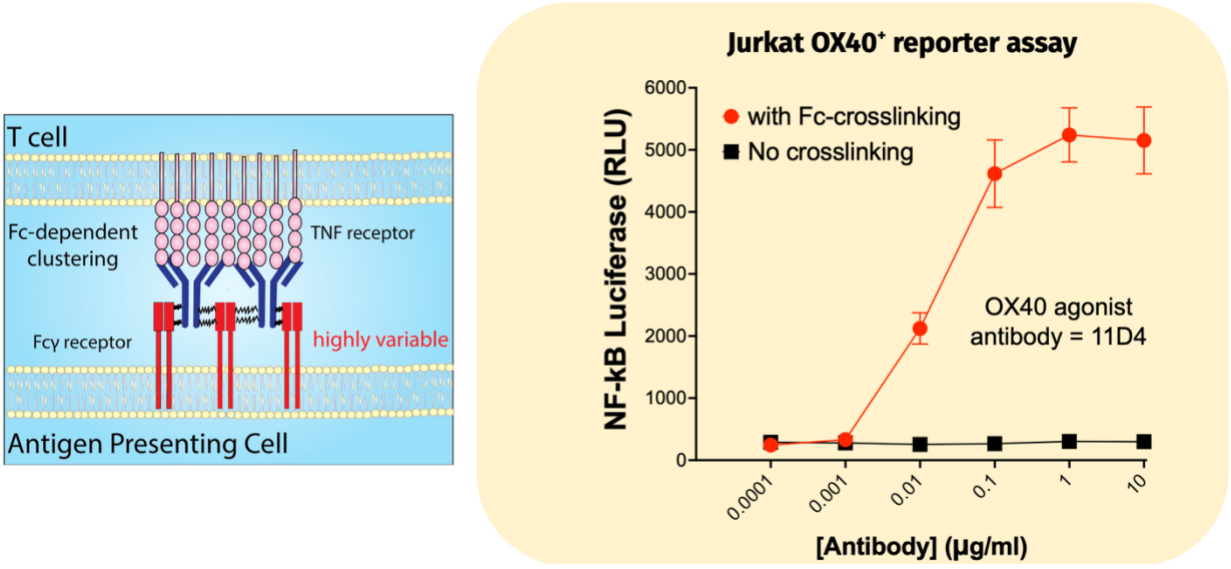

Figure 3-2: The Fc-mediated clustering is critical for activating TNF receptors. (A) the schematic illustration of the dependency of bivalent antibodies on Fc-mediated clustering from FcγR receptors on antigen presenting cells. (B) The Jurkat cell assay in the presence or absence of Fc-mediated crosslinking with CHO-expressing FcγRIIB. The graph demonstrated the Fc-crosslinking is necessary to activate receptors.

To develop potent agonist antibodies, the receptor structure and biology must be at the forefront of agonist antibody development. A greater understanding of the mechanism of receptor activation may open new avenues for generating optimized agonist antibodies. The trimeric formation of TNF receptors is critical for their intracellular receptor signaling; therefore, some antibody molecular formats (e.g. multivalent) are favorable for receptor clustering (4). Additionally, biepitopic antibodies that obviate the need for Fc-mediated crosslinking are expected to improve receptor agonism. Therefore, the objective of this dissertation was to develop improved methods for discovering biepitopic antibodies with the goal of improving T cell immunity using reporter and primary cell culture models.

In the initial part of the project, we developed a high-throughput antibody discovery platform to generate biepitopic antibodies that are robust and easy to use. The conventional biepitopic antibody discovery process is arduous and requires a cumbersome process of animal immunization and epitope binning, along with combining unique antibody pairs to construct biepitopic antibodies. On the other hand, our novel antibody screening approach is time-efficient and uses off-the-shelf antibodies to discover novel antibodies with unique epitopes. The antibody discovery process is relatively simple (i.e., requires minimal training), predicable (i.e., applicable to a broad set of clinical-stage OX40 antibodies), and generalizable (i.e., utilized for discovering CD137 antibodies). Using this technique, we were able to discover novel single-chain antibodies for OX40 and CD137 receptors which are critically important for regulating immune response and have been explored in developing therapies against diseases. Finally, we characterized the novel single-chain antibodies using yeast and mammalian reporter cell systems to examine their binding epitope characteristics with the goal of designing the next generation of agonist therapeutics.

Next, we used molecular cloning techniques to design biepitopic tetravalent antibodies to examine their function in primary T cell assays. Our results showed that biepitopic antibodies mediate significantly improved T cell activation as demonstrated by proliferation and IL-2 ELISA assays. To display the broad applicability of our biepitopic approach, additional clinical-stage antibodies were constructed as biepitopic to highlight the superior nature of these unique antibodies. Interestingly, we also showed that an OX40 antagonist antibody when paired with our novel single-chain antibody showed improved receptor activation due to its ability to mediate enhanced receptor clustering. Finally, the biepitopic antibody engineering approach was generalizable to additional TNF receptors, including CD137, which shows the broad applicability of our novel antibody discovery approach.

Looking forward, we anticipate that our antibody discovery platform will accelerate the discovery of agonist antibodies against a variety of T cell receptors. For example, we believe that this antibody discovery and engineering strategy will most likely be applied to immune receptors where receptor clustering is a dominant form of receptor agonism. Furthermore, antibody development strategies may also be applicable to design novel agonist therapeutics for critical immune checkpoints such as PD-1 and CTLA-4 for applications in autoimmune diseases. Finally, it would be important to evaluate the role of these novel antibodies in the context of tumor biology. Collectively, we expect that the combination of one or more of these optimization approaches can improve agonist function and therapeutic efficacy.

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