High-throughput Development of Fc-independent Biepitopic Antibodies that Activate T cell Receptors

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Summary:

Antibodies that activate immunomodulatory receptors (e.g., the CD137 T cell receptor) to regulate the immune system are a promising class of emerging therapeutics against cancer. However, most "agonist" antibodies have poor therapeutic efficacy due to their reliance on external factors such as Fcγ receptors to mediate sufficient activity. The development of "biepitopic" antibodies targeting two different areas on the same receptor represents a novel approach towards mediating robust activity, but there is a lack of high-throughput methodology to generate such antibodies.
In my capstone project, I intend to utilize a unique methodology for discovering novel epitopes against the CD137 receptor and designing tetravalent antibodies with improved agonist activity. Towards this goal, I will screen antibody libraries to discover variants with unique epitopes compared to the clinical antibody utomilumab. Next, I will generate tetravalent biepitopic or monoeptiopic antibodies by pairing the discovered clones with utomilumab. Finally, I will evaluate the biological activity and characteristics of the antibody constructs using primary T cell assays and binding experiments. My specific contributions to the project are screening antibody libraries for specific clones, developing the gene sequences that encode for the antibody constructs, and characterizing the biological activity of the antibodies.

Introduction:

The tumor necrosis factor receptor superfamily (TNFSR) are a wide and diverse set of receptors responsible for enabling immune cell functions such as cell differentiation and proliferation (Locksley, et al., 2001; Hehlgans, et al. 2005; Mayes et al., 2018). Examples of these receptors include OX40 (TNFSR4) and CD137 (TNFSR9) which have garnered immense interest due to the development of agonist antibodies capable of activating their co-stimulatory functions, inciting their possible use as an immunotherapeutic treatment against various diseases including cancer (Haln et al., 2017; Mayes et al., 2018). Despite their promise, challenges regarding the safety and efficacy of TNFR-targeting agonist antibodies have slowed their clinical translation (Sedger et al., 2014). This could be due to the biological nature of TNFR receptors which have been shown to require the trimerization of individual receptors and subsequent coordination into receptor superclusters to achieve sufficient activation (Chin et al., 2018; Melero et al., 2013). Current antibody formats including bivalent immunoglobulin G (IgG) rely on binding to Fcγ

receptors (FcγRs) to mediate such a degree of clustering, yet this form of improving activity is unreliable due to the varying expression of FcγRs on different immune cells (Furness et al., 2014; Kim et al., 2013; Wilson et al., 2011). The usage of novel strategies towards the robust activation of immune receptors in a FcγR-independent manner represents a desirable pathway to improve the current generation of agonist antibodies.

Several antibody properties have been previously utilized to improve the therapeutic effectiveness and reduce the dependency of FcγR-mediated crosslinking of agonist antibodies. For example, the addition of multiple binding arms to the antibody structure (i.e. multivalency) to bind to multiple receptors simultaneously have proven to be an effective method towards improving antitumor activity in preclinical models (Kucka, Wajant, 2020; Wang, et al., 2021) . Recently, the construction of biepitopic antibodies targeting two different areas on the same receptor has emerged as a unique strategy to facilitate receptor clustering by daisy-chaining receptor superclusters together (Yang et al., 2019). This method was shown to enact potent *in vivo* agonist activity using an tetravalent, biepitopic OX40 antibody construct with four antibody arms targeting two different non-overlapping receptor regions. Furthermore, the improved activity was not lost when mutations abrogating affinity for FcγRs was introduced into the antibody structure, demonstrating the robust agonism and Fc-independent nature of the biepitopic format. However, several limitations towards this method such as the requirement of discovering two antibody variants with non-overlapping epitopes pose a blockade towards the implementation of biepitopic antibodies as a common format.

Therefore, we have sought to develop a high-throughput methodology to greatly simplify the generation of biepitopic antibodies against the TNF receptors OX40 and CD137. In my capstone project and as part of this project, we utilized this unique methodology for discovering novel epitopes against the CD137 receptor and designing tetravalent antibodies with improved agonist activity. Towards this goal, we screened two separate human non-immune antibody libraries to discover variants with unique epitopes compared to the clinical antibody utomilumab. Next, we generated tetravalent biepitopic or monoeptiopic antibodies by pairing the discovered clones with utomilumab . Finally, we evaluated the biological characteristics of the antibody constructs using HEK293 binding experiments (Figure 1). We expect that this methodology can be used to generate biepitopic versions of current preclinical agonist antibodies and provides a facile means to improve their therapeutic efficacy.

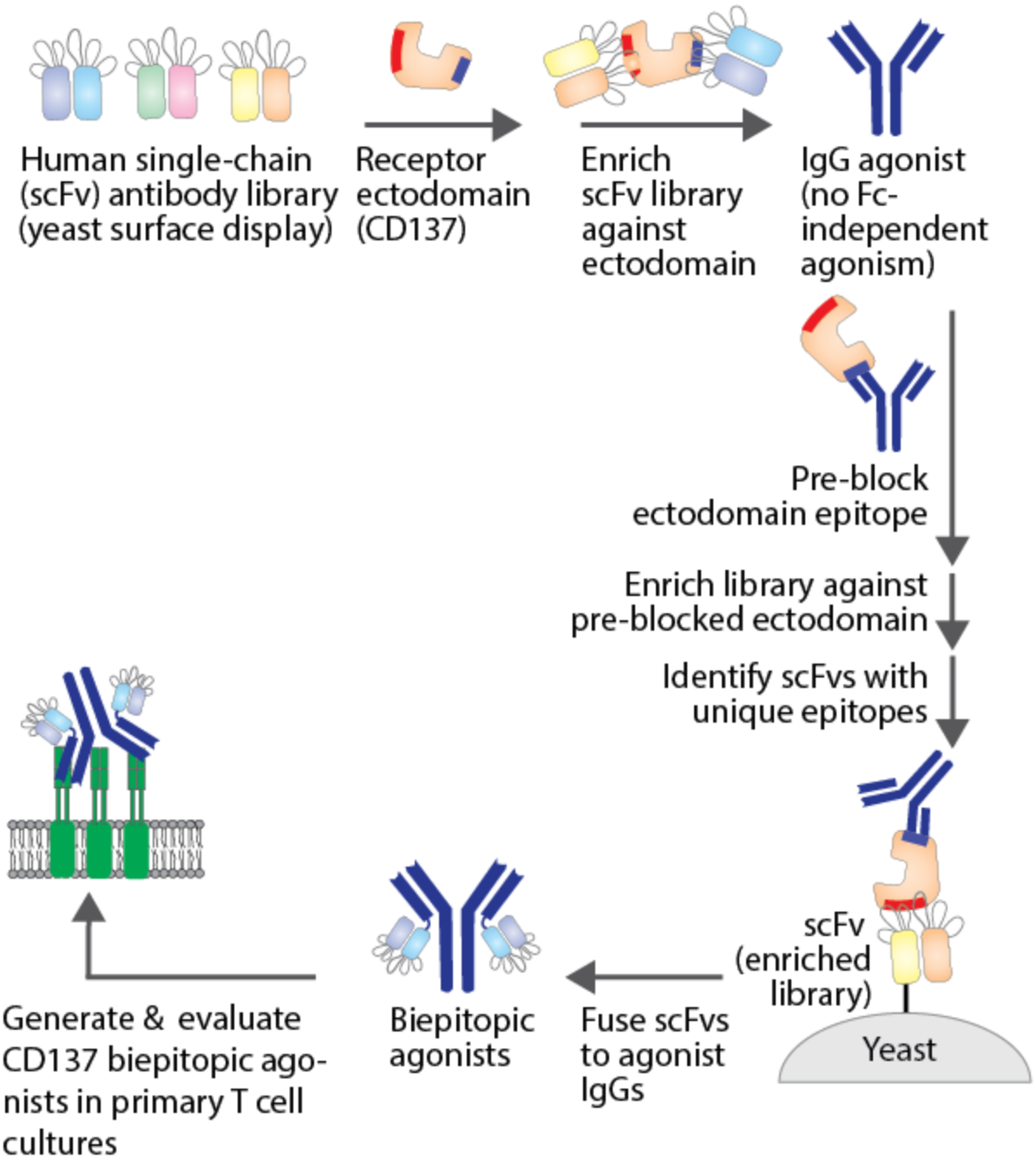

Figure 1: Overview of competition-based discovery of biepitopic agonist antibodies against the CD137 receptor Fc independent agonist activity. A nonimmune human single-chain antibody (scFv) library was first enriched against the target CD137 ectodomain. Next, an existing bivalent anti-CD137 IgG (utomilumab), which lacks Fc independent agonist activity, was used to block its epitope on its cognate receptor ectodomain. The scFv library was further enriched against the ectodomain/IgG mixture to identify scFvs with unique receptor epitopes.

Then, the IgG is fused to the identified scFvs to generate biepitopic/tetravalent agonists with intrinsic, Fc-independent activity.

Methods:

Antibody discovery

The human non-immune library (library #1) (Feldhaus et al., 2003) and synthetic library (library #2) (Kelly et al., 2018) were expressed as Aga2-scFv fusion proteins on the surface of yeast using a standard yeast plasmid (pCTCON2). For library sorting, two initial MACS selections were conducted using CD137-Fc (produced in-house). The antibody libraries were induced in media (SDGCAA) for 1 day at 30 °C to promote the expression of scFvs on the cell surface. 109 yeast cells were incubated with 300 nM of CD137-Fc in 5 ml of 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 750 µl of Protein A microbeads (Miltenyi Biotec, Cat. #130071001) per 109 cells 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 5 mL of SDCAA liquid media (20 g/l of dextrose, 6.7 g/l of yeast nitrogen base without amino acids, 5 g/l of casamino acids, 16.75 g/l of sodium citrate trihydrate, 4 g/l citric acid) at 30 °C. Subsequently, the cells were grown in SDCAA media for 1 days at 30° C. Next, the enriched library was sorted via FACS at antigen concentrations of 300 nM for rounds 1-4 against biotinylated CD137 ectodomain (CD137: Acro Biosystems 41B-H82E6). Following a 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 neutravidin-PE (Invitrogen, Cat. #A2660) at 1:1000 dilution 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 rounds 5-6 (CD137), 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 hrs at room temperature on a rocker prior to labeling with goat anti-mouse AlexaFluor 488 (Invitrogen, A11001) at 1:200 dilution and neutravidin-PE (Invitrogen, Cat. #A2660, 1:300) at 1:1000 (Jackson laboratories, 016-600-084). Finally, the library was sorted using FACS and collected into 2 ml of SDCAA media. The cells were regrown and Sanger sequenced.

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 at 37 °C 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 mg of vector plasmid (7.5 mg of variable light chain (VL) plasmid and 7.5 mg of variable heavy chain plasmid (VH) for bivalent and 11.25 VL plasmid and 3.75 VH plasmid for tetravalent antibodies) and PEI (3-fold excess, 45 mg) 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 min 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.

CD137 HEK-293T binding analysis

HEK-293T cell lines were first engineered to express a stable doxycycline-inducible human CD137 receptor (developed in-house) using a lentivirus delivery system. In 96-well plates, 50,000 CD137 cells were plated with 100 ng/ml doxycycline at 37 °C (5% CO2) to achieve a volume of 150 mL. After 2 days, 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.

Results:

Competition-based selection of CD137 single-chain antibodies

To develop biepitopic CD137 antibodies, we sought to identify single-chain antibodies (scFvs) to pair with a clinical-stage CD137 agonist antibody that lacks intrinsic agonist activity, namely

utomilumab (Chin et al., 2018). To establish proof-of-principle results for our competition-based discovery approach, we had first evaluated the expression and antigen-binding activity of 11D4 as a single-chain Fab (scFab) fragment on the surface of yeast. 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 and CD137 epitopes.

Next, we performed a series of in vitro selections to identify scFvs that recognize unique CD137 epitopes from nonimmune and synthetic human libraries (Figure 1) (Feldhaus et al., 2003). First, the library was enriched for CD137 binding via an initial round of magnetic-activated cell sorting (MACS) against the bivalent CD137-Fc. Following one round of MACS, the yeast population was propagated and evaluated via fluorescence-activated cells sorting (FACS) for binding to biotinylated monovalent CD137. Significant enrichment of the antigen-binding population was observed after each sort (FACS rounds 1-4), as shown in Figure 1.

We next sought to identify single-chain antibody (scFvs) that engaged non-overlapping CD137 epitopes relative to the epitope of utomilumab. We applied a competition-based strategy by screening for antigen binding in the presence of soluble utomilumab IgG. Consecutive rounds of screening were conducted, first at an equimolar concentration of CD137: utomilumab IgG followed by a 10-fold molar excess of 11D4 utomilumab relative to CD137. Encouragingly, we retained a clear binding population after pre-blocking the utomilumab epitope on CD137 (Figure 1, 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 sequenced antibodies from the enriched libraries using Sanger Sequencing and identified three unique variants (CD.F7, CD.F8, CD.K2) for continued evaluation.

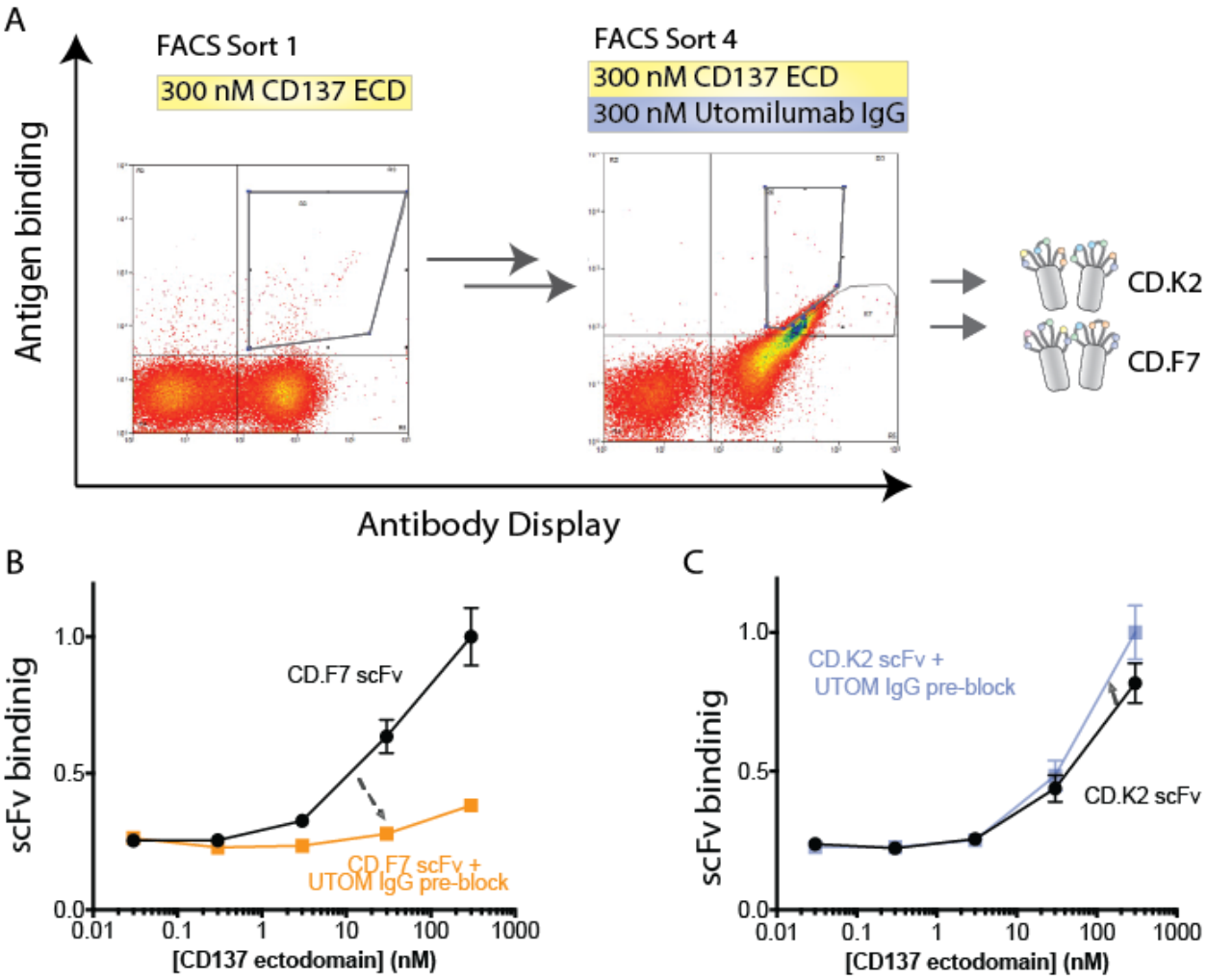

Figure 2: Development of antibody screening system to discover novel antibodies specific for the CD137 receptors. (a) To confirm the functionality of the yeast screening platform, the clinical IgG utomilumab (UTOM) were expressed as scFabs and verified to bind strongly to the CD137 ectodomain. The human single-chain antibody (scFv) library was enriched after 4 rounds of FACS sorting and clones binding to the ectodomain pre-blocked with the clinical IgG were selected. (b-c) Three clones (CD.F7,CD.F8 [Not shown], and CD.K2) demonstrate distinct binding to the CD137 receptor where CD.F7 scFv competes with UTOM for binding (b) while the CD.K2 scFv exhibits strong binding in UTOM-preincubated conditions (c), demonstrating that the CD.K2 targets a unique epitope.

Selected single-chain antibodies engineered as biepitopic antibodies recognize unique epitopes relative to existing CD137 IgG

To evaluate the CD137 binding of the identified scFvs, affinity analysis on yeast was conducted in the presence or absence of 11D4 IgG pre-blocking (Figure 1B-C). Encouragingly, while CD.F7 appeared to bind on an overlapping region of the CD137 receptor (Figure 1B), the CD.K2

clone retained binding to CDF137 in the presence and absence of utomilumab IgG, suggesting that the two scFvs engage distinct epitopes (Figure 2B-D). Interestingly, the CD.K2 scFv exhibited stronger CD137 binding after utomilumab IgG pre-blocking compared to the control without pre-blocking. As a positive control, we confirmed that utomilumab scFv lost binding in the presence of utomilumab IgG pre-blocking (Figure S1). Overall, these results demonstrate that the selected single-chain antibodies engage unique CD137 epitopes compared to the utomilumab IgG.

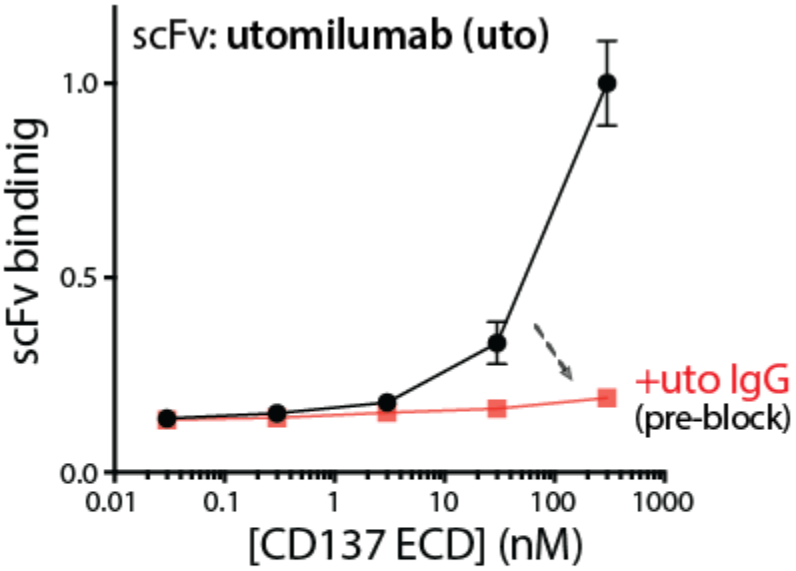

Figure S1: CD137 binding to the utomilumab single-chain antibody on yeast is inhibited by utomilumab IgG. The CD137 ectodomain was pre-incubated with utomilumab IgG (1:5 molar ratio) and then the binding of the CD137/utomilumab complex to utomilumab scFv was evaluated via flow cytometry.

Finally, we generated an IgG-scFv construct using the CD.K2 scFv and tested its ability to bind onto CD137-expressing HEK293T cells (Figure 3A-B). As expected, the biepitopic antibody (CD.K2/Utom) maintained affinity for the CD137 receptor in blocking conditions while the monoepitopic IgG-scFv (Utom/Utom 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.

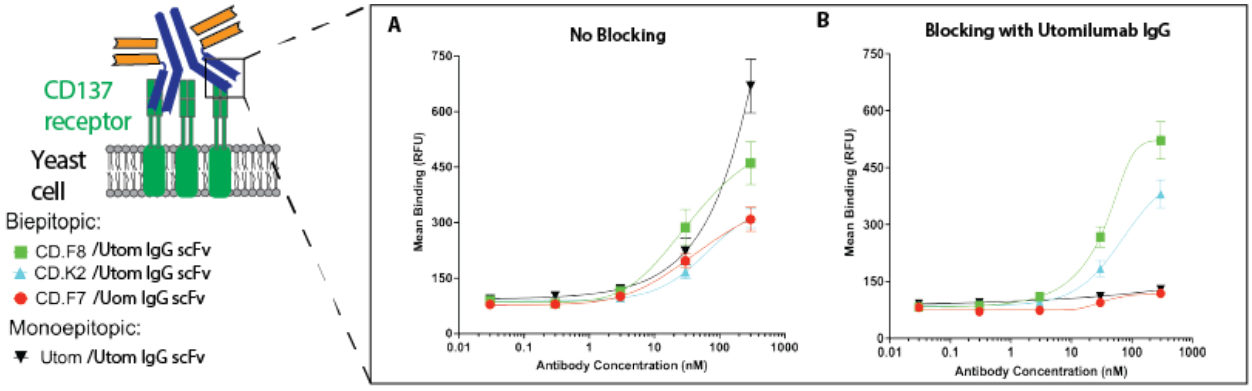

Figure 3: Engineered CD137 agonist antibody constructs mediate Fc-independent T cell activity. (a-b) All selected antibody clones exhibit strong binding to the CD137 receptor alone, but competition analysis reveals that only the CD.F8 and CD.K2 biepitopic antibodies exhibit strong binding to the CD137 receptor preincubated with UTOM, demonstrating that these variants target unique epitopes compared to the UTOM clinical antibody.

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 (Abdiche et al., 2009, 2012, 2014; Anderson et al., 2017). 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 (Bogen et al., 2020). To overcome these challenges, our approach uses previously discovered and clinically relevant 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.

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 (Bogen et al., 2020; Overdijk et al., 2020; Yang et al., 2019). Biepitopic antibodies have been recently generated in a variety of molecular formats, including as tetravalent mAb-scFvs (as used in this work), tetravalent DVDs (Yang et al., 2019), bivalent IgGs (Bogen et al., 2020), and Fc-engineered constructs that non-covalently trimerize upon antigen binding (Overdijk et al., 2020). One such report has shown that biepitopic antibodies in a bivalent IgG format induce receptor clustering of soluble and cell-bound epidermal growth factor receptors (EGFR) (Bogen et al., 2020). 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. We hypothesize that this phenomenon is further enhanced by using a tetravalent antibody format as multiple receptor clusters could be brought together to induce greater receptor activation.

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